Recreating Epidermolysis Bullosa Simplex in zebrafish with transgenesis

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Abstract

Epidermolysis Bullosa simplex (EBS) is a rare genetic disorder that is typically inherited in an autosomal dominant fashion and affects approximately 1 out of 20 000 individuals. This disease is caused by mutations in either the *KRT*14, *KRT*5 or *PLEC* genes. These genes code for proteins involved in the formation of the cytoskeleton in basal keratinocytes, which form the basal layer of the epidermis. The cytoskeleton provides structural support to the basal keratinocytes and mutations in these genes cause cytoskeletal malformations, making these cells more susceptible to physical stress. This results in the cells undergoing lysis under trivial mechanical stress and causing the epidermis to detach from the dermis, the layer immediately below the epidermis. This leads to the primary symptom of EBS: the formation of blisters. The goal of this project is to recreate EBS in zebrafish using transgenesis and to create stable mutant transgenic line. In the future, high throughput drug screening will be done on mutant zebrafish embryos to find potential drug candidates that can alleviate the symptoms of EBS. To accomplish this, missense and deletion mutations in zebrafish *krt5* cDNA using site-directed mutagenesis were performed. It was previously shown that mice models for this disease die shortly after birth and thus no stable mutant lines were able to be created. To ensure embryo survival and avoid a similar fate, mutant *krt5* cDNA was expressed in non-essential tissue, such as the embryonic fin fold using a fin epithelial-specific enhancer named *epi*. These constructs were injected into one-cell stage zebrafish embryos, which were raised and screened for integration of the construct in their germ cells. While results from injected embryos were promising, mutant transgenic zebrafish did not demonstrate any blistering. In an attempt to induce blistering, mutant zebrafish embryos were placed under various environmental stressors known to worsen the symptoms of EBS. This was not successful. Expression of mutant keratin 5 in the basal epidermis of the entire embryo using the 2.3kb upstream region of the zebrafish *krt5* gene to drive expression also did not yield any results. More investigations are needed to determine if it will be possible to use the zebrafish to model EBS.

Table of Contents

Abstract ii

List of tables and figures v

Abbreviations vi

Acknowledgements vii

1. Introduction 1

  1.1 Overview 1

  1.2 The Epidermolysis bullosa (EB) diseases 2

  1.3 The mammalian skin 5

  1.4 The keratin protein family 8

  1.5 Molecular mechanism behind EBS 12

    1.5.1 EBS arising from mutations in the keratin 5 and keratin 14 proteins. 12

    1.5.2 EBS arising from mutations in the *PLEC* gene 15

  1.6 Current treatments for EBS 16

  1.7 Experimental design 18

    1.7.1 Choosing zebrafish to model EBS 18

    1.7.3 EBS was chosen to be recreated in zebrafish using the zebrafish krt5 gene 23

    1.7.4 Transgenesis was used to express zebrafish *krt5* cDNA in non-essential epithelial tissue 28

2. Materials and Methods 31

  2.1 Zebrafish husbandry 31

  2.2 Microinjections 32

  2.3 Screening 32

  2.4 Site-directed mutagenesis 33

  2.5 Cloning 33

  2.6 Immunohistochemistry 34

3. Results 35

  3.1 Selection of the amino acid position to be mutated in zebrafish keratin 5 protein. 35

  3.2 Environmental Stress experiments 41

  3.3 Deletions of the helix initiation motifs and helix termination motifs 45

  3.4 Driving mutant *krt5* cDNA expression with the 2.3kb genomic sequence upstream of the zebrafish *krt5* gene 50

  3.5 Interchanging the 2A linker peptide with P2A 56

3.6 Immunohistochemistry 60
4. Discussion 62
4.1 Overview 62
4.2 I161S and E477K substitution 63
4.3 Environmental stress 67
4.4 Deletions of the helix initiation and termination motifs 69
4.5 Expression of mutant keratins during keratin cytoskeletal formation 72
4.6 Characterization of keratin cytoskeleton 76
4.7 Conclusion and future direction 77

List of tables and figures

Figure 1.2.1 Anatomy of the mammalian skin. 4
Figure 1.3.1. The different layers of the epidermis. 7
Figure 1.4.1. Schematic of the three protein domains found in keratins. 11
Figure 1.5.1.1. Frequency and position of published mutations found in the keratin 14 (K14) and keratin 5 proteins (K5). 14
Figure 1.7.2.1. ISH of 3dpf zebrafish embryos using a *krt91* RNA probe (A) and *krt5* RNA probe (B). 25
Figure 1.7.2.2. Expression of the *KRT5* gene is found in the basal layer of the skin in zebrafish. 27
Figure 1.7.3.1. The *epi + βG* enhancer/minimal promoter was chosen to drive expression of mutant *krt5 cDNA*. 30
Figure 3.1.1. The I161 and the E477 positions were chosen to be mutated 37
Figure 3.1.3. Embryos injected with the mutant keratin constructs displayed blisters. 39
Figure 3.1.4. Mutant I161S and E477K *krt5* transgenic lines. 40
Figure 3.3.1. The helix initiation motif (HIM) and the helix termination motif (HTM) were deleted in the new constructs. 46
Figure 3.3.2. Tg(*epi*+ BG: *krt5Δ2b 2A EGFP)* **at 3dpf.** 48
Figure 3.3.3. The *epi* enhancer + human βG minimal promoter was used to drive expression of mutant keratin 5 and GFP proteins in non-essential *epi*thelial tissue. 49
Figure 3.4.1. Zebrafish embryo injected with keratin 5: *I161S-krt52A-EGFP* demonstrating blisters. 54
Figure 3.4.2. Zebrafish embryos injected with constructs containing mutant *krt5* cDNA under the control of the *keratin5p* regulatory elements at 2-4 dpf. 55
Figure 3.5.1 The P2A cDNA sequence is optimized for use in zebrafish 57
Figure 3.5.2. The upstream region of *krt5* was used to drive expression of mutant keratin 5 and GFP proteins but could not recreate EBS in zebrafish. 59
Figure 3.6.1. Immunohistochemistry of pan type II keratin antibody on wild type zebrafish epithelial cells 61
Table 1.7.1.1 Advantages and disadvantages of the three most commonly used animals in high throughput drug screening compared to mice. 20
Table 3.1.1. Results of transient expression of the transgene in injected embryos 39
Table 3.3.1. Results of transient expression of the transgene in injected embryos for the deletion constructs 47
Table 3.4.1. Results of transient expression of the *keratin5p* constructs injections. 53

Abbreviations

EB: Epidermolysis bullosa

EBS: Epidermolysis bullosa simplex

IF: Intermediate filament

KRT#: Human/mouse *keratin* gene number

Krt#: zebrafish *keratin* gene number

K#: keratin protein number

βG: human beta globin

Dpf: days post fertilization

Hpf: hours post fertilization

EGFP: enhanced green fluorescent protein

Him: Helix initiation motif

Htm: helix termination motif

Keratin5p: The 2.3kb upstream region of the zebrafish *krt5* gene

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1. Introduction

1.1 Overview

The integrity of the skin is paramount for the proper functioning of an individual: it provides a first line of defense against pathogens, allows us to interact with our environment through touch, thermoregulates our bodies through sweating, resists water loss and stores lipids. If this organ was compromised it could lead to a variety of problems, some of which might be fatal to affected individuals. The cytoskeleton, a filamentous structure made of keratin heterodimers among other proteins, gives structural strength to the epidermis, the upper tissue layer of the skin. Without an effectual cytoskeleton, the keratinocyte found in the epidermis will lyse when presented with friction.

Epidermolysis bullosa simplex (EBS) is a genetic disorder caused mutations in the *KRT5, KRT14* or *PLEC* genes and affects the basal keratinocyte layer of the epidermis. The frequency of EBS is currently unknown, however it is estimated that 1 in 30,000 to 50,000 people are affected by this genetic disorder (Abitbol & Zhou, 2009). The most prominent symptom of EBS is blistering of the skin due to the detachment of the epidermis from the more basal skin layers (Lane et al., 1992). The severity of the blistering can vary greatly depending on the type and location of the mutation in these genes and can range from minor blisters in the hands and feet to the death of the individual shortly after birth. There is no effective treatment for this disease other than palliative care; however, progress is being made each year. In my thesis project, I focused on two goals: creating EBS in zebrafish by use of a transgene construct and analysis of the phenotype of mutant fish. The future goal of this project will be using mutant zebrafish embryos in a high throughput drug trial.

1.2 The Epidermolysis bullosa (EB) diseases

The EB diseases, which EBS is a part of, are a group of genetic diseases that result in blisters with varying severity that can be found either locally or throughout the body (Magin et al., 2007). Blisters arise when the epidermis detaches itself from the basement membrane (Figure 1.2.1), which leaves a space that fills with interstitial fluid (Smith et al., 1993). The EB disease group is divided into three main categories based on ultrastructural pathology: junctional epidermolysis bullosa, dystrophic epidermolysis bullosa and epidermolysis bullosa simplex (Smith et al., 1993). Junctional EB arises from mutations in the laminin and collagen protein families and affects the hemidesmosomes that attach the epidermis to the dermis (Pfendner & Lucky, 1993; Smith et al., 1993). Dystrophic EB is caused by a mutation in the *COL7A1* gene, which codes for the protein type VII collagen (Dang & Murrell, 2008). This leads to a weakening of the link between the basement membrane and the upper dermis layer, leading to life threatening blisters (McGrath et al., 1993).

EBS is caused by mutations in the *KRT5, KRT14* or *PLEC* genes (Pfendner & Bruckner, 1993). These genes code for the keratin 5, keratin 14 and plectin proteins respectively, all of whom are expressed in the basal keratinocytes (figure 1.2.1). This form of EB is estimated to affect around 1 in 30,000 to 50,000 people (Abitbol & Zhou, 2009; Magin et al., 2007). This is not considered to be accurate as it is theorized that many of the milder cases go undiagnosed (Coulombe & Lee, 2012). Depending on the mutation, EBS can either be autosomal dominant or autosomal recessive (Batta et al., 2000; Stephens et al., 1995). In addition, the severity of this disease depends on the location and type of mutation (Lane et al., 1992; McGrath et al., 1992). EBS is divided into three groups based on the location and severity of the symptoms. 1) Localized EBS (also known as Weber-Cockayne) is the most common type of EBS and has the

mildest symptoms. Blisters are typically found in the hands and feet and will heal with little to no scarring (Ehrlich et al., 1995). Many cases of localized EBS will go undiagnosed due to the mild nature of the symptoms. 2) EBS-DM (also known as Dowling-Meara) is the type of EBS with the most severe symptoms. Blisters can be found throughout the body, including the mucosa of the mouth and throat (Smith et al., 1993). This type of EBS can be fatal in its most severe form and can cause death shortly after birth (McGrath et al., 1992). Symptoms will appear at around five days after birth and in most cases the symptoms tend to improve with age (McGrath et al., 1992). 3) EBS with mottled pigmentations is also a type of generalized EBS but it is associated with large patches of darker skin colour on the body (Li-Hong Gu & Coulombe, 2007). Like EBS-DM, symptoms appear at birth or in early infancy, can be found throughout the body, and tend to improve with age (Pfendner & Bruckner, 1993). Symptoms, however, tend to be less severe than EBS-DM (Minakawa et al., 2013). Even within these categories, severity of symptoms can vary greatly (Pfendner et al., 2005).

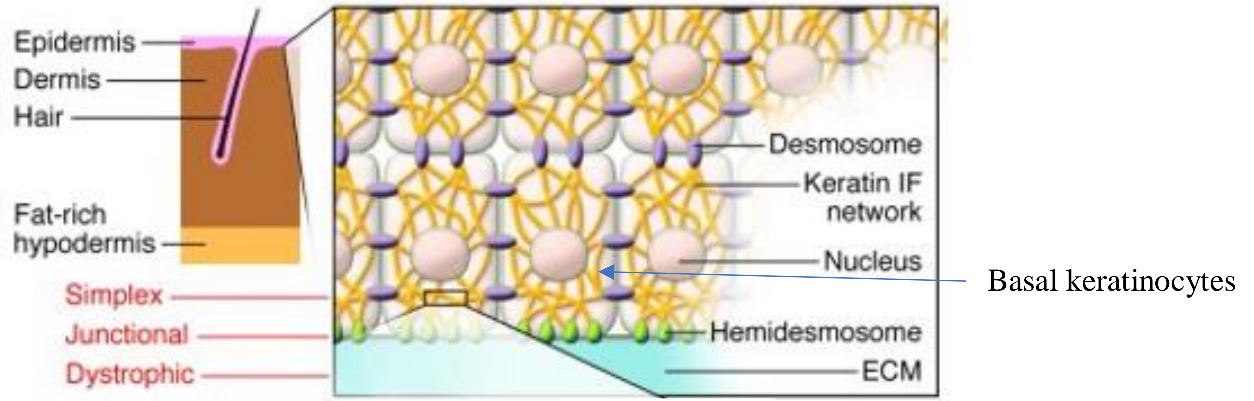


Figure 1.2.1 Anatomy of the mammalian skin. Basal keratinocytes are found at the most basal layer of the epidermis and interact with the basement membrane, which connects the epidermis and the dermis. Each epithelial cell contains a cytoskeleton, the main structural component of which is made of a 10 nm keratin heterodimers polymer, called the 10 nm filament. In the case of the basal keratinocytes, K5 and K14 are the keratins used to make the 10nm filament. This cytoskeleton attaches to the hemidesmosomes which hold the epidermis to the dermis layer. Red text indicates EB diseases and the associated-structure that is affected by each of them (EB simplex affects the cytoskeleton, junctional EB affects the hemidesmosomes and dystrophic EB affects the anchoring fibrils found in the ECM). Adapted from Coulombe et al, 2009.

1.3 The mammalian skin

Mature mammalian skin can be divided into three different layers: the dermis and the epidermis, with the hypodermis underlying these layers (Figure 1.2.1). The hypodermis consists mostly of adipocytes and its primary function is to attach the skin to the underlying bone and muscle as well as supplying the skin with blood vessels and nerves (Ibrahim, 2010). The high fat content of the hypodermis also serves as insulation and padding for the underlying tissue (Ibrahim, 2010). The dermis is found underneath the epidermis and consists of mostly collagenous connective tissue, along with hair follicles, blood vessels, apocrine glands and nerves (Holbrook et al., 1982). The primary function of the dermis is to provide strength and elasticity to the skin (Krieg & Aumailley, 2011). The dermis is divided into two different layers based on the density of connective tissue: the papillary region and the reticular dermis. The reticular dermis is the most basal layer of the dermis and is thicker than the papillary region. It contains a dense concentration of connective tissues such as collagen and elastic fibers to give skin its ability to resist stress and strain (Holbrook et al., 1982). The papillary dermis is the most superficial layer of the dermis and connects to the epidermis through the basement membrane. This layer contains loose connective tissue and blood vessels (Krieg & Aumailley, 2011). The basement membrane is a thin fibrous layer made of several large glycoproteins that is found between the epidermis and dermis. Its role is to provide structural support for the surrounding tissue, modulate cellular function and attach the epidermis to the dermis (LeBleu et al., 2007).

The most superficial tissue of the skin is the epidermis. Its primary function is to protect the body from pathogens and to regulate water loss (Wickett et al., 2006). The epidermis is mostly made of keratinocytes and contains no blood vessels as it receives its oxygen supply through diffusion of the surrounding air (Wessells, 1967). It also contains the Merkle cells, nerve cells involved in the sensation of touch, and immunologically active cells (Wickett et al., 2006).

The epidermis is made from four layers of cells, and each of these layers contain a different stage of keratinocyte differentiation and different expression of *KRT* genes (Figure 1.3.1).

The basal layer is made of proliferating and non-proliferating keratinocytes, called the basal keratinocytes, along with melanocytes (Wickett et al., 2006). It is attached to the dermis through the basement membrane with the aid of hemidesmosomes (Rezniczek et al., 2010). Hemidesmosomes are dynamic transmembrane protein complexes produced by epithelial cells that are involved in the attachment of epithelial cells to basement membranes (Borradori & Sonnenberg, 1999). They also play a role in determining cell polarity, cellular organization and tissue architecture (Todorovićet al., 2013). The basal keratinocytes express keratin 5 and 14 to form their cytoskeleton (Alam et al., 2011). The second most basal layer, the spinous layer, is composed of keratinocytes that are interconnected by desmosomes (Kouklis et al., 1994). Immunologically active Langerhans cells can also be found in this layer (Salmon et al., 1994). Keratinocytes in this layer are at the second stage of keratinocyte differentiation: expression of keratin 5 and 14 is down regulated, while expression of keratin 1 and 10 is upregulated (Moll et al., 2008). The granular layer, the layer superficial to the spinous layer, is where the keratinocytes begin to lose their nuclei and insoluble keratin aggregates, made of keratin 1 and 10, start to form in their cytoplasm (Wessells, 1967). The most superficial layer, the cornified layer, contains the corneocytes, which is the last stage of keratinocyte differentiation (Wickett et al., 2006). The corneocyte forms a heavily keratinized water proof layer that protects the body from pathogens and prevents water loss (Rosso & Levin, 2011).

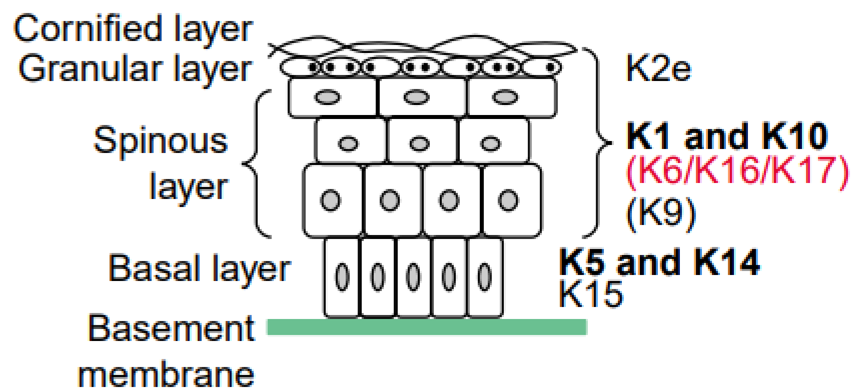


Figure 1.3.1. The different layers of the epidermis. Error! Bookmark not defined. Each layer of the epidermis represents a different stage of the keratinization process, with the superficial layer of the epidermis (the *stratum corneum*) represents the last stage of this process and the basal layer representing the start of the process. While most layers of the epidermis express keratin 1 and 10 (K1 and K10 respectively) among other keratins, the basal keratinocytes express K5 and K14. K2e is expressed in the later stages of keratinocyte differentiation. During the keratinization process, cells gradually lose their organelles and nuclei and become progressively flattened. Their cytoplasm becomes more granular in nature due to the high expression of keratin proteins, which gives the cells the ability to resist mechanical stress and prevent water loss. Adapted from Porter & Lane (2003).

1.4 The keratin protein family

The keratin protein family is a subgroup of the intermediate filament family that plays an important regulatory and structural role in the majority of epithelial tissues (Steinert et al., 1985). They are coded by the *KRT* genes and a total of 54 functional keratin proteins have been identified in humans (Schweizer et al., 2006). Keratins can be divided into two different types based on their sequence homology, protein structure and isoelectric point: acidic type I keratins, which include K9-K10, K12-K28, K31-K40, K33a and K33b; and basic or neutral type II keratins, which include K1-K8, K6a, K6b, K6c, and K71-K86 (Coulombe & Omary, 2002; Schweizer et al., 2006).

