THE ROLE OF INTEGRIN ALPHAVBETA6 IN IMPAIRED WOUND HEALING AND HAIR FOLLICLE REGENERATION

by

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Abstract

Integrin αvβ6 is an epithelial-specific receptor that is absent from the healthy epidermis but synthesized de novo during wound repair. However, its function in wound repair is unknown. Integrin-mediated activation of transforming growth factor-β1 (TGF-β1) is the main activation mechanism of this key cytokine in vivo. It has been previously reported that chronic human wounds continue to express αvβ6 integrin and transgenic mice over-expressing αvβ6 integrin produce chronic hypertrophic wounds that contain high levels of TGF-β1. Therefore, we hypothesize that αvβ6 integrin-mediated regulation of TGF-β1 activity may play a role in the impaired wound healing process. To this end, a well-established dexamethasone-induced mouse impaired wound model was used in β6 integrin-deficient (β6-/-) and WT mice. The dexamethasone-treated β6-/- mice demonstrated an accelerated wound repair and enhanced keratinocyte proliferation in wound epithelium and particularly in the hair follicles, while the production of pro-inflammatory cytokines and TGF-β1 activation were reduced compared to the treated WT controls.

TGF-β1 has been implicated as an endogenous inducer of hair follicle regression. Since hair follicles constitutively express αvβ6 integrin, we tested whether αvβ6 integrin-mediated TGF-β1 signaling regulates hair regeneration and hair follicle involution process. Using a depilation-induced mouse hair cycling model we showed that hair regeneration was accelerated and hair follicle regression retarded in the β6-/- mice

compared to WT controls. These changes were associated with enhanced keratinocyte proliferation in both hair follicles and interfollicular epidermis (IFE). Additionally, the levels of TGF-β1 and Smad2 phosphorylation were significantly reduced in β6-/- follicles during early anagen and anagen-catagen transition. Our study also demonstrated that the expression of integrin αvβ6 was strongly upregulated and specifically enhanced in the bulge stem cell niche during early hair regeneration.

In summary, the results indicate that αvβ6 integrin plays an important inhibitory role in keratinocyte proliferation in both the IFE and hair follicles after wounding. The downregulated TGF-β1 signaling in β6-/- mice may impact epidermal stem cell behavior via modulating the microenvironment of the bulge stem cell niche after injury, which suggests a possible manipulation target in the functions of epidermal stem cells in the future.

Preface

A version of chapter 2 has been published. Xie Y, Gao K, Häkkinen L and Larjava HS. Mice lacking beta6 integrin in skin show accelerated wound repair in dexamethasone impaired wound healing model. Wound Repair Regen. 2009;17(3):326-39. Research question of chapter 2 was identified and project was designed by Dr. Hannu Larjava and Dr. Lari Häkkinen. Yanshuang Xie performed the experiments with the help of Dr. Kai Gao. Data were collected and analyzed by Yanshuang Xie under the guidance of Dr. Hannu Larjava and Dr. Lari Häkkinen. Manuscript was prepared by Yanshuang Xie, edited by Dr. Kai Gao, Dr. Lari Häkkinen, and Dr. Hannu Larjava.

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The University of British Columbia Animal Care Committee has examined and approved the use of animals for the above experimental projects. Animal Care Certificate number: A07-0195.

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BM	basement membrane
BSA	bovine serum albumin
β6-/-	β6 integrin knockout
CAV-9	coxsackievirus-9
DAPI	4′, 6′- diamidino-2-phenylindole
ECM	extracellular matrix
ESC	epidermal stem cell
FMDV	foot-and-mouth disease virus
GR	glucocorticoid receptor
H&E	hematoxylin and eosin
HF	hair follicle
HGF	hepatocyte growth factor
HPEV 1	human parechovirus 1
HPF	high-power field
IFE	interfollicular epidermis
IGF-1	insulin-like growth factor-1
IL-1α	interleukin-1 α
IRS	inner root sheath

KGF	keratinocyte growth factor
LAP	latency-associated peptide
LLC	large latent complex
LRC	label-retaining cell
LTBP	latent TGF-β-binding protein
MMP	matrix metalloproteinase
ORS	outer root sheath
PBS	phosphate buffered saline solution
PDGF	platelet-derived growth factor
PMN	polymorphonuclear leucocyte
SCC	squamous cell carcinoma
SG	sebaceous gland
SLC	small latent complex
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
TβRIII	TGF-β type III receptor
TGF-β1	transforming growth factor-β1
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial growth factor
WT	wild-type

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To

My husband,

who has accompanied me with his love, unlimited patience, understanding and encouragement to pursue academic excellence.

And also to

My parents,

for bringing me up with their endless love and support.

Chapter 1: Review of the literature

1.1 Integrin superfamily

Integrins are trans-membrane receptors that function as both cell anchoring and signaling molecules (Hynes 2002). Structurally, integrins are heterodimers composed of two different, non-covalently associated, α and β subunits. To date, 18 α and 8 β subunits have been identified, each of which can bind to one or several partners that form at least 24 different cell surface receptors (Thomas *et al.* 2006; Bandyopadhyay and Raghavan 2009). Each integrin binds to different ligands and presents distinct functions (Figure 1.1) (Thomas *et al.* 2006). The α and β subunits of integrins span the cell membrane and have a long extracellular domain extending into the extracellular space and a short cytpolasmic tail on the inner side of the plasma membrane. The short cytoplasmic tail of integrins associate and interact with a cohort of signaling and structural proteins and thus activate intracellular signal transduction pathways (Liu *et al.* 2000; Yamada and Even-Ram 2002). Except binding to different ECM proteins, integrins have been shown to interact with cell surface ligands, transmembrane proteins, soluble proteases, pathogens, and growth factors (Sheppard 2000; van der Flier and Sonnenberg 2001; Hynes 2002; Luo *et al.* 2007). The crucial role of integrins in multiple biological processes is appreciated from a variety of severe phenotypes of integrin subunit deficient or transgenic animals (De Arcangelis and Georges-Labouesse 2000; Bouvard *et al.* 2001; Hynes 2002) and from the

pathological consequences following integrin defects (Danen and Sonnenberg 2003). It is evident that integrins are more than simple anchoring molecules by mediating the adhesion of the cellular cytoskeleton to the extracellular matrix (ECM); they act as bidirectional signaling molecules by receiving and transmitting signals from both sides of the plasma membrane, a property generally referred to as outside-in and inside-out signaling thereby regulating a plethora of vital processes like adhesion, proliferation, migration, differentiation and tumor invasion and metastasis (Giancotti and Ruoslahti 1999; Schwartz 2001; Calderwood 2004; Ginsberg *et al.* 2005; Luo *et al.* 2007). These cell functions are critical during embryogenesis and help to maintain tissue homeostasis under normal physiological conditions.

1.2 Integrin αvβ6

The integrin αvβ6 is unique in the integrin superfamily due to its exclusive expression in epithelial cells (Breuss *et al.* 1993; Breuss *et al.* 1995). β6 subunit pairs only with αv unit that combine to form this particular cell surface receptor (Hynes 2002). Integrin αvβ6 recognizes and binds to its ligands through the RGD-motif, a tripeptide amino acid sequence arginine–glycine–aspartate acid (Thomas *et al.* 2006). Fibronectin, tenascin-C, vitronectin and fibrillin-1 are all binding partners of αvβ6 integrin in the ECM (Busk *et al.* 1992; Prieto *et al.* 1993; Huang *et al.* 1998; Annes *et al.* 2004; Jovanovic *et al.* 2007). The αvβ6 integrin also binds to the RGD sequence of latency-associated peptides-1 and 3

(LAP-1 and -3), which are the N-terminal parts of the latent complex of transforming growth factor-β1 and 3 (TGF-β1 and 3) (Munger *et al.* 1999; Annes *et al.* 2002). In addition, αvβ6 integrin is a receptor for the viral capsids of the foot-and-mouth disease virus (FMDV), coxsackievirus 9 (CAV-9) and human parechovirus 1 (HPEV 1) (Miller *et al.* 2001; Williams *et al.* 2004; Seitsonen *et al.* 2010). The RGD-containing ligands of αvβ6 integrin are summarized in Table 1.1.

During embryogenesis αvβ6 integrin is expressed at high levels in the epithelia of the developing kidney, lung and skin. The expression is downregulated and mostly undetectable in fully differentiated resting epithelia of the normal adult tissue (Breuss *et al.* 1993; Breuss *et al.* 1995) with the exception of junctional epithelium of teeth (Ghannad *et al.* 2008), hair follicles (Breuss *et al.* 1995; Xie *et al.* 2009) and ameloblasts (our unpublished observations) that constitutively express this integrin. Despite generally absent from adult epithelia, the level of αvβ6 integrin can be induced during wound healing, inflammation (Breuss *et al.* 1995), and in more severe pathologies, including chronic skin wounds (Hakkinen *et al.* 2004) and tumorigenesis (Thomas *et al.* 2006). A highly upregulated expression of integrin αvβ6 has been reported in a variety of carcinoma tissues, including lung, breast, skin, oral, colon, stomach, esophageal, pancreas, ovary, cervical and endometrium carcinomas (Hamidi *et al.* 2000; Thomas *et al.* 2006; Bandyopadhyay and Raghavan 2009). Moreover, αvβ6 integrin has been shown to upregulate the pro-enzyme form of type IV collagenase, matrix metalloproteinase-9 (MMP-9) through the unique terminal 11 amino acids of its cytoplasmic tail, which leads

to the promotion of tumor cell migration and invasion (Thomas *et al.* 2001; Thomas *et al.* 2001; Thomas *et al.* 2001; Morgan *et al.* 2004; Scott *et al.* 2004). In summary, the characteristic expression of the integrin αvβ6 suggests its functions in regulating epithelial remodeling during development, tissue repair, and neoplasia (Breuss *et al.* 1995).

The role of αvβ6 integrin in the activation of TGF-β1 and in wound healing will be reviewed in a separate sections below.

The study of β6 integrin knockout mice has revealed a role for this integrin in downregulating local inflammation (Huang *et al.* 1996). Increased macrophage infiltration into the skin were observed in the local hair loss area in the juvernile β6-/- mice although the baldness was resolved by ~30 days of age. In addition, there is a persistent accumulation of activated lymphocytes in the lungs, which results in an airway hyperresponsiveness to a bronchoconstrictor, acetylcholine. These results suggest that αvβ6 integrin may function to regulate inflammatory response in the skin and lungs (Huang *et al.* 1996).

It has been demonstrated that the binding of the αvβ6 integrin to some of its RGD-containing ligands, the viral capsid of FMDV (Berryman *et al.* 2005; O'Donnell *et al.* 2005; Ruiz-Saenz *et al.* 2009) and CAV-9 (Stewart and Nemerow 2007; Heikkila *et al.* 2010), induces endocytosis of the integrin-ligand complexes. Foot-and-month disease is a severe vesicular disease of cloven-hoofed animals caused by the infection of FMDV. Studies of the internalization mechanisms of FMDV have showed that FMDV can utilize

αvβ6 integrin as one of its cell entry receptors in epithelial cells. FMDV gets internalized together with αvβ6 integrin through the clathrin-mediated endocytosis pathway, but not caveolae or other internalization pathways (Berryman *et al.* 2005; O'Donnell *et al.* 2005; Ruiz-Saenz *et al.* 2009). In this manner αvβ6 integrin mediates the FMDV infection. CAV-9 has also been shown to utilize αv integrin, particularly αvβ6 integrin as its receptors. The endocytic pathway by which CAV9 enters human cells after the initial attachment to the cell surface is at least partly dependent on the αvβ6 integrin (Stewart and Nemerow 2007; Heikkila *et al.* 2010).

Studies on the mechanism controlling αvβ6 integrin induction have demonstrated that the expression of this integrin could be regulated by certain growth factors including TGF-β1 and tumor necrosis factor-α (TNF-α). TGF-β1 has been shown to induce de novo expression of αvβ6 integrin in normal human keratinocytes (Zambruno *et al.* 1995) and on HaCaT keratinocytes, a non-transformed keratinocyte cell line (Koivisto *et al.* 1999). In addition, the expression of αvβ6 integrin is significantly reduced in TNF-α-/- keratinocytes compared to wild-type (WT) keratinocytes. In contrast, when treated with exogenous TNF-α, the expression is upregulated in both TNF-α-/- and WT keratinocytes (Scott *et al.* 2004). A clearer understanding of the exact mechanisms how expression of this integrin is regulated may provide novel targeting strategies for human diseases that involve the abnormal expression of αvβ6 integrin.

