

Models and treatments of Dominant Dystrophic Epidermolysis Bullosa

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Abstract

Epidermolysis bullosa (EB) is a group of genetic blistering disorders caused by mutations in the structural proteins of the skin. EB is characterised anatomically by skin fragility, blistering and scarring, and frequently results in pain, itch and – in severe cases – death. The dystrophic subtype of EB is caused by mutations in the human *COL7A1* gene which encodes for collagen VII, and can be inherited in either a dominant or recessive manner. Dominant dystrophic epidermolysis bullosa (DDEB) is commonly caused by glycine substitutions within the collagenous domain of collagen VII. Improving understanding of the pathophysiology of DDEB should enable the development of more effective treatments. To this end, having suitable mouse models for DDEB would be helpful, but none have yet been reported.

This thesis describes the generation of two novel mouse lines, each of which carries a modified *Col7a1* allele (p.G2037R and p.G2028R) that was introduced using CRISPR and corresponds to the most common *COL7A1* mutations found in DDEB patients (p.G2043R and p.G2034R). Dominant inheritance of either of these alleles results in a phenotype that closely resembles that seen in DDEB patients. Specifically, mice carrying these alleles show recurrent blistering that is first observed transiently around the mouth and paws in the early neonatal period and then again around the digits from 5-10 weeks of age. Histologically, the mice show micro-blistering and reduced collagen VII immunostaining. Biochemically, collagen VII from these mice displays reduced thermal stability, which we also observed to be the case for DDEB patients carrying the analogous mutations. Unlike previous rodent models of EB which frequently show severe disease and early lethality, these mouse models – which to our knowledge are the first for DDEB – show no impairment in survival and have a relatively mild phenotype, and thus represent a practically and ethically tractable means for better understanding and treating EB.

As proof-of-concept of the utility of these DDEB mice, animals carrying the p.G2037R allele were assessed in standardised pain behavioural tests and observed to be hypersensitive to both mechanical and thermal stimuli using the von Frey and Hargreaves' assays respectively, mimicking what has been observed previously in EB patients. A range of different pharmacological agents – some of which are already used clinically to treat pain in EB patients and others of which are more experimental – were then objectively compared across a range of relevant doses and their analgesic effectiveness in DDEB mice was found to vary. While most drugs provided only a partial reduction in pain hypersensitivity, the non-steroidal anti-inflammatory agent, meloxicam, as well as a novel analgesic agent in early pre-clinical development were both found restore sensitivity to that of wild type mice, thus demonstrating that the DDEB mice may be useful preclinical tools for future drug development in EB.

Declaration

This is to certify that:

- (i) The thesis comprises my original work towards the PhD, except where indicated in the Preface,
- (ii) Due acknowledgment has been made in the text to all materials used, and
- (iii) The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

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January, 2021

Preface

Publication supporting thesis

Chapter 2

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Mouse models for dominant dystrophic epidermolysis bullosa (DDEB) carrying common human point mutations recapitulate the human disease

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I conceptualised the study, designed and conducted the experiments, performed the data analyses, and drafted the initial manuscript, and revised the manuscript co-ordinating the input from the other authors.

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Table of Contents

Abstract.....	i
Declaration.....	ii
Preface	iii
Acknowledgements.....	iv
Table of Contents.....	vi
Figure List.....	ix
Abbreviations.....	xi
1 Chapter 1 Introduction	2
1.1 Skin.....	2
1.1.1 Epidermis	2
1.1.2 Dermis	2
1.1.3 Hypodermis.....	2
1.1.4 Anchoring Proteins.....	3
1.1.5 Pain and nerves.....	3
1.2 EB Overview	4
1.3 EB Subtypes.....	4
1.4 EB Genetics	4
1.5 EB Symptoms	7
1.5.1 Blisters.....	7
1.5.2 Pain.....	7
1.5.3 Itch	8
1.5.4 Death.....	8
1.6 Diagnosis	9
1.6.1 Immunofluorescence Mapping.....	9
1.6.2 Genetic testing	9
1.6.3 Electron Microscopy	9
1.7 Treatments.....	9
1.7.1 Supportive care	9
1.7.2 Curative therapies.....	10
1.8 Animal Models	10
1.9 Hypothesis and Aims.....	12

1.9.1	Hypothesis.....	12
1.9.2	Aims.....	12
Chapter 2 – Development of novel mouse models for DDEB.....		13
2.1	Mouse models for dominant dystrophic epidermolysis bullosa (DDEB) carrying common human point mutations recapitulate the human disease	14
2.2	Abstract.....	14
2.3	Introduction	14
2.4	Results.....	15
2.5	Discussion.....	19
2.6	Materials and Methods.....	20
2.7	References	22
2.8	Supplimental Information.....	23
3	Chapter 3 Characterisation and treatment of pain in DDEB mouse models	33
3.1	Introduction	33
3.2	Methods.....	34
3.2.1	Mice.....	34
3.2.2	Habituation	34
3.2.3	Hargreaves test	34
3.2.4	Manual von Frey	34
3.2.5	Dynamic Plantar Aesthesiometer (DPA)	35
3.2.6	Drugs and administration.....	35
3.2.7	Statistical analyses	36
3.3	Results.....	36
3.3.1	DDEB mice with an 11aa deletion have increased thermal and mechanical sensitivities compared to wild-type.....	36
3.3.2	Use of clinically-relevant analgesics results in partial improvement in mechanical hypersensitivity in DDEB mice with an 11aa deletion	37
3.3.3	Mechanical hypersensitivity in DDEB mice with an 11aa deletion is similar at 8 and 18 weeks of age	38
3.3.4	EB mice carrying the 11aa deletion and G2037R mutation display similar levels of mechanical allodynia.....	39
3.3.5	EB mice carrying the G2037R mutation show a variable response to different analgesic agents	39
3.3.6	EB mice carrying the G2037R mutation can also be used to test novel analgesic agents..	42
3.4	Discussion.....	44

4	Conclusion.....	46
5	Bibliography	48

Figure List

Figure 1.1. Genes associated with forms of Epidermolysis Bullosa and their location within the skin layers. Figure reproduced from (C. Has, Liu, et al., 2020).	5
Figure 1.2 Schematic presentation of different types of mutations within Collagen VII and their outcomes for DEB. Figure reproduced from (Chung & Uitto, 2010)	6
Figure 1.3 Radar diagram of mean pain scores of types of pain reported in patients with Epidermolysis bullosa subtypes	8
Figure 3.1 EB 11aa (p.A2027_G2037del) KI/+ show marked thermal and mechanical allodynia.....	36
Figure 3.2 Assessment of mechanical allodynia in DDEB mice carrying the 11aa deletion following administration of different analgesic agents.....	38
Figure 3.3 Assessment of mechanical allodynia in DDEB mice carrying the 11aa deletion at later timepoints and in comparison to the mice carrying the G2037R mutation.	39
Figure 3.4 Assessment of mechanical allodynia in DDEB mice carrying the p.G2037R mutation following administration of different analgesic agents.	41
Figure 3.5 Assessment of mechanical allodynia in DDEB mice carrying the p.G2037R mutation following administration of novel analgesic agents.	43

Table 1.1 Examples of transgenic mice developed as models for different forms of EB. Reproduced from (L. Bruckner-Tuderman et al., 2010).....	11
Table 4.1 Drugs tested for their analgesic properties	35

Abbreviations

%	Percentage
°	Degrees
bp	Base pairs
BSA	Bovine serum albumin
C	Celsius
CKO	Conditional “knock-out”;
CO ₂	Carbon-dioxide
DEB	Dystrophic Epidermolysis Bullosa
DDEB	Dominant Dystrophic Epidermolysis Bullosa
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxy ribonucleotide triphosphates
EB	Epidermolysis Bullosa
EBS	Epidermolysis Bullosa Simplex
EBS-MD	Epidermolysis Bullosa Simplex with muscular dystrophy
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GKO	Germline “knock-out”
HS	Hallopeau-Siemens variant
HYP0	hypomorphic mouse (10% expression of the Col7a1 in comparison to wild-type mice)
JEB	Junctional Epidermolysis Bullosa
kb	kilo-base pair
KI	“knock-in” of a dominant gene
KS	Kindler Syndrome
NGS	Next Generation Sequencing

PA	pyloric atresia
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
rpm	Rotations per minute
RDEB	Recessive Dystrophic Epidermolysis Bullosa
SCC	Squamous cell carcinoma
sec	Seconds
SD	Standard Deviation
SEM	Standard Error of the Mean
T _m	Melting temperature
μ	Micro
μg	Micrograms
μl	Microlitres
μM	Micromolar
WT	“Wild-type”

Chapter 1 - Introduction

1 Chapter 1 Introduction

1.1 Skin

Our skin plays a vital role as a barrier that protects us from environmental insults such as microbes, chemicals and physical stresses, while at the same time preventing the loss of water and nutrients. It is made up of three layers – the epidermis, dermis and hypodermis – with each layer containing different types of cells and structures (Weller, Hunter, & Mann, 2015).

1.1.1 Epidermis

The epidermis is the outer layer of the skin, and is made up primarily of keratinocytes which exist in various states of differentiation. The basal layer of proliferating keratinocytes is attached to the basement membrane zone by hemidesmosomes. Following proliferation, keratinocytes undergo differentiation and gradually move up to the outer layers of the epidermis. During differentiation, keratinocyte production of the key structural protein keratin shifts from keratins 5 and 14 to keratins 1 and 10. Subsequently, as they age, keratinocytes lose organelles and die and, in doing so, form a lipid layer, which becomes the protective outer barrier of the skin (Weller et al., 2015).

Within the epidermis, other cell types include melanocytes, Langerhans cells, and Merkel cells. Melanocytes are mostly located in the basal region, do not have desmosomes, and contain melanosomes which produce melanin that helps to protect the surrounding keratinocytes from UV damage. Langerhans cells are suprabasal, do not have desmosomes, and play a key immune role by taking up antigens to present to T lymphocytes. Merkel cells are located in the basal region, are attached to neighbouring keratinocytes by some desmosomes, and play a mechanosensory role by detecting fine touch (Weller et al., 2015).

1.1.2 Dermis

The dermis sits under the epidermis, and both supports and nourishes the epidermis. The primary cell type in the dermis is the fibroblast, which is responsible for the synthesis of key extracellular molecules such as collagen, reticulin, elastin, fibronectin, glycosaminoglycans and collagenase. The majority of the dry weight of the epidermis (between 70-80%) is made up of extracellular collagens, with the main collagen subtype in the dermis being collagens I and III. Apart from fibroblasts, the dermis also contains small numbers of resident and transitory mononuclear phagocytes, lymphocytes, dermal dendritic cells and mast cells (Weller et al., 2015).

1.1.3 Hypodermis

The hypodermis is a looser connective tissue found below the dermis. It contains larger blood vessels, nerves and abundant fat which acts as a shock absorber, provides insulation and a connection to bone and muscle. Though in some areas of the body this layer is not present (Hossler, 2014; Weller et al., 2015).

1.1.4 Anchoring Proteins

Connecting the cells of the dermis and epidermis are anchoring proteins that play a key role in preventing the separation of these two skin layers. Chiefly comprising of collagens and laminins, these proteins form chains that span between hemidesmosomes on cells within the epidermis and anchoring fibrils within the dermis. When components of this anchoring system fail, the epidermis and dermis separate, leading to skin blistering (Weller et al., 2015).

Laminins are large glycoproteins produced by keratinocytes. These allow adhesion between the basal layer of cells via integrins and type IV collagen.

Individual collagen molecules have large stretches which contain a repeating glycine motif where every third amino acid is a glycine. This allows them to form a triple helix. These molecules can then form into microfibrils which can then form larger collagen fibres. Within the dermis there are interconnected fibres of mainly collagen which forms bundles. Type I collagen represents most of this skin collagen accounting for around 85%; it is also found in most connective tissues including tendon and bone. Type III collagen is the second most abundant skin collagen and accounts for around 15%; it is also found in blood vessels. Together types I and III collagen form collagen fibrils which are held together in the dermis by various elastin proteins. The collagen fibrils connected to the basale lamina by anchoring fibrils formed from type VII collagen. Monomers of collagen VII are form antiparallel dimers which associate and are processed, wrapping around the collagen fibrils and connecting back to type IV collagen (which makes up the lamina densa) or forming anchoring plaques (L. Bruckner-Tuderman, Höpfner, & Hammami-Hausli, 1999; Weller et al., 2015).

1.1.5 Pain and nerves

As our boundary with the outside world, the skin contains an estimated 1 million nerve fibres. Some of these fibres are free sensory nerve endings called nociceptors and are able to sense mechanical, chemical, or thermal stimuli. However, if the intensity of these stimuli is high enough pain is perceived. Chronic pain can result when nociceptive neurons become hyperresponsive (Pinho-Ribeiro, Verri, & Chiu, 2017), resulting in both allodynia (i.e. pain due to normally innocuous stimuli) and hyperalgesia (i.e. increased pain due to normally noxious stimuli). Nociceptor sensitisation can be caused by a number of processes (Basbaum et al., 2009). The keratinocytes and resident immune cells can produce small molecules (ATP, serotonin), cytokines (IL-1b, IL-6, TNF), peptides (substance P (SP), calcitonin gene-related peptide (CGRP)), proteases, lipids (prostaglandins), and growth factors (nerve growth factor, NGF). These can activate G protein-coupled receptors (GPCRs) and transient receptor potential (TRP) ion channels (TRPV1, TRPA1) on sensory neurons. This activation in turn modulates the activity of ion channels (voltage-gated sodium channels Nav1.1- Nav1.8) to cause increased and sustained nociceptor firing (Pinho-Ribeiro et al., 2017). This can be counteracted by specialised mechanoreceptor cells, (e.g., Merkel cells and Meissner corpuscles) which can inhibit nociception (Melzack & Wall, 1965). As such a disruption of the homeostasis between keratinocytes, immune cells, nociceptors and mechanoreceptors can drive pain sensitisation in the skin.

1.2 EB Overview

Epidermolysis Bullosa (EB) is a group of genetic skin disorders which result in blistering of skin and mucous membranes. The overall prevalence of EB is around 1 in 17,000 live births (J. D. Fine et al., 2014). The extremities of the hands and feet are commonly affected by blistering in response to mechanical stress and can progress to include nail loss and deformity (J. D. Fine et al., 2014; Hsu et al., 2014). Other common symptoms include blisters around the eyes, nose, mouth, and throat, which can result from minor injury, friction, or temperature change, and can be present from birth. Patients also report both pain and itch within the skin whose severity depends on the underlying EB subtype (Schröder, Yuen, & Jonkman, 2018).

1.3 EB Subtypes

EB is broken down into four major subtypes, classified according to the ultrastructural level at which the blisters arise. Epidermolysis Bullosa Simplex (EBS) is characterised by blisters that form in the epidermis. Junctional Epidermolysis Bullosa (JEB) causes a disruption in the lamina lucida between the layers of the dermis and epidermis. Dystrophic Epidermolysis Bullosa (DEB) results from disruption of the anchoring fibrils that secure the epidermis to the basement membrane zone. The final subtype is Kindler syndrome (KS), where blisters form across multiple planes rather than within discrete layers (Leena Bruckner-Tuderman, 2010; J.-D. Fine et al., 2014; Has et al., 2018; Hsu et al., 2014).

1.4 EB Genetics

EB arises due to mutations in one of several different genes that encode structural skin proteins, and the anatomical location of the affected protein determines the EB subtype (Figure 1.1). More specifically, mutations of genes *KRT5*, *KRT14*, or *PLEC1* cause disruptions within the stratum basale result in EBS. Mutations of genes *LAMA3*, *LAMB3*, *LAMC2*, *COL17A1*, *ITGB4*, or *ITGA6* which code for proteins in the lamina lucida result in JEB. Mutations of *COL7A1*, the gene encoding Collagen VII, which extends from the dermal-epidermal basement membrane to the underlying dermis, results in Dystrophic Epidermolysis Bullosa (DEB). Finally, a mutation of *FERMT1* results in KS.

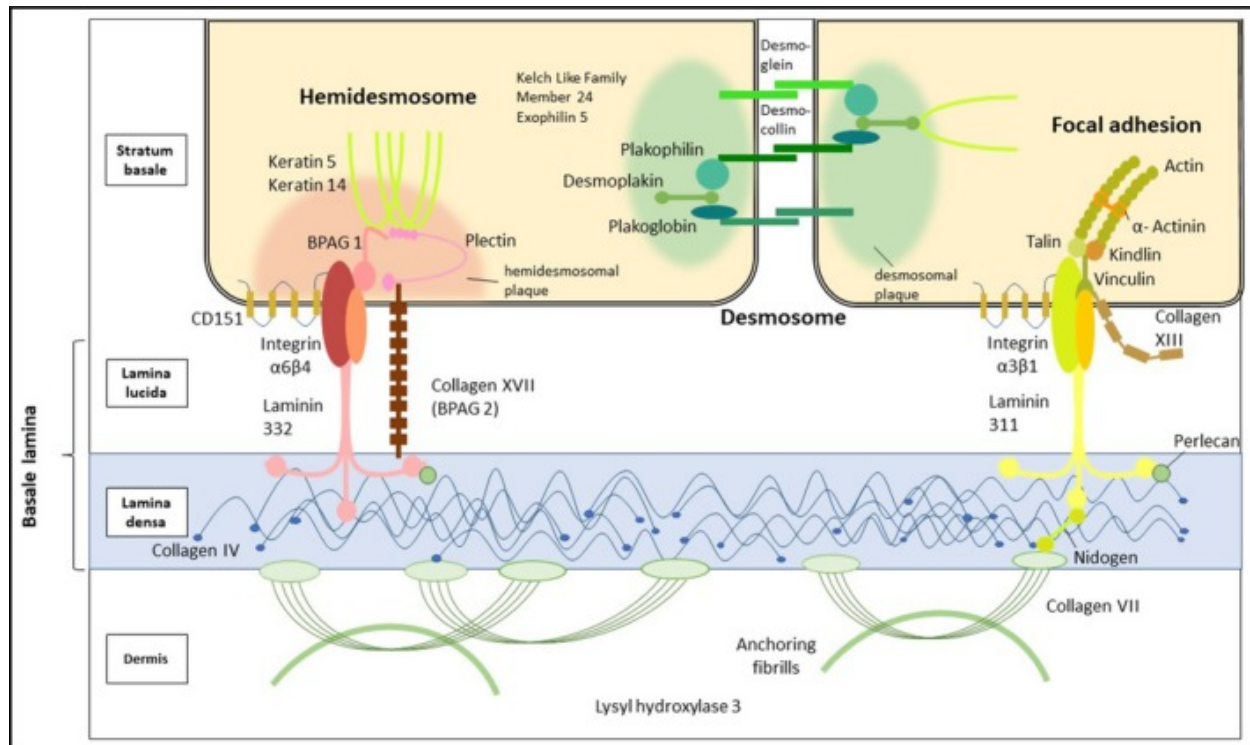


Figure 1.1. Genes associated with forms of Epidermolysis Bullosa and their location within the skin layers. Figure reproduced from (Has, Liu, et al., 2020).