Keratins are obligate heterodimers where one type I and one type II keratin combine in a 1 to 1 molar ratio to form keratin heterodimers that are approximately 10nm in width (Hatzfeld & Franke, 1985). Polymerization of these keratin heterodimers forms a long filamentous network called the 10nm filament, which is an important component of the cytoskeletons found in epithelial cells (Pekny & Lane, 2007; Seltmann et al., 2013). The cytoskeleton not only gives structural rigidity to the epithelial cell, allowing them to resist mechanical stress, but is also involved in cellular transport and signaling (Kim et al., 2006; Windoffer et al., 2011). The cytoskeleton also plays regulatory roles in the cell: they have been implicated in cell migration, cell proliferation, cell differentiation and apoptosis during embryonic development (Coulombe & Omary, 2002; Pan et al., 2013). Due to their heterodimeric nature, keratins are expressed in a pairwise fashion and keratin pairs are specific to different types of epithelial tissue (Gu & Coulombe, 2007). For example, the keratin 8/18 pair is found in squamous epithelial tissue while the keratin 5/14 pair is found in basal keratinocytes (O'Guin et al., 1990). While epithelial cells

always express one type I and one type II keratin, more than one of each type of keratin can be expressed in the same tissue (Hatzfeld & Franke, 1985).

Type I and II keratins have "preferred partners" and bind more strongly to their partner than any others of the same type (Hatzfeld & Franke, 1985). However, if the preferred partner is not present (due to a null mutation for example), keratins of the same type can replace the missing keratin and a functional cytoskeleton can still be formed (Kerns et al., 2007; Lloyd et al., 1995). However, this is just a general rule, and not every combination of type I and type II keratins can form a 10 nm filament (Alvarado & Coulombe, 2014; Hatzfeld & Franke, 1985). Even if a 10 nm filament can be formed, other functions of the cytoskeleton, such as cell motility, cellular organization, distribution of organelles and cell proliferation might be adversely affected (Hatzfeld & Franke, 1985; Paramio & Jorcano, 2002; Yamada et al., 2002).

In terms of protein structure, all keratin proteins share the same three functional domains: the α-helical rod domain flanked on either side by the non-helical head and tail domain (Figure 1.4.1). Each of these domains play important roles in the function of the keratins. The function of the head domain is to stabilize the keratin heterodimer formation as well as aid in the cytoskeletal polymerization (Hatzfeld & Burba, 1994). The non-helical tail domain plays a role in organization of the keratin 10 nm filaments within the cell: mutations causing non-functional tail domains in the K1, K5 and the K14 protein lead to improper keratin filament bundling (Gu & Coulombe, 2005; Sprecher et al., 2001). Furthermore, the tail domain interacts with the central rod domain of other keratins, increasing the stability of the 10 nm filaments (Bragulla & Homberger, 2009). Mutation in the non-helical tail domain may also lead to improper translocation of certain proteins within a cell: research suggest that the keratin cytoskeleton is physically linked to translation of proteins, dictating global and local protein synthesis (Kim &

Coulombe, 2010). One example is the improper translocation of loricrin, an important structural protein found in the corneocytes, due to mutations in the non-helical tail domain of the K1 protein (Sprecher et al., 2001).

The α-helical rod protein domain is highly conserved among epithelial keratins and plays a crucial role in the assembly of the heterodimers as well as the polymerization of the heterodimers to form the 10 nm filament (Rugg et al., 1999). This protein domain contains seven subdomains: the coil subdomains 1A, 1B, 2A, and 2B that are linked by the L1, L12 and L2 linker subdomains (Figure 1.4.1). The coil subdomains of the keratin protein will interact with the corresponding coil subdomains of its partner keratin to form a coiled-coil structure, effectively binding them together (Lee et al., 2012). The ends of the α-helical rod domains are called the helix initiation and helix termination motifs and contain sequences that are highly conserved in all intermediate filament proteins (Figure 1.4.1). They play a crucial role in assembling keratin heterodimers into filaments: mutations in these regions lead to the breakdown of the cytoskeleton into keratin aggregates, which can lead to diseases like EBS (Lane et al., 1992; Mülle et al., 1999; Rugg et al., 1999).

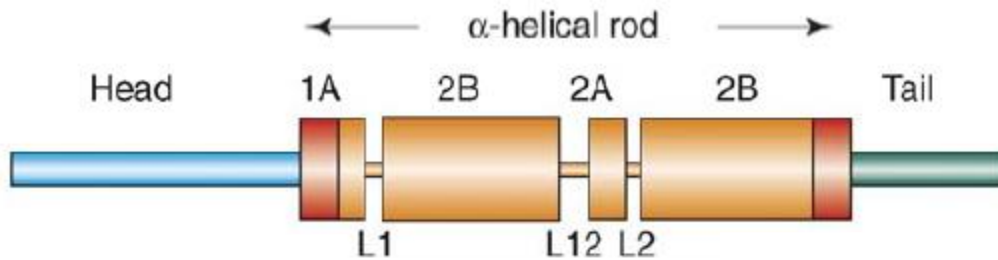


Figure 1.4.1. Schematic of the three protein domains found in keratins. The head (blue) and tail (green) domains form globular structures, while the α-helical rod domain (red and yellow) forms a helical structure. The α-helical rod domain contains the helical subdomains 1A, 1B, 2A and 2B that are linked together by L1, L12 and L2. Indicated in red are regions 15-20 amino acids long that are highly conserved among all IF proteins. These regions play important roles in the polymerization of the keratin heterodimers into keratin filaments. Adapted from Li-Hong Gu & Coulombe (2007).

The keratin proteins are also divided into two different categories based on the tissue they are expressed in and sequence homology. These two categories of keratins are called the hard keratins and soft keratins. Hard keratins, also known as hair keratins, are expressed in epithelial appendages such as hair and nails. Their primary function is to provide structural strength to these appendages. This is reflected in the considerably higher sulfur content in the head and tail domains compared to soft keratins (Coulombe & Omary, 2002). This high sulfur content contributes to their high levels of filamentous cross-linking with keratin-associated proteins, which gives hair and nails their ability to resist high levels of mechanical stress (Coulombe & Omary, 2002; Gu & Coulombe, 2007). The keratins that fall into this category are the type I K21-K28, K31-K40, K33a and K33b and the type II keratins K71-K86 (Schweizer et al., 2006b). Soft keratins are expressed in epithelial tissue such as the epidermis, the mucosa of the mouth and throat and the lining of blood vessels and organs (Coulombe & Omary, 2002). Soft keratins include type I K9-K10 and K12-K20, and type II K1-K8. This includes keratin 5/14 pair who play an important role in EBS.

1.5 Molecular mechanism behind EBS

1.5.1 EBS arising from mutations in the keratin 5 and keratin 14 proteins.

Keratin 5 and keratin 14 are a pair of structural keratin proteins that are co-expressed in the basal keratinocytes (Figure 1.2.1). These keratins are the primary component of the cytoskeleton found in these cells (Kerns et al., 2007). This cytoskeleton maintains the structural integrity of the basal keratinocytes and gives them the ability to resist mechanical stress (Fuchs & Weber, 1994; Pekny & Lane, 2007). Furthermore, the cytoskeleton attaches itself directly to the hemidesmosomes that bind the basal keratinocytes to the basement membrane, stabilizing the connection between these two layers (Kouklis et al., 1994; Müller et al., 1999). Recent research also suggests that K14 and possibly K5 play a role in cell proliferation : inhibition of K14 production by RNA interference causes a significant reduction in cell proliferation and delay in cell cycle progression in cell lines derived from stratified epithelia (Alam et al., 2011). This might be caused by the interaction of the keratin cytoskeleton with Notch1 and members of the 14-3-3 protein family (S. Kim et al., 2006; Lähdeniemi et al., 2017). Abnormal expression levels of keratin 5 and 14 have been also linked with cancer: downregulation of keratin 5 and 14 has been shown to increase the risk of basal keratinocytes metastasizing (Seltmann, et al., 2013).

EBS can arise from either autosomal dominant or autosomal recessive mutations in the *KRT14* or *KRT5* genes depending on the type and location of the mutation (Moll et al., 2008; Rezniczek et al., 2010). The mutations associated with recessive EBS are typically null mutants and are much rarer than the dominant mutations (Alvarado & Coulombe, 2014; Peters et al., 2001). When one of the keratins is not present, heterodimerization cannot take place and no cytoskeleton can form. This will cause the basal keratinocytes to lyse when presented with minimal mechanical stress and cause the detachment of the epidermis from the dermis, which

results in blisters (Tabor et al., 2017). Null *KRT5* mutant mice were found not to be viable and die shortly after birth due to the inability to eat, brought on by the blisters found in the mouth and throat (Peters et al., 2001). Null *KRT14* mutant mice tend to have less severe symptoms than *KRT5* null mutants since a small amount of keratin 15 expression partially compensates for the lack of keratin 14 (Cao et al., 2001; Lloyd et al., 1995). Despite this, null *KRT 14* mutant mice also die shortly after birth (Batta et al., 2000).

EBS caused by dominant mutations can occur in either the *KRT5* or *KRT14* genes and can be caused by either missense or deletion mutation (Pekny & Lane, 2007; Porter & Lane, 2003). Figure 1.5.1.1 illustrates the location and frequency of published mutations found in the *KRT5* and *KRT14* genes that cause EBS in unrelated families before the year of 2003. Since then, the numbers have been updated: for example, the current number of EBS causing in the *KRT5* gene at the I161 location is 15 while it is also 15 at the E477 position (in 2003, the numbers were 8 and 3 for the I161 and E477 positions, respectively). The location of mutations that cause dominantly-inherited EBS tend to cluster in the helix initiation and termination motifs located at the coil 1A and the terminal region of coil 2B protein domains respectively (McGrath et al., 1992). In fact, individuals with deletions of these protein domains have the most severe form of generalized EBS (EBS- DM) (Müller et al., 1999; Rugg et al., 1999). When both the coil 1A and terminal region of the coil 2B domains are functional, elongation of the keratin 10 nm filament occurs normally. However, when one of these domains is non-functional due to a mutation, proper 10 nm growth cannot occur (Gu & Coulombe, 2005). For example, if the coil 1A domain is non-functional, growth of the keratin filament can occur at the function coil 2B end, but cannot continue at the coil 1A end, effectively stopping growth of the 10 nm filament (Cao et al., 2001). This will result in short keratin filament chains that do not form an effective cytoskeleton

(McGrath et al., 1992). Without an effectual cytoskeleton, the basal keratinocytes are rendered fragile (Russell et al., 2004). Additionally, the cytoskeleton will no longer be properly attached to the hemidesmosomes (Figure 1.2.1), causing additional instability (Seltmann et al., 2013). Environmental stress, such as friction or elevated temperatures, cause the basal keratinocytes with mutant keratins to lyse more easily compared to wild type basal keratinocytes (Morley et al., 1995; Russell et al., 2004). Death of the basal keratinocytes detaches the epidermis from the dermis, causing blisters.

The type and location of the mutations can affect the severity of the symptoms. Severity of EBS can differ even if different missense mutation occurs at the same amino acid position. For example, if arginine is substituted for a glycine at position 169 on the K5 protein, localized EBS (EBS-WC) will result (Minakawa et al., 2013). However, if arginine is substituted for a proline, severe generalized EBS (EBS-DM) will result (Müller et al., 2006).

Figure 1.5.1.1. Frequency and position of published mutations found in the central rod domain of the keratin 14 (K14) and keratin 5 proteins (K5) before 2003. Coloured bars represent the different diseases associated with mutations: Green; EBS – Weber Cockayne, Red; EBS – Dowling Meara, Blue; EBS Knöber, black; homozygous and knockout mutations, Purple; type of EBS depends on the substitution. The shortest bars without any numbers represent the discovery of only one mutation. The frequency of mutations found at each position is indicated if more than one mutation was found. Together, the 1A-1B-2A-2B domains, linked by linker domains, make up the highly conserved alpha rod domain. The green coloured boxes represent the helix imitation motif (HIM), found in the 1A domain, while the red boxes represent the helix termination motif (HTM), found at the end of the 2B domain. Adapted from Porter & Lane (2003).

1.5.2 EBS arising from mutations in the *PLEC* gene

The *PLEC* gene, which codes for the plectin protein, is a cytoskeletal protein linker important in the formation of an effectual cytoskeleton (Natsuga et al., 2010). *PLEC* is expressed in stratified epithelium (which includes the basal keratinocytes), as well as in the muscle and simple epithelia (Rezniczek et al., 2010). Eight different isoforms of the plectin protein have been identified, indicating the diverse roles that the *PLEC* gene plays in the body (Natsuga, 2015). The different isoforms are named plectin 1, and plectin 1a – 1g. The plectin 1a isoform plays an important role in anchoring the hemidesmosomes of the basal keratinocytes to the extracellular matrix (figure 1.2.1). Without plectin 1a, the hemidesmosomes do not function properly and mechanical trauma will cause the detachment of the epidermis from the dermis resulting in localized EBS (Walko et al., 2011). Due to the various roles of the *PLEC* isoforms, mutations affecting the whole *PLEC* gene result in a general form of EBS called EBS with muscular dystrophy (EBS-MD) and it is almost always fatal (Natsuga, 2015).

1.6 Current treatments for EBS

There is neither a cure, nor any effective methods to alleviating the symptoms of EBS. Treatment focuses on promoting the healing of blisters and on preventing any further blisters from developing (Pfendner & Lucky, 1993; Tabor et al., 2017). Since blisters present an opportunity for pathogens to infect the body, treatment for EBS also focuses on preventing infections (Abitbol & Zhou, 2009). Some medications have proven useful in alleviating some of the symptoms of EB: injections of Botulinum toxin type A in the feet of an affected individual with localized EBS significantly reduced the amount of blistering (Abitbol & Zhou, 2009). While this result has been recreated in other EBS patient, further trials must be done to assess its effectiveness and safety (Chaptini et al., 2015; Holahan et al., 2016).

One possible cure for recessive EBS caused by null mutations could come from expressing another keratin of the same type with gene therapy: mice studies show that the symptoms associated with recessive EBS caused by *KRT14* knockout are less severe than symptoms associated with *KRT5* knockout because of partial compensation from a small amount of K15 expression (Lloyd et al., 1995). Inducing keratin 17 expression with a Gli2 regulatory region, which directs expression in basal keratinocytes, causes the partial rescue of null *KRT14* mutant mice (Kerns et al., 2007). Keratin replacement has also been shown to work in *KRT5* null mutants: directed expression of a chimeric type II keratin protein has shown to partially rescue the phenotype of *KRT5* null mutant mice (Alvarado & Coulombe, 2014). This chimeric keratin protein is a modified K8 protein with its coil 1A, L2 and coil 2B amino acid sequence replaced with the corresponding K5 protein domains.

Another potential strategy to cure, or at least alleviate the symptoms of recessive EBS caused by null-mutations is to use drugs to activate the production of keratin proteins not

normally found in the basal keratinocytes. One such drug is sulfurophane, which activates the expression of keratin 17 and keratin 16 in the basal keratinocytes (Kerns et al., 2007). Null *KRT14* mutant mice injected with sulfurophane showed considerably less blistering than the non-injected controls (Kerns et al., 2007). Recently, a novel keratin gel that accelerates wound healing and upregulates *KRT* gene expression has been found to help individuals living with EBS manage their symptoms (Denyer, Marsh, & Kirsner, 2015). However, these medications are not effective for every case of EBS and only small limited trials on their effectiveness and safety have been performed. While promising, these strategies only focus on EBS caused by null mutations and not the more widespread forms of EBS caused by dominant mutations (Coulombe & Lee, 2012; Pfendner & Bruckner, 1993). Furthermore, little to no research has been done on remedying EBS caused by mutations in the *KRT5* gene.

1.7 Experimental design

1.7.1 Choosing zebrafish to model EBS

The overall goal of this project is to recreate EBS in a model organism to further characterize the disease in an animal context and, in the future, to perform a high throughput drug screen to find chemical compounds that can alleviate the symptoms of EBS. While the high throughput drug screen is outside the scope of this thesis, it was taken into consideration when designing this project. The following paragraphs outline the factors that were considered when choosing the ideal model organism for this project.

One factor is that the future goal of this project is to perform a high throughput drug screen on this disease model using a chemical library. A chemical library is a random assortment of chemicals that contain many different organic compounds that can be used in a high throughput drug screen. The purpose of a screen is to quickly find novel chemicals that can aid in alleviating the symptoms of a disease. Traditionally, this was done by testing a chemical library on isolated proteins or enzymes to see if any chemical compounds would react with these proteins (Roy et al., 2010). These isolated proteins and enzymes are typically associated with the disease itself or a bodily response to the disease, such as inflation. Recently, small animals have been used in high throughput drug screens instead of isolated proteins or enzymes (Giacomotto & Ségalat, 2010; Strange, 2016). Using small animals in high throughput drug screening has three advantages over using isolated proteins or enzymes: 1) Chemicals can be tested on entire protein pathways instead of one protein, increasing the chances of finding an effective drug (Rennekamp & Peterson, 2015). 2) Drug screening in animals does not depend on the previous identification of a target protein before trials can commence, reducing delays. 3) Chemicals can be screened for their safety profile in an *in vivo* context as well as their effectiveness in alleviating the

symptoms at the same time (Giacomotto & Ségalat, 2010). Some of the disadvantages include the cost of maintaining live animals and the possibility that the organism might not have the gene responsible for the human disease of interest (Giacomotto & Ségalat, 2010).

Currently the three species of animals most widely used for high throughput drug screens are *C. elegans* (roundworms)*, D. melanogaster* (fruit flies)*,* and *D. rerio* (zebrafish) (Strange, 2016). These animals are ideal for this type of drug screening due to their small size and their prolific nature as it makes them compatible with scale of drugs tested in high throughput drug screening (Giacomotto & Ségalat, 2010; Strange, 2016). In addition, the genomes of these species have been sequenced, which allows for the creation of genetic mutants (Strange, 2016). A high throughput drug screen of these mutants can then be used to reveal chemical compounds that can reverse a detrimental phenotype caused by the mutation. Each of these species have advantages and disadvantages in the drug screening process depending on the circumstance (Table 1.7.1.1).

Table 1.7.1.1 Advantages and disadvantages of the three most commonly used animals in high throughput drug screening compared to mice. +, ++, +++, ++++ indicate the relative strength of the model in each category. Mice (M. musculus) were also included as a point of comparison. Adapted from Giacomotto & Ségalat (2010).

Organism	***C. elegans***	***D. melanogaster***	***D. rerio***	***M. musculus***
Generation time	3 to 5 days	10 to 14 days	3 to 4 months	3 to 4 weeks
Embryo size	50 μm	100 μm	1mm	N/A
Adult size	1 mm	3 mm	6 cm	10 cm
Ease of obtaining individuals for testing	++++	++++	+++	+
Resource database	Yes	Yes	Yes	Yes
Ease of storage	++++	++	+++	+
Annual cost	Low	Low	Low to medium	Very high
Orthology to humans (genome)	>50%	>60%	>70%	>90%
Generation of transgenic organism	Weeks	Weeks	Months	Months
Tissue specific promoters	++++	++++	++	+
Equipment cost	Low	Low	Low to medium	Very high
Amount of organisms that can be screened	Medium to high	Low to medium	Low to medium	Very low

Another factor influencing which model organism was chosen was the presence of the same biological conditions that leads to EBS in humans, namely the structural failure of the cytoskeleton in the basal keratinocytes and the detachment of the epidermis from the dermis. Therefore, the model organism must have the *PLEC1*, *KRT5* and *KRT14* genes and a basal keratinocyte layer that connects the epidermis to the dermis.