1.3 Transforming growth factor-βs (TGF-βs) and TGF-β receptors

The three TGF-β isoforms, TGF-β1, TGF-β2 and TGF-β3, are synthesized as homodimeric pro-TGF-β that is covalently linked to the latency associated protein (LAP) (Massague *et al.* 2000). The pro-TGF-β is then intracellularly cleaved by furin-like enzymes, which generates the mature TGF-β (Lawrence *et al.* 1984; Dubois *et al.* 1995). The mature TGF-β remains non-covalently attached to LAP in a linkage forming the so-called small latent complex (SLC). Latent TGF-β either exists in form of the soluble SLC or as a resident of the ECM. Only very few cell types, however, produce and secrete latent TGF-β in the diffusible form (Dallas *et al.* 1994). TGF-β is secreted by the majority of cell types as part of the large latent complex (LLC) (Miyazono *et al.* 1991; Dallas *et al.* 1994; Taipale *et al.* 1998). The LLC is formed by establishing a covalent disulfide bond between the SLC (TGF-β/LAP) and the latent TGF-β-binding proteins (LTBPs) (Saharinen and Keski-Oja 2000; Wipff and Hinz 2008). LTBPs bind to several other ECM proteins, such as fibrillin-1 (ten Dijke and Arthur 2007), fibronectin (Taipale *et al.* 1994) and vitronectin (Schoppet *et al.* 2002). Therefore, the LLC provides a reservoir of latent TGF-β in the ECM that is available when needed (Gleizes *et al.* 1997; Annes *et al.* 2003; Hyytiainen *et al.* 2004; Todorovic *et al.* 2005).

TGF-β is secreted in this latent form that cannot be recognized and bound by its high affinity receptors. The dissociation of TGF-β from LAP is required for the release of biologically active TGF-β. This process is termed latent TGF-β activation (Gleizes *et al.*

1997; Annes *et al.* 2003). Activation of latent TGF-β is a major mechanism to regulate TGF-β functions. Indeed, integrin-mediated TGF-β activation has been shown to be the major activation mechanism of TGF-β in vivo (Yang *et al.* 2007; Aluwihare *et al.* 2009; Nishimura 2009; Margadant and Sonnenberg 2010).

TGF-β isoforms signal through binding to three high-affinity cell surface receptors, the TGF-β type I (TβRI), type II (TβRII) and type III (TβRIII) receptors. The activation of TGF-β leads to the release of TGF-β from the LLC, which then binds to its receptor TβRII (Wipff and Hinz 2008). Binding of active TGF-β to the TβRII phosphorylates TβRI and leads to the recruitment of TβRI into a heterodimeric serine/threonine kinase receptor complex. The serine/ threonine kinase activity of the activated receptor complex then leads to the recruitment and phosphorylation of the intracellular effector proteins Smad2 and Smad3. Phosphorylated Smad2 and Smad3 subsequently both bind to Smad4 and translocate into the nucleus to regulate gene transcription (Feng and Derynck 2005; Massague *et al.* 2005).

In contrast to TβRII and TβRI, TβRIII is a co-receptor of TGF-β superfamily. TβRIII is thought to function through binding TGF-β superfamily ligands, including TGF-β isoforms (Blobe *et al.* 2000), inhibin (Lewis *et al.* 2000; Wiater and Vale 2003) and bone morphogenetic proteins (BMPs) (Kirkbride *et al.* 2008), and presenting them to the appropriate type II or type I TGF-β superfamily receptors (You *et al.* 2009).

In addition to the Smad-dependent canonical pathway, cross talk and signaling through Smad-independent pathways have been reported (Yu *et al.* 2002; Moustakas and Heldin

2005). Mechanisms for TGF-β signaling to these pathways remain to be fully defined.

1.4 The activation of TGF-β1 mediated by integrin αvβ6

Integrin αvβ6 recognizes and binds to a tripeptide amino acid sequence arginine–glycine–aspartate acid (RGD-motif) in its ligands, which include fibronectin, tenascin-C, and vitronectin ((Busk *et al.* 1992; Huang *et al.* 1998; Annes *et al.* 2004) (Table 1.1). The presence of RGD motifs in the pro-peptide of TGF-βs makes them potential integrin ligands that have being investigated by various groups (Munger *et al.* 1999; Thomas *et al.* 2002; Annes *et al.* 2004). A common mechanism that has emerged from different studies is a non-proteolytic latent TGF-β1 activation mechanism mediated by αvβ6 integrin. After the binding of αvβ6 to the RGD motif present in the LAP, TGF-β1 activation occurs through cell traction forces exerted by the actin cytoskeleton. These cell traction forces mediated by αvβ6 integrin are transmited to LAP-β1 in the TGF-β1-LAP-LTBP1 complex (LLC), which leads to a conformational change of the LLC and presentation of active TGF-β1 to its receptor (Annes *et al.* 2004; Fontana *et al.* 2005; Wipff *et al.* 2007; Wipff and Hinz 2008). Hence, non-proteolytic TGF-β1 activation results from a conformational change in the LLC and is dependent on the ability of αvβ6 to binds to the actin cytoskeleton of the cell rather than via cleavage of the peptide. In line with this, cells expressing mutated β6 subunits could still bind LAP, but were unable to connect with actin cytoskeleton and thus could not activate TGF-β1

(Munger *et al.* 1999; Sheppard 2005). TGF-β2 does not contain an RGD sequence and is not a ligand of αvβ6 integrin, whereas the LAP3 of TGF-β3 is also a RGD-containing peptide, and thus is activated in a similar fashion by αvβ6 integrin (Busk *et al.* 1992; Huang *et al.* 1998; Annes *et al.* 2002; Annes *et al.* 2004).

The activation of TGF-β1 by αvβ6 integrin leads to the release of TGF-β1 from the LLC, which then binds to its receptor, thus activating the signaling pathway (Wipff and Hinz 2008). The integrin-mediated activation of latent TGF-β1 has been demonstrated to be important in vivo. Absence of integrin-mediated TGF-β1 activation resulting from the mutation of the RGD site of LAP leads to abnormalities similar to those observed in TGF-β1 knockout mice (Yang *et al.* 2007). Similarly, the phenotype of mice deficient in both the αvβ6 and αvβ8 integrins recapitulates the defects observed in mice that lack TGF-β1 and TGF-β3, suggesting that the integrins αvβ6 and αvβ8-mediated activation of TGF-β is the main activation mechanism of TGF-β1 and TGF-β3 in vivo (Aluwihare *et al.* 2009). Our laboratory has previously reported that the constitutive expression of αvβ6 in the basal layer of the epidermis of the transgenic mice, leads to elevated TGF-β1 activation and the development of spontaneous chronic ulcers with severe fibrosis (Hakkinen *et al.* 2004). In addition, specifically αvβ6 integrin-dependent activation of TGF-β1 has been confirmed to be pivotal in mouse models of TGF-β1-dependent fibrosis in various epithelial organs, including lung (Horan *et al.* 2008; Puthawala *et al.* 2008), kidney (Ma *et al.* 2003; Hahm *et al.* 2007) and liver (Wang *et al.* 2007; Patsenker *et al.* 2008; Popov *et al.* 2008). Moreover, inhibition of αvβ6 integrin prevents the fibrosis

development in different animal models from several groups (Nishimura 2009; Margadant and Sonnenberg 2010), suggesting that this mechanism of TGF-β activation may be of general importance in tissues that express αvβ6 integrin.

1.5 Wound healing process

1.5.1 Overview of wound healing process

The process of cutaneous wound healing is to restore skin damage and to regain, at least in part, lost integrity, tensile strength and barrier function of the skin (Singer *et al.* 2000). The wound healing process includes three continuous and overlapping phases: inflammatory, proliferative and remodeling phase (Werner and Grose 2003). The inflammatory phase is initiated immediately upon injury and lasts up to 4 to 6 days after wounding. A variety of inflammatory cells influx to the wound site to fight infection and remove debris. The proliferative phase is characterized by angiogenesis, fibroplasia and granulation tissue formation, re-epithelialization, and wound contraction (Midwood *et al.* 2004), and lasts from about day 4 to day 14 after wounding. The remodeling phase starts at around day 8 and may last up to a year or even longer (Broughton *et al.* 2006).

1.5.2 Main events in wound healing process

Upon cutaneous wounding and damage to blood vessels, platelets are exposed to the ECM proteins, which then lead to immediate coagulation and fibrin clot formation (Lau

et al. 2009). Mediated by cytokines in the blood clot released by the platelets and mast cells, such as platelet-derived growth factor (PDGF) (Szpaderska *et al.* 2003) and TNF-α (Malaviya *et al.* 1996), polymorphonuclear leucocytes (PMNs) from the peripheral blood migrate into the wound site via diapedesis (Lou *et al.* 2007). PMNs are involved in phagocytosis and eliminating microbes and debris, damaged cells and bacteria (Ehrlich and Krummel 1996; Deodhar and Rana 1997). They also release large amount of inflammatory cytokines and growth factors. About two days after injury, macrophages replace PMNs and become predominant inflammatory cells in the wound site (Hubner *et al.* 1996). In addition to phagocytosis and immune response, macrophages continue to secrete major growth factors such as TGF-β1 and PDGF. Aside from clearance of debris, these inflammatory cells initiate angiogenesis as the wound healing process proceeds to the proliferative phase.

The subsequent proliferative phase is characterized by angiogenesis, fibroplasia and granulation tissue formation, re-epithelialization, and wound contraction (Midwood *et al.* 2004). Low oxygen environment stimulates neovascularization by inducing macrophages and platelets to secrete angiogenic factors such as fibronectin and increased growth factors that attract vascular endothelial cells to the granulation tissue (Grazul-Bilska *et al.* 2003). Vascular endothelial cells give rise to the new blood vessels in granulation tissue. Neovascularization occurs to support the newly formed granulation tissue and to transport circulatory cells to the wound (Li *et al.* 2005).

In fibroplasia and granulation tissue formation, fibroblasts grow and form a new,

provisional ECM. The provisional ECM is mainly composed of fibronectin, type III collage, glycosaminoglycans, hyaluronan, elastin, glycoproteins and proteoglycans, which provide a hydrated wound bed that facilitates cell migration (Midwood *et al.* 2004). The new formed blood vessel, fibroblasts, inflammatory cells, endothelial cells, myofibroblasts, and the provisional ECM constitute the major components of granulation tissue.

Migration of keratinocytes and hence re-epithelialization starts as early as two hours after wounding. Keratinocytes that re-epithelialize the wound are mainly derived from two populations of epidermal stem cells in the skin. One is located in the basal layer of the interfollicular epidermis (IFE) close to the wound edges (Liang and Bickenbach 2002). Another resides in the hair follicle bulge, located in the outer root sheath (ORS) of the permanent portion of the hair follicle where the arrector pili muscle inserts (Cotsarelis *et al.* 1990; Lyle *et al.* 1998; Oshima *et al.* 2001; Morris *et al.* 2004). In response to wounding, these two epidermal stem cells populations provide most if not all keratinocytes that repopulate the wound epithelium (Taylor *et al.* 2000; Ito *et al.* 2005). Keratinocytes secrete proteases and plasminogen activator that activates plasmin, which dissolves the clot, debris, and parts of the ECM and promote cell migration (Ghersi *et al.* 2002; Etscheid *et al.* 2005). Wound contraction by myofibroblasts present in the granulation tissue accelerates wound closure by bringing wound edges closer together (Montesano and Orci 1988). Keratinocytes continue migrating across the wound bed until cells from different sides meet in the middle. Subsequently, new layers of keratinocytes

differentiate and give rise to a stratified epidermis. Fast keratinocyte migration and re-epithelialization often leads to better wound healing outcomes and decreased scar formation (Li *et al.* 2006).

Following complete wound closure, tissue remodeling occurs below the epidermal surface. During remodeling phase, the provisional matrix is replaced with collagen fiber bundles that more closely resemble the healthy non-wounded tissue. Type III collagen, a weaker form of the structural protein that can be produced rapidly and is abundant in the newly formed granulaton tissue, is gradually degraded and is replaced by the stronger and long stranded type I collagen (Risteli *et al.* 1993). Depending on the size and location of the wound, the remodeling phase can last from months up to a year or longer to complete (Gurtner *et al.* 2008).

1.6 The role of TGF-β1 in wound healing

TGF-β1 affects all cell types that are involved in all stages of wound healing (O'Kane and Ferguson 1997). TGF-β1 has been shown to promote cell migration (Santibanez *et al.* 2000), stimulate wound contraction (Montesano and Orci 1988; Desmouliere *et al.* 1993) and promote the production of ECM molecules (Leask and Abraham 2003). Although the beneficial effects of TGF-β1 in wound healing have been well recognized, accumulating evidence suggests that TGF-β1 may also delay wound repair, reflecting the complex nature of the biological functions of TGF-β1. TGF-β1 has been confirmed to be an

inhibitor of keratinocyte growth and delay wound re-epithelialization (Glick *et al.* 1993). Inhibition of epithelial cell proliferation by TGF-β1 involves down regulation of c-Myc leading to upregulation of cyclin-dependent kinase inhibitors p15, p21 and p27, which inhibit the CDK4/6-cyclin D and CDK2-cyclin E-mediated phosphorylation of the retinoblastoma protein (Reynisdottir *et al.* 1995; Robson *et al.* 1999; Massague *et al.* 2000; Moustakas *et al.* 2002; Ten Dijke *et al.* 2002; Frederick *et al.* 2004). In support of this notion, Smad3 is a TGF-β-activated transcription factor that plays an important role in mediating a number of wound repair-associated TGF-β1 responses. TGF-β1 signaling is partially abolished in Smad3 knockout mice and these mice exhibit accelerated wound healing phenotype, characterized by increased keratinocyte proliferation, migration and reduced monocyte infiltration (Ashcroft *et al.* 1999). Consistently, transgenic mice overexpressing TGF-β1 in the epidermis exhibited retarded re-epithelialization due to inhibited keratinocyte proliferation after burn injury (Yang *et al.* 2001). Conversely, TGF-β1 null mice showed accelerated re-epithelialization after incisional wounding (O'Kane and Ferguson 1997; Koch *et al.* 2000). In addition, transgenic mice overexpressing a dominant-negative TGF-β receptor display accelerated re-epithelialization in skin wounds. This was associated with an enhanced proliferation and reduced apoptosis of keratinocytes at the wound edge owing to the resistance of keratinocytes to TGF-β1-mediated growth inhibition and apoptosis induction (Amendt *et al.* 2002).