Each of the major EB subtypes have slightly different patterns of inheritance. KS is exclusively inherited in an autosomal recessive manner. EBS, JEB, and DEB can each also be inherited in this way. EBS and DEB can also both be inherited in an autosomal dominant manner (Leena Bruckner-Tuderman, 2010; Lai-Cheong & McGrath, 2010; Sprecher, 2010; Yancey & Hintner, 2010). Finally, EBS, JEB, and DEB also display *de novo* inheritance patterns that arise when a spontaneous mutation develops in the germline of an unaffected parent.

For DEB, mutations in *COL7A1* can include missense mutations, premature termination codons (PTC) and glycine substitutions. These mutations can occur in isolation with a wild type allele or in various combinations with each other, and the overall disease severity and inheritance pattern will depend on which particular mutations are present and their effect on the integrity of the *COL7A1* anchoring fibrills (Figure 1.2) (Chung & Uitto, 2010).

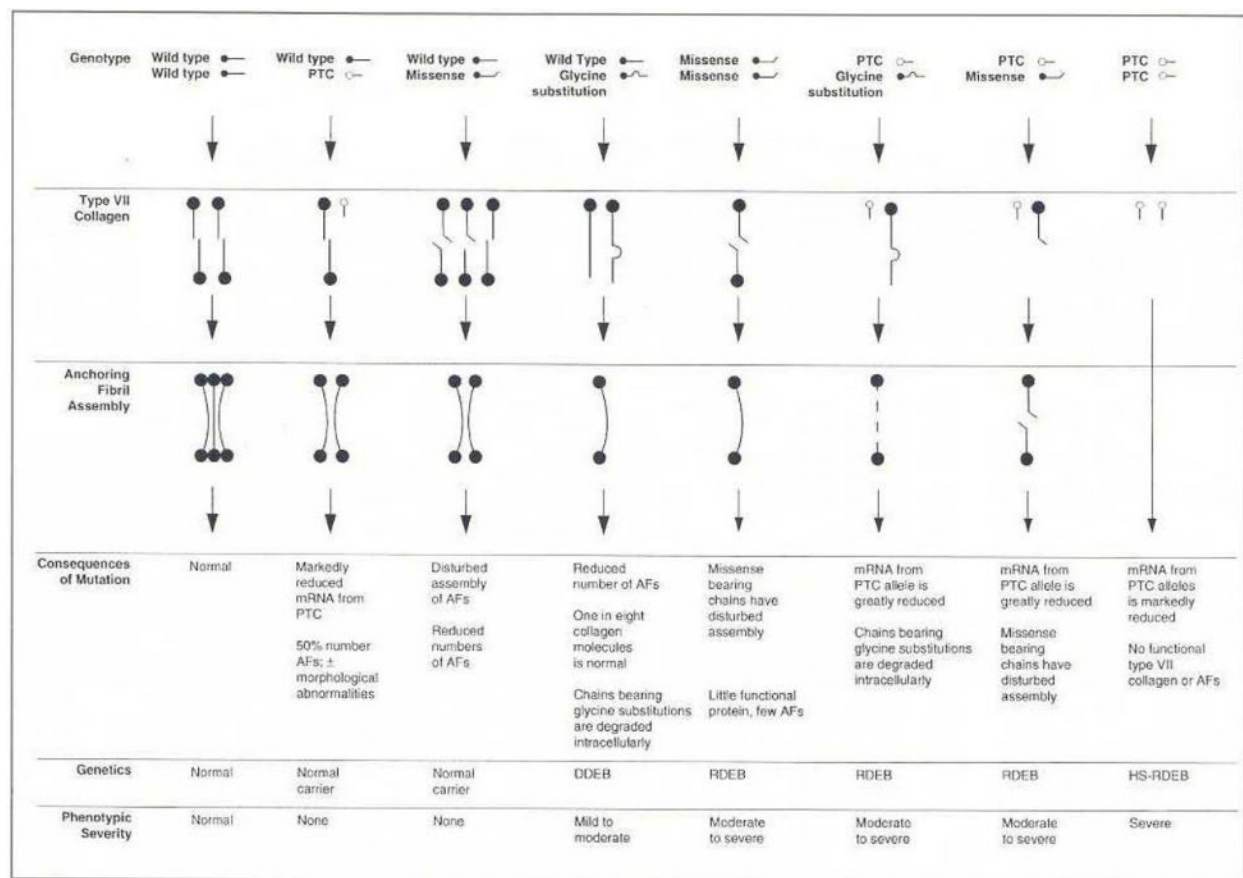


Figure 1.2 Schematic presentation of different types of mutations within Collagen VII and their outcomes for DEB. Figure reproduced from (Chung & Uitto, 2010)

Dominant DEB (DDEB), which is the focus of this thesis, arises due to the presence of dominant negative mutations in the gene encoding collagen VII, *COL7A1*, with particular glycine substitutions usually responsible (Dang & Murrell, 2008). The severity of DDEB varies from patient to patient, and these differences in severity are believed to be due in part to how different mutations affect formation of the collagen VII anchoring fibrils. However the same mutations can result in different clinical phenotypes. For example, heterozygous carriers of the p.1770S mutation may have no symptoms, nail dystrophy, or nail dystrophy, blistering and inflammation (Almaani et al., 2011).

As with all collagens, *COL7a1* contains a triple helical collagenous domain. For *COL7a1*, this domain is 1530 amino acids long and consists of an α -helical chain containing a Gly-X-Y repeating motif as well as a 39 amino acid hinge region. During the assembly of the *COL7a1* fibrils, three pro- $\alpha1$ collagen polypeptides assemble together through folding of the triple helical collagenous domains.

To help improve understanding of DEB, a DEB patient register was established several years ago. It collects and publishes data about DEB patients, including the mutations they carry and their disease severity (van den Akker et al., 2011; Wertheim-Tysarowska et al., 2012). From this register as well as from previous research, it is evident that the majority of mutations that cause DDEB occur just after the

hinge region within exon 73 (Christiano et al., 1994; Chung & Uitto, 2010; Sakai et al., 1986; Varki et al., 2007).

1.5 EB Symptoms

1.5.1 Blisters

As stated above, blistering is the major symptom of EB, and its severity depends on the underlying EB subtype. As well as being problematic in and of themselves, blisters can also cause a variety of secondary problems. Due to the breakdown of the normal protective barrier of the skin, blistering can lead to infections. Repeated blistering can also cause chronic wounds and scarring. When the latter occurs on the hands and feet, this can result in nail loss and pseudosyndactyly, or so-called mitten deformity, where the fingers or toes become covered in an epidermal cocoon (Breitenbach et al., 2015). A complication of the former is squamous cell carcinomas (SCC) which are most common in RDEB patients. (Condorelli et al., 2019; Montaudié et al., 2016). Some patients also experience blistering on their mucosal membranes. For example, oesophageal involvement can lead to difficulty swallowing and strictures, which can in turn cause malnutrition (Salera et al., 2020). Both dental issues such as peri and interoral blisters and ocular issues such as corneal/conjunctival erosions can also occur, and require special management (Feijoo et al., 2011; Fine et al., 2004; Krämer et al., 2012; Laimer, Prodinger, & Bauer, 2015).

1.5.2 Pain

Pain is a frequent complaint of EB patients, and one of the major reasons for a reduced quality of life in EB.

The main impact on the quality of life of EB sufferers is pain, which can be life long and is often severe (Jeon, On, & Kim, 2016). The causes of this pain can be acute or chronic. Acute pain typically arises in the context of blister formation, dressing changes, and surgical intervention. Chronic pain can arise from wounds, contractures, mitten deformities, oral and gastrointestinal ulceration, oesophageal strictures and scarring, as well as from squamous cell carcinomas. Interestingly, even in the absence of these issues, many EB patients appear to experience chronic pain for reasons that are unclear.

Consistent with this, 95% of patients with some forms of dystrophic EB are regularly in pain, with 50% rating their regular pain level ≥ 5 on a 1-10 scale (Goldschneider & Lucky, 2010). Despite chronic pain being a major problem for many EB patients, its pathogenesis and underlying molecular mechanisms remain unclear (Goldschneider et al., 2014). One recent study suggested that chronic pain in EB may be neuropathic in origin, but its cause is likely to be multifactorial (Mack et al., 2015; von Bischoffshausen et al., 2017).

In keeping with a multifactorial origin, the nature of pain experienced by EB patients takes many forms and can differ between patients. For instance, DDEB patients are more likely to describe itchy and throbbing pain, while sharp and shooting pain is predominantly associated with RDEB (Figure 1.3) (Schröder et al., 2018).

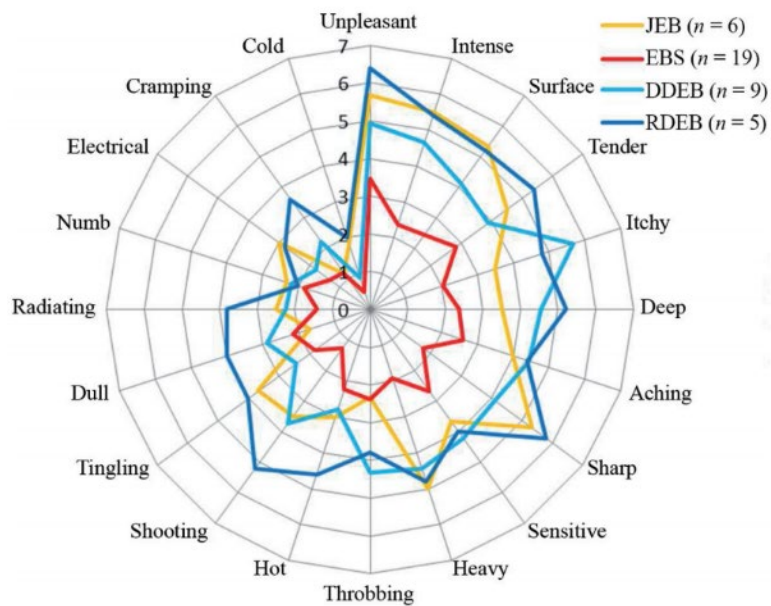


Figure 1.3 Radar diagram of mean pain scores of types of pain reported in patients with Epidermolysis bullosa subtypes

The mean scores (0–10) of pain qualities from the Pain Quality Assessment Scale (PQAS) for four subtypes of EB. Reproduced from (Schröder et al., 2018).

1.5.3 Itch

Itch is another leading cause of reduced quality of life in EB, and is common across EB subtypes. For example with more than 90% of patients with DEB complain of itch (Snauwaert et al., 2014). Itch occurs not only in association with blisters or wounds but also in intact skin. While its pathogenesis remains unclear, a mixed interplay of inflammation secondary to disruption of skin barrier, wound healing cascades, and dysregulated activation of sensory nerve fibres in the skin epidermis has been proposed as being responsible (Papanikolaou et al.). Itch can be aggravated by several factors including wound healing, dryness, infection, stress, heat and humidity (Danial et al., 2015). There is a broad overlap between peripheral mediators and receptors underpinning pain and itch, and mechanisms of neuronal sensitisation in the peripheral and the central nervous system have striking resemblances (Ikoma et al., 2006).

1.5.4 Death

The four major subtypes of EB vary in severity, with death the most severe outcome. Severe JEB and EBS result in fatalities at young ages due to secondary skin infections, and subsequent sepsis. DEB patients often survive to adulthood, but can die of cardiopulmonary and renal complications (Hon et al., 2015). The previously mentioned SCCs and metastases which are more common in RDEB can also lead to death (Fine et al., 2008; Hon et al., 2015; Laimer et al., 2010; Yancey & Hintner, 2010). Even in KS, where blistering tend to reduce with age, squamous cell carcinomas can arise and also cause death (Lai-Cheong & McGrath, 2010).

1.6 Diagnosis

Once EB is suspected immunofluorescence mapping and molecular genetic diagnosis are recommended as soon as possible to allow for subtype classification. These two tests are most likely to result in a diagnosis to assist in therapeutic decisions and prognosis. Following this, a patient's parents might also require testing and genetic counselling. Transmission electron microscopy (TEM) can be used if neither of these previous methods result in a clear answer (Has, Bauer, et al., 2020).

1.6.1 Immunofluorescence Mapping

Assessing the anatomical level of blistering within the skin through immunofluorescence mapping is a useful step in diagnosis. By staining frozen sections for collagen IV (a primary component of the lamina densa see Figure 1.1) the level of blistering can be determined from the location of the staining within the blister and usually provides helpful information about the likely genetic basis. For example, a collagen VII mutation would release collagen IV to the roof of the blister (Has, Liu, et al., 2020).

1.6.2 Genetic testing

For the past few decades, Sanger-based sequencing methods have been used to determine the underlying genetic basis of EB and aid in diagnosis. In recent years, with the advent of next generation sequencing technologies, targeted gene panels via whole exome sequencing have been used for EB and can help accelerate the diagnostic process (Has, Bauer, et al., 2020).

1.6.3 Electron Microscopy

TEM, while more expensive and labour intensive, can also reveal information about the epithelial cell–cell and cell–matrix adhesion structures. Interpreting TEM skin images for the purposes of diagnosing EB requires expert knowledge and training and can be an important adjunct to immunofluorescence mapping and genetic testing (Has, Bauer, et al., 2020).

1.7 Treatments

1.7.1 Supportive care

Currently the mainstay of treatment in EB is supportive care, which takes many forms.

Much care of the EB patients is centred around blister and wound management. This tends to involve regular saline baths and wound dressings. Wound dressing changes can be extensive, depending on the severity of disease. Supportive occupational therapies to prevent wounds are also used in milder cases such as the use of gloves to prevent blistering.

Supportive care also involves management of various complications. Infections are usually treated with antibiotics. Contractures and carcinomas typically require surgical intervention. Nutrition must be carefully managed on a case-by-case basis with nutritional supplements recommended. Though gastrostomy tubes and nasogastric feeding is possible, skin fragility can cause complications (Salera et al., 2020). With pain such a common problem, many EB patients receive analgesics to help relieve their pain. Opioids, tricyclic anti-depressants, anti-convulsants, and nonsteroidal anti-inflammatory agents (NSAIDs) have all been used. Similarly, many patients receive symptomatic support for itch. Most drugs used to treat itch are histamine H1 and H4 blockers that are more effective against acute itch (Ralvenius

et al., 2018). However, given histamine typically has a lesser role in chronic itch, such medications are less effective in the long-term. Surgeries are also used to treat strictures, pseudosyndactyly, and to remove SCCs.

Unfortunately, despite the availability of these various supportive medical treatment measures, many patients remain severely affected, so psychological therapies (e.g. to manage stress and cope with pain) are often a key part of supportive care.

1.7.2 Curative therapies

In recent years, several experimental treatments have been proposed to address the underlying problem in EB and try to achieve a cure, including bone marrow transplantation (Sat et al., 2000), gene therapy, and protein replacement therapy (Hsu et al., 2014; Marinkovich & Tang, 2019). A successful example in recent years was the presented by (Hirsch et al., 2017) where a patient with JEB underwent skin grafting with genetically engineered sheets which over expressed full length LAMB3 transgene, the transplants were still producing protein after 21 months (reviewed in (Marinkovich & Tang, 2019)).

The rationale for the above treatments is to reintroduce normal copies of the defective proteins and thus allow for normal adherent skin structures to be reformed. However, such treatment strategies are unlikely to be effective for the dominant forms of EB, since normal proteins are likely to be compromised by the ongoing presence of the mutant proteins with a dominant negative effect. Another factor in this which affects protein replacement therapies is turnover of the collagen VII with reports suggesting it could be as short as 30 days (Kühl et al., 2016). This would lead to a constant need for new collagen VII to be introduced to the skin meaning such protein replacement therapies would require constant dosing (South & Uitto, 2016). Progress on stem cell therapies is ongoing, though most gene correction focuses on recessive DEB patients (Jacków et al., 2019; Osborn et al., 2013). There has been attempts to correct the mutant allele of DDEB cells wherein the allele was further truncated to prevent mutant collagen VII associating with wild -type or forming triple helical structures (Shinkuma, Guo, & Christiano, 2016). One novel treatment strategy that could help with cases is the use of exon skipping oligos to prevent the translation of glycine substitutions which has been found to occur naturally in some patients (Bremer et al., 2019).

1.8 Animal Models

In order to better understand the pathogenesis of EB – including poorly understood symptoms such as pain and itch – as well as to test and/or compare different therapeutic approaches, animal models for EB can play an important role.

In some cases, these animal models of EB have arisen from spontaneous mutations in non-mouse species. These include dogs, cats, cows and various other domesticated animals with different subtypes of EB (Medeiros & Riet-Correa, 2015), as well as a rat model of DDEB (Nyström et al., 2013).

Given the pre-eminence of the laboratory mouse as a tool to study mammalian biology and as a tractable model for human disease, the vast majority of EB animal models have been developed and characterised in mice. Currently, there are at least 15 mouse models of EB available (Leena Bruckner-Tuderman et al., 2010). These models cover a range of EB subtypes (see Table 1.1) though have some drawbacks. For example, many suffer from early lethality or severe blistering that can pose practical and

ethical challenges for experimentation. In addition to these limitations, another notable gap in the existing mouse models for EB is the lack of any DDEB mice.

Table 1.1 Examples of transgenic mice developed as models for different forms of EB. Reproduced from (L. Bruckner-Tuderman et al., 2010)

Human disease model	Mouse gene defect	Approach	Phenotypic features	References
EBS	<i>Krt5</i>	GKO	Severe phenotype with high postnatal lethality in -/- mice; subtle phenotype in heterozygotes	Peters et al., 2001
EBS-MD	<i>Plec1</i>	GKO	Postnatal lethality	Andrä et al., 1997
EBS-MD	<i>Plec1</i>	CKO	Mosaic deletion of plectin in the skin; localized EBS	Ackerl et al., 2007
EBS-Ogna	<i>Plec1</i>	KI	Viable, fertile, mimics human disease	Wiche et al., unpublished
JEB-Herlitz	<i>Lama3</i>	GKO	Severe blistering, early lethality	Ryan et al., 1999
JEB-Herlitz	<i>Lama3</i>	GKO	Naturally occurring mutation with severe blistering	Kuster et al., 1997
JEB-Herlitz	<i>Lamc2</i>	GKO	Severe blistering with early lethality	Meng et al., 2003
JEB-other	<i>Col17a1</i>	GKO	Skin blistering with survival up to 6 months	Nishie et al., 2007 ; Bubier et al., 2010
JEB-other (PA)	<i>Itgb4</i>	CKO	Skin blistering, loss of hemidesmosomes	Raymond et al., 2005
JEB-other	<i>Itga3</i>	CKO	Skin microblistering, loss of hair	Margadant et al., 2009
RDEB-HS	<i>Col7a1</i>	GKO	Severe blistering of the skin and mucous membranes; death during the first week of life	Heinonen et al., 1999
RDEB-severe generalized	<i>Col7a1</i>	HYPO	Recapitulates features of severe RDEB with a life span up to 150 days	Fritsch et al., 2008
RDEB	<i>Col7a1</i>	CKO	Conditional inactivation either in all cells (β -actin promoter) or in keratinocytes (KRT promoter); localized symptoms of RDEB after treatment with tamoxifen	Bruckner-Tuderman et al., unpublished

Kindler syndrome	<i>Fermt1</i>	GKO	Skin atrophy and intestinal epithelial dysfunction with perinatal lethality	Ussar et al., 2008
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EBS-MD, EB simplex with muscular dystrophy; HS, Hallopeau-Siemens variant; PA, pyloric atresia; CKO, conditional “knock-out”; GKO, germline “knock-out” of the gene by targeted ablation; HYPO, hypomorphic mouse with about 10% expression of the Col7a1 in comparison to wild-type mice; KI, “knock-in” of a dominant gene.