A search of the non-redundant protein sequence proteomic database for amino acid sequence similar to the human plectin, keratin 5 and keratin 14 proteins in the three organisms most commonly used for high throughput drug screening reveal that *C. elegans* and *D. melanogaster* do not possess either the keratin or plectin proteins. Furthermore, a closer look at the skin structure of *C. elegans* reveals that their skin structure does not contain a dermis layer (Chisholm & Xu, 2012). *D. melanogaster* is also a poor candidate in terms of skin structure: instead of skin made from keratinocytes that rely on a keratin cytoskeleton to provide structural support, they possess an exoskeleton which uses chitin as the main structural support molecule (Moussian et al., 2005) . The absence of the *KRT* gene family and differences in skin structures make *C. elegans* and *D. melanogaster* poor candidates for this project.

The most ideal candidate for this project is *D. rerio* (zebrafish) due to its similarity to human biology and genomics. While no orthologs for the human *KRT14* gene has been positively identified in zebrafish, they do possess orthologs for the human *KRT5* and the *PLEC1* genes. The name of the zebrafish ortholog for *KRT5* is *krt5.* Zebrafish have two orthologs for the human *PLEC1* gene, *pleca* and *plecb* (Bührdel et al., 2015). In terms of skin structure, humans and adult zebrafish share many similarities, such as having an epidermal layer made of keratinocytes that is connected to an underlying dermis (Li et al., 2011).The skin structure of zebrafish embryos also mirrors human skin structure: by 6 dpf, the zebrafish embryo has a clearly demarked

basement membrane that separates a two-cell layer epidermis made of keratinocytes, from the collagenous dermis (Li et al., 2011). This is crucial since high throughput drug screening would be done on zebrafish embryos. By adulthood a multilayer epidermis is separated from the dermal layer by a clearly defined basement membrane (Li et al., 2011). An additional similarity between human and zebrafish skin structures are the hemidesmosomes that connect the basal keratinocytes to the basement membrane (Li et al., 2011).

Zebrafish were also considered the ideal candidate for this project for these additional reasons: 1) Alignment of zebrafish keratin 5 amino acid sequences with human keratin 5 demonstrates a high degree of similarity (85%). This indicates that the keratin 5 proteins most likely play similar roles in both organisms. 2) High throughput drug screening using zebrafish is an already established protocol, with more than 60 papers published using this method (Rennekamp & Peterson, 2015). 3) The transparent skin of zebrafish embryos makes visualizing phenotypes easier. This will be useful when characterizing and analyzing the manifestations of EBS in zebrafish as well as screening for potential drug candidates. 4) The main symptom of EBS, blisters, have been recreated in zebrafish (Carney et al., 2010). While the blisters were created using knockdowns of basement membrane proteins, it nonetheless indicates that there is no limitation in biology that would arrest the development of blisters. These factors make zebrafish the ideal model organism for this project.

1.7.3 EBS was chosen to be recreated in zebrafish using the zebrafish krt5 gene

EBS can be caused by mutations in either the *KRT5*, *KRT14* or *PLEC1* genes. Depending on the type of mutation found in these genes, EBS can either be inherited in an autosomal recessive or dominant fashion (Coulombe, 2016). For the purposes of this project, it was decided that recreating EBS with a dominant mutation was preferable due to the prevalence of EBS caused by dominant mutations in the human population and due to a technical consideration that will be explored in the next section. (Coulombe & Lee, 2012). To accomplish this, a mutated version of the zebrafish ortholog for either the *KRT5*, *KRT14* or *PLEC1* gene would need to be expressed in the basal keratinocyte layer of the zebrafish epidermis. This would recreate the genetic conditions that lead to EBS in humans. This section will justify why using zebrafish *krt5* cDNA was the best direction for this project.

First, I will examine why the zebrafish *krt5* gene was chosen EBS caused by dominant mutations are much more prevalent in the human population than the recessive version and are caused by mutations in the *KRT5* or *KRT14* genes (Coulombe & Lee, 2012). A subset of EBS cases are caused by recessive mutations in the *KRT5, KRT14* and *PLEC1* genes (Nakamura et al., 2005). Only a couple cases of EBS caused by a *PLEC* mutation have been recorded (Nakamura et al., 2005; Natsuga et al., 2010). Due to the rarity of EBS caused by mutations in the *PLEC1* gene and the fact that no cases of EBS caused by dominant mutations in the *PLEC1* gene have been found, mutations in the *PLEC1* gene were not considered to recreate EBS in zebrafish.

While many cases of EBS caused dominant mutations in the *KRT14* gene have been previously described, this gene carries its own set of problems, making it unsuitable for this project. The most serious problem is the fact that the zebrafish ortholog of the human *KRT14*

gene has yet to be positively identified. A search in the non-redundant protein sequence database using the human keratin 14 protein as a search query and restricting the search for protein sequences found in zebrafish revealed no conclusive protein sequence that would match to human K14 (Table 1.7.2.1). The top five zebrafish protein sequence results from the search contained three unidentified keratin proteins that could possibly match human K14: zebrafish keratin 97 (K97), 92 (K92), and 91 (K91). Keratin 91 has the highest percent similarity of amino acid sequence to human K14 amino acid sequence at 75%, while K92 and K97 are both at 71%. However, zebrafish K15 and K17, which are the definitive human protein orthologs of human K15 and K17, also display a high level of amino acid sequence similarity with human K14 (both at 75%). In this case, expression data from ISH is useful to identify the most likely ortholog of human *KRT14*. This is because keratins are expressed in pairs (Pan et al., 2013) and expression of the zebrafish ortholog of human *KRT14* should not significantly differ from that of zebrafish *krt5*. *krt5* is expressed in the basal epidermal layer of the skin across the entire body during early embryo development (Thisse & Thisse, 2004). ISH expression data from *krt97* shows that, by 2 dpf, zebrafish expression domains are restricted to the epidermis layer of the dorsal, nose and median and pectoral fin fold of the zebrafish (Thisse & Thisse, 2004). Since their expression pattern differs significantly early in the development of the embryo, *krt97* is not similar enough to zebrafish *krt5* to be considered its partner (Thisse & Thisse, 2004). *krt91* is the most likely candidate to be the zebrafish ortholog human *KRT14* in this respect: ISH of *KRT91* shows that its expression pattern is the same as keratin 5 up to 5 dpf (Figure 1.7.2.1). However, no expression data of *krt92* could be found and it cannot be ruled out as a potential candidate for the zebrafish ortholog for human *KRT14*.

Table 1.7.2.1. Top 5 hits for zebrafish protein for BLASTP alignment of human keratin 14 amino acid sequence. The first three keratin proteins are proteins that are either novel to zebrafish or their human ortholog has not been identified (keratin 91, 92 and 97). Percent positive includes perfectly align amino acid as well as alignment of amino acids that share similar properties.

Protein	Identities (%)	Similarity (%)
Keratin 97	54%	71%
Keratin 92	55%	71%
Keratin 91	57%	75%
Keratin 15	56%	75%
Keratin 17	57%	76%

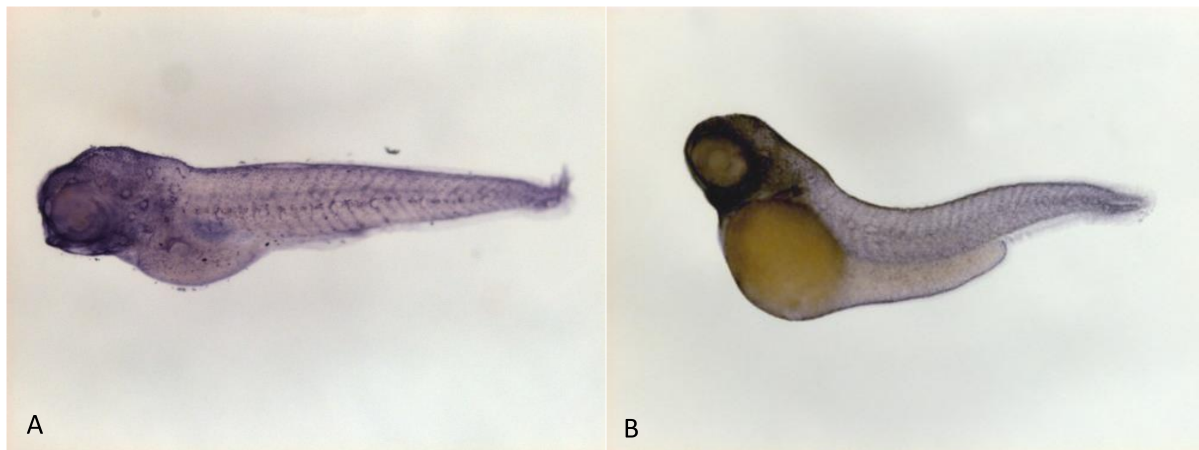


Figure 1.7.2.1. ISH of 3dpf zebrafish embryos using a *krt91* RNA probe (A) and *krt5* RNA probe (B). Areas of purple colouring indicate areas of gene expression. Expression of both genes is found in similar tissue, namely in the basal keratinocyte layer of the epidermis. Adapted from Thisse & Thisse, 2004.

In contrast to the zebrafish ortholog of the human *KRT14* gene, the zebrafish ortholog for the human *KRT5* gene has been identified. Comparing the protein sequence between human and zebrafish K5 using the non-redundant protein sequence database reveals that the percentage of similar amino acid between the orthologs is 86%. Further similarity between the two genes can be seen in their expression patterns: ISH of 5 dpf zebrafish embryo for *krt5* mRNA reveals that expression is found in the epidermal layer of the skin across the entire body (Figure 1.7.2.1). This data is further supported by zebrafish transgenic reporter lines, which demonstrate that the zebrafish 2.3 kb region upstream of the *krt5* gene drives expression in the epidermis (Hu et al., 2010; Wang, 2006). However, it is difficult to discern if expression of *krt5* is found in the basal keratinocyte layer of the two-cell layer epidermis of the embryonic zebrafish due to the small size of the epidermis at this stage. The multiple cell layer epidermis of the adult zebrafish provides more information about the location of expression of *krt5*: ISH of *krt5* on adult zebrafish pectoral fin sections reveal that *krt5* expression is found in the basal keratinocyte layer of the epidermis (Figure 1.7.2.2). This further supports the notion that zebrafish *krt5* plays the same role in zebrafish as it does with humans.

In all, the zebrafish *krt5* gene is a better candidate for mutagenesis than the possible zebrafish ortholog of the human *KRT14.* This is because the zebrafish ortholog of human *KRT*5 shares a higher percent similarity with its human counterpart compared to the most likely zebrafish counterpart of human *KRT14*, *krt91* (86% compared to 75% respectively). In addition, mutations in the *KRT5* gene cause more severe types of EBS phenotype than mutations found in the *KRT14* gene. This suggests that the expression of other type I keratins in the basal keratinocytes compensates for the loss of K14 protein functions, while no such compensations are found for the KRT5 protein mutants (Cao et al., 2001; El Ghalbzouri et al., 2003). Consequently, most

disease models for EBS have been made using *KRT14* mutants (Cao et al., 2001). By creating an EBS disease model using *KRT5*, the role of the KRT5 protein in EBS can be further examined and studied.

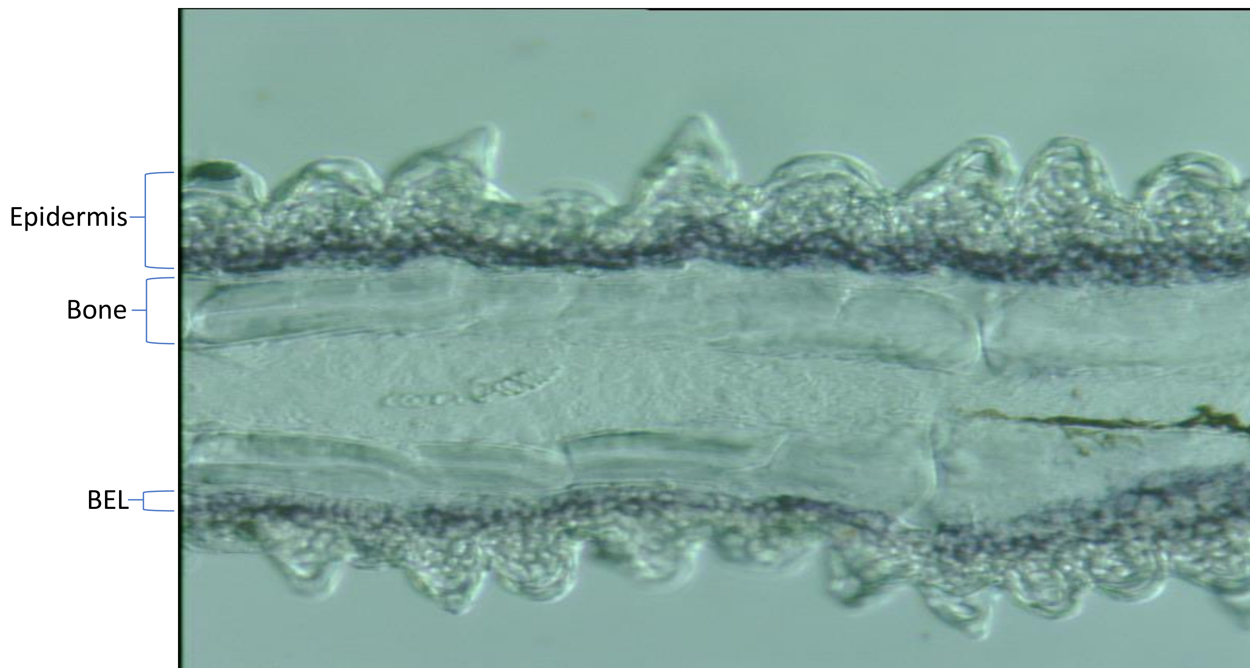


Figure 1.7.2.2. Expression of the *KRT5* gene is found in the basal layer of the skin in zebrafish. *In situ* hybridization was performed on longitudinal cryogenic sections of male adult pectoral fin using zebrafish *krt5* probe to stain the basal epidermal layer (BEL) in purple. The basement membrane, while not visible in this picture, can be found underneath the basal epidermal layer.

1.7.4 Transgenesis was used to express zebrafish *krt5* cDNA in non-essential epithelial tissue

It was decided that transgenesis would be used to recreate EBS in zebrafish. Transgenesis is the process of injecting a DNA construct into an embryo to create a stable transgenic line where the DNA constructs can be passed on to offspring. In this case, the DNA construct would contain a mutated *krt5* cDNA that would be expressed in the epidermis of the zebrafish with the help of regulatory elements. This model is only possible due to the dominant nature of the mutations that lead to EBS, as all endogenous expression of *krt5* would have to be removed if a recessive mutation was chosen instead of a dominant mutation. An added benefit of using transgenesis is that expression of the mutant proteins can be directed in certain tissue by using tissue specific promoters. This is useful as severe mutations found in the *KRT5* gene have been found to be fatal to humans and mice (Kerns et al., 2007; Sathishkumar et al., 2016); driving expression of mutant *KRT5* in non-essential tissue should allow for the creation of stable mutant lines. Non-essential epithelial tissue is tissue that the organisms do not necessarily need to live and produce offspring. Recently, the Akimenko lab found a non-essential tissue specific enhancer, called the *epi* fragment (Lalonde et al., 2016a). The *epi* fragment is an enhancer that is part of the *and1* regulatory elements, which drives expression of the actinodin 1 protein in the basal keratinocyte layer of the median and pectoral fin fold epithelium in the developing zebrafish embryo (Figure 1.7.3.1). The median fin fold is the tissue that will eventually form the tail of the adult zebrafish, while the pectoral fins are a pair of appendages that can be found on either side of the zebrafish and are analogous to the forelimbs of mammals (Lalonde et al., 2016: Figure 1.7.3.1). These tissues are not essential for to survival as stable zebrafish mutants lines without fins have been created (Harris et al., 2008). Since enhancers are unable to drive expression on their own, human beta globin (βG) minimal promoter was used in combination

with the *epi* enhancer to drive expression of zebrafish *krt5* cDNA (Figure 1.7.3.1). The human β globin minimal promoter was chosen for this project as it has been proven effective in driving expression of genes when in conjunction with the *epi* enhancer and among other enhancers (Broyles et al., 2001; Lalonde et al., 2016a).

EGFP cDNA, which codes for green fluorescent protein (GFP), was inserted at the 3'end of the zebrafish *KRT5* cDNA (Figure 1.7.3.1). GFP was used as a fluorescent reporter and allowed for the visualization of transgenic expression under UV light. The *EGFP* cDNA was separated from the mutant *krt5* cDNA by the *2A* cDNA sequence, which codes for the 2A linker peptide (Figure 1.7.3.1). When the transgene is transcribed, a long transcript containing *krt5, 2A* and *EGFP* cDNA sequences is made. During translation, the 2A amino acid sequence will break apart by ribosomal skipping, which leads to the separation of the keratin 5 and GFP proteins (Provost, Rhee, & Leach, 2007). Separation of the two proteins was preferable to expressing a keratin5-EGFP fusion protein, which has been found to affect the integrity and function of proteins in the past (Huang et al., 2014). This might not allow for integration of the mutant protein in the keratin cytoskeleton, leading to the lack of symptoms.