TGF-β1 has been known for its anti-inflammatory role ever since studies have shown that

TGF-β1-deficient mice exhibit inflammation in multiple organs and develop autoimmune conditions (Shull *et al.* 1992; Kulkarni *et al.* 1993). However, accumulating evidence indicates that TGF-β1 may also play a pro-inflammatory role that depends on the cell type and microenvironment and may vary in different organs. TGF-β1 has been shown to be a potent chemotactic cytokine for virtually all leukocytes that are involved in inflammation (Wahl *et al.* 1987; Wahl *et al.* 1993). In support of this notion, transgenic mice overexpressing latent TGF-β1 in the basal keratinocytes and hair follicles spontaneously develop chronic skin inflammatory lesions (Li *et al.* 2004). These transgenic mice exhibited a significant delay in wound healing compared to the WT mice that was associated with profound inflammation throughout all of the stages of wound healing (Wang *et al.* 2006). Similarly, Smad3-null mice also exhibited accelerated wound healing with reduced monocyte infiltration (Ashcroft *et al.* 1999).

In addition to modulating keratinocyte growth and inflammatory response, TGF-β1 promotes ECM synthesis (Taipale *et al.* 1998). TGF-β1 expression is increased in scar formation and numerous fibrotic conditions (Sharma and Ziyadeh 1994), which is associated, at least in part, with the TGF-β1-driven transdifferentiation of fibroblasts into myofibroblasts. These contractile, secretory cells cause an accumulation of ECM with resulting scarring (Powell *et al.* 1999). Additionally, excess activity of TGF-β1 released from platelets and inflammatory cells in the first phase of wound healing, failure to eliminate myofibroblasts from granulation tissue and reduced collagen breakdown at later time points have been considered as conditions that lead to formation of hypertrophic

scars (Desmouliere 1995; Ghahary *et al.* 1996).

1.7 Integrin αvβ6 and wound healing

The αvβ6 integrin is unique in that it is not expressed constitutively in the intact epidermis of nonwounded skin, but is induced upon wounding (Breuss *et al.* 1993). The de novo expression of αvβ6 integrin is confined to the basal keratinocytes along the wound edges (Larjava *et al.* 1993; Watt and Jones 1993; Breuss *et al.* 1995; Haapasalmi *et al.* 1996; Hakkinen *et al.* 2000; Hakkinen *et al.* 2004). *In vitro* studies have showed that β6-/- keratinocytes could not migrate as efficiently on fibronectin or vitronectin as the WT keratinocytes and αvβ6 integrin facilitates keratinocyte adhesion and migration on tenascin-C (Huang *et al.* 1998), all of which are components of the early wound matrix (Busk *et al.* 1992; Koivisto *et al.* 1999; Hakkinen *et al.* 2000). Additionally, αvβ6-dependent upregulation of the type IV collagenase MMP-9 could facilitate cell movement by allowing detachment from the basement membrane (Thomas *et al.* 2001). These data suggest an important role of αvβ6 integrin in keratinocyte migration. On the other hand, *in vivo* studies demonstrated that expression of αvβ6 in wounds is absent in the wound edges during the first days of keratinocyte migration after injury, and maximal expression was seen relatively late in the healing process, when migrating edges of the wound epithelium have fused (Larjava *et al.* 1993; Clark *et al.* 1996; Haapasalmi *et al.* 1996; Hakkinen *et al.* 2000). Then the level of αvβ6 remains high for days (Thomas *et al.*

2006; Eslami *et al.* 2009). Therefore, the de novo, but transient expression of keratinocyte αvβ6 integrin in the relatively late stage of healing process suggests that the primary function of αvβ6 integrin in cutaneous wound healing could be in the regulation of cell proliferation in the epidermis and to stop the re-epithelialization process possibly through controlling the activation of TGF-β1 at this stage rather than to maintain adhesion and migration of keratinocytes. In support of this notion, expression of αvβ6 integrin in β6-transfected cells plated on tenascin-C completely failed to proliferate (Yokosaki *et al.* 1996).

Integrin-mediated activation of latent TGF-β1 plays an important role in vivo (Yang *et al.* 2007; Aluwihare *et al.* 2009; Nishimura 2009; Margadant and Sonnenberg 2010). As a potent endogenous activator of TGF-β1, the function of αvβ6 integrin in wound healing is, however, still poorly defined. To investigate the role of αvβ6 in cutaneous wounds, mice that either lack, or constitutively express β6 integrin have been generated. The β6-/- mice do not have an altered healing rate, but show some local hair loss and inflammation at young age (Huang *et al.* 1996). Interestingly, these inflammatory infiltrates were mainly composed of macrophages resembling those found in the TGF-β1 null transgenic mouse (Shull *et al.* 1992). The rate of wound closure in the transgenic mice with constitutive expression of αvβ6 in the basal layer of the epidermis is also unaltered and the mice healed without significant scarring, however, developed spontaneous chronic skin ulcers (Hakkinen *et al.* 2004). These chronic lesions contained areas with severe fibrosis and numerous activated macrophages and fibroblasts expressing high levels of

TGF-β1. Consistently, human chronic wounds show prolonged expression of αvβ6 integrin. These findings suggest that increased αvβ6 integrin in keratinocytes plays an active part in abnormal wound healing possibly through a mechanism involving increased activation of TGF-β1 (Hakkinen *et al.* 2004).

Although studies of acute wound healing in young β6 integrin deficient (β6-/-) (Huang *et al.* 1996) and β6 integrin overexpressing mice (Hakkinen *et al.* 2004) have found no wound healing abnormalities. Interestingly, a somewhat improved healing response has been, however, reported in old hydrocortisone-treated αvβ6 integrin overexpressing mice (AlDahlawi *et al.* 2006).

In addition, it has been proposed that αvβ6 integrin could regulate inflammation via TGF-β1 signaling. Interestingly, increased macrophages infiltration were observed in both the hair loss area in the juvernile β6-/- mice (Huang *et al.* 1996) and in the spontaneous chronic fibrotic ulcers in the transgenic mice (Hakkinen *et al.* 2004). A blockade of αvβ6 integrin reduces the expression and activity of TGF-β1 and leads to an inhibition of the pro-inflammatory effect of TGF-β1 in the kidney (Hahm *et al.* 2007).

1.8 Impaired wound healing and glucocorticoids

More than 50 years after their discovery, glucocorticoids have remained one of the most frequently used classes of drugs. About 30 million patients in the United States receive treatment with exogenous steroid agents (Ettinger *et al.* 2001). Their therapeutic

indications are widespread, the side effects, however, are common and of great importance to the patients. Chronic glucocorticoid therapy induces detrimental effects in several tissues, including the skin where it causes skin atrophy. When encountering an accidental or surgical trauma, impaired tissue repair capacity is one of the serious complications of glucocorticoid therapy.

Glucocorticoids affect almost every phase of wound healing because of their inhibitory effect on gene expression in various cells. In the early phase after injury, dexamethasone inhibits recruitment of various inflammatory cells and the expression of a variety of genes at the wound site that encode key proteins in the wound healing process, including pro-inflammatory cytokines interleukin-1 α and β (IL-α and β), TGF-β1, 2, and 3 and their receptors, TNF-α, keratinocyte growth factor (KGF), PDGF and their receptors, tenascin-C, etc (Hubner *et al.* 1996; Beer *et al.* 2000; Wen *et al.* 2002). During the proliferative and remodeling phases, dexamethasone can inhibit the synthesis of several dermal ECM proteins, and delay re-epithelialization and fibroplasia (Beer *et al.* 2000). In contrast, the glucocorticoid-induced impaired wound repair could be reversed by treatment with growth factors, including the TGF-β1 and 2 (Pierce *et al.* 1989; Beck *et al.* 1991). Therefore, a better understanding of the cellular and molecular mechanisms during impaired wound healing will be instrumental for the development of effective therapies for patients with aberrant wound healing.

Substantial evidence has shown that epidermal stem cells reside in not only the interfollicular epidermis, but also hair follicle bulge. Chebotaev et al. have reported that

keratinocytes of the interfollicular epidermis and keratinocytes of hair follicle bulge respond differently to glucocorticoid treatment (Chebotaev *et al.* 2007). It is well known that cell desensitization to continued glucocorticoid therapy is mediated via temporary downregulation of glucocorticoid receptor (GR) expression. GR expression was significantly reduced in the basal keratinocytes of the interfollicular epidermis after each glucocorticoid treatment. Many bulge keratinocytes, however, retained GR in the nucleus. Consequently bulge keratinocytes appeared to develop resistance more slowly and thus are more sensitive to the antiproliferative effect of glucocorticoids than the basal keratinocytes as a result of the incomplete process of desensitization. Additionally, these findings suggest that bulge keratinocytes appeared not to significantly contribute to the repair of the atrophic epidermis after steroid-induced hypoplasia. Stem cells residing in the interfollicular epidermis potentially are a major source for the regeneration of the epidermis during the glucocorticoid-induced skin atrophy. Thus, epidermal stem cells from the interfollicular epidermis and hair follicle bulge could be different cell populations with distinct properties and roles in the skin homeostasis and regeneration.

1.9 The hair cycle

The hair follicle undergoes periodic cycles of involution and regeneration throughout adult life, which can be divided into three phases, including anagen (growth), catagen (involution) to telogen (resting) phases. This process depends on epidermal stem cells

contained in hair follicles, residing in a region of the ORS termed the “bulge” (Cotsarelis *et al.* 1990; Paus and Cotsarelis 1999; Stenn and Paus 2001; Cotsarelis 2006). Accumulated evidence has confirmed that the bulge is the repository of multipotent epidermal stem cells in skin. Epidermal stem cells maintain the capability of self-renewal and multilineage differentiation, which is regulated by the surrounding microenvironment, or niche (Blanpain *et al.* 2004; Moore KA 2006). In mice, the first hair follicle cycling is initiated about 17 days post partum, starting from a rapid organ involution (catagen). This first catagen lasts two to three days, and is followed by the phase of relative quiescence (telogen). The first growth phase of the hair cycle (anagen) occurs after 4 weeks post partum (Muller-Rover *et al.* 2001).

1.9.1 Anagen

Anagen is the growth phase in which the hair shaft, inner root sheath (IRS), ORS, and new hair matrix are produced from tip to root (Figure 1.2). Anagen follicles are long, straight, but are angled to permit the hair coat to lie flat along the body surface. In anagen, keratinocytes in the hair matrix, which are referred to as transient amplifying cells derived from epidermal stem cells in the bulge, proliferate intensively and then differentiate into distinct epithelial hair lineages (Muller-Rover *et al.* 2001; Stenn and Paus 2001; Legue and Nicolas 2005; Fuchs 2007; McElwee KJ 2008). From outermost to innermost, anagen follicles are composed of three layers of IRS, including Henley, Huxley and cuticle layers, and three layers of the hair shaft, including the cuticle, cortex

and medulla layers. The duration of anagen determines the length of the hair and is dependent upon continued proliferation and differentiation of the keratinocytes in the hair matrix (Alonso and Fuchs 2006; Krause and Foitzik 2006).

1.9.2 Catagen

Catagen is a phase of epithelial regression driven by apoptosiss and is the dynamic transition between anagen and telogen (Muller-Rover *et al.* 2001). The keratinocytes in the hair matrix undergo a limited number of cell divisions before differentiating. As the supply of matrix cells declines, hair shaft and IRS differentiation slow and the follicle enters a destructive phase - catagen. The progression of catagen is evident from the color of the skin in pigmented mice, which changes from the dark grey to black of anagen to pale pink of telogen (Alonso and Fuchs 2006; Krause and Foitzik 2006).

During catagen, the lower two-thirds of 'cycling' portion of each hair follicle rapidly regresses mainly by apoptosis of matrix, IRS and ORS keratinocytes (Lindner *et al.* 1997), while bulge hair follicle stem cells escape apoptosis. Eventually, the lower hair follicle recedes to a temporary structure - the epithelial strand - which is unique to catagen (Figure 1.3), bringing the dermal papilla into close proximity of the bulge, located in the permanent, non-cycling upper follicle, where it remains anchored during telogen. Catagen lasts 3-4 days in mice (Muller-Rover *et al.* 2001; Stenn and Paus 2001; Alonso and Fuchs 2006; Krause and Foitzik 2006).