1.9 Hypothesis and Aims

1.9.1 Hypothesis

Introducing common DDEB human point mutations into mouse Col7a1 will create novel mouse models that recapitulate the biology and clinical features of DDEB and can be ultimately used to better understand the pathophysiology of DDEB and to improve its treatment.

1.9.2 Aims

As described in the following chapters, my PhD studies have sought to fill the gap in DDEB mouse models and, having done so, have strived to develop a model to improve understanding and treatment of its associated pain. More specifically, the three aims of my studies were to:

1. Establish the first mouse models of DDEB;
2. Characterise pain related behaviours in DDEB mice; and
3. Test and compare different drugs for their analgesic properties in DDEB mice.

Chapter 2 – Development of novel mouse models for DDEB

RESEARCH ARTICLE

Mouse models for dominant dystrophic epidermolysis bullosa carrying common human point mutations recapitulate the human disease

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ABSTRACT

Heterozygous missense mutations in the human *COL7A1* gene – coding for collagen VII – lead to the rare, dominantly inherited skin disorder dominant dystrophic epidermolysis bullosa (DDEB), which is characterised by skin fragility, blistering, scarring and nail dystrophy. To better understand the pathophysiology of DDEB and develop more effective treatments, suitable mouse models for DDEB are required but to date none have existed. We identified the two most common *COL7A1* mutations in DDEB patients (p.G2034R and p.G2043R) and used CRISPR-Cas9 to introduce the corresponding mutations into mouse *Col7a1* (p.G2028R and p.G2037R). Dominant inheritance of either of these two alleles results in a phenotype that closely resembles that seen in DDEB patients. Specifically, mice carrying these alleles show recurrent blistering that is first observed transiently around the mouth and paws in the early neonatal period and then again around the digits from 5–10 weeks of age. Histologically, the mice show micro-blistering and reduced collagen VII immunostaining. Biochemically, collagen VII from these mice displays reduced thermal stability, which we also observed to be the case for DDEB patients carrying the analogous mutations. Unlike previous rodent models of epidermolysis bullosa, which frequently show early lethality and severe disease, these mouse models, which to our knowledge are the first for DDEB, show no reduction in growth and survival, and – together with a relatively mild phenotype – represent a practically and ethically tractable tool for better understanding and treating epidermolysis bullosa.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Epidermolysis bullosa, Mouse model, Skin, Blistering

INTRODUCTION

Epidermolysis bullosa (EB) is a group of genetic skin fragility conditions caused by disruption of epithelial adhesion proteins in the skin. Mutations in the gene coding for collagen VII (*COL7A1*) lead to a disease type known as dystrophic epidermolysis bullosa (DEB), which can be inherited in either a dominant (DDEB) or recessive (RDEB) manner (Fine et al., 2014; Has et al., 2020). Collagen VII forms anchoring fibrils, critical structural components of the skin's basement membrane that anchor it to the dermis (Sakai et al., 1986). Collagen VII contains a triple-helical domain, which consists largely of a G-X-Y repeating motif (where G is glycine, and X and Y are different amino acids) that is critical for its structural integrity. Autosomal-dominant mutations causing DDEB are manifold, but typically involve glycine substitutions that disrupt this G-X-Y motif and lead to dominant-negative interference during anchoring fibril assembly and reduced triple-helix stability. Owing to this reduced stability, the structural integrity of collagen VII and the basement membrane are compromised (Chung and Uitto, 2010). The epidermis and dermis thus separate more readily. This separation can occur spontaneously or in response to triggers such as mechanical friction, humidity or temperature change. Clinically, this manifests as skin and mucosal blisters and erosions, which heal with atrophic scarring, milia formation, nail dystrophy and alopecia. Over time, repeated epithelial wounding and scarring can lead to joint contractures and mucosal strictures (Bruckner-Tuderman and Has, 2014; Watkins, 2016).

To better understand the pathophysiology of epidermolysis bullosa (EB) and develop more effective treatments, a number of pre-clinical animal models of different EB subtypes have been developed. These include knockouts or conditional knockouts (Nyström et al., 2013a; Heinonen et al., 1999) and hypomorphic mouse models of RDEB (Fritsch et al., 2008), for which clinical presentation is generally more severe than that for DDEB, which arise due to mutations causing complete or almost complete loss of collagen VII. Although a spontaneously arising mutation in *Col7a1* led to the existence of a rat model for DDEB (Nyström et al., 2013b), there is currently no published mouse model of DDEB (Bruckner-Tuderman et al., 2010; Has and Nyström, 2015). This is problematic, because the development of novel therapies for DDEB requires different strategies from that for RDEB. Specifically, although methods such as gene therapy, protein replacement or stem cell transplantation offer hope of providing a source of functional collagen VII for RDEB, they will not remove the defective collagen VII of DDEB that interferes with the assembly of normal anchoring fibrils.

The aim of this study was to develop the first mouse model of DDEB. Traditionally, generating a new mouse model presents many

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challenges, requiring significant investment of time and effort. However, creating genetically modified mice has become easier with the advent of CRISPR-Cas9-mediated genome editing, which allows for targeted mutations – both deletions and point mutations – to be readily introduced into specific genes of interest.

Here, we describe the successful development and characterisation of multiple novel mouse models of DDEB via CRISPR-Cas9. Using a publicly available database, we selected the two most commonly reported *COL7A1* mutations resulting in DDEB – both of which occur in a region that is highly conserved between human and mouse *COL7A1* genes – and introduced these separately into mouse embryos. These mouse models closely recapitulate the dominantly inherited blistering phenotype of patients with DDEB and represent new pre-clinical models for improving our understanding and treatment of DDEB.

RESULTS

Selection of targeted DDEB mutations

The publicly available DEB Register collects DEB patient data globally, including information on clinical features and specific mutations (www.deb-central.org/). Using this registry, we identified that the majority of mutations reported in DDEB occur within exon 73, just after the hinge region of the triple-helical domain and within the G-X-Y repeating motif. In particular, two mutations that cause replacement of the glycine residue at amino acid positions 2034 and 2043 account for the majority of DDEB mutations (Fig. 1A,B). Because this region is highly conserved between the mouse and human genomes, we decided to directly target the homologous glycine (G) residues in the mouse genome (at amino acid positions 2028 and 2037, respectively) and replace them with arginine (R) residues.

Generation of novel *Col7a1* alleles using CRISPR-Cas9

Using CRISPR-Cas9-mediated genome editing, we introduced two separate mutations, p.G2028R and p.G2037R, into the genomes of early-stage mouse embryos and confirmed the success of this procedure by next-generation sequencing of affected pups. In addition to these two new alleles, which we designated *Col7a1*^{em1Kepa} and *Col7a1*^{em2Kepa} (Fig. 1C), three other novel *Col7a1* alleles were inadvertently generated as a by-product of the CRISPR-Cas9 endonuclease and comprised small, in-frame microdeletions of six, 11 and 15 amino acids (p.I2035_G2040del, p.A2027_G2037del and p.G2028_A2042del), which we

designated *Col7a1*^{em3Kepa}, *Col7a1*^{em4Kepa} and *Col7a1*^{em5Kepa}, respectively (Fig. S1).

Each novel *Col7a1* mouse line displays dominantly inherited blistering

Male mice hemizygous for each of these novel *Col7a1* alleles were bred to C57Bl/6J female mice, and genotyping of the resultant offspring at 3 weeks of age showed that mice carrying the affected alleles were recovered alive at the expected 1 in 2 Mendelian ratio (Table S1).

Visual inspection of these mice revealed mild to moderate blistering that was dominantly inherited. At postnatal day (P)1, mice carrying the targeted mutations (Fig. 2A) or the microdeletion alleles (Fig. S2A) displayed blisters on the paws and around the mouth, which disappeared by P7. Blisters then re-appeared around the digits from ~5 to 10 weeks of age, depending on the underlying allele (Fig. 2B,C; Fig. S2B,C), with the p.G2037R point mutation showing much greater penetrance than p.G2028R among the two targeted mutations. By 12 weeks of age, loss of nails also started to be evident (Fig. 2D; Fig. S2D), with the frequency again allele dependent. Taken together, the macroscopic changes observed among these mice are directly reminiscent of those seen in patients with p.G2034R and p.G2043R (corresponding to mouse p.G2028R and p.G2037R, respectively) mutations, who similarly show signs of blistering and nail loss (Fig. 2E). Of note, mice carrying these novel *Col7a1* alleles showed normal weight gain during development, and there were no signs of increased mortality even up to 6 months of age, with 3.0% of adult mice (nine of 300) requiring euthanasia due to ill health (most commonly malocclusion), a rate consistent with the known survival rates of wild-type C57BL/6 animals (Turturro et al., 2002).

Col7a1 mutations lead to microscopic changes in the skin

In addition to these macroscopic changes, light microscopy of Haematoxylin and Eosin (H&E)-stained skin sections taken from the back at 12 weeks of age revealed the presence of microscopic blisters at the dermal-epidermal junction of mice carrying the targeted mutations (Fig. 3A) as well as the microdeletion alleles (Fig. S3A).

To better understand the structural basis for the macroscopic changes observed above, electron microscopy (EM) and immunostaining for collagen VII was performed on skin sections

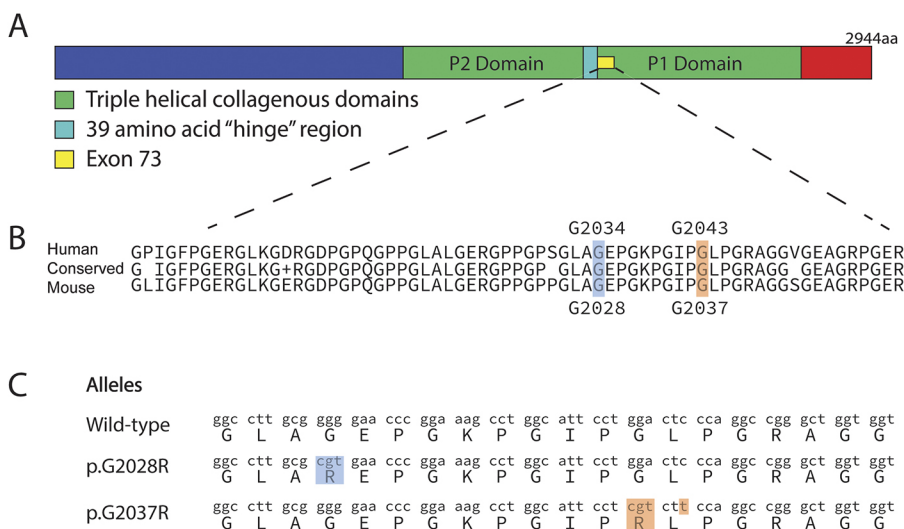


Fig. 1. Targeted *Col7a1* mutations introduced via CRISPR-Cas9 editing. (A) Collagen VII has a triple-helical domain, interrupted by a 39-amino acid 'hinge' region. Exon 73 codes for the region directly upstream of the hinge region. aa, amino acids. (B) Most mutations causing dominant dystrophic epidermolysis bullosa (DDEB) are within exon 73, which is highly conserved between human and mouse. The two most frequently mutated amino acids in human DDEB are G2034 and G2043. These glycines (and the amino acids surrounding them) are highly conserved and correspond to G2028 and G2037, respectively, in mice. (C) Using CRISPR-Cas9, we introduced *Col7a1* mutations (p.G2037R and p.G2028R) in mice (designated *Col7a1*^{em1Kepa} and *Col7a1*^{em2Kepa}, respectively).

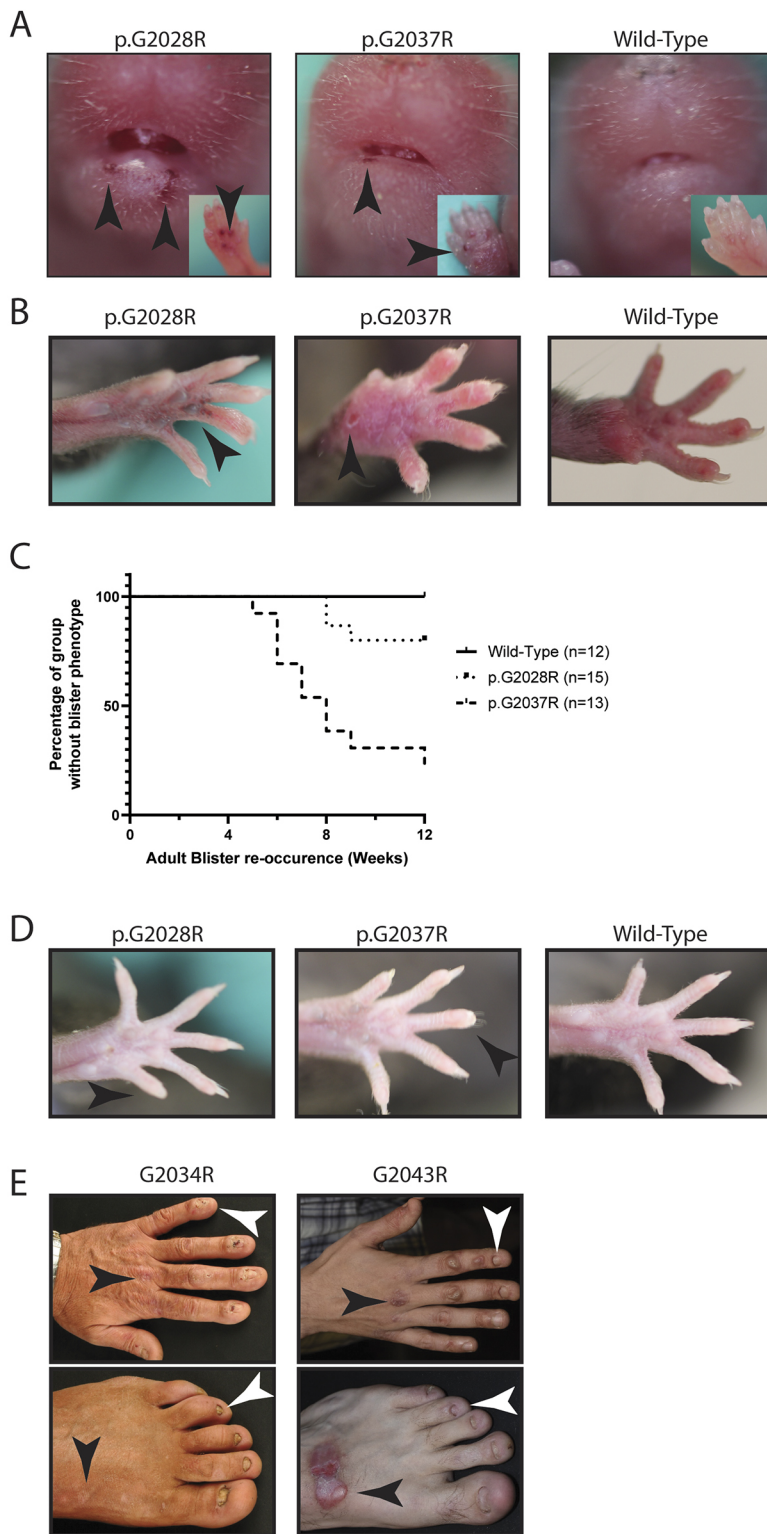


Fig. 2. The two targeted mutations lead to blistering with varying penetrance of re-occurrence. (A) One-day-old mouse pups carrying the targeted alleles show varying amounts of blisters (indicated by black arrowheads) around the mouth and on the paws (insets). (B) These blisters (indicated by black arrowheads) faded by the time the mice are 1 week old and re-occur as the mice age, affecting the paws and generally becoming visible from 6-10 weeks of age. (C) Graph showing adult blister occurrence with different patterns of onset between p.G2028R and p.G2037R alleles, with the earliest occurrence at 5 weeks of age. Mice with the p.G2028R mutation show a later onset of blistering and lower penetrance, whereas mice with the p.G2037R mutation have earlier onset and higher penetrance, with most mice affected by 12 weeks of age. (D) At 12 weeks of age, the mice start to lose nails (indicated by black arrowheads). (E) Patients with the p.G2034R (mouse p.G2028R) and p.G2043R (mouse p.G2037R) mutations display nail dystrophy and loss (indicated by white arrowheads) (similar to that of the mice shown in D) as well as acral blistering and scarring (indicated by black arrowheads) (similar to that of the mice shown in A and B).

from the backs of 12-week-old mice. EM sections taken from mice carrying the two targeted mutations showed putative separation below the epidermal basement membrane (Fig. 3A) and were suggestive of possible disruption of the anchoring fibrils (Fig. 3B). Formal quantification of the latter showed a significantly lower number of anchoring fibrils compared to wild-type tissues (Fig. 3C), with skin from the p.G2037R animals most affected. In contrast, we could not discern consistent ultrastructural differences between wild-type mice

and those carrying the microdeletion alleles (Anton-Lamprecht, 1992; Chmel et al., 2018; Winberg et al., 1997) (Fig. S3). Meanwhile, immunostaining for collagen VII showed a reduction in staining intensity for each of the two targeted alleles compared to wild type (Fig. 4A). Formal quantification of collagen VII staining intensity (Fig. 4B) confirmed this impression, and revealed that skin carrying the G2037R mutation had a more severe reduction in expression. For the microdeletion alleles, immunostaining results also differed from