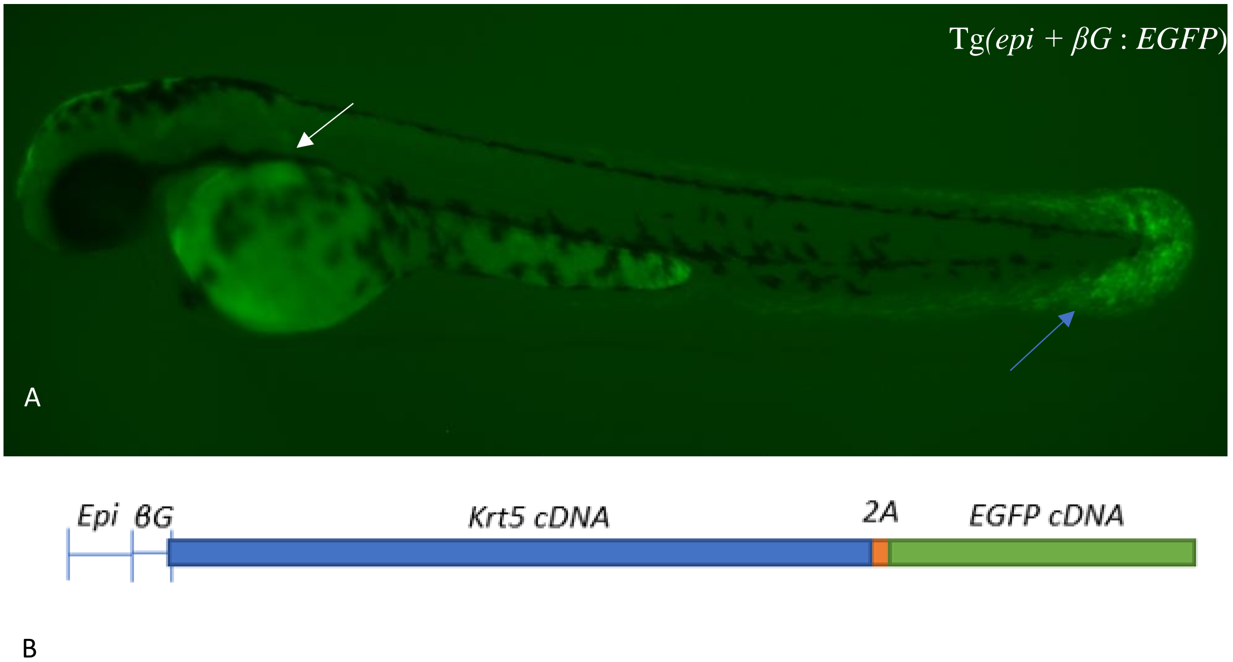


Figure 1.7.3.1. The *epi + βG* enhancer/minimal promoter was chosen to drive expression of mutant *krt5 cDNA*. (A) 2dpf zebrafish embryos containing the *EGFP* cDNA under the control of the *epi* + *βG* regulatory elements. Expression is found in the epithelium of the median fin fold (blue arrow) and the pectoral fins (white arrow). Figure taken from Lalonde 2017. (B) Basic plasmid map of *epi* + *βG*: *krt5-2A-EGFP* in Tol 2 vector.

2. Materials and Methods

2.1 Zebrafish husbandry

All adult zebrafish and embryos were maintained at 28.5°C. Zebrafish were kept in a photoperiod cycle of 14 hours of light followed by 10 hours of dark as described by Westerfield (2000). For breeding purposes, a perforated plastic container was placed inside a tank filled with water. Two female adults and one adult male fish were placed inside this tank and kept separated with a divider for the night. The following morning the water was exchanged for fresh water and the divider was taken out to allow the fish to breed. Embryos would fall through the holes of the plastic container and gather at the bottom of the tank. These embryos were collected and placed in a petri dish containing E3 embryo medium (5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, 0.33mM $MgSO_4$, 0.005% methylene blue) and were incubated at 28.5°C. Between 28 – 40 hours post-fertilization embryos were placed in a 0.003% bleach solution (NaOCl) for 10 minutes. The embryos were taken out of the solution and washed with 0.05% sodium thiosulfate ($Na_2S_2O_3$) solution for 1 minute to neutralize residual bleach. Following neutralization, they were washed twice in 1X E3 embryo medium solution, each 2 minute long. Embryos were placed back into petri dishes containing 1X E3 embryo medium and incubated at 28.5°C.

2.2 Microinjections

Injection solution, containing RNAse free water, the desired construct (final concentration of 200ng/µL), 0.5% phenol red and transposase RNA (final concentration of 50ng/µL) were made fresh the day of injections and kept on ice. Microinjection needles were made by pulling borosilicate glass (dimensions: 1 mm diameter, thickness 0.5 mm, 10 cm length, from Sutter instruments– item no B100-50- 10) in the flaming/brown micropipette puller (from Sutter instruments - model no P-87). Using the micropipette and a gas dependent microinjector, one-cell stage (0-30 minutes after spawning/fertilization) embryos were collected and injected with the injection solution. The preferred location of injection was just outside the cell, in the yolk sack. Injected embryos were incubated overnight and bleached the next day. Primary-injected embryos were then screened for the presence of enhanced green fluorescent protein (GFP) using a UV light attached to a Leica MZ FLIII microscope. The developmental stage at which embryos were screened depended on which regulatory elements were used: embryos injected with *epi + βG* constructs were screened at 2-3 dpf while embryos injected with 2.3kb region upstream of *krt5* (also known as *keratin5p*) were screened at 1 dpf.

2.3 Screening

GFP positive embryos obtained from microinjections were raised until they reached sexual maturity. Primary-injected fish were then mated with either other primary-injected fish or WT fish depending on gender ratios. Embryos were collected, bleached and screened for presence of EGFP fluorescence using the UV light on the Leica microscope. Transgenic embryos were raised in the same fashion as the primary-injected embryos.

2.4 Site-directed mutagenesis

To create the *krt5Δ1A* and *krt5Δ2B* deletion mutants, site-directed mutagenesis was performed using the Q5® Site-Directed Mutagenesis kit and protocol (New England BioLabs) on the tol2 (*epi* + βG: WT *krt5 – 2A – EGFP) tol2* plasmid. This involves amplification of all the plasmid except for the desired regions to be deleted through PCR. The primers used to create the *krt5Δ1A* mutant were the forward primer 5' GACAAGGTGCGCTTCCTGGAACA 3' and reverse primer 5' GACAACTTGGATGTTGGGGTCGATC 3'. For the *krt5Δ2B* mutant, the forward primer 5' TCCAGAATTGCATCTGGTGGCAATACTG 3' and the reverse primer 5' AATGTCCAAGGCCAGTTTGACGTTCA 3' were used. The ends of the linearized plasmid were then ligated together to form the final circular plasmid. After transformation and multiplication of the plasmid in bacterial vectors the plasmid was purified and ready to be injected. The plasmid was also sequenced to ensure that the DNA was correctly added to or deleted from the plasmid.

2.5 Cloning

Cloning and subcloning of the constructs were done according to the protocols established by Sambrook, J., & Russel (2001). The e*pi+βG* fragment in *tol2* vector was provided by the Akimenko lab. Also provided by the Akimenko lab was the wild type *krt5* cDNA along with the *E477K* and *I161S* mutant *krt5* cDNA. The *2A-EGFP* cDNA fragment in *pdrive* was provided by the lab of Dr. M. Ekker, University of Ottawa, while the *P2A-EGFP* cDNA in *pdrive* was provided by the Akimenko lab. The 2.3kb region upstream of the *krt5* gene was amplified from the *keratin5p:EGFP* fragment in pcmini vector provided by the laboratory of Dr. Lee from the North Carolina Central University. The 2.3kb upstream fragment was then cloned

into *pdrive* and used to replace the e*pi+βG* fragment in the e*pi*+βG: *Krt5-2A-EGFP*, e*pi*+βG: *I161S Krt5-2A-EGFP*, and e*pi*+βG: Δ1A *Krt5-2A-EGFP* constructs.

2.6 Immunohistochemistry

3-5 dpf zebrafish embryos were fixed in Dent's reagent (20% DMSO, 80% methanol) and left at 4°C overnight. After washing the embryos 3 times in 1X PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.1% tween 20, adjusted to a final pH of 7.4) for 5 minutes each, the embryos were placed in an acetone solution at -20°C for 20 minutes. After two 10-minute PBST washes, the embryos were placed in blocking solution (10% bovine serum albumin, 10% calf serum in 1X PBST) for 1-3 hours. The embryos were then placed in staining solution (1X PBST) containing a 1:10 dilution of anti-Cytokeratin antibody Ks pan 1–8 (Progen Biotechnik) at 4°C overnight. They were then washed 4 times for 15 minutes with 1X PBST. Embryos were placed in staining solution (1X PBST) containing a 1:500 dilution of the secondary antibody Alexa Fluor® 594 goat, anti-mouse IgG (Life technologies) for three hours at room temperature. After another four washes in 1X PBST for 15 minutes each, Zeiss LSM 510/AxioVert 200 confocal and zen2009 software were used to visualize and take pictures of the embryos. The software ImageJ and Adobe Photoshop were then used to process the images.

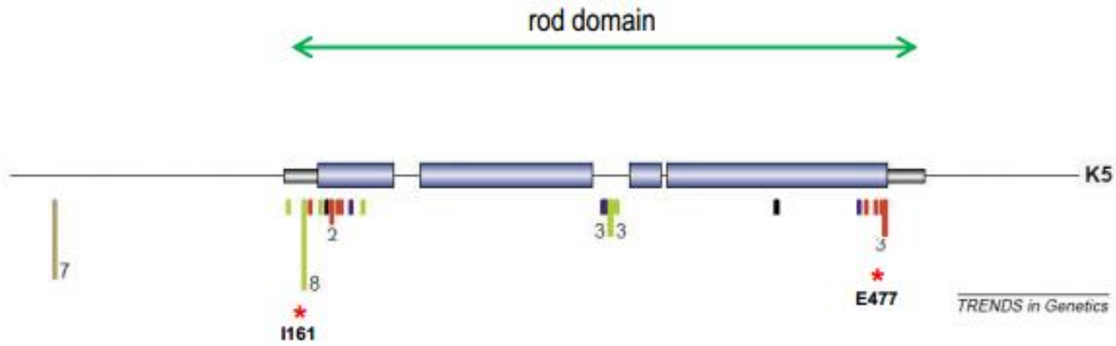
3. Results

3.1 Selection of the amino acid position to be mutated in zebrafish keratin 5 protein.

Three conditions were met to find suitable *krt5* point mutations to recreate EBS in zebrafish: 1) they must create EBS in humans, 2) they must be inherited in an autosomal dominant fashion, and 3) the positions are conserved in zebrafish keratin 5 protein so that the same mutation can be recreated. Another favorable criterion is that the same mutation was found in multiple unrelated families as it would indicate that the position is critical to the proper functioning of the keratin 5 protein. A search on http://www.interfil.org/, a database on human intermediate filaments and their associated disease, revealed many different mutations that satisfied the first two conditions outlined. Out of the many different possible positions, the amino acid positions I161 and E477 were chosen due to their prevalence in creating EBS in unrelated families (Figure 3.1.1). The I161 position is found in the helix initiation motif while the E477 position is found in the helix termination motif (Figure 3.1.1). The helix initiation and termination motifs play crucial roles in the polymerization of the keratin heterodimers to make the 10nm filament (Sprecher et al., 2001; Wilson et al., 1992). The most prevalent mutation found in the I161 position is the I161S mutation, which is associated with EBS-WC, a mild localized version of EBS (Schwarz et al., 2016). As for the E477 position, the E477K mutation is the most prevalent and it is associated with EBS-DM, a severe generalized version of the disease (Ehrlich et al., 1995; Pfendner et al., 2005).

In addition, these positions also satisfy the third criteria, as alignment of zebrafish and human keratin 5 amino acid sequences reveal that these positions are conserved in zebrafish (Figure 3.1.1). This suggests that generating identical mutations found in human patients in zebrafish keratin 5 protein could recreate the symptoms of EBS.

A



B

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    afish-Krt5      GGAGFGGGFG-PGG-VVP---ITAVTVNQNLLAPLNLEIDPNIQVVRTQEKEQIKTLNNR 167
Human-KRT5          GGAGFGGGFGGPGFPVCPPGGIQEVTVNQSLLTPLNLQIDPSIQRVRTEEREQIKTLNNK 178
                    *********** **   * *   *  *****.**:****:***.** ***:*:********:

Zebrafish-Krt5      FASFIDKVRFLEQQNKVLETKWSLLQEQ--TTTRSNIDAMFEAYIANLRRQLDGLGNEKM 225
Human-KRT5          FASFIDKVRFLEQQNKVLDTKWTLLQEQGTKTVRQNLEPLFEQYINNLRRQLDSIVGERG 238
                    ******************:***:*****   .*.*.*:::.:** ** *******.:  .*:

Zebrafish-Krt5      KLEGELKNMQNLVEDFKNKYEDEINKRAAVENEFVLLKKDVDAAYMNKVELEAKVDSLQD 285
Human-KRT5          RLDSELRNMQDLVEDFKNKYEDEINKRTTAENEFVMLKKDVDAAYMNKVELEAKVDALMD 298
                    :*:.**:***:****************::.*****:*********************:* *

Zebrafish-Krt5      EINFLRAIFEEELRELQSQIKDTSVVVEMDNSRNLDMDAIVAEVRAQYEDIANRSRAEAE 345
Human-KRT5          EINFMKMFFDAELSQMQTHVSDTSVVLSMDNNRNLDLDSIIAEVKAQYEEIANRSRTEAE 358
                    ****:: :*: ** ::*:::.*****:.***.****:*:*:***:****:******:***

Zebrafish-Krt5      SWYKQKFEEMQSSAGKYGDDLRNTKAEIADLNRMISRLQNEIEAVKGQRANLEAQIAEAE 405
Human-KRT5          SWYQTKYEELQQTAGRHGDDLRNTKHEISEMNRMIQRLRAEIDNVKKQCANLQNAIADAE 418
                    ***: *:**:*.:**::********* **:::****.**: **: ** * ***:   **:**

Zebrafish-Krt5      ERGELAVKDAKLRIKDLEDALQRAKQDMARQVREYQELMNVKLALDIEIATYRKLLEGEE 465
Human-KRT5          QRGELALKDARNKLAELEEALQKAKQDMARLLREYQELMNTKLALDVEIATYRKLLEGEE 478
                    :*****:***: :: :**:***:******* :********.*****:**************

Zebrafish-Krt5      SRIASGGN-TATIHIQESSSSSG-GGGGGFGYGGGSGYGG--GSGFGGGSG--YGGGSGF 519
Human-KRT5          CRLSGEGVGPVNISVVTSSVSSGYGSGSGYGGGLGGGLGGGLGGGLAGGSSGSYYSSSSG 538
                    .*::. *   ...* :   ** *** *.*.*:* * *.* **   *.*:.***.  * ..*.
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Figure 3.1.1. The I161 and the E477 positions were chosen to be mutated. (A) Frequency and position of published mutations found in keratin 5 amino acid sequence. Coloured bars represent the different disease associated with mutations: Green; EBS – Weber Cockayne, Red; EBS – Dowling Meara, Blue; EBS Knöber, and black; homozygous and knockout mutations. The green arrow represents the rod domain, a highly conserved sequenced that is important in heterodimer formation. The red asterisks represent point mutations that were found in high frequency and were considered for this project. Adapted from Porter & Lane, (2003). (B) Alignment of human and zebrafish keratin 5 amino acid sequence. The symbol beneath each position indicates the degree of similarity between each aligned amino acid (star = exact match, semi-colon = high degree of similarity, period = low degree of similarity, no symbol = no degree of similarity). The area shaded in grey indicates the central rod domain which demonstrates an 83.2% homology between the species. The highlighted amino acids position was mutated in zebrafish *krt5* cDNA to recreate EBS.

Through site-directed mutagenesis on zebrafish *krt5* cDNA, the Akimenko lab was successful in generating the I161S and E477K mutant zebrafish *krt5* cDNAs. The mutant *krt5* cDNAs and wild type *krt5* cDNA were placed under the control of the e*pi* + βG regulatory elements. The *epi* fragment is an enhancer that drives expression in the median and pectoral fin folds, while the *βG* (human β globin) region acts as a minimal promoter, which works in conjunction with the *epi* enhancer to drive expression of the *krt5* cDNA (Lalonde et al., 2016). To detect transgene expression, the *2A-EGFP* cDNA sequence was inserted at the 3' end of the *krt5* cDNA through cloning. Transcription of the transgene will result in an mRNA containing the *krt5*, *2A* and *EGFP* sequences. During translation, the 2A peptide undergoes ribosomal skipping, which breaks the 2A peptide into two pieces (Provost et al., 2007). This results in separate keratin 5 and GFP protein as the 2A peptide is found between these two protein sequences. GFP was used as a fluorescent reporter used to screen zebrafish embryos for transgene expression (Gong et al., 2002).

These constructs were injected into wild type one-cell stage zebrafish. At 3 dpf, the injected zebrafish embryos screened for fluorescence (Table 3.1.1). Mosaic GFP expression was detected in the median and pectoral fin folds of injected embryos indicating transgene expression (Figure 3.1.3.). Transgene mosaicism in primary injected zebrafish embryo has been widely documented and was expected (Ung et al 2015; Urasaki et al., 2008). While zebrafish embryos injected with the mutant *krt5* cDNA construct did demonstrate blistering (Figure 3.1.3), it was seen in only a fraction of GFP+ embryos (Table 3.1.1). The rate of GFP+ fish with skin defects was slightly higher when mutant *krt5* construct was injected compared to when WT *krt5* plasmid was injected. Severity of the skin defects between embryos injected with the same constructs varied greatly, and observation of the blisters indicated that skin defects in the embryos injected

with the E477K mutant construct were no more severe than embryos injected with the I161S mutant construct. This was not expected as the E477K mutation in humans creates a much more severe type of EBS then the I161S mutation.

Table 3.1.1. Results of transient expression of the transgene in injected embryos. One-cell stage embryos were injected with 100ng/μl of plasmid. Embryos were analyzed between 2 and 4 days post fertilization for GFP and fin defects. Skin defects included significant damage to the skin or the formation of blisters in the median fin folds.

	WT krt5	*E477K krt5*	*I161S krt5*
GFP+ fish/ total number of fish injected (% of GFP+ fish)	25/90 (27.8%)	44/211 (20.9%)	125/264 (47.3%)
GFP+ fish with skin defects	4/25 (16.0%)	11/44 (25.0 %)	26/125 (20.8%)

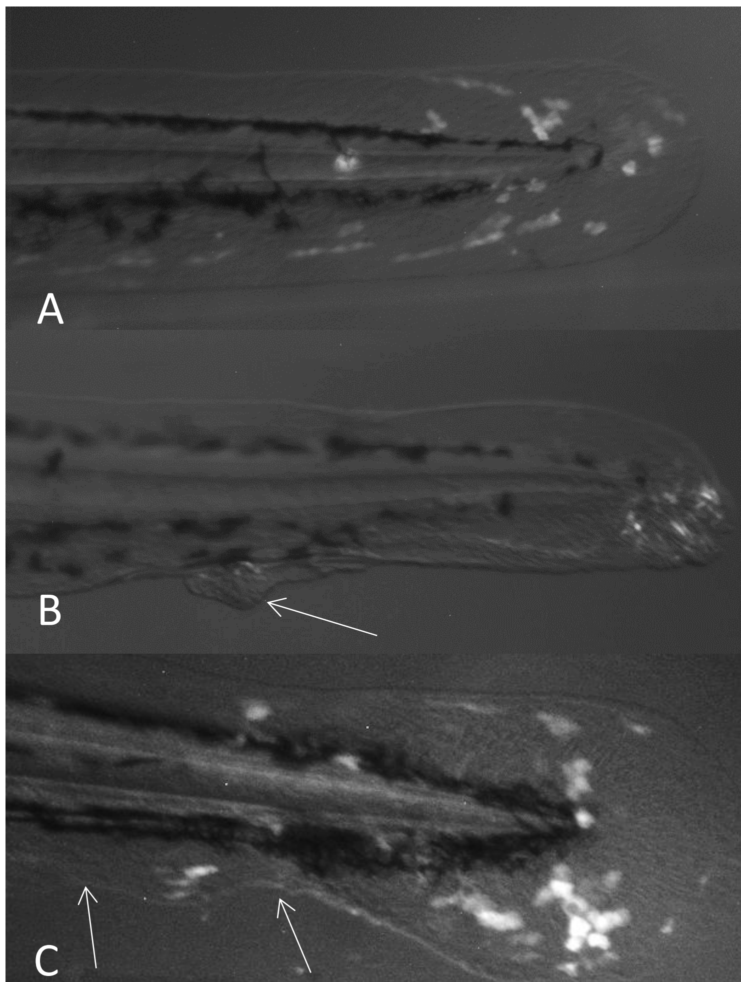

Figure 3.1.3. Embryos injected with the mutant keratin constructs displayed blisters. Photos of the median fin fold of 2-4 dpf primary injected embryos injected with (A) wild type *krt5*, (B) *krt5 I161S*, and (C) *krt5 E477K* cDNA constructs under the control of *epi* + *βG* regulatory elements observed using a fluorescence microscope. White cells indicate areas of GFP expression. Arrows in (B) and (C) highlighted areas of tissue damage in median fin fold. (B) Has a clearly defined blister.