1.9.3 Telogen

Following catagen, hair follicles enter a phase of relative quiescence (telogen). In mice, the first telogen is short, lasting only 1 or 2 days, from approximately 19 to 21 days post partum in the mid back. The second telogen, however, lasts more than 2 weeks, beginning around 42 days post partum. Hair follicle cycling slows down remarkably in aging animals with each additional cycle the duration of telogen expands.

1.10 Molecular regulators of the anagen-catagen transition

Although some molecular regulators of the anagen-catagen transition have been identified through mouse mutants with defects in hair follicle cycling, and by characterizing gene profiles of distinct murine hair cycle stages (Stenn and Paus 2001; Lin *et al.* 2004; Paus and Foitzik 2004), the molecular mechanisms that drive hair regression remain obscure.

Mouse mutants have demonstrated that Wnt/β-catenin, BMP antagonists (e.g. Noggin) and Shh induce anagen, while FGF5 is a key inducer of catagen (Hebert *et al.* 1994; Stenn and Paus 2001; Paus and Foitzik 2004; McElwee KJ 2008). FGF5-deficient mice showed a prolonged anagen phase resulting in an angora hair phenotype (Hebert *et al.* 1994).

TGF-β1 has been implicated as an endogenous inducer of hair follicle regression in vivo possibly through the inhibition of keratinocyte proliferation and induction of apoptosis

(Foitzik *et al.* 1999; Foitzik *et al.* 2000; Soma *et al.* 2003). TGF-β1-/- mice displayed a delayed catagen development and more ki-67-positive cells in hair follicles than WT follicles at identical stages. In contrast, Injection of TGF-β1 into the back skin of the WT mice induced premature catagen development and hair follicle dystrophy (Foitzik *et al.* 2000).

In addition to TGFβ-1, molecules that have been described to promote the transition to catagen include the growth factors TGF-β family members such as BMPRIa (Andl *et al.* 2004), and BMP2/4 (Stenn and Paus 2001; Paus and Foitzik 2004), EGF (Hansen *et al.* 1997), neurotrophins such as NT-3, NT-4 and BDNF, interleukin-1β, p53 and TNF-α (Stenn and Paus 2001; Paus and Foitzik 2004; Schmidt-Ullrich and Paus 2005).

Conversely, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1) (Paus and Foitzik 2004), SGK3 (Alonso *et al.* 2005)), and Msx2 (Ma *et al.* 2003) are known to maintain anagen.

Moreover, the molecular crosstalk between keratin-17 and downstream effectors of TNF-α signaling may be in part responsible for the control of catagen entry by regulating the rate of apoptosis (Tong and Coulombe 2006).

Although these molecular regulators of the anagen-catagen transition have been identified, how they work together to terminate anagen and promote catagen is, however, not yet understood (Schneider *et al.* 2009).

1.11 Hair follicle stem cells and the hair cycle

During the telogen-to-anagen transition, hair follicle stem cells are activated to initiate a new round of hair growth. Studies have shown that maintenance of the hair follicle cycle is largely dependent on different epidermal stem cell populations capable of giving rise to the different epithelial components of the hair follicle. In 1990, Costarelis et al. demonstrated that, in rodent hair follicles, label-retaining cells (LRCs), the slowly cycling keratinocytes, residing in a prtion of the ORS of the hair follicle, termed the "bulge", represent hair follicle stem cells (Cotsarelis *et al.* 1990). The bulge region of the hair follicle marks the bottom of the permanent portion of the follicle during cycling, just below the sebaceous gland and at the insertion site of the arrector pili muscle level (Cotsarelis *et al.* 1990; Taylor *et al.* 2000; Ohyama *et al.* 2006). The bulge area has been referred to as the reservoir of multipotent epidermal stem cells and is currently the best characterized site of epidermal stem cell populations (Kloepper *et al.* 2008; Abbas and Mahalingam 2009), which are activated at the start of a new hair cycle and upon wounding to provide cells for hair follicle regeneration and repair of the epidermis (Taylor *et al.* 2000; Morris *et al.* 2004; Tumbar *et al.* 2004; Ito *et al.* 2005).

1.12 Hair follicle stem cells and the contribution of hair follicle to wound repair

1.12.1 Stem cells in the hair follicle bulge are not necessarily required for epidermal homeostasis

During physiological skin renewal, stem cells in the hair follicle bulge do not contribute to the IFE. Normal IFE homeostasis is controlled by its own unipotent progenitor cells that reside within the IFE to ensure tissue renewal in the absence of injury (Morris *et al.* 2004; Ito *et al.* 2005; Levy *et al.* 2005; Blanpain and Fuchs 2006), as evidenced by the fact that epidermal homeostasis and wound repair can still take place in non-hair-bearing palmoplantar skin and in the skin of a number of genetic hair disorders such as hairless mice (Montagna *et al.* 1954; Blanpain and Fuchs 2006).

1.12.2 The significance of hair follicle stem cells to cutaneous wound repair

The significance of hair follicles to cutaneous wound repair has been well recognized, as exemplified by the clinical observations that skin grafting is not required for burn wounds that only destroy the epidermis but leave hair follicles intact (Green 1991). In contrast, skin grafting will be necessary for deeper burns in which the hair follicles are destroyed as the IFE cannot be repopulated by the hair follicle, except from the wound edges (Green 1991; Alonso and Fuchs 2003; Lau *et al.* 2009).

As early as several decades ago, studies involving re-epithelialization during wound repair have led researchers to suppose that hair follicles may have the capacity to

regenerate epidermis upon injury (Argyris 1976). More recently, studies have demonstrated that ablating early hair follicle stem cells in neonatal mice models led to severe compromised regeneration of IFE (Nowak *et al.* 2008), whereas cutaneous wounds heal with an acute delay in reepithelialization in adult mutant mice that lack hair follicle development (Langton *et al.* 2008) .

It has been discovered that cutaneous wounds stimulate proliferation in neighboring hair follicles resulting in the migration of keratinocytes in the hair follicle bulge along the infundibulum to the site of the lesion (Taylor *et al.* 2000; Ito *et al.* 2005; Levy *et al.* 2007). Additionally, studies have revealed that hair follicle-derived keratinocytes, as evidenced by in vivo lineage tracing technique, contributed long-termly to the newly formed epidermis, suggesting that the hair follicle stem cells can undergo reprogramming upon wounding and adopt the characteristic fate of epidermal cells (Ito *et al.* 2005; Levy *et al.* 2007). Therefore, as a major local reservoir for several different progenitor cell populations, the multipotent hair follicle bulge stem cells play key roles in wound healing.

1.12.3 Clinical applications of hair follicle stem cells to wound healing

These findings mentioned above make the hair follicle a fascinating target for regenerative medicine. In recent years, a number of stem cell treatment strategies have been developed and applied to animal models and clinical trials in the stem cell research field. Positive results in all phases of skin wound healing have been reported from

various treatments with the applications of hair follicle stem cells to animal models (Lau *et al.* 2009). Clinical trials on wound patients using stem cells from the hair follicles also have shown to improve and cure acute and chronic wounds, burn wounds (Liu *et al.* 2003; Lau *et al.* 2009) and chronic lesions like ulcers (Kurata *et al.* 1994; Limat *et al.* 1996; Limat *et al.* 2003; Tausche *et al.* 2003; Navsaria *et al.* 2004; Tiede *et al.* 2007). More recently, researchers have found that the pluripotency and high proliferative potential of hair follicle stem cells of mice even can rival embryonic stem cells in the regenerative potential, as exemplified by rejoining severed nerves using hair follicle stem cells in mouse wound models (Hoffman 2006; Hoffman 2007). These studies suggest that hair follicle stem cells can provide an effective, easily accessible and abundantly available autologous source of adult stem cells for regenerative medicine including therapeutic application on cutaneous wound repair (Richardson *et al.* 2005; Waters *et al.* 2007; Hunt *et al.* 2008; Lau *et al.* 2009).

1.13 The expression of integrin αvβ6 in hair follicle

During embryogenesis, the αvβ6 integrin is expressed at high levels in the multiple developing epithelial organs including skin, localized in both the basal layer of epidermis and the developing hair follicle. The expression of αvβ6 integrin was no longer detectable in the epidermis by week 21 of gestation, in the newborn and adult. However, the expression was still high at week 21 of gestation in the hair follicle. In normal adult

skin, although absent from the healthy epidermis, the αvβ6 integrin was still detectable at a low level in hair follicles (Breuss *et al.* 1995).

To date, the constitutive expression of αvβ6 integrin in adult hair follicles has been reported in human (Hakkinen *et al.* 2004), mice (Tumbar *et al.* 2004; Bandyopadhyay and Raghavan 2009), sheep (Brown *et al.* 2006) and pig (our unpublished observations). In 2004, Tumbar and colleagues conducted gene profiling analysis of hair follicle stem cells and revealed that β6 integrin transcripts were among the genes involved in TGF-β pathway that were strongly and selectively upregulated in a specific portion of ORS in hair follicle (Tumbar *et al.* 2004). This portion of hair follicle, termed "bulge", has been defined as the niche of epidermal stem cells in skin (Cotsarelis *et al.* 1990; Morris and Potten 1999; Taylor *et al.* 2000; Tumbar *et al.* 2004). Epidermal stem cell populations residing in the bulge area are slow cycling and express elevated transcripts encoding cell cycle regulatory proteins, and particularly, keratinocyte growth inhibitors involved in TGF-β signaling. Among them, LTBP-1 is required for latent TGF-β activation and is selectively and strongly localized to the bulge (Tumbar *et al.* 2004). Activated TGF-β/Smad target transcripts, protein, and downstream interacting proteins were also upregulated in the hair follicle bulge. More prevalent phospho-Smad2 immunoreactivity was observed in the bulge, suggesting a higher level of TGF-β activation in bulge stem cells than their progeny cells (Tumbar *et al.* 2004). Moreover, TGF-β1 has been implicated as an endogenous inducer of hair follicle regression in vivo (Foitzik *et al.* 1999; Foitzik *et al.* 2000; Soma *et al.* 2003) possibly through the inhibition of

keratinocyte proliferation and induction of apoptosis. As a potent endogenous activator of TGF-β1, the function of αvβ6 integrin in hair follicles remains, however, still unknown. As a cell surface receptor that is exclusively expressed by epithelial cells, αvβ6 integrin is only constitutively expressed in either self-renewing structures in skin (Paus and Cotsarelis 1999), the hair follicles, or de novo synthesized in the basal keratinocytes in response to wound stimuli, both conditions involved the essential participation of the biological functions of epidermal stem cells. These findings suggest a possible role of αvβ6 integrin in the biological functions of the epidermal stem cells.

1.14 Mouse model of depilation-induced hair regeneration / cycling

In the research field of hair biology, a highly standardized C57BL/6 mouse model of depilation-induced hair cycling has been widely used for the analysis of hair follicle growth and the pathophysiology of hair loss disorders (Paus *et al.* 1990; Paus *et al.* 1994; Muller-Rover *et al.* 2001; Porter 2003). To induce the hair cycling, firstly, a mixture composed of wax and rosin is applied on the back skin of seven-week-old mice with all hair follicles in telogen stage, which can be evidenced by the homogeneous pink skin color in the pigmented mice or confirmed by histological analysis in non-pigmented mouse strains. Removal of the mixture will lead to the removal of all hair shafts and immediately induces homogeneous development of three consecutive hair growth cycle stages, anagen, catagen and telogen, over the entire depilated area of the mouse back skin

(Muller-Rover *et al.* 2001).

Hair plucking generates numerous “micro-wounds” in the hair follicles of the depilated area and induces a wound healing response immediately after the depilation (Argyris 1968; Muller-Rover *et al.* 2000; Muller-Rover *et al.* 2001). This leads to profound alterations in the thickness and architecture of almost all skin compartments in the mice. Among them, the most evident alteration are the substantial, hair cycle-associated fluctuations in the skin thickness (Paus *et al.* 1990; Muller-Rover *et al.* 2001). The depilation-induced anagen hair follicles reach their maximal length after 9 days of depilation (Slominski *et al.* 1991; Paus *et al.* 1998; Muller-Rover *et al.* 2001). Around day 17 after depilation, the catagen-associated morphological changes first appear in the neck region. In the pigmented mice, the onset of catagen can also be macroscopically recognized by a skin color change from black to gray-pink. Around day 19, the catagen development wave reaches the tail region of the mouse (Muller-Rover *et al.* 2001).