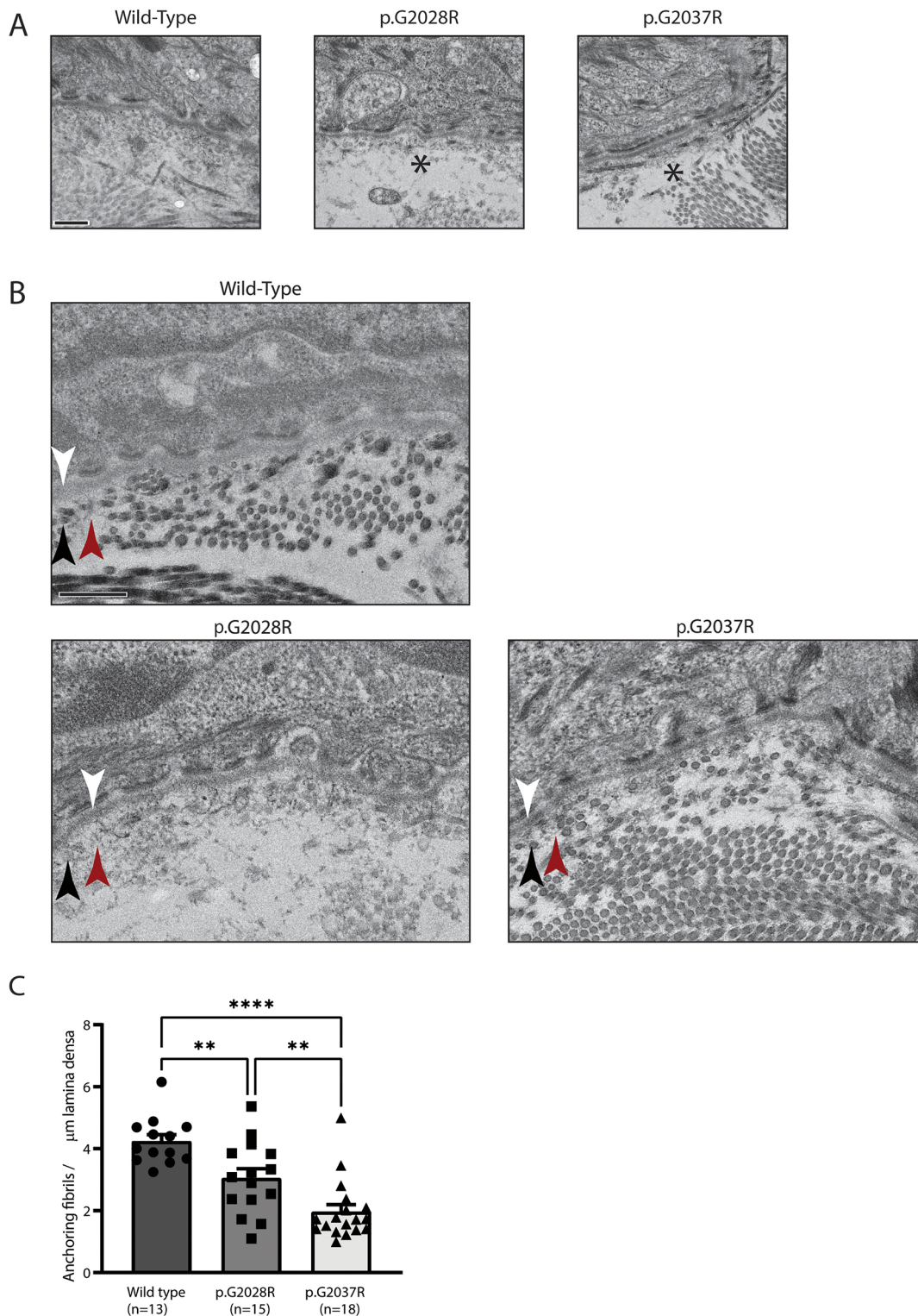


Fig. 3. Electron microscopy reveals putative separation and reduced anchoring fibrils. (A) Electron microscopy of 12-week-old back skin shows putative separation below the lamina densa of the epidermal basement membrane, indicated by asterisks. Data are representative of multiple fields from a single biological replicate. Scale bar: 400 nm. (B) Electron microscopy also shows possible changes to the anchoring fibrils (indicated by red arrowheads) that sit below the lamina densa (indicated by white arrowheads) and among the interstitial collagen fibres (indicated by black arrowheads). Data are representative of multiple fields from a single biological replicate. Scale bar: 400 nm. (C) Quantification of anchoring fibrils per μm lamina densa, showing a reduction in both mutant mice. Number of random fields per genotype is indicated in the figure. Analysis by one-way ANOVA, ** $P < 0.01$, **** $P < 0.0001$.

wild type, but the most consistent difference was an increase in the width of the collagen VII layer (Fig. S4) that was not observed for the targeted alleles (Fig. 4C).

The stability of mutant collagen VII is reduced

Given the changes in collagen VII staining intensity observed in mice carrying the two targeted *Col7a1* alleles, we next sought to

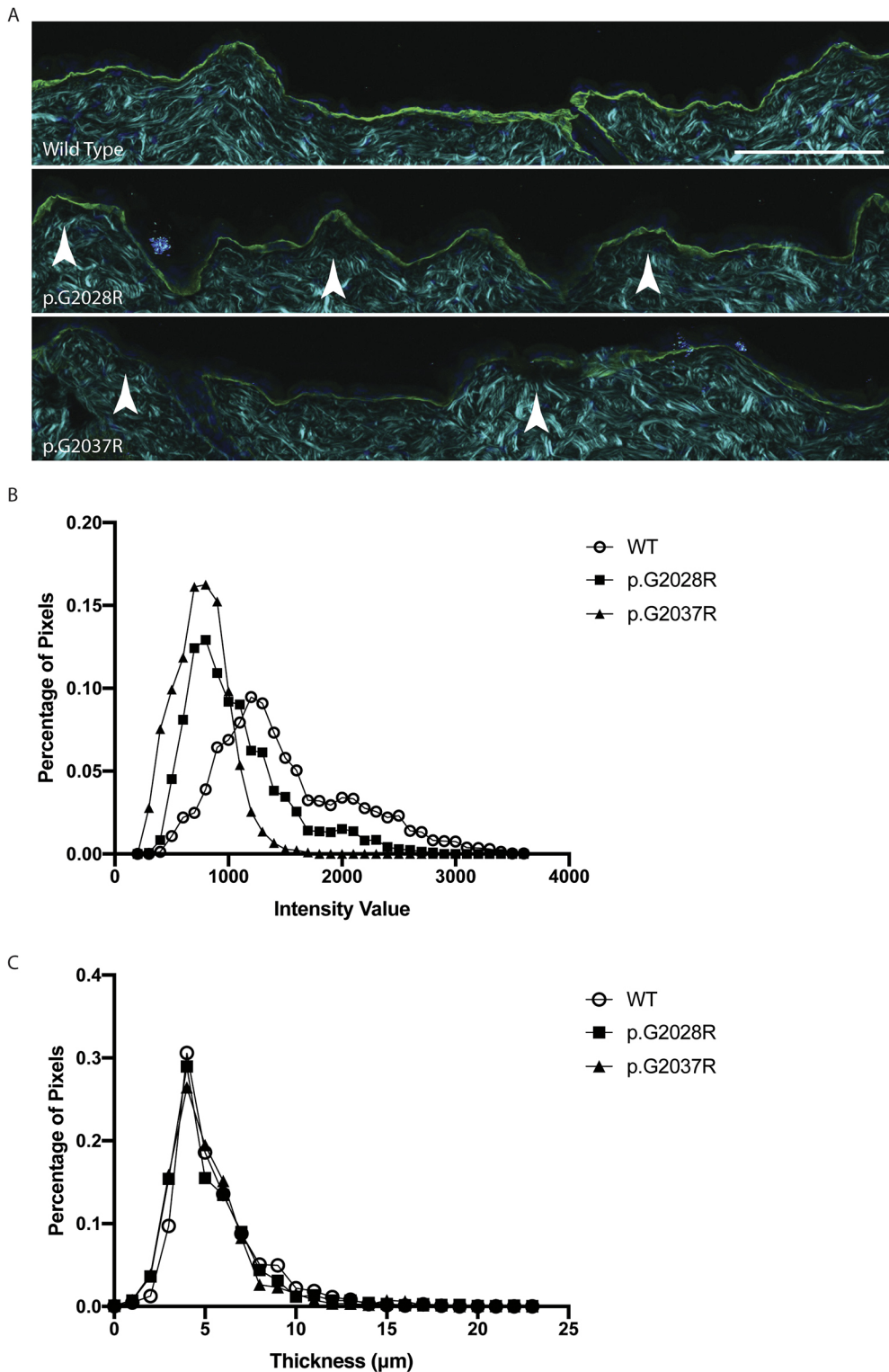


Fig. 4. Immunofluorescence staining shows changes in both collagen VII expression and basement membrane structure. (A) Immunofluorescent staining of collagen VII (green) and DAPI (dark blue) in conjunction with second-harmonic imaging (cyan), which shows the structure of ordered collagens, in sections from 12-week-old mouse back skin. Changes in the intensity and thickness of collagen VII in the mutant sections are apparent when compared with wild-type skin, as is the presence of micro blisters (indicated by white arrowheads) below the basement membrane. Scale bar: 100 μ m. **(B)** Analysis of the stained sections (five images per replicate, from three biological replicates per genotype) shows that the intensity of collagen VII staining varies across the mutants, with p.G2028R, and p.G2037R showing slightly less intensity than wild type (WT). **(C)** Analysis of the thickness of collagen VII staining shows that the p.G2028R and p.G2037R have a similar thickness to wild type.

determine via quantitative RT-PCR (qRT-PCR) whether there were any differences in *Col7a1* mRNA abundance as a result of these mutations. Using back skin as a source of RNA, we found that *Col7a1* mRNA levels were comparable across wild-type and mutant alleles (Fig. 5A; Fig. S5), suggesting that *Col7a1* transcription rates and RNA stability are not impaired by the mutations. In contrast, western blotting of mouse embryonic fibroblast (MEF) protein lysates from the two targeted mutant alleles revealed a reduction in the levels of secreted collagen VII present in the media (Fig. 5B),

consistent with our immunostaining results. However, intracellular collagen VII was significantly increased in cell lysates from MEFs carrying either of the targeted alleles (Fig. 5B). Collectively, these data suggested impaired secretion of collagen VII due to misfolding and intracellular retention, but it was possible that a reduction in protein stability of the secreted mutant collagen VII might also be important.

To explore this possibility, limited trypsin digest assays were performed and showed markedly reduced stability of the collagen

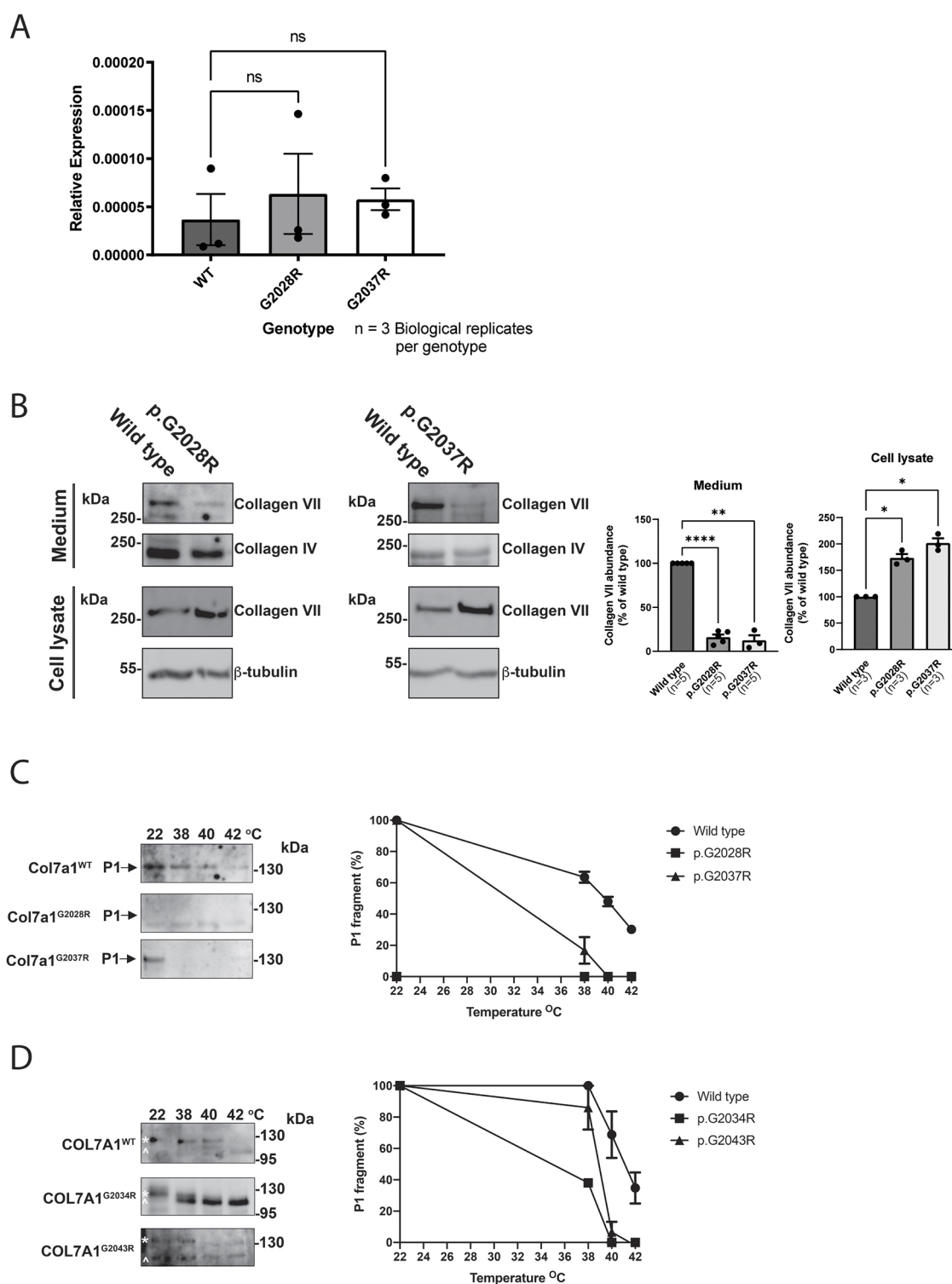


Fig. 5. See next page for legend.

VII triple helix from MEFs carrying the p.G2028R and p.G2037R mutations (Fig. 5C). This was consistent with samples from patients, which also showed a decrease in collagen VII stability for the homologous p.G2034R and p.G2043R mutations (Fig. 5D).

DISCUSSION

Here, we describe the generation of multiple novel DDEB mouse models, two of which not only carry a mutation directly

homologous to the two most common mutations found in DDEB patients but also closely recapitulate the human disease. All these mutations lie within the collagen VII triple-helical region, and, together, our data reaffirm the importance of the triple helix for DDEB pathogenesis.

Among our two targeted models, mice carrying the p.G2037R allele displayed the more severe phenotype and also the greater reduction in collagen VII expression as revealed by skin

Fig. 5. *Col7a1* changes appear to be the result of increased intracellular retention and reduced extracellular stability. (A) qRT-PCR of *Col7a1* mRNA relative to the geometric mean of *Gapdh* and lamin A mRNA shows that there is no significant change at the mRNA level between wild-type and mutant back skin (individual data points represent biological replicates, and mean $\pm$ s.e.m. are shown). Analysis by one way ANOVA; ns, not significant. (B) 3 million wild-type and mutant MEFs were cultured, and the intracellular and extracellular collagen VII protein levels were assessed from the cell lysates and culture media, respectively. Western blot (left panels) show that the amount of secreted collagen VII in the medium is reduced in both mutants, whereas the amount of intracellular collagen VII is increased in the mutants compared to wild type. These experiments were conducted three times, and quantification was performed (right panels) using the intracellular housekeeper protein, β -tubulin and the secreted protein, collagen IV, as normalisation controls for the cell lysates and culture media, respectively (individual data points represent biological replicates and mean $\pm$ s.e.m. are shown). Analysis by one-way ANOVA, * P <0.05, ** P <0.01, **** P <0.0001. (C) Left: limited trypsin digests of the collagen VII protein from mouse mutants show digestion at lower temperatures compared with wild-type controls. Right: quantification of digest data, showing the percentage of P1 fragment remaining at different temperatures. All digest experiments were performed three times, and each time 40 μ l per lane was loaded (from a total of 200 μ l precipitated medium dissolved in TBS, which was in turn derived from precipitation of 10 ml medium). Data are representative of three experiments, and mean $\pm$ s.e.m. are shown. (D) Left: limited trypsin digests of the collagen VII protein from human patient samples also show decreased collagen VII stability for the p.G2034R (mouse p.G2028R) and p.G2043R (mouse p.G2037R) mutations. Asterisks indicate collagen VII collagenous P1 domain; carets indicate intermediate degradation product. Right: quantification of digest data, showing the percentage of P1 fragment remaining at different temperatures. All digest experiments were performed three times, and each time 40 μ l per lane was loaded (from a total of 200 μ l precipitated medium dissolved in TBS, which was in turn derived from precipitation of 10 ml medium). Data are representative of three experiments, and mean $\pm$ s.e.m. are shown.

immunostaining. This suggests that the level of expression of collagen VII in DDEB skin is likely to be an important factor in determining the severity of disease, and is consistent with clinical observations that RDEB patients with more severe disease are those that completely lack functional collagen VII protein. However, we cannot exclude the possibility that the glycine at position 2037 might be more structurally critical than the one at position 2028. Looking ahead, it would be interesting to determine whether the difference in severity between these two alleles is also observed between DDEB patients carrying mutations at their corresponding positions of G2034 and G2043. In practice, however, this might be difficult, because phenotypic differences in such patients are likely to be confounded by differences in genetic background and environmental exposures. Another potential avenue for further study would be to generate mice homozygous for the two targeted alleles, so as to check for a gene-dosage effect.

The models described here represent to our knowledge the first mouse models for DDEB. A rat model of DDEB (Nyström et al., 2013b) – which arose from a spontaneous glycine to aspartic acid substitution at position 1867 (in exon 69) – has previously been described by members of our team. Like our mice, the rat model displays dominantly inherited blistering and nail dystrophy. Although both the rat and mouse models will be useful for studying DDEB, we believe that our two mouse models carry several advantages over the rat model in this regard. First, our mice directly model the two most frequent human mutations seen in DDEB (van den Akker et al., 2011; Wertheim-Tysarowska et al., 2012) and, as such, carry strong construct validity (McGraw et al., 2017). Second, the common genetic aetiology between these mice and DDEB patients may make them a more useful pre-clinical

model for therapeutic development. For example, a clinical trial is currently underway to test the effectiveness of an oligonucleotide that promotes skipping of exon 73 in RDEB patients (<https://clinicaltrials.gov/ct2/show/NCT03605069>), and such an oligonucleotide might also be worth testing in our mice. After all, the mutations in our mice are all in exon 73, which is highly conserved between humans and mice, and recent *in vitro* models suggest that oligonucleotides may also be useful for DDEB (Bornert et al., 2020). Finally, the abundance of genetic tools and experimental methods that are available for mouse-based research – as well as the ability to easily generate sufficient numbers of the animals for well-powered experimental studies – make our DDEB mice well suited to address mechanistic questions related to DDEB pathogenesis. This includes the opportunity to explore the role of inflammation in DDEB, for which we have preliminary evidence in our mice Fig. S6).