Primary-injected zebrafish embryos who were GFP+ were raised until adulthood and screened for transmission of the transgene. Multiple transgenic lines were obtained for both mutants and wild type constructs: *n=2* for Tg(*Epi*+ βG: *E477K krt5-2A-EGFP), n*=3 for *Tg(Epi+βG: I161S krt5-2A-EGFP)* , and *n=2* for *Tg(Epi+βG: WT krt5-2A-EGFP*). Unlike primary-injected embryos, transgenic lines do not exhibit mosaicism and the transgene was present in all the cells of the embryo (Figure 3.1.4). GFP expression in the transgenic lines was seen in the median and pectoral fin fold and was comparable to *Tg(Epi+βG: EGFP*). While GFP expression indicated the expression of mutant *krt5* in all mutant transgenic lines, no blistering or skin defect was found in the transgenic lines despite analysing 50-200 embryos for each line (Figure 3.1.4).

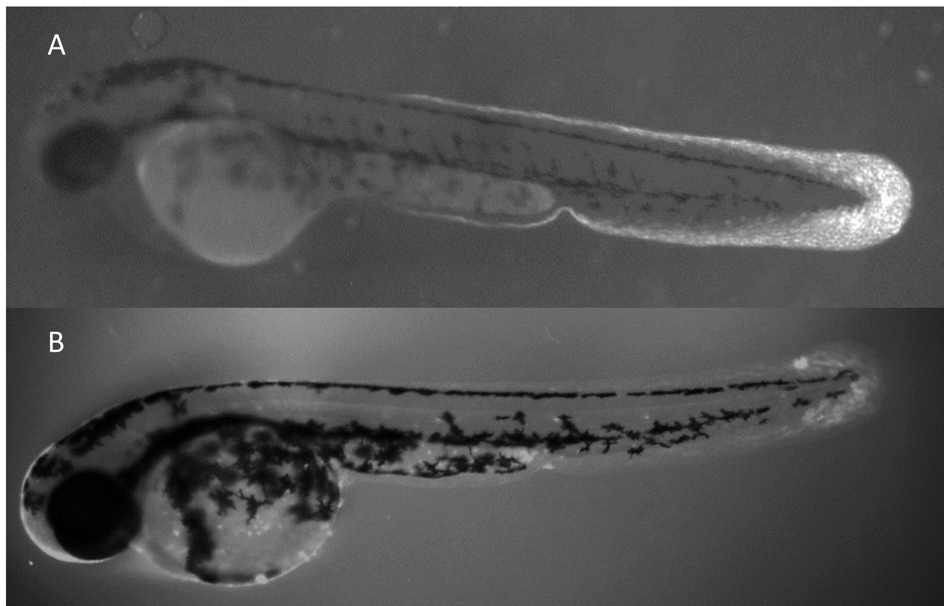

Figure 3.1.4. Mutant I161S and E477K *krt5* transgenic lines. (A) Tg(*epi*+ βG: *krt5 I161S-2A-EGFP)* at 2dpf. Strong GFP expression is found in the median fin fold but no EBS symptoms were present in the transgenic line. (B) Tg(*epi*+ βG: *krt5 E477K-2A-EGFP)* at 2dpf also did not show any EBS-like symptoms.

3.2 Environmental Stress experiments

Environmental factors play a large role in the development of the symptoms of EBS (McGrath et al., 1992; Morley et al., 1995). The two most important environment stressors that create the symptoms of EBS are friction and elevated temperatures (McGrath et al., 1992; Russell et al., 2004; Toivola et al., 2010). These stressors were recreated in the zebrafish mutant transgenic lines in the hopes to induce symptoms that are EBS-like.

To recreate frictional stress, heated semi-liquid agar was poured into a petri dish. Sand was poured on the solidifying agar to create a rough surface on the surface of the agar. Embryo media was poured into the petri dish to form a shallow layer of water allowing for embryos to swim only near the surface of the sand-embedded agar. 3-6 dpf E477K and I161S mutant and WT embryos zebrafish embryos were placed on the agar plate and swam for varying amount of times. To induce further frictional stress, the petri dishes were placed on a shaker and shaken at varying speeds and for varying amounts of time (1 hour to 18 hours). To eliminate the possibility that different agar plates might bring about different results due to differences in the quantity and distribution of sand, an equal amount of non-mutant WT controls and mutant fish were placed in the same agar dish ($n>10$ for WT and both mutant zebrafish lines). Results for this experiment were inconclusive: the shaking and the shallow depth of the embryo media caused the zebrafish embryos to develop blisters across their bodies. However, there was no discernable difference between the wild type (WT) zebrafish and the mutant transgenic lines in the severity or frequency of blistering. In addition, this test was also performed on zebrafish under the influence of a muscle relaxer, tricaine. This was done to prevent the zebrafish embryos from swimming away from the sand-embedded agar surface. Tricaine did not seem to make a difference in the frequency and severity of the blistering for both mutant and WT zebrafishes ($n>10$ for each line).

To recreate environmental stress caused by elevated temperature, 3-6 dpf WT and mutant zebrafish embryos were heated to 37°C for an hour in an incubator. The method used to recreate stress caused by elevated temperatures was previously described to activate the production of HSP (heat shock proteins) in zebrafish (Shoji & Sato-Maeda, 2008). The production of HSP is thought to be a contributing factor in the exasperation of EBS symptoms of affected individuals in warmer climates (Toivola et al., 2010). After removing the embryos from the incubator, they were cooled to room temperature for an hour to recover. This was repeated up to three times. Both non-mutant WT and mutant transgenic fish were able to survive this experiment with no visible impairments (n>50). However, no skin defects were detected in the WT, I161S or E477K zebrafish embryos when inspected under a microscope.

A possible problem in producing an ineffectual cytoskeleton to recreate EBS is the difference in temporal expression between the endogenous WT *krt*5 and the mutant *krt5* cDNA under the influence of the *epi* enhancer. Endogenous zebrafish WT *krt5* expression starts as early as 5.25 hours post fertilization (Hu et al., 2010; Thisse & Thisse, 2004), while the *epi* enhancer activity starts at 24 hours post fertilization (Lalonde et al., 2016). It is possible that the mutant keratins were unable to disrupt cytoskeletal function because an insufficient amount of mutant proteins were able to integrate within the already formed keratin cytoskeleton. Taking advantage of zebrafish larvae's ability to regenerate their median fin fold after amputation, an experiment was devised for simultaneous onset of expression of mutant and endogenous keratin 5 proteins inside developing epithelial cells. As the expression of *and1* is an important step for regeneration of the median fin, the *epi* enhancer originating from the *and1* gene will activate soon after amputation (Northorp, unpublished and Figure 3.2.1). When activated, the *epi* enhancer will drive expression of the *krt5* cDNA in the regenerating fin fold. The expression of the *krt5* cDNA

will coincide with the expression of endogenous WT *krt5,* since WT *krt5* expression is also activated shortly after amputation (Padhi et al., 2004).

Tg(epi+βG:E477K krt5-2A-EGFP) mutant zebrafish embryos were first chosen for this experiment because their mutation causes a more severe type of EBS than the I161S mutation in humans and therefore should create an more easily identifiable phenotype. The most posterior part of the median fin fold of mutant *Tg(epi+βG:E477K krt5-2A-EGFP)* and wild type siblings were cut at 48 hours post fertilization and allowed to regenerate until the median fin fold fully healed. Fluorescence at the distal tip of the developing fin in *Tg(epi+βG:E477K krt5-2A-EGFP)* zebrafish embryos indicate that mutant keratin proteins were produced during regeneration (Figure 3.2.1). However, the fins in the mutant fish regrew normally at a pace comparable to the WT embryos. They also did not present EBS-like symptoms (Figure 3.2.1). This experiment was also performed with the I161S (*n=3)* mutant zebrafish line but results did not differ from the E477K mutants (results not shown).

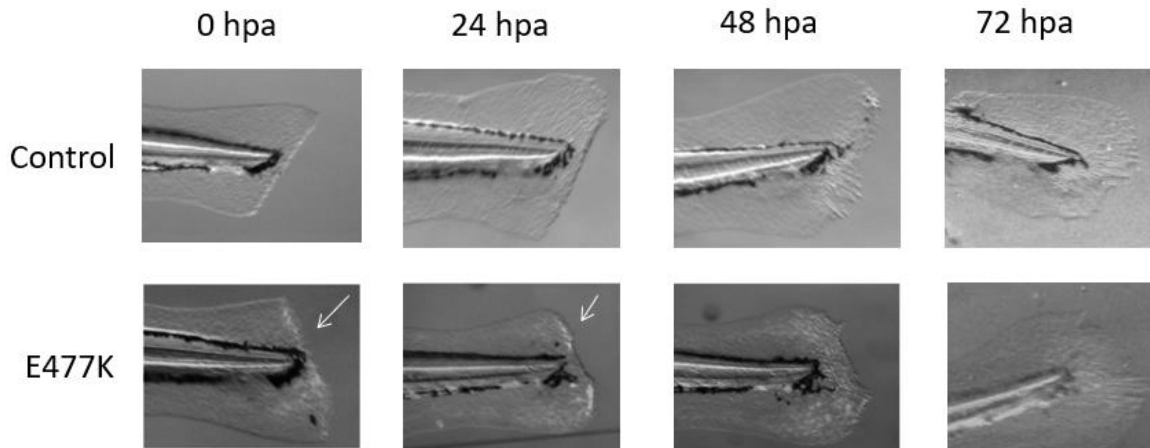

Figure 3.2.1. The median fin folds of both WT and E477K mutant fish regrew normally. The median fin fold of Tg(*epi+ BG*: *krt5 E477K -2A- EGFP)* (*n=3)* and wild type sibling (*n=3)* were cut at 48 hours post fertilization and allowed to regenerate for 72 hours post amputation (hpa). White arrows point to *GFP* expression at the site of injury, indicating that mutant keratin 5 proteins were being expressed. This most likely means that mutant keratin 5 proteins were being incorporated during the creation of new keratin cytoskeletal elements. However, no blistering or skin defects were observed.

3.3 Deletions of the helix initiation motifs and helix termination motifs

The failure to recreate EBS-like symptoms in zebrafish could be attributed to the fact that the positions chosen (I161 and E477) might not play an essential role in the assembly of the 10 nm filament in zebrafish. To eliminate the possibility that the mutations were not significant in the zebrafish, two new mutations were made. These deletion mutations targeted the helix initiation motif (him) and helix termination motif (htm), which are protein subdomains essential in the polymerization of the keratin heterodimers to form the 10nm keratin filament (Gu & Coulombe, 2005; Rugg et al., 1999). The him and htm are found in the coil 1A and end of coil 2B protein domains, respectively (Figure 3.3.1). The zebrafish helix initiation motif shares an 84.2% amino acid identity with its human K5 counterpart. Furthermore, the zebrafish helix termination domain shares 100% amino acid identity with its human counterpart (Figure 3.3.1). This indicates that these domains most likely function similarly to their human counterparts. Without these domains, the mutant keratin 5 protein should still be able to form a heterodimer with the zebrafish equivalent of *krt*14 but would be unable to polymerize into the 10nm keratin filament, preventing the formation of an effectual cytoskeleton.

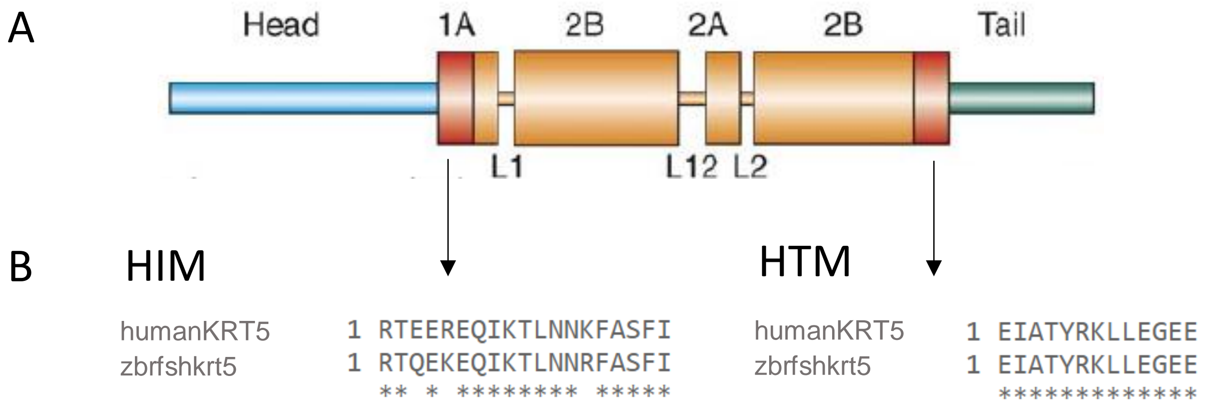

Figure 3.3.1. The helix initiation motif (HIM) and the helix termination motif (HTM) were deleted in the new mutant keratin 5 constructs. (A) Schematic of the proteins domains found in the keratin proteins. Orange-coloured areas shaded in yellow indicates the α – helical rod, a highly conserved region essential in the formation of the coiled-coil keratin heterodimers. Indicated in red are the helix initiation motif (HIM) and the helix termination motif (HTM) that play an important role in the polymerization of the keratin heterodimers into keratin filaments. These regions were deleted in three new mutant *krt* cDNA called the *krt5Δ1A*, *krt5Δ2B* and *krt5Δ1AΔ2B* and were placed under the control of the *epi + BG* regulatory elements to drive expression. Adapted from Gu & Coulombe, (2007). (B) Alignment of the human and zebrafish keratin 5 helix initiation motif (HIM) and helix termination motif (HTM) amino acid sequences. The zebrafish HIM shares an 84.2% identity with its human counterpart while the HTM shares a 100% identity between the two species.

Three different mutant *krt5* cDNAs were made through site-directed mutagenesis: *krt5Δ1a,* where 19 amino acids corresponding to the helix initiation motif in the coil 1A protein domain were deleted*; krt5Δ2b*, where the 13 amino acids corresponding to the helix termination motif in the coil 2B protein domain were deleted; and the *krt5Δ1a Δ2b* mutant cDNA where both the HTM and HIM were deleted (Figure 3.3.1). These mutated *krt5* cDNA were placed in the same basic constructs as the other *krt5* mutants (Figure 3.3.3). They were then injected into one-cell stage zebrafish embryos. Mosaic expression of GFP was seen in the median and pectoral fin folds. In terms of the % of GFP+ embryos with skin defects, the deletion constructs closely matched the results seen in the I161S and E477K injections (Table 3.3.1). Again, the severity of the blisters greatly varied from embryo to embryo. In addition, the severity of the blisters found in the deletion constructs (*krt5Δ1a, krt5Δ2b* and *krt5Δ1a Δ2b)* were not visibly more severe than the blisters found in the I161S and E477K constructs.

Table 3.3.1. Results of transient expression of the transgene in injected embryos for the deletion constructs. One-cell stage embryos were injected with 100ng/µl of plasmid. Embryos were analyzed between 2 and 4 days post fertilization for fin defects and blisters.

Deletion	*krt5Δ2b*	*krt5Δ1a*	*krt5Δ1a Δ2b*
GFP+ fish/ total number of fish injected (% of GFP+ fish)	22/80 (27.5%)	15/48 (31.5%)	27/86 (31.3%)
% of GFP+ fish with skin defects	7/22 (31.8%)	3/15 (21.4%)	4/27 (14.8%)

GFP+ embryos were grown until sexual maturity and screened for transmission of the transgene. Two lines of mutant zebrafish for Tg(*epi* + βG: *krt5Δ2b-2A-EGFP)* were obtained. The mutant zebrafish were bred with WT zebrafish to produce mutant embryos. 50-100 embryos were produced by each of these mutant lines but did not demonstrate EBS-like symptoms. (Figure 3.3.2). No transgenic lines could be obtained for the *krt5Δ1a* and *krt5Δ1a Δ2b* construct*s*. However, the similar injection results and the fact that no EBS-like symptoms were present in two transgenic lines for Tg(*epi* + βG: *krt5Δ2b-2A-EGFP)* suggests that another factor is responsible for the lack of skin defects.

Figure 3.3.3 summarizes the constructs that have been made, the number of transgenic lines obtained, and the number of primary injected fish raised to adulthood and screened for transgenesis for each construct.

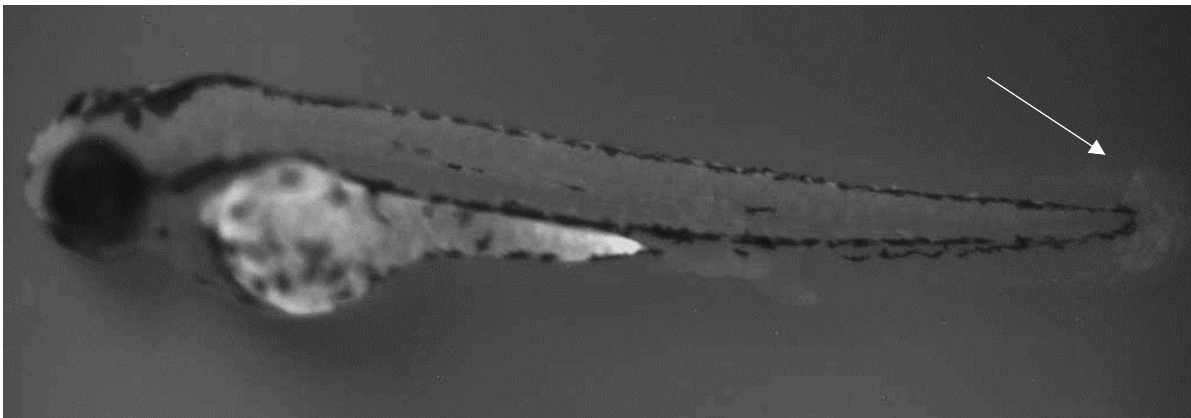

Figure 3.3.2. Tg(*epi*+ BG: *krt5Δ2b 2A EGFP)* at 3dpf. The median fin fold shows very weak GFP expression (white arrow). No blistering was found on any of the mutant embryos.