The cycling hair follicle has been used as an excellent model to study the basic biology of organ regeneration, stem cell properties and a potential source of adult stem cells for regenerative medicine for the following reasons (Porter 2003; Yu *et al.* 2008; Al-Nuaimi *et al.* 2010). First of all, this model of depilation-induced hair cycling is a wound healing response of the hair follicle caused by a plucking trauma. (Chase *et al.* 1951; Argyris 1968; Slominski *et al.* 1991; Slominski and Paus 1993; Paus 1998; Muller-Rover *et al.* 2000). Hair regeneration is triggered immediately after depilation and depilation induced anagen is fully synchronized over the entire plucked area. In contrast, spontaneous

anagen develops in a wave-like pattern. Hair follicle morphogenesis and consecutive stages of the hair cycle develop following a rather precise time-scale, which makes a highly predictable development of three different stages of hair cycle possible (Foitzik *et al.* 2000; Muller-Rover *et al.* 2001). This provides a window for manipulation of hair cycle by treatment agents. Secondly, the mouse hair cycle is only taking about 3 weeks. The short and synchronized hair cycle allows hair follicles to be easily harvested and analyzed at specific time points of the hair cycle. Thirdly, the distinct stages of the hair cycle have been well characterized and classified in mice. For example, anagen has been divided morphologically into six different sub-stages and catagen into eight sub-stages (Muller-Rover *et al.* 2001), which provide pragmatic criteria for the recognition of distinct stages of the hair cycle. Finally, the depilation-induced anagen hair follicles do not show significant morphological differences compared with spontaneously developing anagen follicles.

Therefore, the mouse model of depilation-induced hair cycling provides a useful tool to identify the morpgological abnormalities of hair growth cycling in a highly reproducible, quantifiable, and easily applicable manner and is an ideal model system for studying the hair regeneration (Muller-Rover *et al.* 2001; Porter 2003).

1.15 Hypotheses and objectives

Hypothesis 1: The integrin αvβ6-mediated regulation of TGF-β1 activity plays a role in impaired wound healing induced by dexamethasone.

Objective 1: To investigate the healing rate in the untreated and dexamethasone-treated WT mice and β6-/- mice at different time points after wounding.

Objective 2: To evaluate re-epithelialization, granulation tissue formation, collagen organization and basement membrane regeneration in the untreated and dexamethasone-treated WT mice and β6-/- mice at different time points after wounding.

Objective 3: To assess cell proliferation and inflammatory response in the untreated and dexamethasone-treated WT mice and β6-/- mice at different time points after wounding.

Objective 4: To determine and compare the levels of TGF-β1 expression and activation in the non-wounded and wounded skin at different time points in the untreated and dexamethasone-treated WT mice and β6-/- mice.

Objective 5: To examine the expression patterns of αvβ6 integrin in the non-wounded and wounded skin at different time points under the untreated and dexamethasone-treated conditions.

Objective 6: To investigate whether any compensatory changes in the expression of other integrins in the β6-/- mice.

Hypothesis 2: Integrin αvβ6-mediated TGF-β1 signaling regulates hair regeneration and hair follicle involution.

Objective 1: Using WT FVB mice and β6-/- mice with the same genetic background to establish depilation-induced hair growth cycle model; To determine and compare hair follicle morphology and the development of hair growth cycle stages between the different mouse strains of WT FVB mice and C57BL/6 mice.

Objective 2: To investigate the spatio-temporal expression patterns of αvβ6 integrin in different stages of hair growth cycle.

Objective 3: To assess and compare the dynamic morphological changes of the regenerating hair follicles and IFE at different time points through the complete hair growth cycle in WT mice and β6-/- mice.

Objective 4: To assess and compare the basement membrane regeneration in WT mice and β6-/- mice at different time points after depilation.

Objective 5: To determine the influence of deficiency of the αvβ6 integrin on hair density before and after depilation.

Objective 6: Using histomorphometry of hair growth cycle stages to evaluate and compare catagen development in WT mice and β6-/- mice.

Objective 7: To assess and compare keratinocyte proliferation in WT mice and β6-/- mice at different time points after depilation.

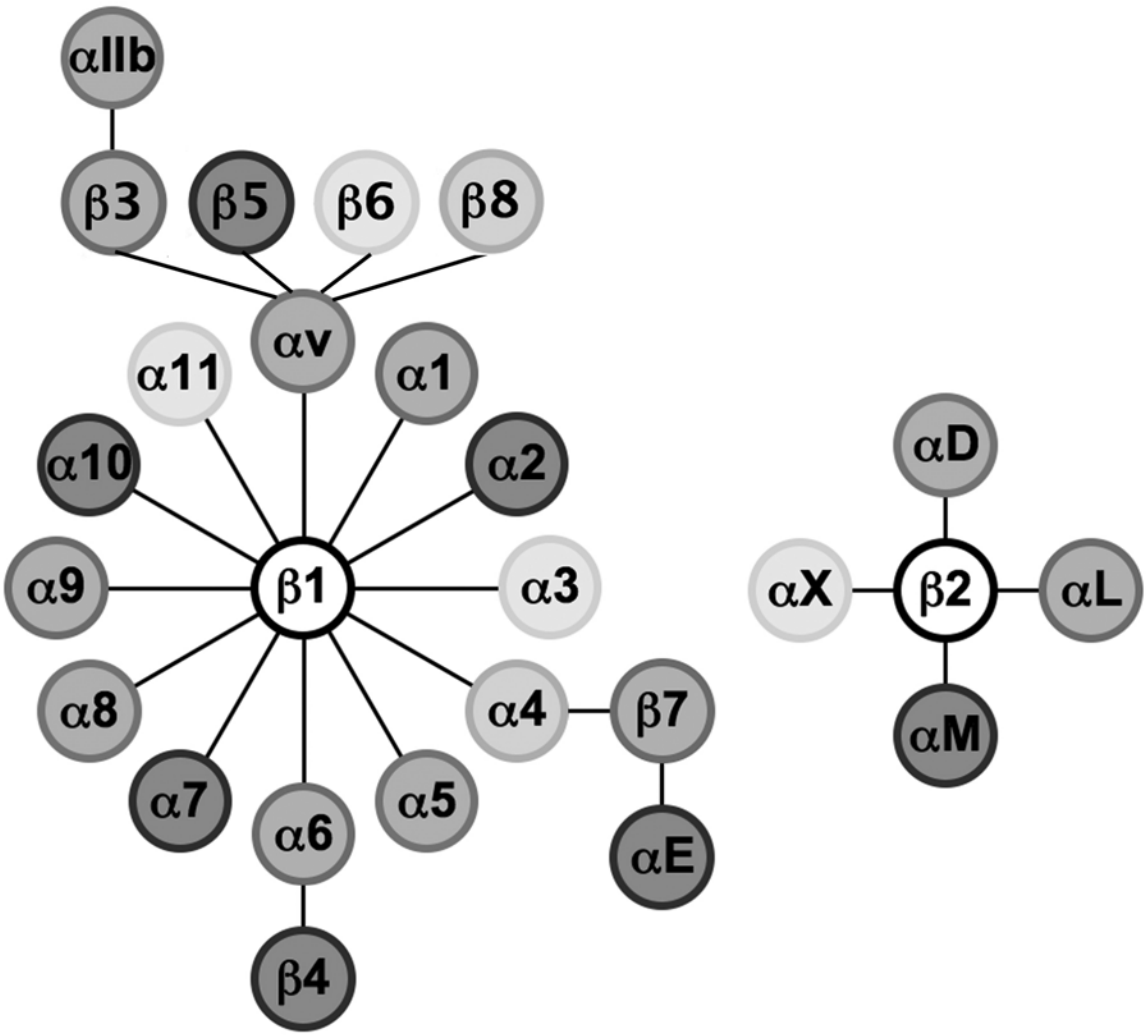
Objective 8: To examine the spatio-temporal expression and activation patterns of

TGF-β1 in different stages of hair growth cycle in WT mice and β6-/- mice; To quantify and compare the relative level of TGF-β1 expression and activation in WT mice and β6-/- mice at different time points after depilation.

Table 1.1 RGD-containing ligands of αvβ6 integrin

Type of ligand	Ligand	Reference
ECM protein	Fibronectin	Busk *et al*. 1992
	Tenascin-C	Prieto *et al*. 1993
	Vitronectin	Huang *et al*. 1998
	Fibrillin-1	Jovanovic *et al*. 2007
Cytokine	Latency associated peptide (LAP) of TGFβ-1	Munger *et al*. 1999
	Latency associated peptide (LAP) of TGFβ-3	Annes *et al*. 2002
Viral capsid	Foot-and-mouth disease virus (FMDV)	Miller *et al*. 2001
	Coxsackievirus 9 (CAV-9)	Williams *et al*. 2004
	Human parechovirus 1 (HPEV 1)	Seitsonen et al. 2010

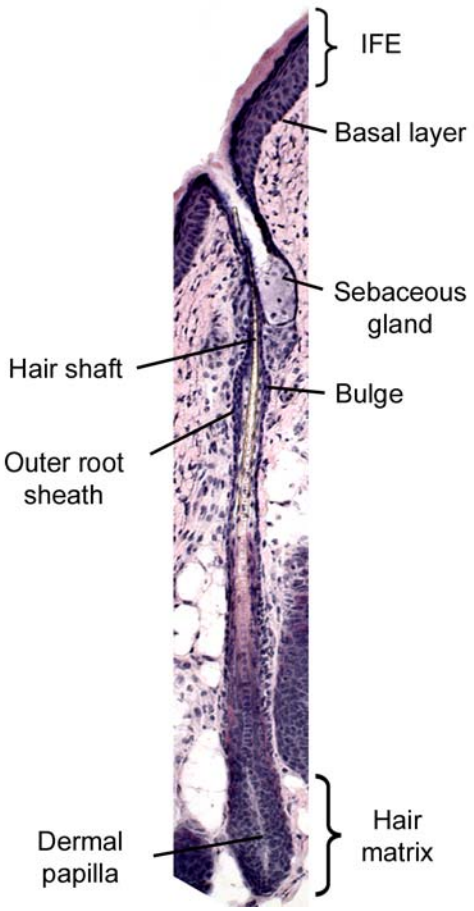
Figure 1.1 The integrin superfamily



The eighteen α and eight β subunits have been identified in the integrin superfamily that form at least 24 distinct heterodimeric integrins. The different subunits selectively bind to their partners. Integrin β6 subunit can only pair with αv subunit.

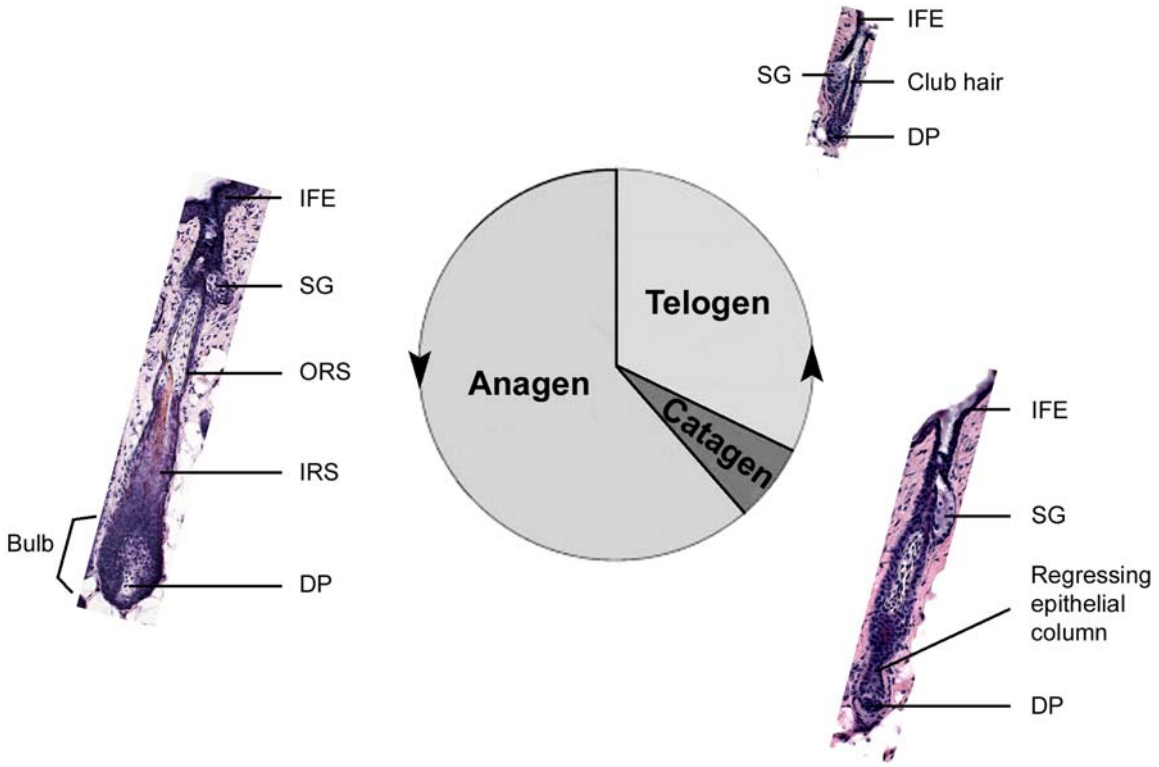
Modified from A. Bandyopadhyay and S. Raghavan, 2009.

Figure 1.2 Hair follicle structure



Hematoxylin/eosin (H&E) staining of a hair follicle in anagen stage from mouse back skin. IFE: interfollicular epidermis.

Figure 1.3 The hair cycle



Hair follicles cycle through alternating phases of anagen, catagen and telogen. IFE, interfollicular epidermis; SG, sebaceous gland; ORS, outer root sheath; IRS, inner root sheath; DP, dermal papilla.