Although mouse models for other forms of EB do exist (e.g. junctional EB, EB simplex, RDEB, etc.), such animals typically demonstrate severe disease and early lethality (Bruckner-Tuderman et al., 2010; Uitto et al., 2016), which can present practical obstacles to using them as models to better understand and treat EB. In contrast, our DDEB mice showed no impairment in growth and no observable differences in survival, which – together with their relatively mild phenotype – make them well suited as an experimental model to better study EB.

Looking ahead, we therefore plan to use these mice to further explore the pathogenesis of DDEB and to investigate novel treatments for this disease. Included among our planned studies are investigations of itch and pain, which are common and significant clinical problems in patients with DDEB, although studying these symptoms in mice can be challenging.

MATERIALS AND METHODS

Mice and genotyping

The *Col7a1* mice used in this study were generated on a C57BL/6J background by the MAGEC laboratory at the Walter and Eliza Hall Institute (WEHI) using CRISPR-Cas9-based methods that have been previously described (Kueh et al., 2017). Specifically, to generate mice carrying the p.G2028R allele (*Col7a1*^{em1Kepa}), 20 ng/ μ l Cas9 mRNA, 10 ng/ μ l sgRNA (CTGGCCACCCGGCCTTGCG) and 40 ng/ μ l oligonucleotide donor (CAGGGGCTCCTGGCCTGGCCCTGGGTGAGAGGGGGCCACCTG-GCCACCCGGCCTTGCGCGTGAACCCGGAAGCCTGGCATT-CCTGGACTCCAGGCCGGGCTGGTGGTTCAGGGGAAGCA) were injected into the cytoplasm of fertilised one-cell-stage embryos. The same method was used to generate mice carrying the p.G2037R allele (*Col7a1*^{em2Kepa}), using the sgRNA (CCAGCCCGCCTGGGAGTCC) and oligonucleotide donor (TGAGAGGGGGCCACCT-TGGCCACCCGGCCTTGCGGGGGAACCCGGAAGCCT-GGCATTCTCGTCTTCCAGGCCGGGCTGGTGGTTCAGGGGAAG-CAGGAAGGCCAGGAGAGAGGgtgagcctggg). Twenty-four hours after injection, two-cell-stage embryos were transferred into the oviducts of pseudo-pregnant female mice. Because CRISPR-Cas9-mediated genome editing typically results in not only alleles carrying the intended targeted mutation but also alleles carrying other incidental changes, viable offspring were genotyped by next-generation sequencing to determine the range of resultant alleles. MiSeq next-generation sequencing was performed with a standard protocol with the forward primer (5'-GTGACCTAT-GAACTCAGGAGTCCGAGGCTGCTTTTCTTCG-3') and reverse primer (5'-CTGAGACTTGACATCGCAGCAACTCGTCTCTCTCTCTC-3'). Mice carrying the correctly targeted p.G2028R (*Col7a1*^{em1Kepa}) and p.G2037R (*Col7a1*^{em2Kepa}) alleles as well as three additional missense alleles containing small, in-frame deletions of six amino acids, p.I2035_G2040del (*Col7a1*^{em3Kepa}), 11 amino acids, p.A2027_G2037del (*Col7a1*^{em4Kepa}), and 15 amino acids, p.G2028_A2042del (*Col7a1*^{em5Kepa}), were identified and continuously backcrossed with wild-type C57BL/6 mice to remove any

off-target mutations (for the experiments performed in this study between two and six backcrosses had already been performed), and maintained in the animal facilities at the WEHI according to national and institutional guidelines for animal care. Subsequent genotyping of each line of mice was performed by Transnetyx, using real-time, allele-specific PCR. Both male and female animals were used in experiments.

Characterisation of macroscopic skin changes

To identify macroscopic skin changes, mice carrying each of the novel *Col7a1* alleles and their wild-type littermates were inspected throughout the first week of postnatal life and then weekly thereafter. To document these changes, photographs were routinely taken at P1, P7, and 3, 6, 9 and 12 weeks of age using a Canon EOS 550D with a Canon Macro EFS 35 mm lens.

Transmission EM

For light microscopic assessment of skin, mice were sacrificed at 12 weeks of age via CO₂ inhalation. Back fur was removed with clippers followed by treatment with Nair™ (Church & Dwight, Sydney, Australia) hair removal cream as per the manufacturer's instructions to establish hairless skin, and then the underlying skin was removed and fixed in PBS/4% paraformaldehyde overnight. Skin samples were subsequently embedded in paraffin blocks, sectioned, mounted onto glass slides, stained with H&E, and finally imaged using a 40×/0.75 NA objective lens attached to an Axioplan 2 microscope (Carl Zeiss MicroImaging).

For transmission EM, mice were sacrificed at 12 weeks of age via CO₂ inhalation. Back fur was removed with clippers followed by treatment with Nair™ hair removal cream as per the manufacturer's instructions to establish hairless skin, and then the underlying skin was removed and fixed in 2% glutaraldehyde for >2 days at 4°C, washed twice in 0.1 M cacodylate buffer and incubated for 1 h in 1% osmium tetroxide solution. After dehydration in ethanol (25, 50, 75, 90 and 100%) and propylene oxide, samples were embedded in an epoxide resin. Sections of 70 nm thickness were mounted on microscopy grids, stained with 5% uranyl acetate and Reynold's solution, and examined with an electron microscope JEM1400 (JEOL) equipped with a 2K TVIPS CCD camera TemCam216.

Immunofluorescence

Back skin was removed from mice at 12 weeks of age as described above and embedded in optimal cutting temperature (OCT) compound prior to freezing on dry ice. Frozen sections were cut on a cryostat, transferred to pre-cooled glass slides, warmed to room temperature for 30 min, washed twice in PBS containing 0.1% Tween 20 (v/v) (PBS-T) twice for 2 min, fixed in ice-cold acetone for 5 min, air dried for 30 min, washed twice again in PBS-T, before blocking in 3% BSA/PBS-T for 20 min. Slides were then incubated overnight at 4°C in 3% BSA/PBS-T containing 1:10,000 rabbit antibody LH7.2, which recognises collagen VII (Bornert et al., 2019). On the following day, slides were washed three times in PBS-T for 5 min each, incubated in 3% BSA/PBS-T containing 1:1000 Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Invitrogen) for 1 h at room temperature, washed again in PBS-T three times, and then cover-slipped with ProLong Gold Antifade Solution with DAPI (Invitrogen, Carlsbad, CA, USA).

Images were acquired on a Leica SP8 using a 20× HC PL APO CS2 0.75 NA objective. Excitation was provided by a pulsed 1030 nm, 750 nm or constant-wave 488 nm laser for second harmonic, DAPI and Alexa Fluor 488, respectively. Emission was collected through 400–445 nm detection gates for second harmonic and DAPI and 495–550 nm for Alexa Fluor 488. Five images per biological replicate were analysed, with three biological replicates per genotype.

Image analysis

Quantification of anchoring fibrils in randomly selected areas and of western blots was performed using ImageJ software (National Institutes of Health). Image analysis of collagen VII staining intensity was performed in the Fiji distribution of ImageJ (Schindelin et al., 2012) with a custom-written macro (available upon request). The macro, in brief, used a user-defined region of interest of the membrane that was then used to measure the width and intensity of the staining every 1.5 µm along the length traced.

Cell lines

Primary MEFs carrying wild-type and novel *Col7a1* alleles were created as previously described (Durkin et al., 2013), frozen in liquid nitrogen after the first passage, then subsequently expanded as needed for qRT-PCR, western blotting and trypsin digest assays.

qRT-PCR

Back skin (30 mg) was removed and snap frozen in liquid N₂, then crushed with a hammer on a metal plate, while cooled on dry ice between overhead projector transparencies. Skin RNA was then isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and cDNA was synthesised with Superscript II reverse transcriptase (Invitrogen) and oligo(dT)15 primers (Promega, Madison, WI, USA). *Col7a1* qPCR for an amplicon spanning exons 1 and 2 was then performed using SYBRGreen Master Mix (Bioneer, Daejeon, South Korea) on a LightCycler®480 II (Roche, Basel, Switzerland) (Fwd primer, 5'-CTGCAGAGATCCTGATGGGA-3'; Rev primer, 5'-GATCATCAC-TGTACTGCACT-3'). The geometric mean of lamin A and *Gapdh* expression was used for normalisation purposes (lamin A Fwd primer, 5'-TTCGAGTGACTGTGACACTGG-3'; lamin A Rev primer, 5'-AACCCGCTGAGTACAACCT-3'; *Gapdh* Fwd primer, 5'-CAAC-TTTGTCAAGCTCATTTCCTG-3'; *Gapdh* Rev primer, 5'-CCTCTCTTG-CTCAGTGTCTCT-3').

Western blotting and limited trypsin digestion assay

MEF cell lysates were extracted with NP-40 lysis buffer. Lysates were boiled in sample buffer containing 8 M urea, separated on 7% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes. The membranes were blocked in 5% milk in TBS-T, incubated with primary antibodies [either rabbit polyclonal murine anti-collagen VII (NC1 reactive) (Bornert et al., 2019), anti-collagen VII P1 domain (NC2-10) (Bornert et al., 2016), rabbit polyclonal anti-collagen IV (Thermo Fisher Scientific; PA5-104508) or β-tubulin (Abcam; ab6046); all diluted 1:2000] in the same blocking buffer, washed and probed with horseradish peroxidase (HRP)-conjugated secondary antibodies. The blots were developed with ECL (Fermentas Thermo Fisher Scientific) and detected using a chemiluminescence detection system (Pierce, Erlangen, Amersham Imager 600, GE Healthcare, Freiburg, Germany).

For the limited trypsin digestion assay to assess collagen VII stability, MEFs were grown to confluence in Dulbecco's modified Eagle medium (DMEM): F12+10% foetal calf serum, after which cells were washed in PBS and serum-free DMEM:F12 with 50 µg/ml ascorbic acid added. The cells were incubated for 2 days with ascorbic acid added fresh every day. After 2 days, the media were collected and precipitated with 20% saturated ammonium sulphate for 1 h. After centrifugation, pellets were dissolved in TBS, the cell layer and extracellular matrix were extracted in NP-40 buffer, and proteins were heated for 1 min to the indicated temperatures and allowed to cool down to room temperature for 10 s. Trypsin (Serva, Heidelberg, Germany) to a total volume of 0.1% v/v was added to the protein lysates for 30 s before the reaction was stopped. The samples were then analysed by western blotting. The collagen VII P1 collagenous domain was detected using the rabbit polyclonal anti-collagen VII antibody NC2-10 (Bornert et al., 2016).

Ethics

All mouse-related procedures were conducted in accordance with National Health and Medical Research Council guidelines and approved by the animal ethics committee at the WEHI (#2015.19 and #2019.11). Patient photos and keratinocytes isolated from biopsies were collected in the Department of Dermatology, Medical Center, University of Freiburg, after informed consent was provided as previously described (Kern et al., 2006), according to the Declaration of Helsinki and in agreement with institutional ethics regulations (166/03).

Statistics

For comparison of anchoring fibril numbers across genotypes, one-way ANOVA with Bonferroni's multiple comparisons test was used. For comparison of *Col7a1* mRNA and protein levels between wild-type and

mutant alleles, ANOVA with Dunnett's multiple comparisons test was performed. Without a pre-specified effect size, sample numbers were based on animal availability.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: B.R.C.S., J.S.K., K.C.P.; Methodology: B.R.C.S., J.S.K., K.C.P.; Software: C.J.N.; Validation: B.R.C.S., A.N.; Formal analysis: B.R.C.S., A.N., C.J.N.; Investigation: B.R.C.S., A.N., I.H., C.G., C.H., J.S.K.; Resources: A.N., I.H., C.H.; Writing - original draft: B.R.C.S.; Writing - review & editing: B.R.C.S., A.N., S.J.R., G.A.V., J.S.K., K.C.P.; Visualization: B.R.C.S.; Supervision: G.A.V., J.S.K., K.C.P.; Project administration: B.R.C.S., J.S.K., K.C.P.; Funding acquisition: J.S.K., K.C.P.

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Alleles	JAX Mouse designation
Wild-type	ggc ctt gcg ggg gaa ccc gga aag cct ggc att cct gga ctc cca ggc cgg gct ggt ggt G L A G E P G K P G I P G L P G R A G G
p.G2028R	ggc ctt gcg ggt gaa ccc gga aag cct ggc att cct gga ctc cca ggc cgg gct ggt ggt G L A R E P G K P G I P G L P G R A G G Col7a1 ^{em1Kepa}
p.G2037R	ggc ctt gcg ggg gaa ccc gga aag cct ggc att cct cgt ctt cca ggc cgg gct ggt ggt G L A G E P G K P G I P R L P G R A G G Col7a1 ^{em2Kepa}
6aa (p.I2035_G2040del)	ggc ctt gcg ggg gaa ccc gga aag cct ggc G L A G E P G K P G cgg gct ggt ggt R A G G Col7a1 ^{em3Kepa}
11aa (p.A2027_G2037del)	ggc ctt G L ctc cca ggc cgg gct ggt ggt L P G R A G G Col7a1 ^{em4Kepa}
15aa (p.G2028_A2042del)	ggc ctt gc G L A t ggt ggt G G Col7a1 ^{em5Kepa}

Fig. S1. Col7a1 mutations introduced via CRISPR-Cas9 editing.

In addition to the two targeted mutants, three alleles with deletions were generated, as off target effects of the CRISPR-Cas9 method. Each of these comprise small, in-frame deletions of six, eleven and fifteen amino acids (p.I2035_G2040del, p.A2027_G2037del and p.G2028_A2042del) and were designated *Col7a1*^{em3Kepa}, *Col7a1*^{em4Kepa} and *Col7a1*^{em5Kepa} respectively.

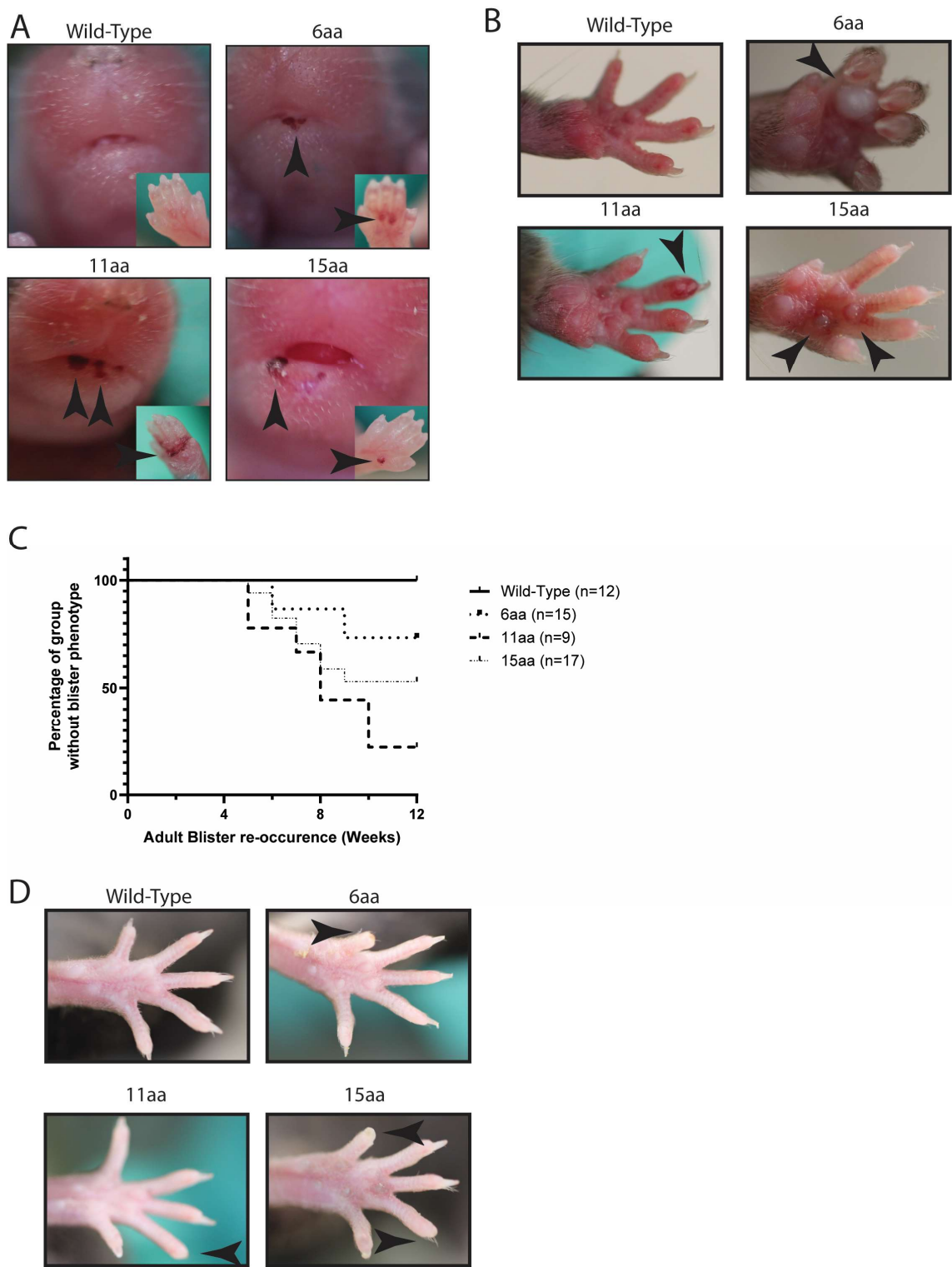


Fig. S2. In-frame Col7a1 microdeletions lead to blistering with varying penetrance of re-occurrence.

One day old mouse pups carrying the in-frame deletion alleles show varying amounts of blisters (indicated with arrows) around the mouth and on the paws (inset) (**A**). These blisters fade by the time the mice are 1 week old, and re-occur as the mice age, affecting the paws and generally becoming visible from 6-10 weeks of age (**B**). Graph showing adult blister occurrence with different patterns of onset between the in-frame deletion alleles, with the earliest occurrence at 5 weeks of age. Mice with the 6aa deletion (p.I2035_G2040del) generally show lower penetrance, while those with the 11aa deletion (p.A2027_G2037del) show greater penetrance, with most mice affected by 12 weeks of age. Those with the 15aa deletion (p.G2028_A2042del) have an intermediate phenotype (**C**). At 12 weeks of age the mice start to lose nails (indicated by arrows) (**D**).