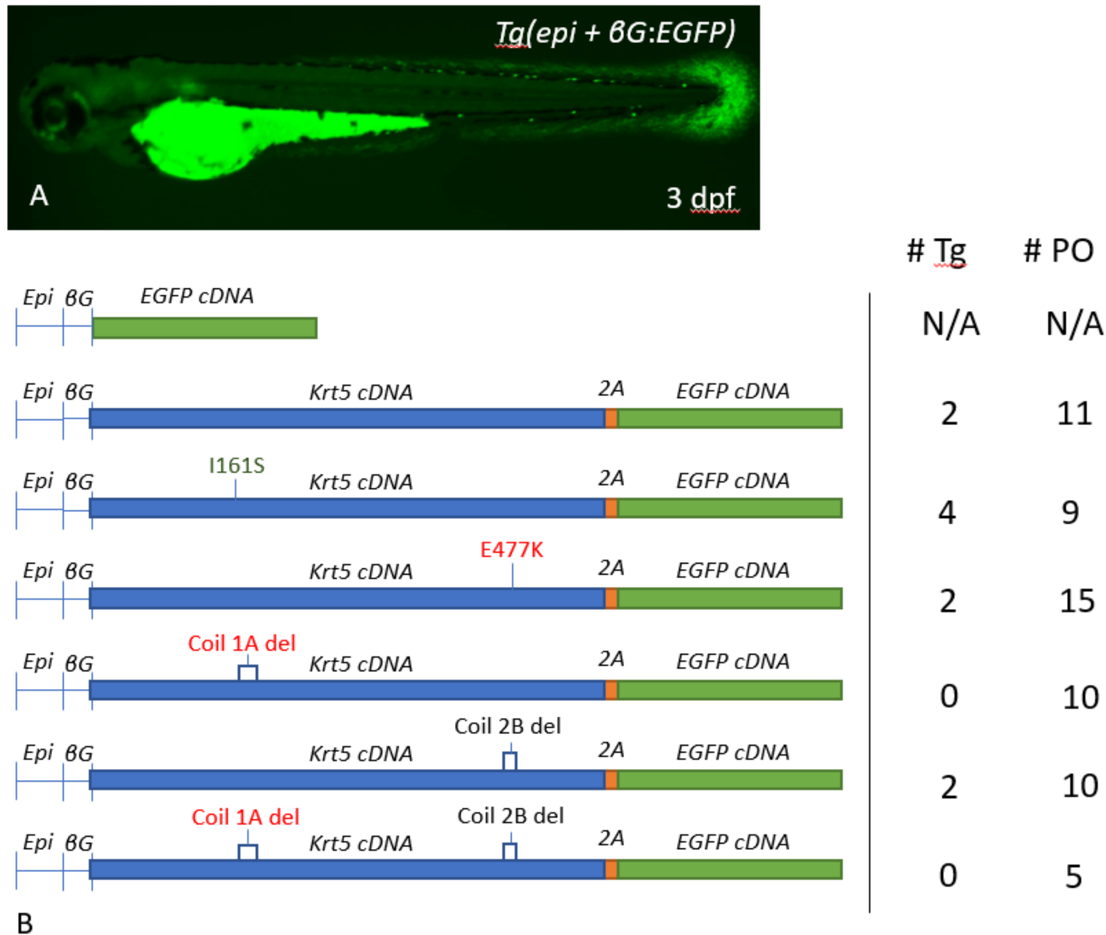

Figure 3.3.3. The *epi* enhancer + human βG minimal promoter was used to drive expression of mutant *krt5* and *EGFP* cDNA in non-essential epithelial tissue. (A) GFP expression in the fin fold epithelium in the reporter transgenic line driven by the *epi* enhancer region of the *and1* gene with the human βG minimum promoter at 3 dpf (Lalonde et al., 2016). (B) Schematic representation of the various mutant *krt5* cDNA constructs along with the relative location and size of the mutation inside the *krt5* cDNA. These mutations include the I161S and E477K single nucleotide substitution, and the *krt5Δ1A*, *krt5Δ2B* and *krt5Δ1AΔ2B* deletions. During translation of the transgene, the keratin 5 and GFP proteins are separated by a sequence coding for the 2A peptide. The numbers on the right represent the number of transgenic lines (# Tg) obtained and analyzed along with the number of zebrafish embryos injected with the construct who were screened for transmission of the transgene (# PO). At least 100 embryos were screened for each primary injected fish before they were considered negative.

3.4 Driving mutant *krt5* cDNA expression with the 2.3kb genomic sequence upstream of the zebrafish *krt5* gene

Mutant transgenic lines using the *epi+βG* regulatory elements to drive expression of mutant *krt5* cDNA were unable to recreate EBS-like symptoms in zebrafish. One potential reason for this failure could be that the mutant keratin 5 proteins were unable to integrate themselves inside the keratin cytoskeleton due to the delay in expression of mutant *krt5* cDNA. As mentioned in the experimental design section, the *epi+βG* regulatory elements drives expression at 72 hours days post fertilization, while endogenous *krt5* onset of expression occurs at 5.25 hours post fertilization (Hu et al., 2010). To test whether simultaneous expression of mutant and endogenous *krt5* could recreate skin defects, the 2.3kb genomic sequence upstream of the zebrafish *krt5* gene (called *keratin5p* henceforth) was used to drive expression of mutant *krt5* cDNA. This genomic fragment contains the promoter of the *krt5* gene and has previously shown to drive reporter expression in the epidermal of the zebrafish embryo (Hu et al., 2010). Unlike the *epi+βG* regulatory elements, the *keratin5p* region drives expression in all of the zebrafish's epidermal layer and is not restricted to the epidermis of the median and pectoral fin folds like the *epi+βG* regulatory elements. A greater proportion of the epidermis would therefore be affected by the possible cytoskeletal defects, increasing the chances of seeing major skin defects. This is also what differentiates this experiment from the previous experiment where the median fin folds of E477K mutant embryos were cut and allowed to regenerate.

The mutant keratins *krt5 I161S* and *krt5Δ1a,* as well as a WT *krt5* cDNA were cloned into transgenic constructs under the control of the *keratin5p*. Mice with severe case of EBS die shortly after birth (Batta et al., 2000). Since the *kerating5p* region drives expression in essential epithelial tissue, this could also happen to the mutant zebrafish. The mutant keratins were chosen with this in mind: *krt5 I161S* creates a mild EBS phenotype in humans (Ehrlich et

al., 1995), therefore embryos with this mutation may also be affected by a mild form of EBS and possibly survive. This would allow for the creation of transgenic mutant lines and analysis of skin defects. The *krt5Δ1a* mutant was chosen since it causes severe cases of EBS in human patients (Rugg et al., 1999). This mutant was chosen to examine if a severe EBS phenotype could be recreated. Wild type *krt5* was chosen to be a control. Like the previous constructs, *2A EGFP* cDNA was added to the 3' end of the mutant *krt5* cDNA for screening purposes. These constructs were injected into one-cell stage zebrafish embryos (Figure 3.4.2).

The percentage of injected embryos that expressed GFP was considerably higher for the *kerating5p* constructs then the *epi+βG* constructs (Table 3.4.1). However, embryos injected with the *keratin5p*: *krt5* WT and keratin 5p: *krt5* I161S constructs demonstrated GFP in only a small fraction of the epidermal tissue (Figure 3.4.2). In some cases, only 1 to 10 cells in the epidermis would fluoresce. Only embryos with more than 15 – 20 GFP positive cells were considered when counting GFP positive embryos for skin defects (Table 3.4.1). While it is currently unclear how many basal keratinocytes need to express mutant keratin to cause cytoskeletal collapse and create blisters, it is unlikely that a single isolated mutant keratinocyte could form a blister. Therefore, any blisters found in primary injected embryos with only a few GFP+ cells would likely be the result of another factor and not the expression of mutant keratin. Furthermore, only embryos with more then 15–20 GFP positive cells were grown to adulthood and screened for transmission of the transgene. This is because, in general, the less uniform the transgene mosaicism is, the less likely it is for a stable transgenic line to be obtained (Clark et al., 2011). To increase the number of GFP+ cells, injection mixes for *keratin5p*: *krt5* WT-2A-*EGFP* and *keratin5p*: *krt5* I161S-2A-*EGFP* were cleaned and purified with a DNA purification kit. However, no improvements in the proportion of the epidermis that was GFP positive could be seen.

In contrast, most of the epidermis in zebrafish embryos injected with *keratin5p*: *EGFP* and *keratin5p*: *krt5 Δ1A* demonstrated GFP expression (Figure 3.4.2). Embryos injected with the *keratin5p*: *krt5* I161S-2A-*EGFP* and the *keratin5p*: *krt5Δ1A*-2A-*EGFP* constructs did show some defects never observed with the other constructs (Figure 3.4.1 and Figure 3.4.2), albeit at a lower defect rate than embryos injected with the e*pi* + βG constructs (Table 3.4.1). While most symptoms in the e*pi+βG* constructs were focused on the tail, a blister was found near the head of a *keratin5p*: *Krt5 I161S-2A-EGFP* primary injected fish and on the yolk sac of an embryo injected with *keratin5p*: *Krt5Δ1a-2A-EGFP* (Figure 3.4.1.). In addition, these blisters co-localized with expression of GFP, which indicates expression of mutant *krt5*. However, GFP expression also indicates that the keratinocytes may be alive, which contradicts what occurs in humans and mice where the death of basal keratinocytes causes the blisters. A possible explanation for this is that GFP could have persisted in the surrounding tissue after the mutant cell lysed. It was expected that the overall number of blisters per embryo and percentage of GFP+ injected embryos with blisters would be greater for the *keratin5p* then the *Epi+βG* constructs. This is due to the relative increase of tissue possibly affected by mutant *krt5* expression during the initial formation of the keratin cytoskeleton. However, the number of GFP+ primary injected embryos with blisters for each of the *keratin5p* constructs was much lower than what was seen in the *epi*+βG constructs (Table 3.4.1).

Primary injected fish for all the *keratin5p* constructs were raised until they reached sexual maturity and crossed with either other primary injected fish or wild type adult zebrafish to screen for transmission of the transgene. However, no transgenic lines were found. This may be because only a fraction of the epidermis expressed the transgene in the embryos injected with the *keratin5p*: *Krt5 I161S-2A-EGFP* and *keratin5p*: WT *Krt5-2A-EGFP*. In addition, all the embryos

injected with *keratin5p*: *Krt5Δ1A-2A-EGFP* died due to technical difficulties before they could be screened.

Table 3.4.1. Results of transient expression of the *keratin5p* constructs injections One cell stage embryos were injected with 100ng/μl of plasmid. Embryos were analyzed between 2 and 4 days post fertilization for fin defects. Skin defects included significant damage to the skin or the formation of blisters.

Keratin mutation	*krt5 WT*	*krt5 I161S*	*krt5Δ1a*
GFP+ fish/total number of fish injected (% of GFP+ fish)	68/75 (90.7%)	90/94 (95.7%)	44/51 (86.3%)
GFP+ fish with more then 15-20 GFP +cells	15/68 (22.1%)	47/90 (52.2%)	44/44 (100%)
GFP+ with more then 15-20 GFP+ cells with skin defects	0/15 (0%)	1/47 (2.1%)	2/44 (4.5%)

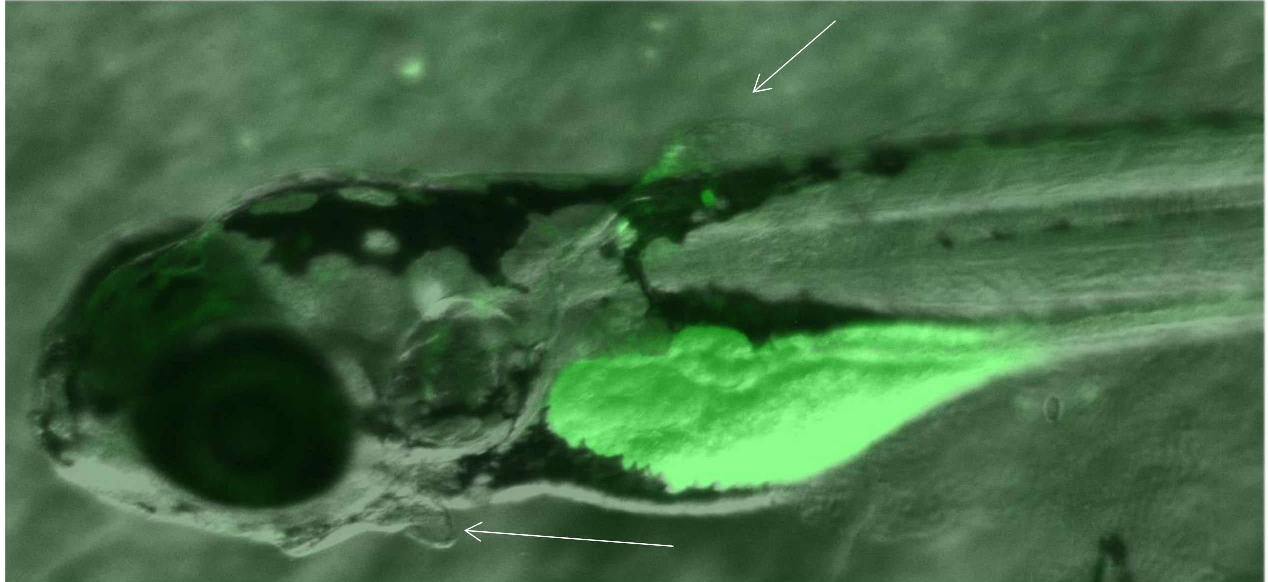

Figure 3.4.1. Zebrafish embryo injected with keratin 5: *I161S-krt52A-EGFP* demonstrating blisters. Arrows point to a blister on its back and near the head of this 3dpf embryo. The blister on the head co-localises with the expression of GFP.

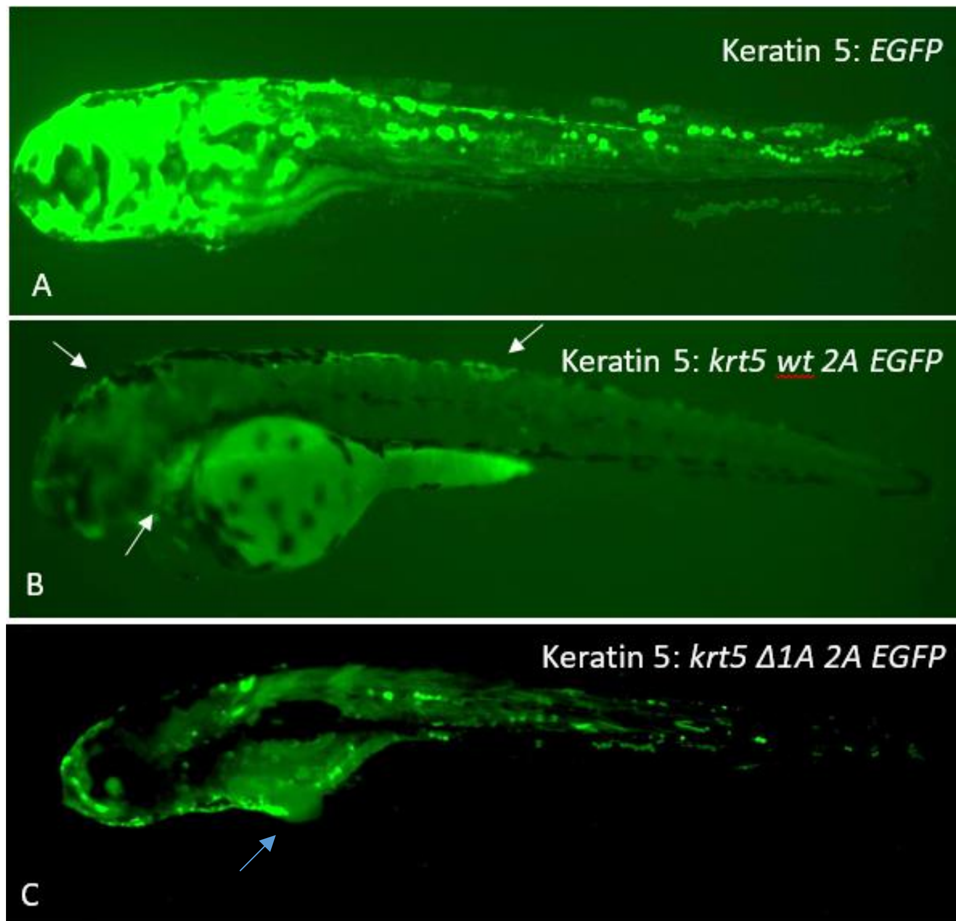

Figure 3.4.2. Zebrafish embryos injected with constructs containing mutant *krt5* cDNA under the control of the *keratin5p* regulatory elements at 2-4 dpf. Zebrafish embryos were injected with *keratin5p*:*EGFP* (A), which expresses GFP in the epidermal layer of the skin in a mosaic fashion. WT *krt5* (B) and *krt5Δ1A* (C) under the control zebrafish *keratin5p* regulatory elements were also injected into zebrafish embryos. In contrast to the *epi+βG* regulatory elements, the *keratin5p* regulatory elements drive expression in all of the epidermal layer. White arrows in (B) indicate areas of GFP expression. The blue arrow in (C) marks a blister that was not found in the *epi* + βG constructs.

3.5 Interchanging the 2A linker peptide with P2A

Due to the repeated failure to recreate EBS in zebrafish the transgenic construct used in the previous experiments were closely examined to see if they could be improved. Data obtained from this experiment, experiments from other researchers from the Akimenko lab, and literature indicated that the 2A peptide sequence used in all the constructs so far was not the best linker peptide available for zebrafish (Kim et al., 2011). To improve the construct a new peptide sequence, P2A, was chosen to replace the 2A peptide as it is superior to the 2A peptide in zebrafish for two reasons: 1) While 2A and P2A have the same amino acid sequence, the P2A considers the codon usage bias in zebrafish. Changing the codons of a gene without changing the resulting amino acid sequence can significantly alter the expression levels of genes (Carlini & Stephan, 2003; Dong et al., 1996; Slimko & Lester, 2003). The 2A linker peptide is not optimized for the codon bias used by zebrafish as 9 out of the 19 amino acids do not use the preferred codon. In contrast, the P2A peptide considers the zebrafish codon bias and uses only the most preferred codon for each of its amino acids. 2) The amino acid sequence GSG was added to the N terminus of the P2A amino acid sequence, which improves its cleavage efficiency (Kim et al., 2011). Consequently, the combination of these factors should increase the number of transgenic transcripts that are made and translated amino acid chains which separate into mutant keratin 5 and GFP proteins. This relative increase of mutant keratin 5 proteins could lead to a more severe collapse of the cytoskeleton, which might cause the development of EBS-like symptoms (Coulombe et al., 1990). The new linker peptide was cloned into the *keratin5p*: *krt5 E477K – 2A – EGFP* plasmid construct to make the *keratin5p*: *krt5 E477K – P2A – EGFP* plasmid to replace 2A (Figure 3.5.1).

Keratin5p: *krt5 E477K – P2A – EGFP* was injected into one-cell stage zebrafish embryos. While the proportion of injected embryos that were GFP+ was lower (73.5% *n*=49) than with the other previously described constructs using the 2A peptide sequence *keratin5p* constructs, there was a visible improvement in the proportion of keratinocytes that were GFP+ when compared to zebrafish injected with tol2 *keratin5p*: *krt5* WT-2A-*EGFP* and *keratin5p*: *krt5* I161S-2A-*EGFP* (Figure 3.4.2). Surprisingly, no blistering could be seen in any of the GFP positive fish (*n*=36), even if significant proportions of their epithelium were expressing mutant keratin 5.

GFP+ primary injected fish were raised and screened for transmission of the transgene. One transgenic line was found but expression was relatively weak and mostly found in the head region, with little to no expression that could be seen in the trunk or median fin region (Figure 3.5.2). Again, of the 50-100 mutant zebrafish embryos analyzed, none demonstrated skin defects.