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Chapter 2: Mice lacking β6 integrin in skin show accelerated wound repair in dexamethasone impaired wound healing model*

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2.1 Introduction

More than 50 years after their discovery, glucocorticoids have remained one of the most frequently used classes of drugs. About 30 million patients in the United States receive treatment with exogenous steroid agents (Ettinger *et al.* 2001). Their therapeutic indications are widespread, the side effects, however, are common and of great importance to the patients. Chronic glucocorticoid therapy induces detrimental effects in several tissues, including the skin where it causes skin atrophy. When encountering an accidental or surgical trauma, impaired tissue repair capacity is one of the serious complications of glucocorticoid therapy. A better understanding of the cellular and molecular mechanisms during impaired wound healing will be instrumental for the development of effective therapies for patients with aberrant wound healing.

The wound healing process includes three continuous and overlapping phases: inflammatory, proliferative and remodeling phase. The inflammatory phase is initiated immediately upon injury and lasts up to 4 to 6 days after wounding. A variety of inflammatory cells influx to the wound site to fight infection and remove debris. The proliferative phase is characterized by the formation of granulation tissue, epithelialization and angiogenesis, and lasts from about day 4 to day 14 after wounding. The remodeling phase starts at around day 8 and may last up to a year or even longer (Broughton *et al.* 2006). Glucocorticoids affect almost every phase of wound healing because of their inhibitory effect on gene expression in various cells. In the early phase

after injury, dexamethasone inhibits recruitment of various inflammatory cells and gene expression of many key wound healing cytokines and growth factors (Beer *et al.* 2000). During the proliferative and remodeling phases, dexamethasone can inhibit the synthesis of several dermal extracellular matrix proteins, and delay re-epithelialization and fibroplasias (Beer *et al.* 2000).

Integrins are heterodimeric adhesion receptors that mediate cell-cell and cell-extracellular matrix interactions. Integrin αvβ6 is an epithelial cell-specific receptor that is not constitutively expressed by normal epidermis. Its expression, however, is induced during wound repair (Breuss *et al.* 1993; Larjava *et al.* 1993). Integrin αvβ6 recognizes a tripeptide amino acid sequence arginine–glycine–aspartate acid (RGD-motif) in its ligands, which include fibronectin, tenascin-C, vitronectin and the latency-associated peptide (LAP) of transforming growth factor (TGF)-β1 and TGF-β3 (Busk *et al.* 1992; Huang *et al.* 1998; Annes *et al.* 2004). In addition to mediating cellular adhesion to these extracellular matrix proteins, αvβ6 integrin activates latent TGF-β1 (Munger *et al.* 1999; Annes *et al.* 2004). Integrin-mediated activation of latent TGF-β1 plays an important role in vivo, as the absence of integrin-mediated TGF-β1 activation recapitulates the phenotype of TGF-β1-null mice (Yang *et al.* 2007). Specifically αvβ6 integrin-dependent activation of TGF-β1 has been confirmed to be pivotal in mouse models of TGF-β1-dependent fibrosis in various epithelial organs, including lung (Horan *et al.* 2008), kidney (Hahm *et al.* 2007) and liver (Wang *et al.* 2007), suggesting that this mechanism of TGF-β activation may be of general importance in tissues that express

αvβ6 integrin. As a potent endogenous activator of TGF-β1, the function of αvβ6 integrin in wound healing is, however, still poorly defined. Our laboratory has previously reported that human chronic wounds show prolonged expression of αvβ6 integrin and transgenic mice over-expressing β6 integrin in skin develop spontaneous chronic fibrotic wounds that contain high levels of TGF-β1 (Hakkinen *et al.* 2004). These studies indicate that αvβ6 integrin-mediated regulation of TGF-β1 activity may play a role in the pathophysiology of the impaired wound healing. Studies of acute wound healing in young β6 integrin deficient (β6-/-) and β6 integrin overexpressing mice have found no wound healing abnormalities (Huang *et al.* 1996; Hakkinen *et al.* 2004). Interestingly, a somewhat improved healing response has been, however, reported in old hydrocortisone-treated αvβ6 integrin overexpressing mice (AlDahlawi *et al.* 2006).

TGF-β1 affects all cell types that are involved in all stages of wound healing. TGF-β1 has been shown to promote cell migration, stimulate wound contraction and promote the production of extracellular matrix molecules. Although the beneficial effects of TGF-β1 in wound healing have been well recognized, accumulating evidence suggests that TGF-β1 may also delay wound repair, reflecting the complex nature of the biological functions of TGF-β1. Smad3 is a TGF-β-activated transcription factor that plays an important role in mediating a number of wound repair-associated TGF-β1 responses. TGF-β1 signaling is partially abolished in Smad3 knockout mice and these mice exhibit an accelerated wound healing phenotype, characterized by increased keratinocyte proliferation, migration and reduced monocyte infiltration (Ashcroft *et al.* 1999).

Consistently, transgenic mice overexpressing TGF-β1 in the epidermis exhibited retarded re-epithelialization due to the inhibited keratinocyte proliferation after burn injury (Yang *et al.* 2001). Conversely, TGF-β1-null mice showed accelerated re-epithelialization after incisional wounding (Koch *et al.* 2000). In addition, transgenic mice overexpressing a dominant-negative TGF-β receptor display accelerated re-epithelialization in skin wounds. This was associated with an enhanced proliferation and a reduced apoptosis of keratinocytes at the wound edge owing to the resistance of keratinocytes to TGF-β1-mediated growth inhibition and apoptosis induction (Amendt *et al.* 2002).

In the present study, to determine the function of αvβ6-integrin in impaired wound healing caused by glucocorticoids, an impaired wound model of full-thickness, excisional wounds was established by dexamethasone treatment. Multiple wound parameters, keratinocyte proliferation, inflammation and TGF-β1 activation were assessed in both dexamethasone-treated and untreated wild-type (WT) and β6-/- mice.

2.2 Materials and methods

2.2.1 Animals

All animal studies were conducted in compliance with Canadian Council on Animal Care guidelines and approved by The University of British Columbia Animal Care Committee. Fifty-six adult male WT FVB and sex-matched β6-/- mice with the same genetic background were used in this study (generous gift from Dr. Dean Sheppard, University of California, San Francisco). Each experimental group was subdivided into dexamethasone-treated and untreated (control) groups.

2.2.2 Excisional wound healing

The treatment groups received intraperitoneal injections of 1 mg kg^{-1} per day of dexamethasone (D4902, Sigma-Aldrich, St. Louis, MO) dissolved in ethanol and further diluted in phosphate buffered saline solution (PBS, pH 7.4). The drug was administered daily at 9 am from 7 days before wounding until the end of the experiment as described previously (Beer *et al.* 2000). The untreated control groups received intraperitoneal daily injections of similar volumes of PBS. On the day of wounding, mice were anaesthetized with inhalation of isoflurane (Baxter Corporation, Mississauga, ON, Canada) and the dorsal skin was shaved and depilated (Reckitt Benckiser NA Inc., Parsippany, NJ). Four full-thickness, 4 mm excisional biopsy wounds were created on the dorsal skin of each

mouse by using a sterile, disposable biopsy punch (Miltex Inc., York, PA). Wounds were left to heal for up to 10 days and during that period mice were caged separately and had free access to water and food.

2.2.3 Wound area and wound closure analysis

At the time of wounding and at every day post-wounding, images of all the wounds were recorded by a digital camera (Nikon Coolpix 995, Tokyo, Japan). All wound pictures were standardized according to a measurement ruler included in the images using Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA). The wound surface area quantification analysis was performed on a Macintosh computer using the public domain NIH ImageJ program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) and the wound size at each time point was expressed as a percentage of the original wound area at day 0. Wound closure rate was also evaluated by daily application of 3% H_2O_2 (Fisher Scientific, Pittsburgh, PA). Each wound received 40 µl of H_2O_2 that was allowed to react for 1 minute. Bubbles forming in the center of the wound, indicating incomplete wound closure, were recorded as a positive response. This test is based on catalase enzyme activity in the underlying connective tissue, which liberates oxygen and water from hydrogen peroxide. If the epithelial barrier is intact, hydrogen peroxide does not diffuse deep enough to release oxygen (Padgett *et al.* 1998).

2.2.4 Histology

Mice were euthanized by CO_2 inhalation. Wound biopsies were harvested using 4 mm (at day 0) or 6 mm (at day 5, 10 post-wounding) biopsy punches. From each mouse, two wound biopsies were mounted in Tissue-TEK (Sakura, Torrance, CA), snap-frozen on dry ice immediately after collection, and used later for frozen sectioning. Another two wound biopsies were snap-frozen in liquid nitrogen for ELISA.

Histological sections (7 μm) were prepared from frozen wound tissues and stored at -80°C. Every tenth section was stained with Harris' hematoxylin and eosin (H&E) to determine the localization of the wound center. Sections taken from the center of each wound were further analyzed histologically. A set of 10-day-old mid-wound sections was fixed with 10% buffered neutral formalin and stained with modified Movat's pentachrome staining for histological evaluation of the extracellular matrix components in the wounds. To quantify the difference in the granulation tissue formation between the groups, the granulation tissue was scored in the H&E stained samples using the following scale: 0 = No granulation tissue formation; 1 = Granulation tissue formation is visible, but with a gap in the middle, 2 = A lot of granulation tissue formation, without a gap in the middle. The number of wounds with different scores was expressed relative to the total number of wounds in each group. To quantify the difference in the rate of re-epithelialization between the groups, the 10-day wounds were categorized as being completely closed or open in the H&E stained samples. The number of open or

completely closed wounds was expressed relative to the total number of wounds in each group.

2.2.5 Immunohistochemistry

Frozen sections taken from the center of each wound were fixed with ice-cold acetone at room temperature for 5 minutes. For Ki67 and cleaved caspase-3 staining, samples were fixed with 10% buffered neutral formalin at room temperature for 10 minutes. Sections were rinsed in PBS containing bovine serum albumin (BSA, 1 mg ml^{-1}) and incubated with normal blocking serum (Vector Laboratories, Burlingame, CA). Sections were then incubated with the primary antibody raised against collagen IV (PS057; Cedarlane Laboratories, Hornby, ON, Canada; 1:200), laminin-1 (L9393; Sigma-Aldrich, St. Louis, MO; 1:100), Ki67 (ab15580; Abcam, Cambridge, MA; 1:1000), cleaved caspase-3 (Asp175, 5A1; Cell Signaling Technology, Danvers, MA; 1:800), F4/80 (RM2900; Caltag Laboratories, Burlingame, CA; 1:200), CD45 (30-F11; BD Biosciences Pharmingen, Frankin Lakes, NJ; 1:200), CD4 (H129.19; BD Biosciences Pharmingen; 1:50), CD8a (53-6.7; BD Biosciences Pharmingen; 1:25), phospho-Smad3 (ab51451; Abcam; 1:50), β6 integrin (β6B1; a generous gift from Dr. Dean Sheppard, University of California–San Francisco; 1:10) or β1 integrin (MB 1.2; a generous gift from Dr. Bosco Chan, University of Western Ontario, Canada; 1:300) diluted in PBS/BSA in a humidified chamber at 4°C overnight. After washing with PBS/BSA, species-appropriate

biotinylated secondary antibodies were incubated at room temperature for 60 minutes, followed by the Vectastain ABC reagent (Vector Laboratories) according to the manufacturer's instructions. The reaction was visualized with either a VIP or a DAB substrate kit (Vector Laboratories). As negative controls, sections were treated with PBS instead of the primary or secondary antibodies to rule out non-specific immunostaining. In all cases, they showed no positive staining.

To quantify the difference in the regeneration of BM between groups, BM in the 10-day-old wounds was categorized as being continuous or discontinuous in the type IV collagen or laminin-1 immunostained samples. The number of wounds with discontinuous or continuous BM was expressed relative to the total number of wounds in each group. To compare the inflammatory response between groups, the positive reaction of specific markers to different inflammatory cells was scored using the following scale according to the intensity of the staining: 0, 1+, 2+, and 3+. The area populated by F4/80-positive cells out of the total area of the granulation tissue was assessed using the following scale: 1 (0-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The sum of the intensity and the percentage scores was used as the final staining score as described previously (Gao *et al.* 2006). To assess cell proliferation, the number of Ki67-positive keratinocyte nuclei was counted in consecutive high-power fields (HPFs, ×200 magnification). In the wound area of each section, a total of five HPFs from either the leading wound margin or the wound center (for closed wounds) were counted. In hair follicles surrounding the wound, five HPFs adjacent to the newly-formed granulation

tissue (no mature hair follicles were present within granulation tissue, except some occasional round structures close to the neo-epidermis representing newly forming hair follicles from epidermis) were counted in each section. All data were expressed as the mean number of positive cells per HPF ± standard deviation of the mean. To assess TGF-β1 activation, the number of phospho-Smad3-positive keratinocyte nuclei was quantified using the same method as we used to evaluate cell proliferation in the wound epithelium (see above).