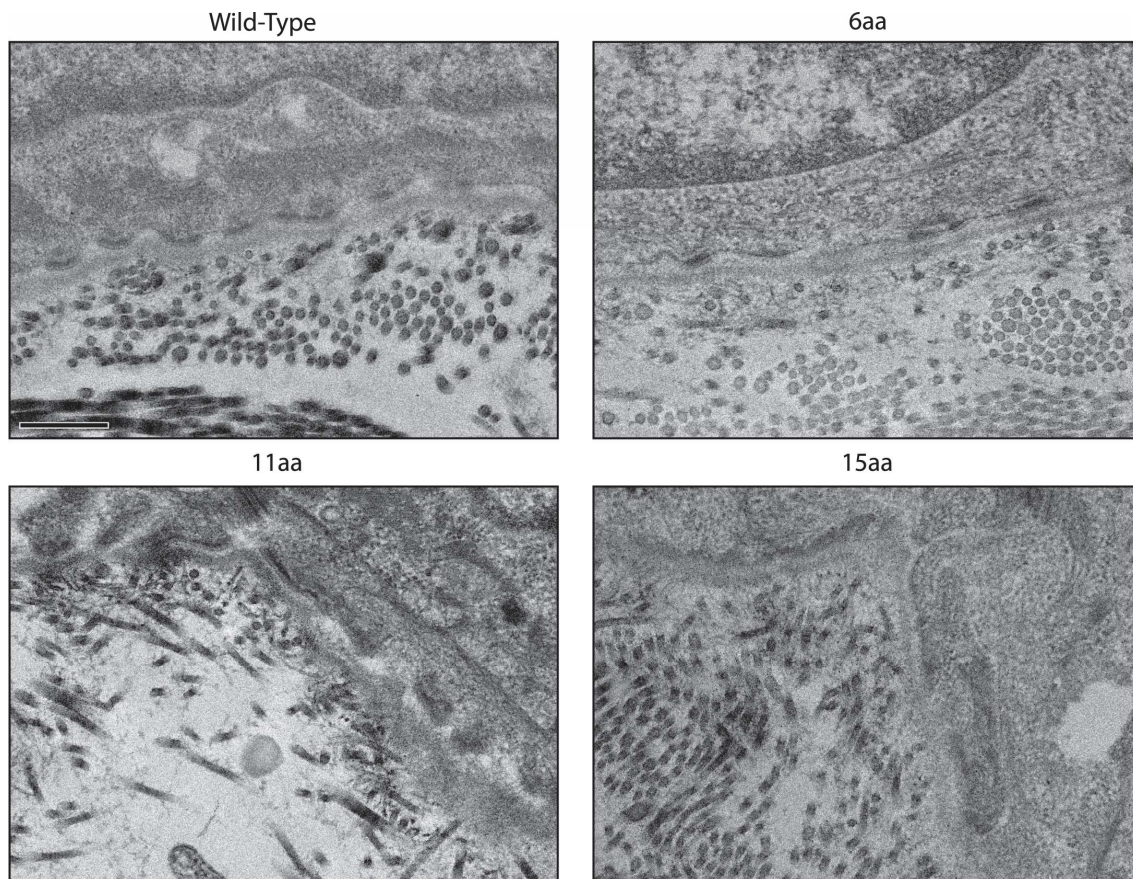


Fig. S3. Electron microscopy of skin from mice carrying in-frame Col7a1 microdeletions.

Electron microscopy of 12-week-old back skin shows possible minor disruption of anchoring fibrils within the basement layer (scale bar = 400nm)

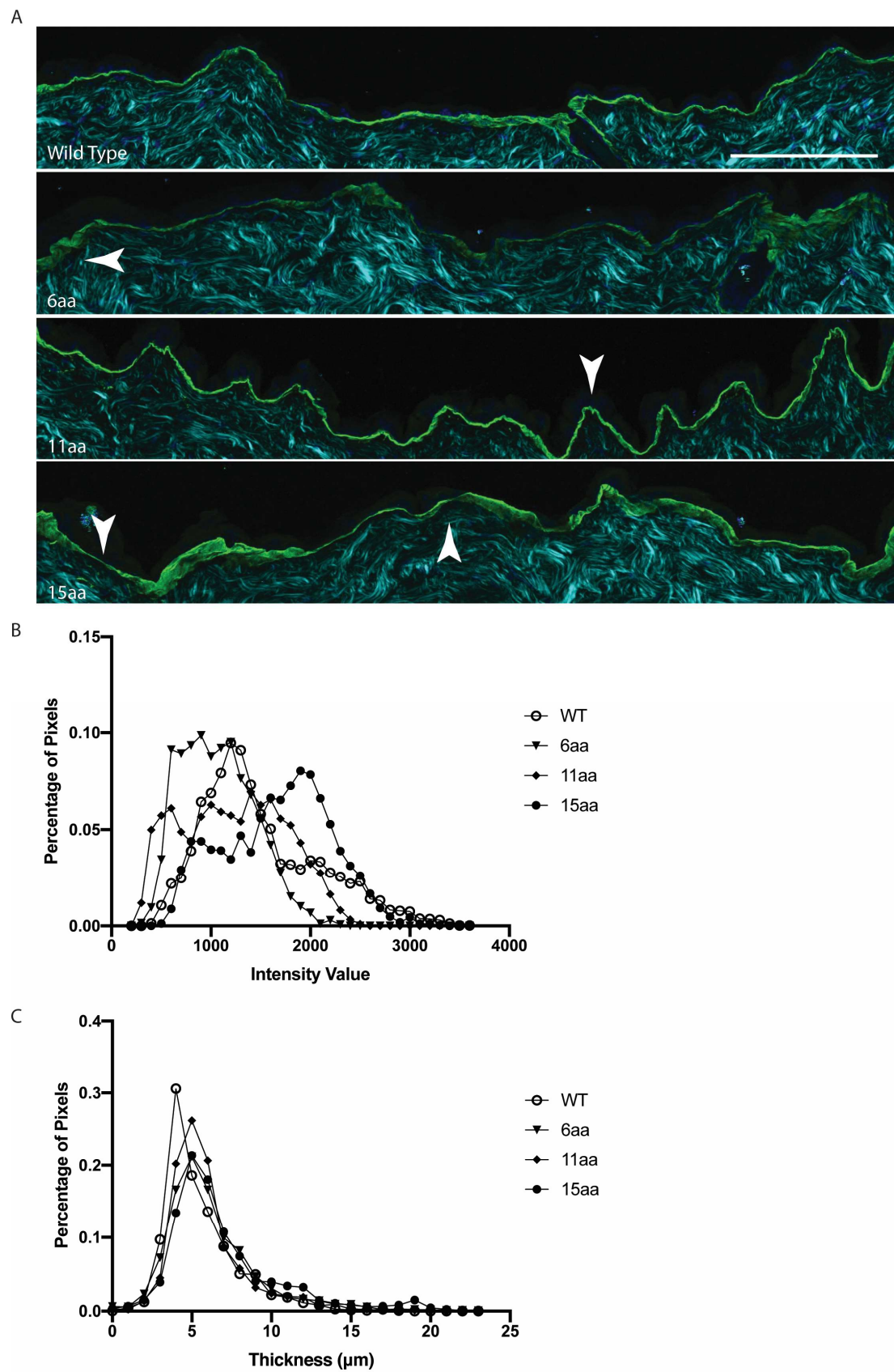


Fig. S4. Immunofluorescence staining of collagen VII in mice carrying in-frame Col7a1 microdeletions.

Immunofluorescent staining of collagen VII (green) and DAPI (dark blue) in conjunction with second harmonic imaging (cyan), which shows the structure of ordered collagens, in sections from 12-week-old mouse back skin. Changes in the intensity and thickness of collagen VII in the mutant sections are apparent when compared with wild-type skin, as is the presence of micro blisters (white arrows) below the basement membrane (scale bar: 100 μ m) (**A**). Analysis of the stained sections (5 images per replicate, from 3 biological replicates per genotype) shows that the intensity of collagen VII staining varies across the mutants. Mice with the 6aa deletion showing slightly less intensity than wild-type, while those with the 11aa and 15aa deletions have an intensity that is close to or above wild-type levels (**B**). Analysis of collagen VII thickness shows that mice with the 6aa, 11aa, and 15aa deletions have slightly thicker layers of collagen VII. (**C**).

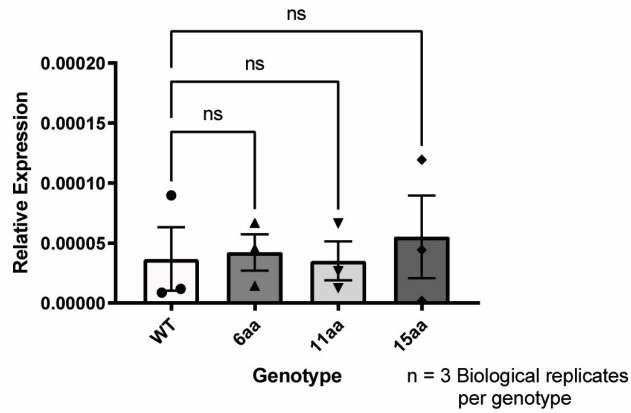
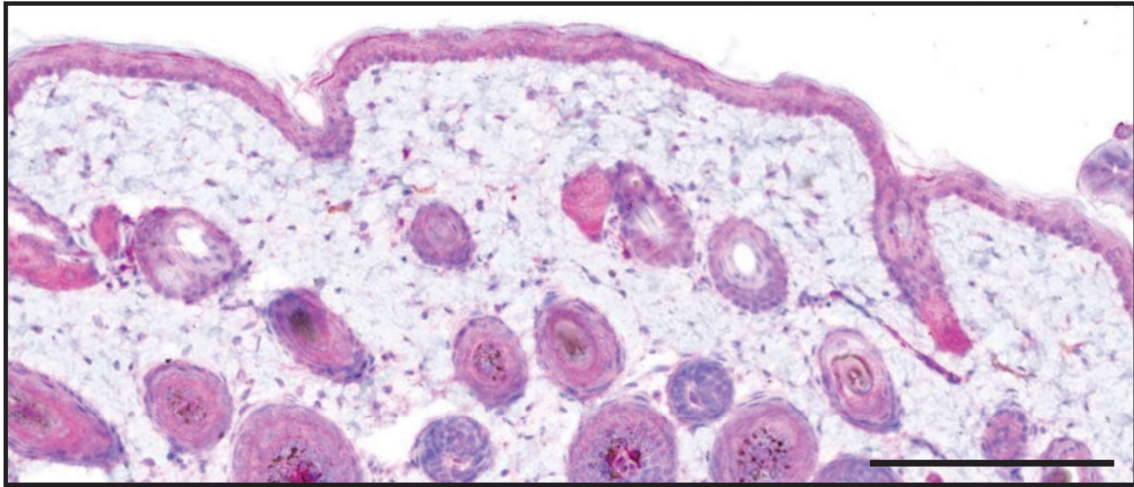


Fig. S5. qRT-PCR of *Col7a1* mRNA from skin of mice carrying in-frame *Col7a1* microdeletions.re S5.

qRT-PCR of *Col7a1* mRNA relative to the geometric mean of Gapdh and LaminA mRNA shows that there is no significant change at the mRNA level in the back skin of wild-type mice and those carrying microdeletions (individual data points represent biological replicates, and mean ± SEM are shown).

Wild-Type



p.G2037R

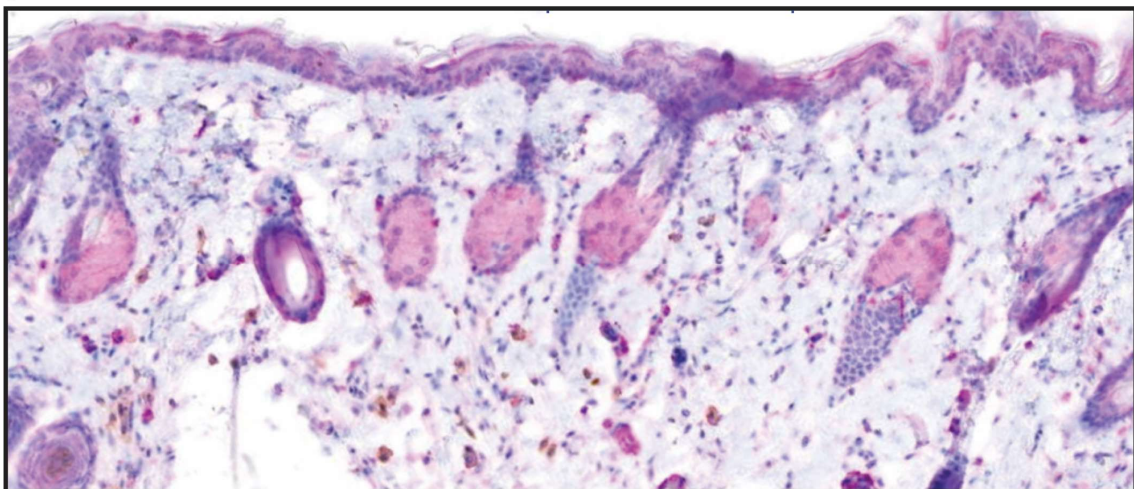


Fig. S6. Immunohistochemical staining of skin from mice carrying in-frame Col7a1 microdeletions shows an apparent increase in CD45+ and S100+ cells.

Immunohistochemical staining of formalin-fixed, paraffin-embedded hind footpad skin from wild-type and pG2037R mice showing expression of CD45 (pan-immune cell marker; shown in brown) and S100 (inflammatory marker; shown in red) with H&E counterstaining. The dermis of pG2037R animals shows an apparent increase in CD45+ and S100+ cells compared to wild-type. Scale bar = 200nm.

Table S1. Recovery of mice carrying Col7a1 mutations at weaning

Mutant allele*	Recovered wild-type mice at weaning	Recovered mutant mice at weaning	Chi-square p-value
p.G2028R	118	125	0.7
p.G2037R	217	220	0.9
6 aa deletion (p.I2035_G2040del)	140	116	0.3
11 aa deletion (p.A2027_G2037del)	163	140	0.3
15 aa deletion (p.G2028_A2042del)	113	103	0.6

* To assess for possible in utero or neonatal lethality, we determined the numbers and genotypes of mice that survived to weaning for each of our mutant strains. Consistent with our strategy of breeding a hemizygous animal with a wild type one, mice carrying the mutant alleles were all recovered at the expected ratio of around 1:1

Chapter 3 - Characterisation and treatment of pain in DDEB mouse models

3 Chapter 3 Characterisation and treatment of pain in DDEB mouse models

3.1 Introduction

As mentioned in the Introduction chapter, pain is one of the main reasons for reduced quality of life in EB patients, and is often life long and frequently severe (Jeon et al., 2016). The causes of pain in EB can be both acute and chronic. Acute pain typically arises in the context of blister formation, dressing changes, and surgical intervention. Chronic pain can arise from wounds, contractures, mitten deformities of the hands and feet, oral and gastrointestinal ulceration, oesophageal strictures and scarring, as well as from squamous cell carcinomas. Interestingly, even in the absence of these issues, many EB patients appear to experience chronic pain for reasons that are unclear.

Consistent with this, 95% of patients with some forms of DEB are regularly in pain, with 50% rating their regular pain level ≥ 5 on a 1-10 scale (Goldschneider and Lucky, 2010). Despite chronic pain being a major problem for many EB patients, its pathogenesis and underlying molecular mechanisms remain unclear (Goldschneider et al., 2014). One recent study suggested that chronic pain in EB may be neuropathic in origin, but its cause is likely to be multifactorial (Mack et al., 2015; von Bischoffshausen et al., 2017).

EB-related pain is currently managed through various treatment options. Acute severe pain (e.g., following interventions such as dressing changes) can require intravenous analgesia with non-steroidal anti-inflammatory drugs (NSAIDs) and/or opioids. Chronic pain is often managed with the same classes of agents (including, for example, methadone) given orally in the long-term. Where pain is thought to be neuropathic in origin, gabapentin and tricyclic antidepressants are often used. Apart from lacking efficacy in many cases, these medications carry a high risk of adverse effects especially when used at high doses. For example, opioids can induce dependence, sedation, impaired functioning, constipation and itch (Goldschneider et al., 2014; Mack et al., 2015). Not surprisingly, in the face of ineffectual and problematic analgesia, psychological interventions and the development of coping strategies become a critical adjunct to pain management for many EB patients.

Given the clinical heterogeneity of EB and the *ad hoc* nature of analgesic prescribing, it remains unclear what the most effective analgesics are in the treatment of EB-related pain. Having a better understanding of which analgesics are best suited to treating pain in EB would therefore be an important advance that would enable more rational use of analgesics in EB patients in the future. In this regard, the creation of a preclinical rodent model to objectively test and compare different analgesic options should be valuable.

At the same time, given the pathophysiology of EB-related pain remains poorly understood, having mouse models of EB also provide an opportunity to explore the underlying mechanisms by which chronic pain arises in EB. To date, however, there have been no reported studies investigating pain in EB animal models.

As described in the preceding chapter, we recently developed and characterised several novel mouse models for dominant DEB. As with other forms of EB, one of the main causes of morbidity in DEB is pain. A study of RDEB patients has suggested that chronic cutaneous injury can lead neuropathic pain (von Bischhoffshausen et al., 2017). Further to this DDEB patients also report pain though with a different profile (Schröder et al., 2018).

Given the current gaps in knowledge concerning the pathophysiology and treatment of EB-related pain, the aims of this chapter were therefore to use our novel DDEB mouse models to:

1. determine whether our DDEB mice can be used as a model to investigate EB-related pain by assessing whether they demonstrate increased pain-related behaviours;
2. objectively compare the effectiveness of existing analgesics commonly used to treat EB-associated pain, and
3. identify alternative options for treating EB-associated pain by assessing the analgesic effectiveness of various novel drugs.

3.2 Methods

3.2.1 Mice

C57/Bl6 mice containing either a single p.G2037R mutation or an 11 amino acid deletion (p.A2027_G2037del), both of which give rise to DDEB as described in the previous chapter, were used for the specified experiments, with wild-type C57/Bl6 littermates used as controls.

3.2.2 Habituation

Mice undergoing behavioural pain testing (see below) were first habituated on a daily basis to the behavioural test environment for up to 4 hrs/day beginning 3 days prior to study. This involved placing mice in the test environment for progressively longer periods over the course of the three days.

3.2.3 Hargreaves test

The Hargreaves test assesses thermal allodynia in rodents. Mice were placed onto a glass plate in secure cages and an infrared light source was focused onto one paw. When the mouse perceived the heat it withdrew its paw and the latency of withdrawal was measured. The temperature of the light applied to the plantar surface of the mouse foot was limited to $\leq 45^{\circ}\text{C}$. The Hargreaves apparatus used to perform these experiments has an automatic cut off at 20s which prevents overheating and prolonged exposure of the paw to heat. For Hargreaves testing, a Ugo Basile (Italy) instrument (catalog number: 37370) was used to measure paw withdrawal thresholds (in milliseconds) for both wild-type and DDEB mice.