Figure 3.5.3 summarizes the constructs that have been made using the *keratin5p* to drive expression, the number of transgenic lines obtained, and the number of injected zebrafish embryos raised to adulthood and screened for transmission of the transgene each construct.

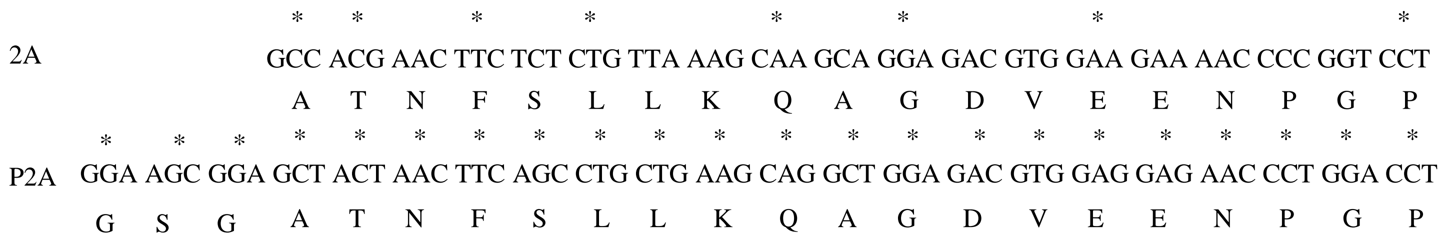

Figure 3.5.1 The P2A cDNA sequence is optimized for use in zebrafish. The 2A and P2A cDNA sequence are grouped into codons. The corresponding amino acid are indicated below

each codon. The P2A includes the amino acid sequence GSG at the N-terminal region, which increases the efficiency of cleavage (J. H. Kim et al., 2011). Stars above the codons indicate that the codon used is the most frequently used codon for that particular amino acid in zebrafish. 19/19 of the codons used in the P2A sequence are the most frequently used codons for the specific amino acids in zebrafish, while only 8/19 codons used in the 2A sequence are the most frequently used.

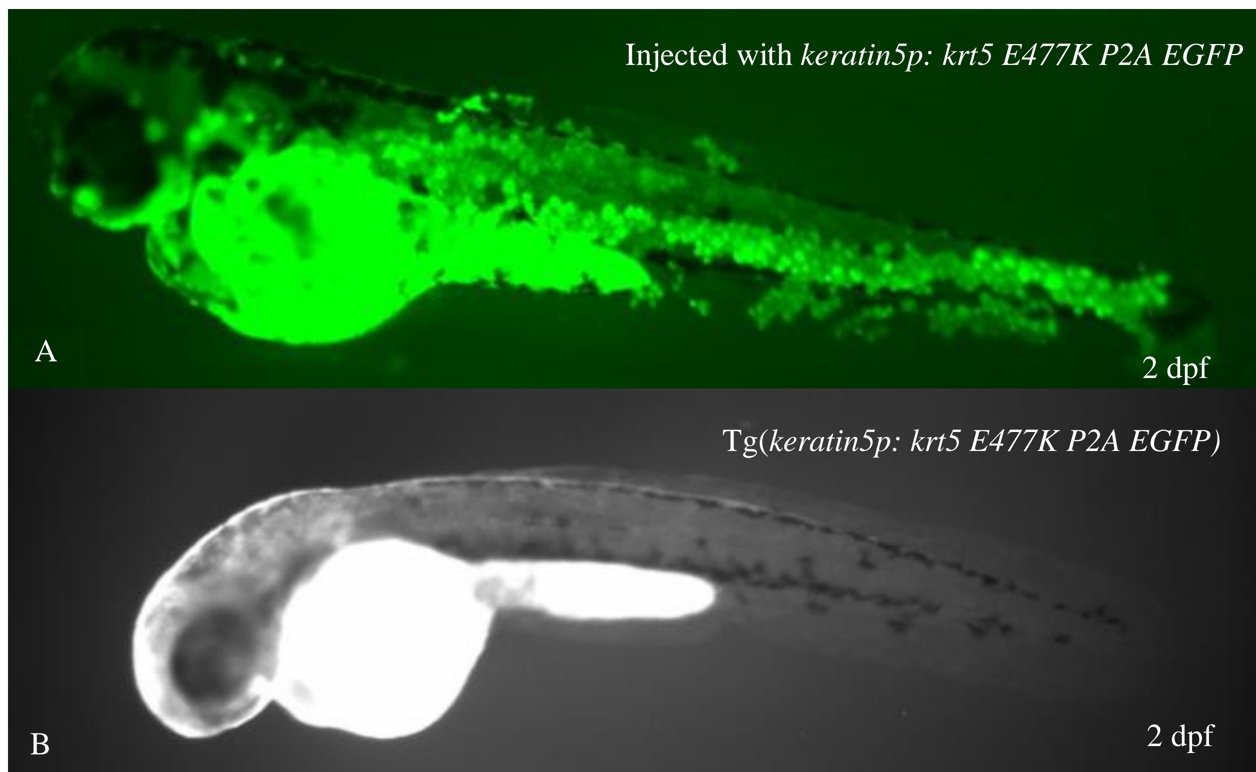

Figure 3.5.2. The new P2A signal peptide was not able to recreate EBS-like symptoms in zebrafish. The *keratin5p: krt5 E477K P2A EGFP* construct was injected into embryos (A), who were subsequently grown to adulthood and screened for transmission of the transgene. One mutant line was obtained (B), but expression was found to not be uniform across the embryo in contrast to what was expected. It is instead concentrated on the surface of the head with little expression in the posterior region of the fish. No EBS-like symptoms were found in the injected or mutant embryos.

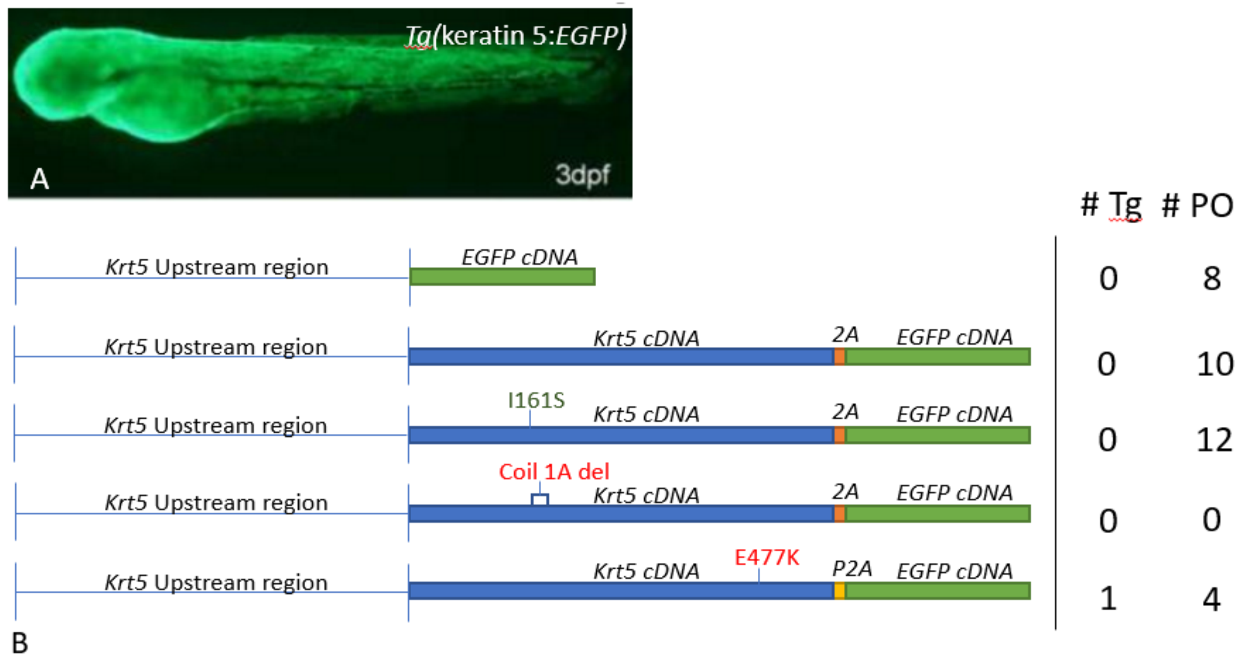

Figure 3.5.3. The upstream region of *krt5* was used to drive expression of mutant keratin 5 and GFP proteins but could not recreate EBS in zebrafish. (A) Example of GFP expression in the entire epidermis layer in the reporter transgenic line driven by the *keratin5p* regulatory elements at 3 dpf (adapted from Hu et al. 2010). (B) Schematic representation of the various mutant *krt5* cDNA constructs that were placed under the control of the *keratin5p* regulatory elements along with the relative location and size of the mutation inside the *krt5* cDNA. These mutations include the I161S and the E477K single nucleotide substitution, and the *krt5Δ1A* deletion. The numbers on the right represent the number of transgenic lines (# Tg) obtained and number of embryos injected with the construct who were screened for transmission of the transgene (#PO).

3.6 Immunohistochemistry

It was reported that basal keratinocytes with severe EBS were unable to form a filamentous keratin cytoskeleton; instead the keratin heterodimers formed proteins aggregates (Chamcheu et al., 2011; Gu & Coulombe, 2005). These aggregates are detectable by immunohistochemistry in immortalized keratinocytes from EBS patients using keratin antibodies (Chamcheu et al., 2011). It was hypothesized that immunohistochemistry might aid in visualizing and characterizing the possible changes in the keratin cytoskeleton in mutant zebrafish. If protein aggregates could be seen, then it would follow that this disease functions pathologically similarly in zebrafish and humans. As antibodies for many of the keratin zebrafish proteins are not yet available, two different keratin antibodies specific to mice keratin proteins were used to stain the keratin proteins in zebrafish. The antibody used was an anti-pan keratin type II antibody, which stains for all type II keratins (keratin 1-8) and that has been shown to work in zebrafish (Reischauer et al., 2009). The other antibody used was an antibody that stains for mice keratin 5. This antibody was chosen as it would most accurately demonstrate what occurs in the basal keratinocytes and the effects mutant keratin 5 on the cytoskeleton. Unlike the anti-pan keratin type II antibody, this antibody has not been shown to function in zebrafish.

Results from the immunohistochemistry on WT zebrafish embryos were inconclusive: no fluorescence could be detected using the anti-keratin 5 antibody despite many attempts (results not shown). In contrast, fluorescence could be detected in the epidermis when using the anti-pan keratin type II antibody after optimization experiments were done. However, a clear and defined cytoskeleton could not be seen (Figure 3.6.1). Without a clear cytoskeleton, it would be difficult to discern if any aggregate formed due to the presence of mutant keratin 5. This would likely mean that it would not be possible to detect keratin protein aggregates if this experiment was

used on the mutant transgenic lines. No further immunohistochemistry was performed due to this problem.

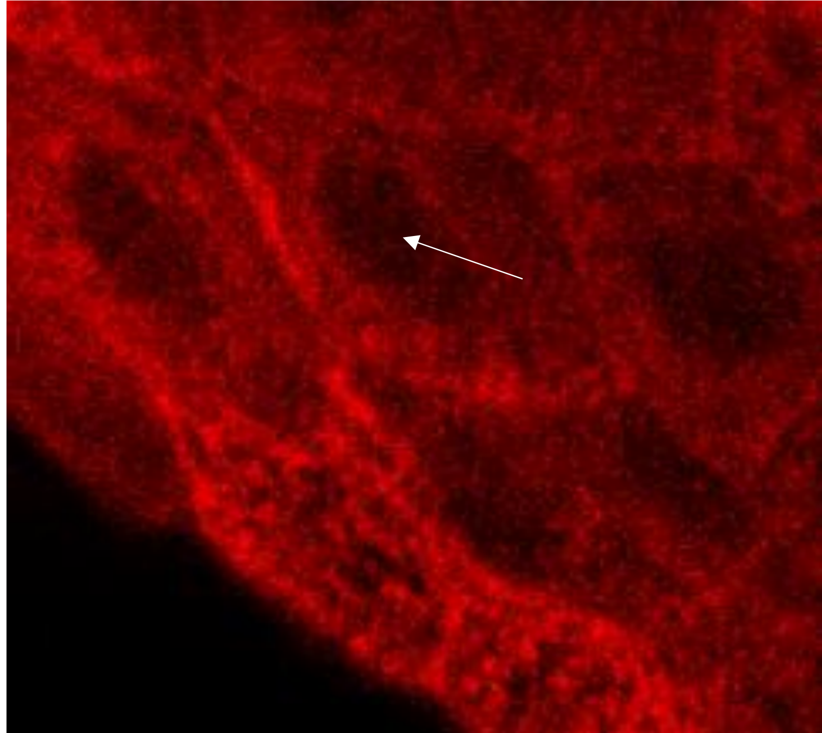

Figure 3.6.1. Immunohistochemistry of anti pan type II keratin antibody on 3 dpf WT zebrafish embryo epithelial cells . The white arrow indicates the location of the nucleus inside of the epithelial cell. Despite optimization on the immunohistochemistry procedure, no clear cytoskeleton could be seen, limiting this experiment's value in terms of analysing and characterizing the effects of the expression of mutant keratin 5.

4. Discussion

4.1 Overview

The primary goal of this project is to recreate EBS in zebrafish to perform a high throughput drug screen to find drugs that could be used to alleviate the symptoms of individuals affected with this genetic disorder since current treatment is only palliative (Abitbol & Zhou, 2009; Tabor et al., 2017). If successful, individuals with this condition, especially people with severe generalized EBS, would be able to better manage their symptoms and live a more normal life. A successful drug could also be tested against several other skin disorders with similar symptoms such as junctional EB and dystrophic EB. A crucial difference between this project and previous research on effective methods to treat EBS is the use of a high throughput drug screen instead of genetic therapy. Another difference is the use of an autosomal dominant mutation to recreate EBS and the use of the K5 gene instead of the K14 gene. The K14 protein was predominantly used in previous research as the null mutant and dominant K14 mutations would demonstrate less severe symptoms than the K5 mutants and thus was easier to study (Cao et al., 2001; Kerns et al., 2007). Additionally, no study has attempted to recreate EBS in zebrafish. Therefore, the secondary goal of this project is to characterize and analyze EBS caused by autosomal dominant mutations in the zebrafish *krt5* gene. To accomplish the above-mentioned goals, transgenic constructs expressing mutant zebrafish *krt5* cDNA were injected into zebrafish embryos to create mutant transgenic lines. It was hypothesized that mutant keratins would disrupt the keratin cytoskeleton in zebrafish and cause blisters just as in humans and mice. This is due to the similarity between zebrafish and human skin structure, homology between the *krt5* and *KRT5* genes, and similar location of keratin 5 protein expression.

4.2 I161S and E477K substitution

The first K5 mutations tested were the I161S and E477K amino acid substitutions under the control of the *epi* + *βG* regulatory elements. The I161S mutation is found in the non-helical head domain of the K5 domain, a domain that has been linked with the assembly of the keratin heterodimers into filaments (Wilson et al., 1992). The E477K mutation is found in the highly conserved R/K L L E G E motif—a motif that is nearly perfectly conserved across all IF proteins (Wilson et al., 1992). This motif plays a crucial role in the proper assembly of the keratin heterodimers into filaments (Lee et al., 2012; Wilson et al., 1992). Previous research demonstrate that most of the mutations that cause EBS can be found in these regions, highlighting the importance of these regions in keratin heterodimer polymerization (Porter & Lane, 2003). Since these regions are conserved in zebrafish K5 protein, it was expected that these mutations would cause EBS-like symptoms in mutant zebrafish lines.

While injection results were promising, transgenic lines showed no signs of blisters or skin defects. A potential explanation for this is that skin defects found in injected embryos were caused by the microinjection process itself and not by the expression of mutant keratins. As for the transgenic mutants, it was unsurprising that the I161S mutant did not cause skin defects. This is because the same mutation in humans creates a mild localized version of EBS, and symptoms only present themselves in tissues exposed to high levels of mechanical stress such as the hands and feet (Ehrlich et al., 1995; Pfendner et al., 2005). As zebrafish do not experience the mechanical stress of walking or manipulating objects, it seemed unlikely that skin defects would appear spontaneously.

In contrast, the E477K mutation causes severe generalized EBS in humans. The E477K mutation has been associated with some of the severest cases of generalized EBS. Symptoms

appear at birth and can be found covering most of the body including the mucosa of the throat and mouth (Pfendner et al., 2005). Unlike other strains of severe generalized EBS, the E477K mutation causes symptoms that do not tend to improve significantly over the lifetime of affected individuals (Müller et al., 2006; Pfendner et al., 2005). While friction is still needed to create these blisters, the amount needed is significantly less then localized EBS and blisters are considered to appear spontaneously (McGrath et al., 1992). The fatality rate in infants before the age of one affected by severe generalized EBS is 2.8%. However, five out of eight infants with different familial background in the United Kingdom affected by the de novo E477K mutation died within six months after birth (Sathishkumar et al., 2016). This makes the fact that no blisters or skin defects were found in the E477K zebrafish mutant even more unexpected, since the blisters need very little friction to appear. The death of the basal keratinocytes caused by trivial mechanical stress is the ultimate cause of the blistering and skin defects in humans afflicted by EBS (Minakawa et al., 2013). However, cell lysis of the basal keratinocytes does not seem to occur in the E447K mutant zebrafish. This can be seen in two distinct ways: no obvious blistering or skin defect can be seen, and mutant cells continually express EGFP, a feat that could not be done if the basal keratinocyte underwent lysis (Figure 3.1.4; Figure 3.4.2). To conclude, the expression of mutant keratin was not enough to spontaneously produce EBS-like symptoms in mutant zebrafish lines.

One possible reason for the failure to recreate EBS in zebrafish is that keratins perform a different function in zebrafish compared to humans. Other roles keratins play include cell signalling, cell proliferation, wound healing and cell motility among others (Alam et al., 2011; Moll et al., 2008; Paramio & Jorcano, 2002). However, I do not believe that a difference in keratin's function is the problem for the following reasons. Sequence homology between human

and zebrafish *krt5*, especially in the central rod domain, which is important in the formation of the keratin heterodimer and filament assembly, is very high (83.2%). Furthermore, the high conservation of the helix initiation and termination motifs between zebrafish and human keratin 5 suggests that these motifs play the same role in keratin heterodimer polymerization. It is unlikely that keratin 5 would function differently given this high homology between the two distantly related species. Furthermore, the expression patterns are similar among the two species, suggesting that they play the same role in epidermis.

Another possible reason for the failure to recreate EBS in zebrafish could be that the zebrafish basal keratinocytes might be stabilized by an additional factor not present in mammals. Every species of mammal is affected differently by EBS. For example, null *KRT14* mutant mice will die shortly after birth, while humans affected by the same condition can live until their seventies (Batta et al., 2000; Kerns et al., 2007). While this theory cannot be discounted, it is nonetheless unlikely as some form of blisters are always found in affected individual regardless of the species, especially in severe generalize EBS. The basal keratinocyte in the mutant zebrafish, however, might still be susceptible to environmental stress that worsens EBS symptoms.