2.2.6 Cytokine ELISA

To compare the levels of cytokines in wound samples, the 6 mm wound samples were snap-frozen in liquid nitrogen and stored at -80°C until used. For protein extraction, the wound samples were weighted and homogenized in the presence of a fixed weight-per-volume ratio of homogenization buffer containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolia, IN) and a 0.1% pyrogen-free BSA (A4919, Sigma-Aldrich) by disposable hard-tissue Omini-tips (OMNI International, Marietta, GA) attached to the rotor-stator homogenizer (Hakkinen *et al.* 2004). The homogenate was cleared of tissue debris by centrifugation. The resulting supernatant was further filtered using a 0.22 μm syringe filter. Finally, the protein extract was aliquoted and stored at -80°C until use. The levels of IL-1β, IL-6, TNF-α and total TGF-β1 in the wound tissue was measured by ELISA with mouse IL-1β, IL-6, TNF-α and TGF-β1 Quantikine

immunoassay kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.2.7 Statistical analysis

The statistical differences in the wound area at each day after wounding were determined by Student's *t*-test. The same test was used to compare the numbers of Ki67-positive proliferating cells, F4/80 scores, the expression levels of cytokines and the number of phospho-Smad3-positive keratinocytes. Chi-square test was used to compare the non-parametric data collected from the hydrogen peroxide test. Comparison of re-epithelialization, regeneration of BM and granulation tissue formation scores were performed by Fisher's exact test. The statistical analyses were done by SPSS 11.5 software for Windows and $p < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 β6-/- mice display an accelerated wound healing in dexamethasone-induced impaired wound model

To determine the importance of β6 integrin for wound repair, we analyzed full-thickness excisional wounds from β6-/- and WT mice at various time points after wounding. In untreated WT and β6-/- groups, the wound area displayed significant differences at the early stage of healing (days 1 to 4). However, wound healing proceeded at a similar rate at the later stage of healing in both groups. After 10 days of healing, the average wound area was 2.3% and 1.6% of the original wound size in untreated WT and β6-/- groups, respectively, and statistical analysis did not show any significant difference between these two groups (Fig. 2.1F). Dexamethasone treatment significantly delayed the wound healing rate. Most wounds in the dexamethasone-treated mice were still open whereas most untreated wounds were closed 10 days after wounding (Fig. 2.1A, G, I). This trend was more pronounced between the treated and untreated WT groups as compared to β6-/- mice (Fig. 2.1C, E). A statistically significant delay existed from day 3 through day 10, especially at the final stage of the healing. For example, at day 10, the average wound size was reduced to 2.3% of the original size in the untreated WT group compared to 30% in the treated WT group ($p<0.001$), which represents over four days of healing delay in this model (Fig. 2.1B).

Based on gross morphology (Fig. 2.1), the wound area measurement (Fig. 2.1B, D, F, H)

and the wound closure evaluation using the hydrogen peroxide test (Fig. 2.1C, E, G, I), an accelerated rate of wound healing was readily apparent in the dexamethasone-treated β6-/- mice at different time points post-wounding compared to the treated WT animals (Fig. 2.1H, I). At day 10, the average wound area was reduced to 14% of the original area in the treated β6-/- group compared to 30% in the treated WT group. Treated β6-/- mice also exhibited relatively mild delayed wound repair when compared to the untreated β6-/- mice, and only showed a significant delay at the later stage of healing (from day 9 to 10, Fig. 2.1D).

A peroxide test was used as a biochemical indicator of wound closure. For the first 4 days of healing, all wounds showed positive test results (Fig. 2.1 C, E, G, I). At day 10, 96% and 82% of the wounds in the untreated β6-/- and WT mice were negative in the test, respectively (Fig. 2.1G). The majority of wounds (70%) in the treated β6-/- group also presented a negative response (Fig. 2.1I). In contrast, 67% of the wounds in the treated WT mice showed a positive reaction. In summary, both the quantitative wound area measurement and the qualitative peroxide test showed an accelerated wound repair in the dexamethasone-treated β6-/- mice compared to the treated WT mice (Fig. 2.1H, I).

2.3.2 Enhanced granulation tissue formation and re-epithelialization in dexamethasone-treated β6-/- wounds

To evaluate granulation tissue formation and re-epithelialization, we further analyzed 5- and 10-day-old wounds histologically. After 10 days of healing, granulation tissue was

still missing in the wound center in 66.7% of the treated WT wounds and therefore there was a gap between the two sides of the wound granulation tissue, while only 16.7% of the treated β6-/- wounds still had a gap in the middle of the wound (Fig. 2.2A, B). Statistical analysis of the 10-day-old wounds showed significantly different granulation tissue scores ($p<0.05$) between the two dexamethasone-treated groups, but not between the two untreated groups (Fig. 2.2B). Re-epithelialization was examined in the H&E-stained sections of the 10-day-old wounds (Fig. 2.2A) and showed that 70% of the wounds in the untreated WT group and 100% of the wounds in the untreated β6-/- group were completely re-epithelialized. In comparison to the untreated groups, 92% of the wounds in the dexamethasone-treated WT group and 42% of the wounds in the treated β6-/- group were still open. Statistical analysis showed a significant difference ($p<0.05$) in the number of wounds showing complete or incomplete re-epithelialization between the two dexamethasone-treated groups in the 10-day-old wounds, but not between the two untreated groups (Fig. 2.2C).

2.3.3 β6-/- mice display higher density of collagen fibers and more organized granulation tissue

To further assess the formation of granulation tissue and collagen organization, modified Movat's pentachrome staining was performed on a set of 10-day-old mid-wound sections (Fig. 2.3). Compared to the corresponding deaxamthasone-treated wounds, abundant,

newly- formed, lightly stained, bluish-green collagen fiber bundles and spindle-shaped fibroblasts were observed in the granulation tissue of the untreated wounds. These collagen fiber bundles were less organized compared to the darkly stained, bright yellow bundles of the unwounded connective tissue at the wound edge. Wounds in both the untreated and treated β6-/- mice showed a higher density of and more organized newly-formed collagen fiber bundles in the granulation tissue compared to the corresponding WT mice at 10 day post-wounding.

2.3.4 Increased rate of BM regeneration in dexamethasone-impaired β6-/- wounds

Type IV collagen and laminin-1 are two major BM constituents. In 10-day-old wounds, we observed type IV collagen or laminin-1 in the BM zone of the epidermis and in the blood-vessel walls of the granulation tissue in all animal groups (Fig. 2.4A, B). At day 10, 70% of the untreated WT wounds and 90% of the untreated β6-/- wounds showed continuous, linear and well-defined type IV collagen and laminin-1 staining at the BM zone. In contrast, in 100% of the dexamethasone-treated WT wounds, the staining of these BM components was still discontinuous while 50% of the wounds in the treated β6-/- mice showed a linear and continuous staining pattern of type IV collagen and laminin-1. Statistical analysis demonstrated a significant difference ($p<0.05$) in the regeneration of BM in the 10-day wounds between the two treated groups, but not between the two untreated groups (Fig. 2.4C).

2.3.5 Lack of β6 integrin in skin leads to enhanced keratinocyte proliferation during wound healing

Having established that the wound healing rate of dexamethasone-treated mice was significantly accelerated in the β6-/- group, we investigated whether the improved healing is due to a difference in cell proliferation. We analyzed keratinocyte proliferation by immunohistochemical staining of the specific proliferating cell marker Ki67 in 0-, 5- and 10-day-old wounds. There were no differences between the groups in keratinocyte proliferation in the unwounded normal skin epidermis and hair follicles (data not shown). In the 10-day-old wounds, however, the number of Ki67-positive basal keratinocytes at either the wound center (for closed wounds) or at the leading wound margin in the dexamethasone-treated β6-/- mice was significantly increased compared to the treated WT mice (Fig. 2.5A). The same trend was observed in the 5-day-old wounds (data not shown). The largest difference in cell proliferation was observed, however, in the hair follicles of the wound margin, characterized by the presence of distinct numbers of proliferating cells at different hair cycle stages (Fig. 2.5B). The hair bulbs of the untreated β6-/- mice contained approximately a 5-fold increase in the number of Ki67-positive cells compared to the WT mice (Fig. 2.5D). Dexamethasone treatment caused significant hair follicle regression in both groups of mice. There were only a few Ki67-positive cells in the hair follicles and they were only located in the area near the papillary dermis surrounding the treated WT wounds. In the treated β6-/- mice, however,

many hair follicles with a significant number of Ki67-positive cells were still present (Fig. 2.5B, D). A notable increase in Ki67-positive cells in the untreated β6-/- mice was mainly localized in the outer root sheaths of the hair follicles (Fig. 2.5B). Taken together, these results indicate that a β6 integrin-deficiency in the skin potently enhances the proliferation of hair follicle keratinocytes during wound healing. To rule out that increased proliferation may lead to increased apoptosis of activated keratinocytes, we identified apoptotic cells by an antibody against activated caspase-3. The results showed no significant difference in the numbers of apoptotic cells between the β6-/- and WT mice (data not shown).

2.3.6 Local inflammatory response and production of pro-inflammatory cytokines is reduced in wounds of dexamethasone-treated β6-/- mice

We next determined if the accelerated re-epithelialization in the dexamethasone-treated β6-/- mice is associated with an altered inflammatory response. The day 5 and day 10 wounds revealed no significant differences in the infiltration of PMN leukocytes (data not shown). Macrophage staining (F4/80) of the 5-day-old wounds indicated a notable increase in the infiltration of macrophages in the treated WT wounds compared to the treated β6-/- wounds (Fig. 2.6A). In the 10-day-old wounds, however, the number of macrophages declined in all groups compared to the 5-day-old wounds and showed no significant difference between the β6-/- and WT mice (data not shown). In both the 5-day

and 10-day-old wounds, only small numbers of $CD4^+$ and $CD8^+$ T lymphocytes were present with no significant differences between the groups (data not shown).

Because pro-inflammatory cytokines regulate wound healing, we investigated their local production in the wound tissue. IL-1β showed a remarkable increase in the 5-day-old wounds compared to the non-wounded tissue in all groups (Fig. 2.6B). The levels of IL-1β were still elevated in the 10-day-old wounds compared to the non-wounded tissue in the dexamethasone-treated mice, while in the untreated animals, the levels had returned to the baseline. The expression of IL-1β was significantly reduced in the wounds of the dexamethasone-treated β6-/- mice compared to the treated WT mice at both the 5-day and 10-day time points (Fig. 2.6B). Both IL-6 and TNF-α ELISA in the 5-day-old wound tissue also demonstrated a significantly reduced expression in the treated β6-/- mice compared to the treated WT mice (Fig. 2.6C, D).

To determine whether accelerated wound repair was associated with an altered TGF-β1 expression, the level of total TGF-β1 was measured in 0-, 5- and 10-day-old wound tissue of the dexamethasone-treated and untreated WT and β6-/- mice by ELISA (Fig. 2.7). In all the 5- and 10-day-old wound tissue, the expression of TGF-β1 was significantly elevated compared to the non-wounded day-0 tissue. Dexamethasone-treated wounds in the β6-/- mice showed no significant differences at the TGF-β1 levels compared to PBS-treated wounds. However, TGF-β1 was significantly elevated in the 5-day-old dexamethasone-treated WT wounds compared to treated β6-/- wounds.

2.3.7 TGF-β1 activation is reduced in dexamethasone-treated β6-/- wound tissue

To determine whether the level of TGF-β1 activation is altered in keratinocytes of the β6-/- wounds, we detected phospho-Smad3 as an indirect measurement of TGF-β1 activation. The positive staining for phospho-Smad3 was detected in both the WT and β6-/- mice, indicating that the β6 integrin independent mechanisms for the activation of TGF-β1 also exist in the wounded skin of the β6-/- mice. In the wound epithelium, the positive staining was mainly detected in the cell nuclei of basal and one to two suprabasal cell layers (Fig. 2.8A). Quantification of positive staining showed that the level of phospho-Smad3 in the treated β6-/- wounds was significantly reduced compared to the treated WT wounds (Fig. 2.8B).

2.3.8 Expression of β6 and β1 integrins in wound tissue

Our study also further delineated the spatial and temporal expression of β6 integrin in the non-wounded and wounded skin at different time points. The β6-/- mice showed a complete lack of staining for β6 integrin in all specimens examined (Fig. 2.9). In the WT mice, keratinocytes in the non-wounded, intact epidermis did not express β6 integrin. Some expression in the cells of the outer root sheath of hair follicles was, however, noted (data not shown). After wounding, the expression of β6-integrin was induced in WT mice and localized in both the epidermis along the wound edges and in the hair follicles. In the epidermis, β6 integrin immunostaining was localized to the cell membrane of the basal

and one to two suprabasal cell layers (Fig. 2.9A). In the hair follicles, β6 integrin-positive staining was mainly confined to the cell membrane of the outer root sheath keratinocytes (Fig. 2.9B). A relatively higher intensity of β6 integrin staining in the hair follicles, especially in the outer root sheath portion below the sebaceous glands, compared to the epidermis was also noted (data not shown). In addition, β6 integrin showed a remarkable up-regulation in the epidermis of the wounded area compared to the wound periphery (data not shown). The localization, distribution and intensity of β6 integrin staining in the dexamethasone-treated WT wounds was similar to that in untreated WT wounds (data not shown), suggesting that dexamethasone did not regulate the expression of αvβ6 integrin. To study whether the wounds in β6-/- mice healed faster because of compensatory changes in the expression of other integrins, we investigated the expression of β1 integrins in the non-wounded and wounded tissue at all time points. The results indicateded that expression of β1 integrins showed no significant differences at different time points between the β6-/- and WT mice (data not shown).