3.2.4 Manual von Frey

The von Frey test assesses for mechanical allodynia in rodents. Mice were placed in a cage with a wire mesh floor. A series of nylon filaments, also known as a von Frey hairs and manufactured to be varying diameters, were then used to assess for paw withdrawal. More specifically, individual filaments were

advanced – starting with the one with the smallest diameter - so that it pressed upon the plantar surface of each animal’s paw. Advancement of the filament continued until either i) the mouse perceived it to be painful and then withdrew its paw or ii) the hair was pressed against the skin with enough force so that the hair buckled and formed a S-shape, in which case the next largest filament was tested in an identical manner. Once a test resulted in paw withdrawal, the previous filament was tested again: assuming this resulted in no withdrawal response, the level of withdrawal was thus determined; if however a withdrawal response was noted, the filament which preceded that one would be tested again and, in the case of no response, the force would be increased again with this result being the recorded response. Given that the force required for each filament to bend is different and depends on the size of the filament, these hairs can be used to apply an accurate force to each paw. The maximum force applied to each paw was 2 g (filament size of 4.31).

3.2.5 Dynamic Plantar Aesthesiometer (DPA)

The DPA is an automated version of the von Frey test, and uses a Ugo Basile (Italy) DPA test apparatus (catalog number: 37450). This apparatus became available to me during the course of my PhD, after which it was used in preference to the manual von Frey test to provide greater reproducibility and throughput. As with the manual von Frey test, mice were placed in a cage with a wire mesh floor, but in this case a single filament was advanced at a constantly increasing force per second (0.5g/s) as it pressed on the plantar surface of each animal’s paw. Advancement continued until the mouse withdrew its paw. This automatically stopped an internal timer within the apparatus, and this time was translated into a specific force.

3.2.6 Drugs and administration

The following drugs were assessed for their analgesic properties:

Table 4.1 Drugs tested for their analgesic properties in EB mice

Drug	Dose	Route	Vehicle
Amitriptyline	up to 45mg/kg	Intraperitoneal	Saline
Bupicavaine	up to 8mg/kg	Subcutaneous	Saline
Buprenorphine	0.05-0.1mg/kg	Subcutaneous	Saline
Losartan	100mg/kg	Intraperitoneal	PBS
Gabapentin	Up to 300 mg/kg	Intraperitoneal	Saline
HOE-140 (Icatibant)	1µmole/kg	Intraperitoneal	PBS
Meloxicam	1-2mg/kg	Subcutaneous	Saline
Paracetamol	up to 100mg/kg	Intraperitoneal	Saline

3.2.7 Statistical analyses

Statistical analysis was conducted using relevant tests (multiple t-tests, 2-way ANOVA, Tukey's multiple comparisons test) via GraphPad Prism (version 8.3.1 for macOS, GraphPad Software, San Diego, California USA).

3.3 Results

3.3.1 DDEB mice with an 11aa deletion have increased thermal and mechanical sensitivities compared to wild-type

Using both the Hargreaves apparatus (Figure 3.1 A) and the manual von Frey filaments (Figure 3.1 B), 8 week old male and female mice carrying the COL7A1 11aa (p.A2027_G2037del) mutation were tested for paw withdrawal thresholds. At the time of testing, there was no obvious blistering in any of these mice.

In the Hargreaves test, wild type mice showed no sex differences (mean $\pm$ SEM for males: 14.02 $\pm$ 0.16s; mean $\pm$ SEM for females: 13.55 $\pm$ 0.32s) and had significantly higher withdrawal times compared to the 11aa (p.A2027_G2037del) EB mice, which did show a mild sex difference (males: 6.38 $\pm$ 0.26s; females: 5.02 $\pm$ 0.21s).

Similar results were obtained in the von Frey assay (Figure 3.1 B). Specifically, wild type mice showed much higher withdrawal thresholds (males: 0.98 $\pm$ 0.03g; females: 1.02 $\pm$ 0.02g) compared to the 11aa (p.A2027_G2037del) EB mice, which again showed mild sex differences (males: 0.47 $\pm$ 0.00g; females: 0.32 $\pm$ 0.05g).

Together, these results therefore indicated that DDEB mice carrying the 11aa (p.A2027_G2037del) mutation have thermal and mechanical allodynia.

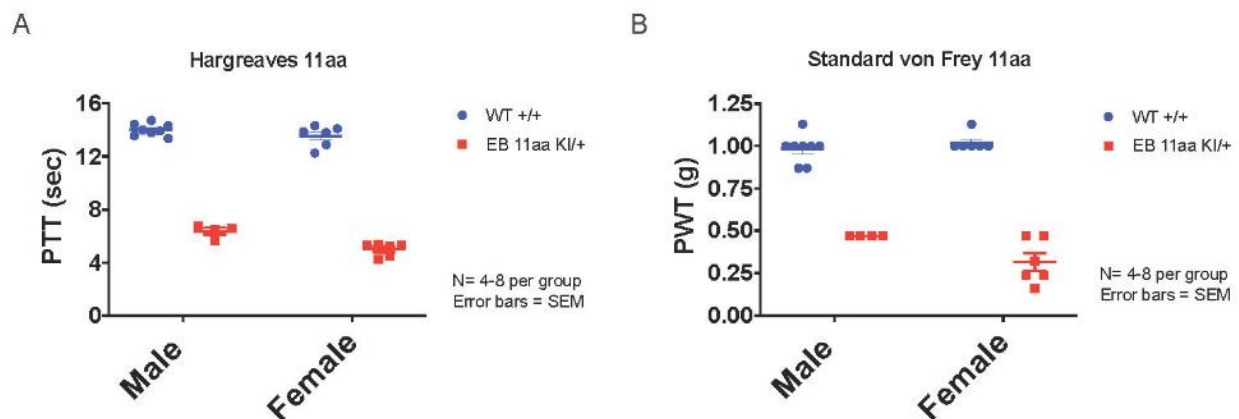


Figure 3.1 EB 11aa (p.A2027_G2037del) KI/+ show marked thermal and mechanical allodynia.

Male and female wild type mice as well as mice carrying the 11aa (p.A2027_G2037del) mutation were tested for paw withdrawal thresholds using a Hargreaves test prior to the onset of any obvious blistering at 8 weeks of age, 2-way ANOVA showed no difference by sex for wild-type, while genotype being highly

significant, minor sex difference was seen in the EB mice. (A). Male and female mice were also tested for paw withdrawal thresholds using a manual von Frey assay, again prior to the onset of any obvious blistering at 8 weeks of age 2-way ANOVA showed no difference by sex for either genotype, with genotype being highly significant (B).

3.3.2 Use of clinically-relevant analgesics results in partial improvement in mechanical hypersensitivity in DDEB mice with an 11aa deletion

Given that it is difficult to know if mice experience pain in the same way as humans, it can be challenging to determine if behavioural experiments that assess pain sensitivity in mice provide useful insights into pain experienced by people. One way to determine whether mouse models are likely to be useful in assessing different behaviours is to examine their predictive validity, which is the ability of a model to accurately predict whether clinically relevant treatments provide a similar benefit to those observed in patients.

As an initial proof-of-concept, we therefore tested three drugs – each of which represent analgesics that are used in EB patients and show varying degrees of clinical effectiveness – in mice carrying the COL7A1 11aa (p.A2027_G2037del) mutation to determine whether they can ameliorate the mechanical allodynia observed in these animals.

Buprenorphine, an opioid analgesic, was tested at a single standard dose of 0.1mg/kg delivered via subcutaneous injection (Figure 3.2 A). While wild type mice showed no change in their paw withdrawal response from baseline (1.02 ± 0.06 g) to 1 hour post injection (1.04 ± 0.13 g), the 11aa deletion DDEB mice showed an increase in their paw withdrawal threshold from baseline (0.24 ± 0.08 g) to 1 hour post injection (0.66 ± 0.19 g), although this improvement was insufficient to reach wildtype thresholds.

Meloxicam, a non-steroidal anti-inflammatory drug, was tested at a single standard dose of 2mg/kg delivered via subcutaneous injection (Figure 3.2 B). At 1 hour post injection, there was no change in the paw withdrawal response in wildtype mice compared to baseline (1.02 ± 0.06 g vs 1.00 ± 0.00 g). In comparison, 11aa (p.A2027_G2037del) EB mouse showed a significant increase in their paw withdrawal threshold from baseline (0.22 ± 0.085 g) to 1 hour post injection (0.70 ± 0.22 g), although this improvement was again insufficient to reach wildtype thresholds.

Finally, bupivacaine, a local anaesthetic that inhibit nerve transmission by blocking Na⁺ channels, was tested at a single standard dose of 0.4mg/kg and mice were assessed 20 minutes post subcutaneous injection (Figure 3.2 C). In this case, wildtype mice showed an increase from their baseline paw withdrawal threshold (1.04 ± 0.09 g) following bupivacaine injection (1.360 ± 0.410 g), and the 11aa (p.A2027_G2037del) EB mice also significantly increased their baseline paw withdrawal threshold from 0.21 ± 0.07 g to 1.24 ± 0.22 g, with the latter exceeding wildtype baseline levels.

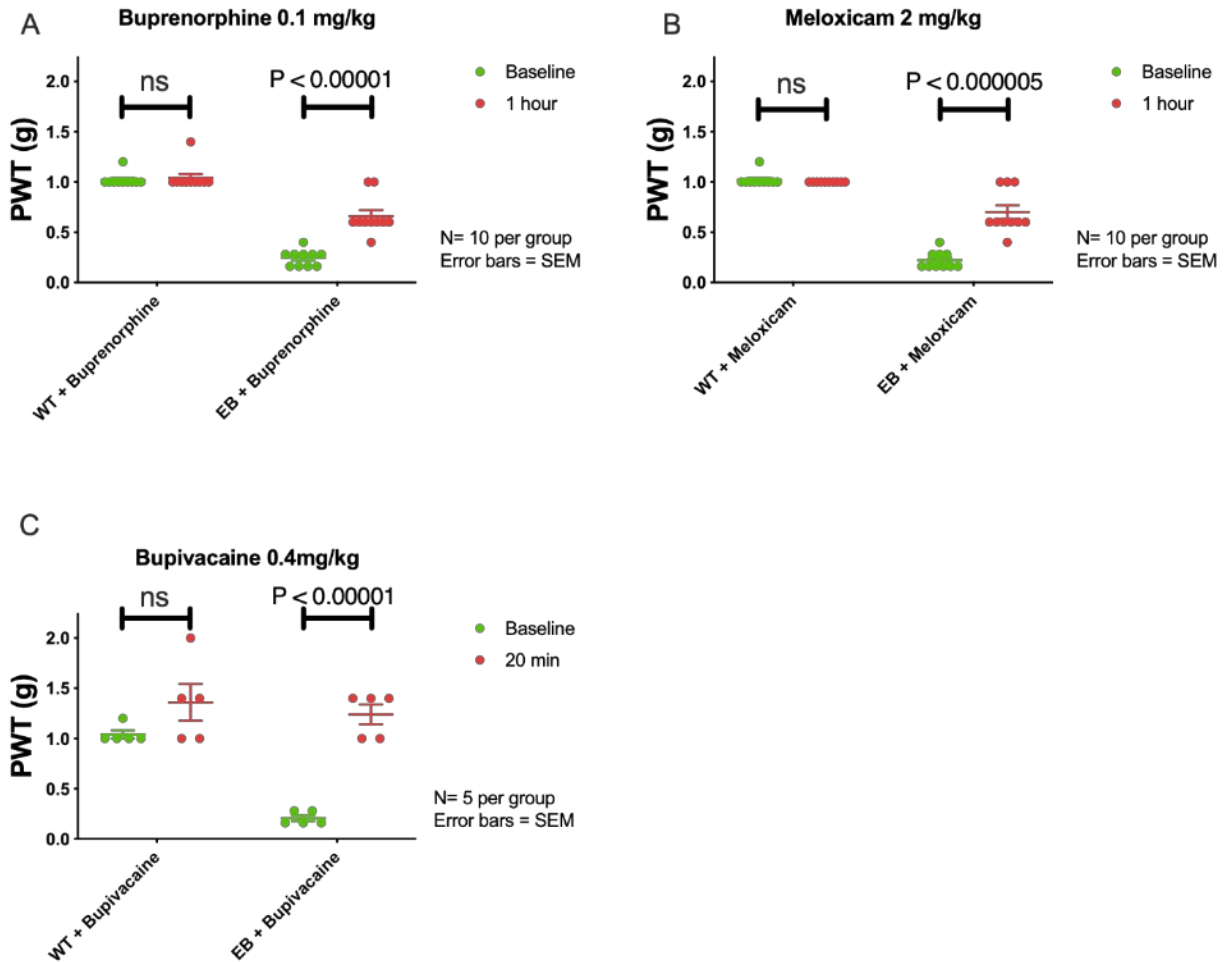


Figure 3.2 Assessment of mechanical allodynia in DDEB mice carrying the 11aa deletion following administration of different analgesic agents.

Wild type and 11aa (p.A2027_G2037del) EB mice were assessed using mechanical von Frey filaments following subcutaneous administration of buprenorphine at 0.1mg/kg (A), meloxicam at 2mg/kg (B), and bupivacaine at 0.4mg/kg (C). Mean $\pm$ SEM are shown in addition to data points for individual animals. P values from multiple t-tests shown.

3.3.3 Mechanical hypersensitivity in DDEB mice with an 11aa deletion is similar at 8 and 18 weeks of age

As described in Chapter 2, our DDEB mice show a temporal progression of their disease, with a greater incidence of blistering and the onset of nail loss and digit shortening over time. To determine if the mechanical hypersensitivity in DDEB mice with an 11aa deletion also progresses over time, we compared 8 and 18 week old DDEB mice with an 11aa deletion using a manual von Frey assay. Taking care to only use mice that did not have any evidence of overt paw blistering, we observed that the mechanical hypersensitivity in both wild type and 11aa deletion mice did not increase with time (Figure 3.3 A).

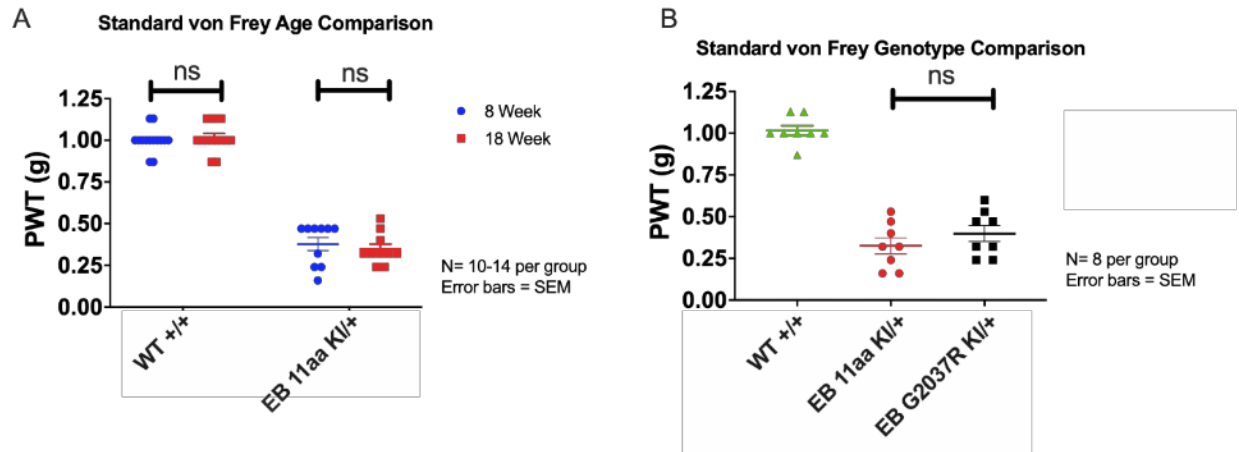


Figure 3.3 Assessment of mechanical allodynia in DDEB mice carrying the 11aa deletion at later timepoints and in comparison to the mice carrying the G2037R mutation.

(A) Wild type and 11aa (p.A2027_G2037del) EB mice were assessed using mechanical von Frey filaments at 8 and 18 weeks of age. Wildtype 8 week old (1.00 ± 0.02 g) and 18 week old (1.02 ± 0.02 g) mice showed no difference in their paw withdrawal thresholds. Similarly, no difference was observed between 8 week old (0.38 ± 0.04 g) and 18 week old (0.35 ± 0.03 g) 11aa deletion EB mice aging mice were not significantly different from their younger counterparts, (multiple t-tests) (B). Paw withdrawal thresholds were also compared between wildtype animals (1.02 ± 0.08), EB mice carrying an 11aa deletion (0.33 ± 0.14) and EB mice carrying a G2037R mutation (0.40 ± 0.14). There was no statistically significant difference between the two EB mouse lines, but both had significantly lower paw withdrawal thresholds than wildtype animals ($p < 0.0001$ Tukey's multiple comparisons test) (error bars = SEM, $N = 8$).

3.3.4 EB mice carrying the 11aa deletion and G2037R mutation display similar levels of mechanical allodynia

Since the p.G2037R mutation is directly homologous to a mutation commonly found in DDEB patients (and therefore has arguably greater construct validity), we were keen to determine whether mice carrying this mutation also displayed mechanical allodynia. We therefore compared the paw withdrawal thresholds of wildtype animals (1.02 ± 0.08 g), EB mice carrying the 11aa (p.A2027_G2037del) deletion (0.33 ± 0.14 g), and EB mice carrying the p.G2037R mutation (0.40 ± 0.14 g) (Figure 3.3 B), and observed similar levels of mechanical allodynia in the two EB mouse lines.

3.3.5 EB mice carrying the G2037R mutation show a variable response to different analgesic agents

For subsequent experiments, we decided to focus on mice carrying the G2037R mutation given its greater relevance to patients. Instead of using manual von Frey filaments, we also started using the DPA to assess mechanical allodynia, given its greater degree of automation, its ability for higher throughput and the likelihood that it would be less subject to operator-based variation.

To begin – and building upon the initial proof-of-concept studies done in the EB mice carrying the p.A2027_G2037del mutation - multiple clinically relevant analgesic agents were tested across a range of doses and time periods to more comprehensively compare their ability to alleviate mechanical allodynia in mice carrying the G2037R mutation.

Firstly, amitriptyline, a tricyclic anti-depressant that is commonly used for EB-related pain believed to be neuropathic in origin, was tested and observed to induce a peak paw withdrawal threshold of 3.50 ± 0.20 g at 30mg/kg, 0.75 hours after intraperitoneal administration (Figure 3.4 A).

Secondly, buprenorphine was tested and observed to induce a peak paw withdrawal threshold of 3.20 ± 0.38 g at 0.1mg/kg, 1 hour after subcutaneous administration (Figure 3.4 B).

Thirdly, gabapentin, an anti-convulsant that is also commonly used for EB-related pain believed to be neuropathic in origin, was observed to induce a peak paw withdrawal threshold of 3.82 ± 0.20 g at 100mg/kg, 1 hour after intraperitoneal administration (Figure 3.4 C).

Fourthly, paracetamol, which is also commonly used to treat EB-related pain, was tested and observed to induce a peak paw withdrawal threshold of 2.70 ± 0.63 g at 100mg/kg, 1 hour after intraperitoneal administration (Figure 3.4 D).