Additionally, the mutations used might not be as significant to the polymerization of the keratin heterodimers as other possible mutations. It is possible that the specific positions used simply do not play the same role in the zebrafish as they do in human K5. Previous studies found that deletions, especially of the coil 1A and terminal end of the coil 2B domains were able to cause severe cytoskeleton disruption in transfected keratinocyte cell lines (Rugg et al., 1999; Wilson et al., 1992). Deletions of these important domains may be able to recreate EBS in zebrafish.

A fourth possible cause of the failure is that the expression of the mutant keratins might occur after the formation of the keratin cytoskeleton as endogenous expression commences at 5.25 hpf, while the *epi* + *βG* regulatory elements drive expression at 24 hpf. The mutant keratins might not be able to integrate themselves in sufficient numbers to disrupt the keratin cytoskeleton.

The ratio of mutant keratins to WT keratins can play a significant part in the creation of blisters (Cao et al., 2001). In humans with autosomal dominant EBS, roughly half of all keratins are mutant. It is possible that the number of mutant keratins in the mutant zebrafish does not reach the required ratio to attain a phenotype.

To find the cause behind the failure to recreate EBS in zebrafish, these possibilities were explored.

4.3 Environmental stress

Friction, a factor known to worsen EBS symptoms, was tested on the mutant zebrafish with inconclusive results. This was done to test if the mutants were susceptible to frictional stress and if this could create blisters in the median and pectoral fin fold. The ideal result would have been that the mutant zebrafish would have had blisters only where mutant keratins were expressed and that the WT controls would have no blisters. However, this did not occur: blistering was found on all the zebrafish including the WT controls during certain tests, on none of zebrafish during other tests and on only some of the zebrafish in other circumstances. They appeared regardless of whether they were mutant or WT embryos. This means that all zebrafish embryos responded to frictional stress in the same fashion. A general weakness of this experiment is the lack of control on the amount and location of the frictional stresses. As expected, injuries could be seen on the body of both mutant and WT zebrafish, indicating that the frictional stress was not directed specifically to the median fin fold. Considering that the primary objective of this project was to do a high throughput drug screen, which could include the use of thousands of mutant zebrafish, the fact that this method could not create consistent blisters in mutant zebrafish calls its value into question. Using this method, the lack of consistency would make it impossible to know if a drug had alleviated the symptoms of EBS, or if the embryo had simply never developed any blisters.

Another environmental factor known to worsen the symptoms of EBS is heat. Individuals with EBS have reported that high temperatures exacerbate their symptoms. Heat causes major rearrangements of the keratin cytoskeleton and the formation of keratin aggregates in basal keratinocytes affected with EBS (both localized and generalized) but not in WT basal keratinocytes (Morley et al., 1995). However, this rearrangement is temporary and the

cytoskeleton will return to normal after the temperature lowers (Morley et al., 1995). One hypothesis is that the production of heat shock proteins (HSP) causes the cytoskeleton rearrangement (Shyy et al., 1989; Toivola et al., 2010). A method previously described to activate the HSP promoter in zebrafish was used on mutant zebrafish embryos to see if blisters or skin defects could be obtained (Shoji & Sato-Maeda, 2008). One benefit of this method is that raising and lowering the temperature is a more consistent process than the friction method mentioned above. However, high temperatures have less of an influence over creating blisters than friction (Pfendner & Bruckner, 1993). Nevertheless, heat was unable to recreate EBS in the mutant zebrafish. A combination of heat and friction was also tested, but the method led to the same problems of inconsistency as the friction test.

4.4 Deletions of the helix initiation and termination motifs

To address the possibility that the I161S and the E477K mutations were not severe enough to cause EBS in zebrafish, mutant *krt5* cDNA with either the sequence corresponding to the helix initiation or termination motifs were deleted and cloned into the existing construct. These new constructs were injected into one-cell stage zebrafish embryos but were unable to recreate EBS-like symptoms. Two transgenic lines expressing the *krt5Δ2B* mutant cDNA were also unable to recreate EBS.

Previous research on EBS that was caused by K14 dominant mutations suggests that the deletion of these motifs causes the collapse of the cytoskeleton and dramatic shortening of the keratin filaments (Coulombe et al., 1990). Additionally, *in vivo* studies found that the deletion of the coil 1A would cause a more severe phenotype compared to the deletion of the 2B coil in the K14 protein (Coulombe, 2016). A similar study was done on the *KRT5* gene; however, the deletions performed only affected the highly conserved R/K L L E G E motif at the end of the coil 2B domains (Wilson et al., 1992). Nonetheless, it was found that this motif was necessary to the proper assembly of the keratin heterodimers (Lee et al., 2012). Part of the sequence deleted in the *krt5Δ2B* mutant cDNA corresponds to this motif, indicating that the assembly of the keratin heterodimers into filaments should be adversely affected.

A study into a family affected by severe generalized EBS found that a deletion of 22 amino acids in the K5 protein at the same location where the *krt5Δ1A* deletion occurred was the cause of their severe blistering (Rugg et al., 1999). One difference between the two deletions is that the family has three more amino acids deleted compared to the *krt5Δ1A* mutant. Other than this, the deletions were the same: the 19 amino acids deleted in the human family who correspond to the amino acids deleted in the *krt5Δ1A* mutant demonstrates a 100% amino acid

identity. These two deletions effectively remove the helix initiation motif from the K5 protein, a motif that is important in assembling the keratin heterodimers (Rugg et al., 1999). This supports the theory that a significant disruption of the keratin cytoskeleton should occur when the mutant *krt5Δ1A* is integrated inside the keratin cytoskeleton.

Zebrafish embryos injected with the *krt5Δ2B* and *krt5Δ1A* mutant constructs had a similar rate of EGFP+ embryos with blisters compared to the I161S and E477K mutant construct injections (Table 3.1.1; Table 3.3.1). EGFP expression could be seen in mutant basal keratinocytes. Again, this affirms the viability of the basal keratinocytes that should have been adversely affected by a disrupted cytoskeleton, as previous research and observation suggest. One possible reason for these cells not undergoing lysis is the mosaic nature of injections: it is commonly seen that EGFP+ basal keratinocytes are surrounded by WT basal keratinocytes where the construct was unable to integrate. It is possible that not enough of the epidermal tissue was affected by an ineffective cytoskeleton to cause significant damage to the tissue as a whole and cause blistering. However, almost all of the basal keratinocytes found in the median fin fold of E477K mutant embryos were EGFP+. This implies that they all produce mutant keratinocytes despite no skin defects being seen. Therefore, it is likely that another factor is preventing blister formation.

The expression of EGFP was weak in the *krt5Δ2B* transgenic mutants when compared to the I161S and E477K mutants (Figure 3.1.4; figure 3.3.2). This raises the possibility that not enough mutant keratins are being produced to create significant disruption of the keratin cytoskeleton. Previous research suggested that depending on the mutation, only 1 in 100 keratins need to be a mutant keratin to cause the collapse of the cytoskeleton into keratin aggregates (Coulombe et al., 1990). Deletions that affect the coil 1A and coil 2B regions were found to only

need a mutant to WT keratin ratio of 1:100 to cause cytoskeletal disruption when compared to point mutations that required a much higher ratio (Coulombe et al., 1990; Wilson et al., 1992). However, the severity of the symptoms in all cases increased as the ratio of mutant keratins to WT keratins increased (Cao et al., 2001; Coulombe et al., 1990). While this relationship is well described in cell lines, more needs to be done to understand what ratio of the mutant keratin needs to be achieved for individual mutations before blisters start to appear. Another possible reason is that the mutant keratins cannot integrate themselves in the cytoskeleton once it is already formed. To conclude, it seems like another factor is preventing the formation of blisters and the nature of the mutations is not primary reason.

4.5 Expression of mutant keratins during keratin cytoskeletal formation

Perhaps the most likely reason for the failure to recreate EBS is the delay in expression between mutant and endogenous keratin. The *epi + βG* regulatory elements drive expression at 24 hours post fertilization, while endogenous keratin 5 expression starts at 5.25 hours post fertilization (Lalonde et al., 2016; Thisse & Thisse, 2004). This means that the keratin cytoskeleton is already formed by the time the mutant keratins are expressed. The keratin skeleton is a dynamic structure, constantly degrading and reforming while the epithelial cells are motile (Windoffer et al., 2011). However, when the epithelial cells are immobile and attached to other tissues through desmosomes and hemidesmosomes, the keratin cytoskeleton is mostly stable and unchanging (Windoffer et al., 2011). The basal keratinocytes of the zebrafish embryo fall into the latter category due to their immobile nature (Reischauer et al., 2009). Therefore, the mutant keratins might not have the chance to integrate themselves in sufficient numbers to create disruption in a stable keratin cytoskeleton. To address this, a fin cutting experiment involving E447K mutant embryos was performed as well as driving mutant K5 expression with the zebrafish *krt5* 2.3 kb upstream region.

The fin cutting experiment sought to recreate an environment in which endogenous keratin 5 would be co-expressed with mutant keratin 5 when the keratin cytoskeleton would be at its most dynamic. In addition, the keratin cytoskeleton is more dynamic during mitosis (Izawa & Inagaki, 2006). In fact, it has been suggested that the decrease in mitotic index could mitigate the symptoms of EBS as affected individuals age, reducing the severity of their symptoms (Morley et al., 1995). The combination of a dynamic cytoskeleton and the expression of mutant keratins during the initial formation of the cytoskeleton was theorized to allow integration of mutant keratins and subsequent collapse of the cytoskeleton. However, the mutant zebrafish's median

fin regrew normally without any defects (Figure 3.2.1). A weakness of this experiment is that keratin cytoskeleton of the daughter cells is based on the pre-existing cytoskeleton of the parental cells. Since the pre-existing cytoskeleton are seemingly stable, it is possible that they pass on their stable cytoskeleton to their daughter cells, explaining the lack of defect. This experiment also shares the same weakness of the friction experiment, but to a lesser extent, namely its impracticality due to the inconsistency of fin cutting. Moreover, he quantity of embryos that would need to be amputated might not be feasible when considering a high throughput drug screen.

Previous mouse studies using a severe dominant mutation in the *KRT14* gene demonstrated that affected mice pups would die within a week (Cao et al., 2001). This was the initial reasoning behind using the *epi + βG* enhancer/minimal promoter complex to drive expression in non-essential tissue. This was done to create stable transgenic lines to perform high throughput drug screening. However, to ensure that the delayed expression of mutant keratin did not cause the lack of EBS symptoms, the zebrafish *kerating5p* regulatory elements was used to drive expression of mutant keratins. This promoter drives the expression of the endogenous *krt5,* meaning that both the mutant and endogenous keratins 5 proteins would be expressed at the same time and location. Expression of EGFP in zebrafish embryos injected with *keratin5p*: WT *krt5* - 2A - EGFP was seen between 5.25 hours and 6 hours, confirming transgenic and endogenous K5 expression commences at the same time.

To test if a mild version of the disease with a low risk of mortality could be created, zebrafish embryo was injected with *keratin5p*: I161S mutant construct. The rate of blisters in EGFP+ embryos was lower than with the previous e*pi + βG* mutant constructs (2.1% compared to 20.8% for I16IS). One primary-injected zebrafish demonstrated a blister on its head that was

co-localized with EGFP expression (Figure 3.4.1). However, the presence of EGFP could indicate that the basal keratinocytes were alive and expressing the transgene. Conversely, mutant keratinocyte expressing GFP could have died and leaked out GFP. The fluorescence seen could simply the remnants of these dead cells. However, this is unlikely as the expression of GFP remained stable in the same areas of the epidermis for days. In addition, fluorescence can be seen in the shape of a hexagon, the shape of a healthy epidermal cell (Yokouchi et al., 2016). It is unlikely that GFP would take such a shape if it had leaked out form a dying mutant keratinocyte cell. Therefore, the most likely explanation for this blister is a defect caused by microinjection. The failure to create EBS symptoms for the *keratin5p*: I161S - 2A - EGFP mutant construct could be twofold: 1) The number of EGFP+ cells were considerably lower when compared to injections of *Keratin5p*: EGFP (Figure 3.4.2). It may be possible that not enough of the epidermis is affected by the mutant keratin 5 to cause blisters. 2) The I161S mutation creates a mild localized version of EBS in humans. Previous research in the effects of different mutations in the *krt5* gene on the structural integrity of the keratin cytoskeleton shows that the less severe a mutation is, the higher the ratio of mutant keratins to WT keratins needs to be to cause the collapse of the cytoskeleton (Coulombe et al., 1990). It may be that simply not enough mutant keratins were created to have a strong effect on the structural integrity of the keratin cytoskeleton. It was concluded that a more severe mutation would be more likely to create blisters.

Zebrafish embryos injected with the *keratin5p*: *krt5Δ1A* – 2A- EGFP mutant construct also had a low rate of blister formation (4.5%). Unlike the *keratin5p*: I161S mutant construct, a larger amount of the basal keratinocytes were EGFP+ but still was not comparable to injections of *kerating5p*: EGFP (Figure 3.4.2). A primary-injected fish demonstrated a blister on the yolk

sac; however, EGFP expression could be seen on top of this blister (Figure 3.4.2). This indicates that the blister arose below the basal keratinocytes and was most likely the result of a microinjection defect and not from disruption of the keratin cytoskeleton. The most probable cause for the failure to recreate EBS with this construct is that not enough mutant keratins were made to cause the collapse of the cytoskeleton (Coulombe, 2016).

It is possible that increasing the concentration of mutant keratins might be able to cause the collapse of the cytoskeleton in the basal keratinocytes (Coulombe et al., 1990). To accomplish this, the 2A linker peptide was changed for the P2A linker peptide to make the *Keratin5p*: *E477K-P2A-EGFP* mutant construct. The *P2A* cDNA sequence considers the zebrafish's codon bias unlike the *2A* cDNA sequence. It has been shown that zebrafish, like all other organisms, have a preference to use specific codons instead of using all of the synonymous codon equally, and that the codon usage can affect the stability of the mRNA (Bazzini et al., 2016). In addition, previous studies have shown that the expression of the same protein can be significantly affected based on the codons used (Carlini & Stephan, 2003; Dong et al., 1996; Slimko & Lester, 2003). This is because gene expression might be hindered by the low availability of tRNA for rare codons (Bazzini et al., 2016).

Visual observation of embryos injected with *keratin5p*: *krt5 E477K-2PA-EGFP*, which used the new P2A sequence, seem to indicate that a larger proportion of the basal keratinocytes were EGFP+ and were comparable to *Keratin5p*: EGFP injections (Figure 3.4.2). However, no blister in any of the embryos injected were seen, despite large portions of basal keratinocytes expressing EGFP (Figure 3.4.2). The Tg(*Keratin5p*: E477K-P2A-EGFP) mutant line also did not demonstrate any blistering. The amount of fluorescence was rather weak in this line and EGFP could only be seen in the head and body region (Figure 3.4.2).

4.6 Characterization of keratin cytoskeleton

To understand if the mutant keratins could create a cellular phenotype, like what was seen in the cell line studies, immunohistochemistry against the keratin 5 and keratin 1-8 was performed. This could help detect if keratin aggregates were formed, which would indicate that the lack of phenotype could be due to another unrelated factor like a compensatory mechanism not seen in mammal. However, immunohistochemistry using the pan keratin 1-8 antibody was unable to accurately stain the keratin cytoskeleton in WT zebrafish embryos, while fluorescence was undetectable when keratin 5 antibody was used. The results for the pan keratin 1-8 are in accordance with previous research using the same method and antibody (Reischauer et al., 2009). The most probable reason for the poor results is that the antibodies were unable to bind strongly enough to the keratin network as they were produced to bind to mouse keratin 1-8. To see a clear cytoskeleton, it is likely that antibodies specific to zebrafish keratins would have to be produced.

Another method that could be used to see the keratin cytoskeleton is to tag the mutant keratins with a fluorescent protein such as EGFP. This method has proven effective in the past with human keratinocytes transfected with keratin 14 tagged with GFP (Pekny & Lane, 2007). In the present study, the 2A and P2A linker protein was used to stop potential interference of the EGFP protein in the integration of the mutant keratins in the cytoskeleton. However, a fusion protein would have many advantages over immunohistochemistry in the context of this project. The most significant advantage of using a fusion protein is the ability to perform real time cell imaging to characterize the effects of the mutant keratins.

4.7 Conclusion and future direction

It is not clear if autosomal dominant mutations in the K5 protein can cause EBS in zebrafish. Perhaps the most probable reason for this failure is that not enough mutant keratins are made in the transgenic lines to disrupt the function of the keratin cytoskeleton in the basal keratinocytes. More research needs to be done to understand how the ratio of mutant to WT keratin affects the keratin cytoskeleton in the basal keratinocytes. In the future, a knockout of the *krt5* gene using the CRISPR system in zebrafish could be useful in two different ways. First, a null *krt5* zebrafish mutant could be characterized. This will be useful in determining if blisters appear in the null mutant as they do in the mice model. If no blisters are found, then it is probable that a compensatory mechanism for the disruption of the keratin cytoskeleton exists in zebrafish that is not found in mice or humans. Second, a *krt5 +/-* mutant could be crossed with a zebrafish expressing a mutant keratin under the control of the *krt5* 2.3kb upstream region. This would result in offspring heterozygous for the *krt5* gene and expressing mutant keratins. The advantage of this is that the ratio of mutant keratins to WT keratins would increase as only one functional allele for the endogenous *krt5* gene would be present. This could cause the death of transgenic embryos as it does in mice models but could also demonstrate the presence of a compensatory mechanism not found in humans if no blisters are found.

As mentioned above, a possible compensatory mechanism found in zebrafish might be able to arrest the development of EBS-like symptoms. For example, the zebrafish proper cytoskeleton formation might still occur if the basal keratinocytes are able to detect mutated keratin proteins and sequester them for destruction. Another possibility that would account for proper cytoskeletal formation would be by increasing production of another type II keratin to compensate for the mutant keratin 5. A compensatory mechanism could also be found at the

tissue level: some structures found in zebrafish embryo not found in mammals such as the actinotrichia could provide enough support to the basal keratinocytes in the event of a cytoskeletal collapse. A network of extracellular proteins could also compensate for the lack of a functioning cytoskeleton by providing additional support to the basal keratinocytes. Further research would have to be done to ascertain how the zebrafish and human basal keratinocytes differ in terms of basal keratinocyte cellular stability in order to understand if the zebrafish have a compensatory mechanism that prevents the development of EBS-like symptoms.

In all, while using zebrafish to study and understand EBS could have great benefits, many difficulties must be overcome to make the zebrafish into a working model for this disease.

Contributions

The E477K, I161S, and WT *krt5* cDNA in tol2 vectors were obtained from the Akimenko laboratory. The *epi+βG* regulatory elements were given by Robert Lalonde, a PhD student in the Akimenko laboratory. The *2A* cDNA sequence was obtained from Dr. M. Ekker from the Univeristy of Ottawa and was cloned into the *2A-EGFP* fragment in pdrive by Eileen-Hue Phan, a MSc student in the Akimenko laboratory. The pmini Tol2 (*Keratin5p*: *EGFP*) plasmid was graciously given by the Dr Lee laboratory from the North Carolina Central University. The P2A-EGFP in the pdrive vector was also obtained from the Akimenko laboratory. Photos of the immunohistochemistry using the confocal microscope was performed by Jing Zhang, MSc and Eileen-Hue Phan, MSc student in the Akimenko lab.

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