Finally, meloxicam was found to induce a peak paw withdrawal threshold of 4.53 ± 0.05 g at 2mg/kg, 1.5 hours after subcutaneous administration (Figure 3.4 E).

Taken together, it was thus evident that each of the analgesic agents showed dose-dependent amelioration of the mechanical allodynia observed in EB mice carrying the p.G2037R mutation, but only meloxicam was able to completely normalise the paw withdrawal threshold of these mice to wild type levels.

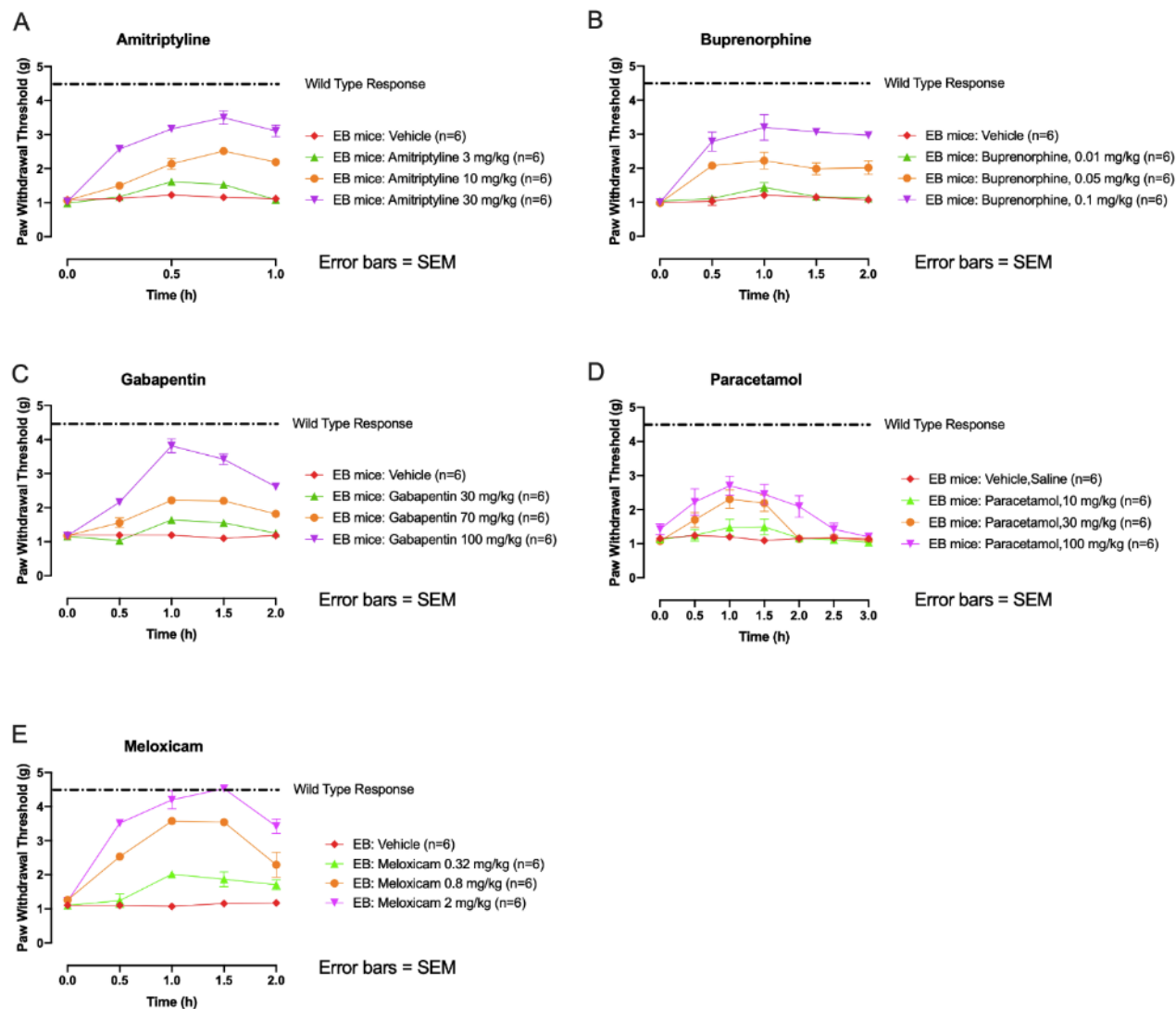


Figure 3.4 Assessment of mechanical allodynia in DDEB mice carrying the p.G2037R mutation following administration of different analgesic agents.

EB mice with the p.G2037R mutation were tested for mechanical hypersensitivity using a DPA in response to several commonly used analgesic agents, including amitriptyline (A), buprenorphine (B), gabapentin (C), paracetamol (D) and meloxicam (E). For each analgesic, a dose range was selected based on previously reported concentrations and animals were assayed for a duration chosen in light of the known pharmacokinetic profiles of each drug. N=6 mice were used per group, and data were plotted as mean $\pm$ SEM.

3.3.6 EB mice carrying the G2037R mutation can also be used to test novel analgesic agents

To demonstrate the ability of our EB mice to assess potentially novel analgesic agents, several different that are not currently used to treat EB were also evaluated for the ability to improve the mechanical allodynia of the EB mice carrying the p.G2037R mutation.

Firstly, based on mass spectrometry data from a collaborator which suggested a disruption in bradykinin-related signalling in DEB mouse skin, we tested HOE140, a specific antagonist of bradykinin B2 receptors, and observed a peak paw withdrawal threshold of $3.26 \pm 0.35\text{g}$ at $1\mu\text{mol/kg}$, 2.5 hours after intraperitoneal administration (Figure 3.5 A).

Secondly, we tested losartan, an angiotensin II receptor antagonist that is currently being assessed in clinical trials for EB, and observed that it induced a peak paw withdrawal threshold of $2.28\text{g} \pm 0.40\text{g}$ at 100mg/kg , 2 hours after Intraperitoneal administration (Figure 3.5 A).

Finally, we tested two novel first-in-class drugs, simply referred to here as drugs X and Y given commercial sensitivities, and both resulted in a rapid improvement in paw withdrawal thresholds. For drug X, the paw withdrawal threshold increased to $3.69 \pm 0.67\text{g}$ within 15 minutes of being injected IP at its highest dose (Figure 3.5 B). For Drug Y, results were even better, with the paw withdrawal threshold increased to a level equivalent to wild type animals ($4.61 \pm 0.08\text{g}$) within 15 minutes of being injected IP at its highest dose (Figure 3.5 C).

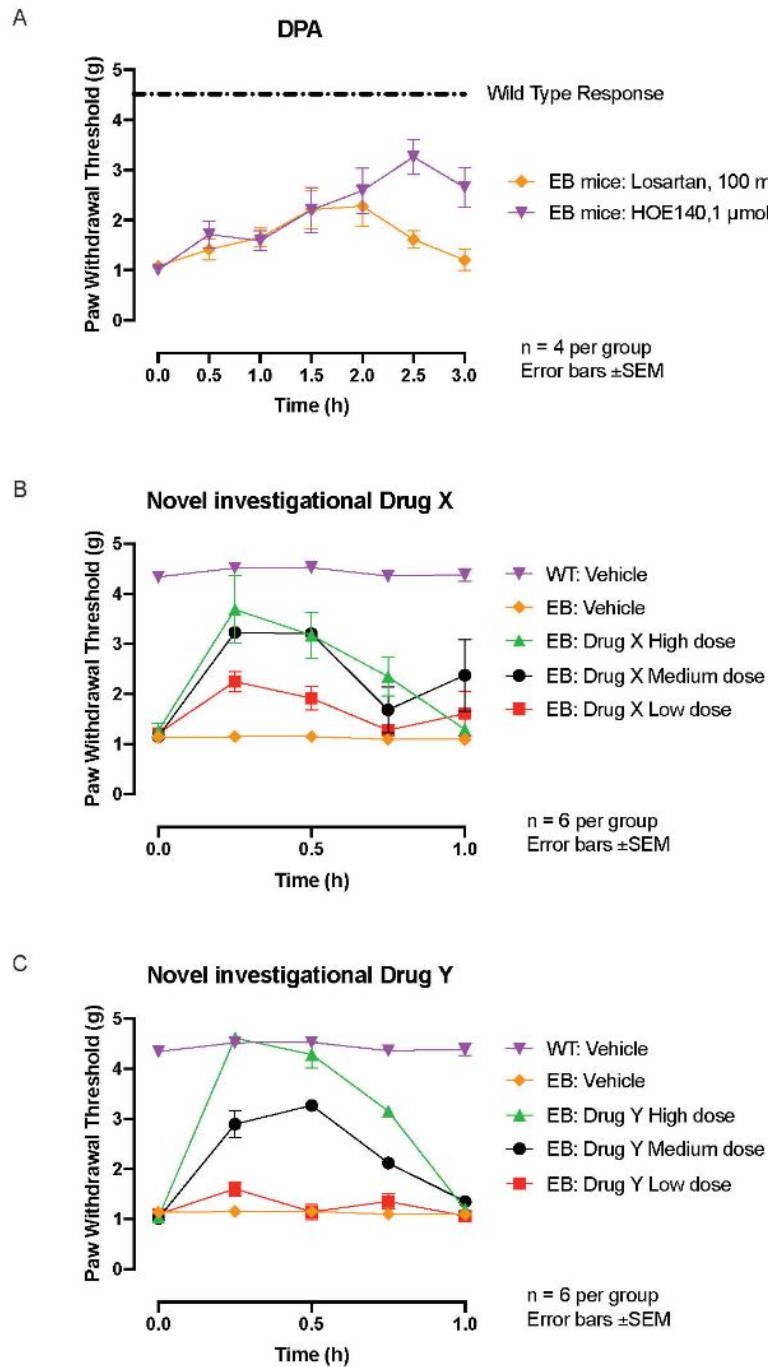


Figure 3.5 Assessment of mechanical allodynia in DDEB mice carrying the p.G2037R mutation following administration of novel analgesic agents.

EB mice with the p.G2037R mutation were tested for mechanical hypersensitivity using a DPA in response to (A) HOE140 and Losartan, (B) investigational drug X, and (C) investigational drug Y. 4-6 mice were used per group, and data were plotted as mean $\pm$ SEM.

3.4 Discussion

The data presented in this chapter suggest that our EB mice carrying p.A2027_G2037del and p.G2037R mutations may be useful models for investigating and treating EB-related pain.

Although it is difficult to know whether any mouse disease models truly recapitulate the pain that humans experience, it is worth bearing in mind that the best animal models display three types of validity – construct, face, and predictive (Crawley 2004) – and our EB mice display each of these types of validity.

Construct validity requires that an animal model is generated with the same underlying biological cause as that seen in humans, whether that be a genetic mutation or environmental factor. In this regard, our DDEB mice carrying the p.G2037R mutation were created using CRISPR to carry the specific glycine mutation most commonly observed in DDEB patients (at position 2043 within the collagen VII protein) and thus display very strong construct validity (see Figure 1 in Chapter 2).

Face validity requires that symptoms, signs and pathology displayed in an animal model are analogous to those observed in humans with the same disease. Our DDEB mice display face validity in multiple ways. Firstly, the mice demonstrate contact-dependent blistering and nail-related changes that are highly similar to those seen in patients with DDEB (see Figure 2 in Chapter 2). Secondly, the mice display pain hypersensitivity to mechanical and thermal stimuli in an identical manner to that previously observed in RDEB patients (von Bischoffshausen et al, 2017).

Predictive validity requires that treatments that are efficacious for treating the human disease are similarly efficacious in reversing symptoms in an animal model. In this regard, we tested a wide range of different analgesic agents used to treat EB-related pain. These include amitriptyline, buprenorphine, gabapentin, meloxicam, and paracetamol. Dose titration studies with each of these agents showed that each had some analgesic activity in our DDEB mice. Notably, however, none were completely effective in reversing the pain hypersensitivity of the mice for a prolonged duration, consistent with what is observed clinically in DEB patients.

Notably, the mechanical and thermal allodynia observed in our EB mice occurred in the absence of the onset of any overt blistering in the paws. This is important, since it suggests that pain hypersensitivity is occurring in the absence of any direct macroscopic skin damage and suggests that such damage is not required to cause chronic pain in EB patients. Understanding the mechanisms that underlie such pain will be important to enable more targeted treatments for EB-related pain, and our mouse models are likely to provide a strong opportunity to investigate this further. For example, EB is commonly associated with increased skin inflammation, which can itself drive pain hypersensitivity, and our DDEB models should enable a comprehensive examination of whether inflammation is responsible for the allodynia observed in our mice.

Using the p.A2027_G2037del EB mice, we observed a mild sex difference in mechanical and thermal allodynia, with female mice having even lower paw withdrawal thresholds. It is unknown if such sex differences are also observed in EB patients, but it might be interesting to explore the cause for such differences. One possibility may be estrus cycle effects, although previous studies have failed to observe

an effect of the estrus cycle across various pain-related assays, including the tail flick test, hot plate test, and von Frey assay (Meziane et al.; Sanoja et al). Whether such sex differences are also apparent in mice carrying the p.G2037R mutation is something we have not looked at. Using the p.A2027_G2037del EB mice, we also tested to see if there was a change in mechanical allodynia as the mice age (Figure 3.3 A), and observed no difference between 8 and 18 weeks of age. However, we have not yet looked any earlier or later in life, which would be important to do to confirm our initial result.

Our EB mouse models also allowed us to test the analgesic effects of several novel agents. For example, HOE140 (also known as Icatibant) was able to partially ameliorate the mechanical allodynia seen in mice carrying the p.G2037R mutation (Figure 3.5A). Although only a single dose was tested and further dose titration experiments have not been conducted, this suggests that bradykinin signalling may play a role in EB-related pain hypersensitivity. Given HOE140 is already approved for clinical use in hereditary angioedema, in theory it could also be used off-label to try to treat EB-related pain that is refractory to other treatment options. In a similar way, using other drugs with defined mechanisms of action may improve understanding of the signalling pathways that are important for pain transmission in EB. Finally, we also tested two first-in-class experimental agents that have yet to be tested in clinical trials (Figure 3.5 B), one of which was able to restore paw withdrawal thresholds to wild type levels. These results invite further preclinical development of these agents as potential analgesic agents for EB – which as a paediatric orphan disease carries regulatory incentives from agencies such as the FDA - and also indicate that our models may be useful for testing other novel analgesic agents for EB.

Finally, it is important to note various limitations to the current studies. Firstly, while the von Frey, DPA and Hargreaves assays are all commonly used to assess pain hypersensitivity in rodents, it would be important in the future to confirm our observations using other more naturalistic methods for assessing pain, such as mouse grimace scales and gait analysis (Deuis, Dvorakova, & Vetter, 2017; Tappe-Theodor, King, & Morgan, 2019). Secondly, we have chosen to focus on assessing pain in the absence of any overt blistering. This is likely to be a good model for chronic pain hypersensitivity but does not directly relate to the acute pain that many EB patients experience secondary to blisters, wounds, dressing changes, and post-surgical pain.

4 Conclusion

The advent of CRISPR-Cas9 editing has revolutionised biological research. Experiments which would once have taken years to accomplish can now be performed with relative speed. Harnessing this technology, we were able to generate five new mouse models of DDEB where previously there were none. Two of these mouse lines carry mutations that are directly analogous to the two most common human mutations responsible for DDEB. Three others are novel deletions which show similar patterns of inherited blistering. These mice are a new resource which the EB research community will be able to use to learn more about EB and hopefully develop more effective treatments. With nearly 3000 congenital genetic diseases in existence (<https://rarediseases.info.nih.gov/diseases/diseases-by-category/5/congenital-and-genetic-diseases>), there are many more possibilities for CRISPR-Cas9 methods to be applied to generate relevant mouse models, and my work in Chapter 2 describing the creation of novel DDEB mouse models directly speaks to the feasibility of this approach.

The potential utility of these DDEB mouse models is directly demonstrated in Chapter 3, which provides a foundation for future studies to improve understanding of the mechanisms by which chronic pain arises in EB. While meloxicam was most effective at improving pain hypersensitivity in our DDEB mice among agents already being used as analgesics in EB, the panel of analgesics that we tested was not exhaustive and it would be important to test other agents in the future. Similarly, future work is required to test pharmacological antagonists of specific pathways and processes known to be involved in pain signalling. For example, is the increased pain the result of mutant collagen VII changing the microenvironment in which nerve fibres develop? Or could it be that the immune system is detecting stress from cells and becoming overactive, leading to an excess activation of pain pathways? Applying advanced microscopy techniques and different omics technologies, we expect to be able to answer such questions in the next few years. Doing so should deepen understanding of the pathophysiology of EB-related chronic pain and ultimately inform the development of more targeted and effective analgesic options for EB patients.

Like pain, itch is a significant clinical problem in EB, and the DDEB mice may provide a tool to better understand and treat EB-related itch. Looking ahead, we therefore plan to use relevant itch-based assays to determine if DDEB mice have a higher propensity to scratch themselves compared with wild-type counterparts. Assuming they do, we can then objectively compare a range of drugs to determine which are most likely to provide effective itch relief for EB patients. As with pain, improved relief from itch would improve the lives not just for those with EB but also the families who care and support them.

Finally, we also expect our mice to present unique opportunities to examine a range of different therapeutic approaches for DDEB that would simply not be possible with existing EB animal models. For example, protein replacement therapies currently being investigated in clinical trials which seek to provide collagen VII supplementation into the skin makes theoretical sense for patients with RDEB who lack collagen VII protein; for DDEB, however, it is unclear whether such a strategy will help patients given the underlying problem is due the existence of a mutant form of collagen VII rather than a lack of the wild-type protein per se. Testing whether collagen VII protein replacement helps improve clinical

outcomes in our DDEB mice would be relatively straightforward and would also allow for detailed investigation of different timing and dose options, which would not be feasible in clinical trials given the rarity of the disease and small patient numbers. As another example, our mice also provide an opportunity to test the effectiveness and safety of an oligonucleotide that is currently in clinical trials and was designed to promote skipping of exon 73 in RDEB patients, but which has yet to be tested in any preclinical models of DDEB ("Topical QR-313 in Dominant Dystrophic Epidermolysis Bullosa (DDEB) or Recessive Dystrophic Epidermolysis Bullosa (RDEB) Due to Mutation(s) in Exon 73 of the COL7A1 gene,").

Lastly, our mice could also be used to explore novel treatments that are directly aimed at treating DDEB rather than RDEB. For example, RNA interference should theoretically allow for the direct targeting of mutant Col7a1 transcripts via short interfering RNAs, and it should be relatively simple to test the efficacy of such RNA therapeutics in our mice. Similarly, while CRISPR-cas9 editing was used to create these models, this technology could also be used to provide a gene editing-based cure, and our mice could be used to explore this strategy. Having said that, it is important to remember that mice are not humans, and therefore the success of a treatment in a mouse does not guarantee success in a human.

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