2.4 Discussion

In the present study, we showed that wounds heal significantly faster in dexamethasone-treated β6-/- mice compared to similarly treated WT animals. In fact, the healing rate in these treated β6-/- mice was remarkably close to the untreated animals. This is a novel and significant finding because dexamethasone is widely used in medicine and has serious side effects, including delayed wound closure in skin. In contrast to the dexamethasone-treated animals, no significant difference was observed in the wound closing rates between untreated WT and β6-/- animals after 10 days of healing. This observation is in agreement with previous studies that examined younger and older, female and male β6-/- mice and reported no abnormalities in the healing response to experimental excisional wounding (Huang *et al.* 1996; AlDahlawi *et al.* 2006). In the present study, we used only male mice because they show slower wound repair compared to females due to the beneficial effects of estrogen on wound healing (Ashcroft *et al.* 1997).

Our findings suggest that the accelerated healing in the β6-/- mice resulted from an increased proliferation of keratinocytes at the wound epithelium and hair follicles. This is likely linked to a reduced anti-proliferative effect of TGF-β1 on keratinocytes as αvβ6 integrin is a potent activator of latent TGF-β1 (Munger *et al.* 1999; Annes *et al.* 2004). TGF-β1 has been confirmed to be a negative regulator of cell proliferation in the basal layer of the epidermis and delay wound re-epithelialization (Glick *et al.* 1993). Inhibition

of epithelial cell proliferation by TGF-β1 involves down regulation of c-Myc leading to upregulation of cyclin-dependent kinase inhibitors p15 and p21, which inhibit the CDK4/6-cyclin D and CDK2-cyclin E-mediated phosphorylation of the retinoblastoma protein (Robson *et al.* 1999; Ten Dijke *et al.* 2002). Our findings in the dexamethasone-treated β6-/- mice are consistent with the transgenic mice overexpressing TGF-β1 in the epidermis. These mice show a decreased rate of re-epithelialization, due to reduced keratinocyte proliferation in the basal layer of the neo-epidermis (Yang *et al.* 2001). These observations are also in line with studies using Smad3-null mice that demonstrate disrupted signaling of TGF-β1 and an increased rate of re-epithelialization after incisional wounding (Ashcroft *et al.* 1999). In addition, a TGF-β1 antagonist accelerates wound re-epithelialization (Huang *et al.* 2002). All of this evidence suggests that reduced TGF-β1 signaling, including elimination of TGF-β1 activator αvβ6 integrin, speeds up the healing process through accelerated keratinocyte proliferation. In the present study, the largest difference in keratinocyte proliferation between the β6-/- and WT mice was observed in the hair follicles. The contribution of hair follicles to cutaneous wound repair has been recognized for decades (Argyris 1976; Taylor *et al.* 2000). In addition to the basal layer of the epidermis, hair follicles have been confirmed to be another source of epidermal stem cells, residing in a region of the outer root sheath called the bulge. After wounding, stem cells from the hair follicle bulge were activated, exited their niche, migrated and proliferated to repopulate the interfollicular epidermis (Taylor *et al.* 2000; Ito *et al.* 2005; Levy *et al.* 2007). Studies have shown that a significant portion

of the newly-formed epidermis originates from the bulge cells of the hair follicles after excisional wounding (Ito *et al.* 2005). Our data demonstrated that, in contrast to the wound epithelium, a relatively higher expression of αvβ6 integrin was specifically localized to the outer root sheath of the hair follicles that fuses to the basal keratinocyte layer of the epidermis. Interestingly, a notable increase of Ki67-positive proliferating cells in the outer root sheath of the hair follicles surrounding the wounded skin in the β6-/- mice was observed. These findings suggest a cause-and-effect relationship between lacking β6 integrin and enhanced keratinocyte proliferation in the hair follicles after wounding. Similarly, an increased number of Ki67-positive cells in the hair follicles of TGF-β1-/- mice has been reported (Foitzik *et al.* 2000). TGF-β1 may act as an endogenous inducer of hair follicle regression (catagen) in vivo (Foitzik *et al.* 2000). It is also well know that dexamethasone is an exogenous inducer of hair follicle regression (Paus *et al.* 1994). The synergistic effect of dexamethasone and αvβ6 integrin (via TGF-β1) to suppress cells in the hair follicles probably explains why enhanced wound healing was observed mainly in the dexamethasone-treated β6-/- mice, and only at the early time points in the untreated β6-/- mice. Additionally, to evaluate the effect of αvβ6 integrin on keratinocyte migration with or without dexamethasone treatment, we used HaCaT (Human epidermal keratinocyte cell line) cells to perform scratch wound assays. Our results suggested that loss of αvβ6 integrin has little if any effect on the keratinocyte migration with or without dexamethasone treatment in vitro (data not shown).

Our data also showed a decrease in the pro-inflammatory cytokine levels and macrophage

infiltration that might be associated with a lower level of total TGF-β1 and reduced TGF-β1 activation in the dexamethasone-treated β6-/- wound tissue compared to the treated WT wound tissue. Paradoxically, we also observed an increased inflammatory response in the dexamethasone-treated animals. These differences might be an indirect reflection of the apparently delayed inflammatory phase of wound healing induced by dexamethasone in the treated groups. The reduced inflammation in the dexamethasone-treated β6-/- wounds could be associated with the accelerated healing rate in the treated β6-/- mice. Alternatively, αvβ6 integrin could regulate inflammation via TGF-β1 signaling. In support of this latter notion, a blockade of αvβ6 integrin reduces the expression and activity of TGF-β1 and leads to an inhibition of the pro-inflammatory effect of TGF-β1 in the kidney (Hahm *et al.* 2007). TGF-β1 has been known for its anti-inflammatory role ever since studies have shown that TGF-β1-deficient mice exhibit inflammation in multiple organs and develop autoimmune conditions (Shull *et al.* 1992; Kulkarni *et al.* 1993). However, accumulating evidence indicates that TGF-β1 may also play a pro-inflammatory role that depends on the cell type and micro-environment and may vary in different organs. TGF-β1 has been shown to be a potent chemotactic cytokine for virtually all leukocytes that are involved in inflammation (Wahl *et al.* 1987; Wahl *et al.* 1993). In support of this notion, transgenic mice overexpressing latent TGF-β1 in the basal keratinocytes and hair follicles spontaneously develop chronic skin inflammatory lesions (Li *et al.* 2004). These transgenic mice exhibited a significant delay in wound healing compared to the WT mice

that was associated with profound inflammation throughout all of the stages of wound healing (Wang *et al.* 2006). Similarly, Smad3-null mice also exhibited accelerated wound healing with reduced monocyte infiltration (Ashcroft *et al.* 1999). Based on the above studies, the reduced expression and activation of TGF-β1 might contribute to the alleviated inflammatory response in the dexamethasone-treated β6-/- mice, which subsequently contributes to the accelerated granulation tissue formation in the wound sites, while the accelerated healing rate in the treated β6-/- wounds could also be responsible for this effect. The actual mechanism of the pro-inflammatory response of TGF-β1 needs further investigation.

Activation of latent TGF-β1 by αvβ6 integrin is a highly localized process and likely occurs under strict spatio-temporal regulation. We used immunohistochemistry to examine the level of phospho-Smad3 as an indirect measurement of TGF-β1 activation. The results of the phospho-Smad3 immunostaining suggest that the presence of αvβ6 integrin contributes to the activation of TGF-β1 in the basal keratinocytes in the presence of dexamethasone, leading to reduced keratinocyte proliferation and subsequently slower migration and wound closure. Reduced TGF-β1 signaling may also reflect total TGF-β1 levels in these wounds. Taken together, our data suggest a possible link between TGF-β1 and the observed effects, although other mechanisms of TGF-β1 activation might also play a role during the cutaneous wound repair.

We also observed a profound change in collagen expression and organization after dexamethazone treatment in β6-/- wounds, which could not only been explained by the

defects in the αvβ6 null keratinocytes. It is obvious that other cell types are also involved. Endothelial cells have been reported to express αvβ6 integrin in multiple human organs after infection by human cytomegalovirus (Tabata *et al.* 2008). Noninfected endothelial cells, however, do not express αvβ6 integrin. Consistent with these findings, we have not observed any expression of αvβ6 integrin in the vascular endothelial cells of the normal or wounded mouse skin. Since the wounds healed with different speed, the increased accumulation of collagen is most likely caused by an indirect effect of the alleviated inflammation and improved healing in β6-/- wounds although we can not rule out other explanations such as altered keratinocyte-fibroblast interaction.

Taken together, our findings indicate that αvβ6 integrin functions to reduce keratinocyte proliferation in both the wound epithelium and especially in the hair follicles surrounding the wounded skin. This reduction has only a mild effect on wound healing unless an additional inhibitory factor is introduced (dexamethasone). Therefore, suppression of αvβ6 integrin function by specific inhibitors may enhance keratinocyte proliferation and down regulate the inflammatory response in the wound site and may provide a specific target for future therapeutic intervention in impaired wound healing.

2.5 Acknowledgments

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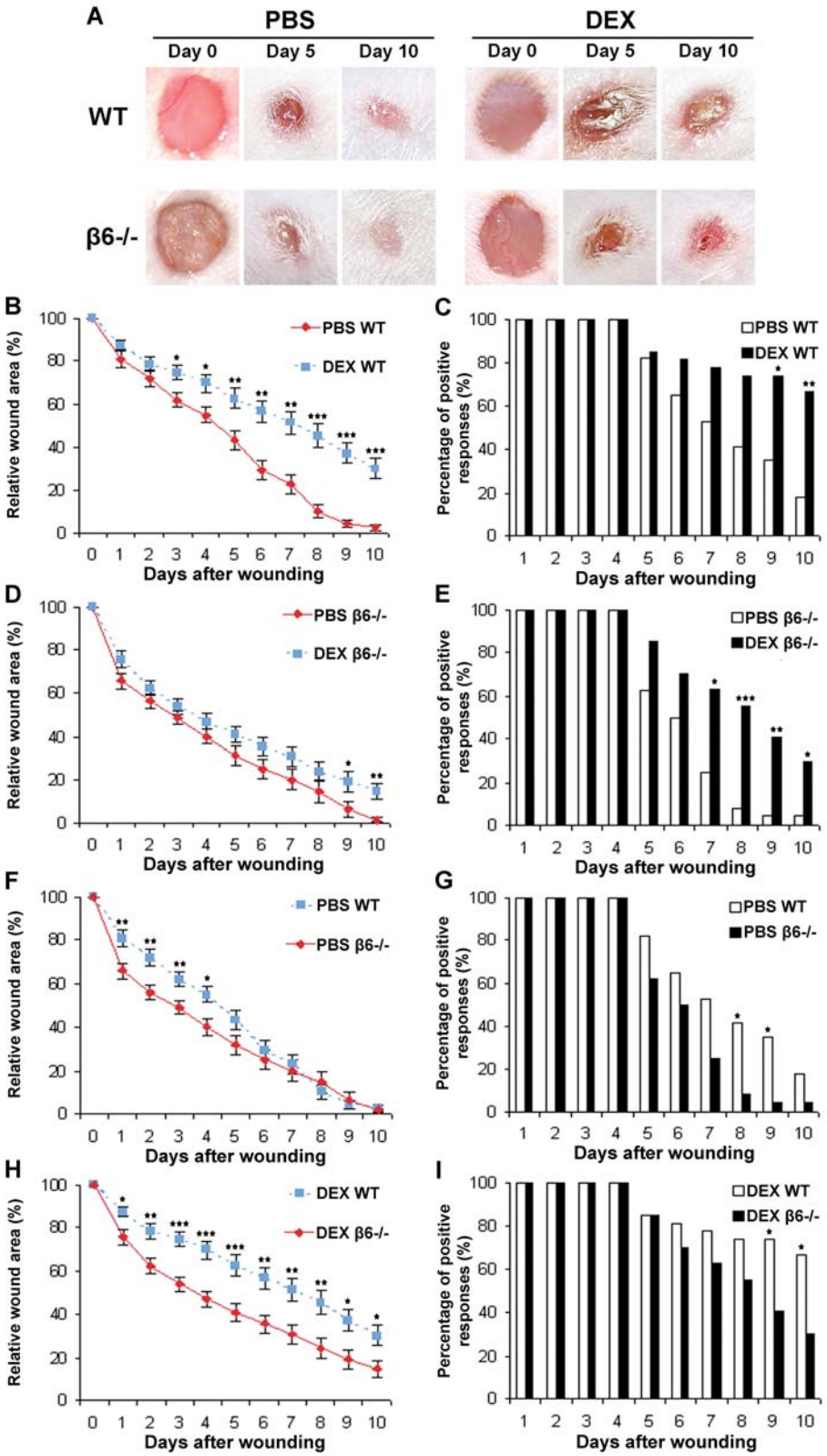
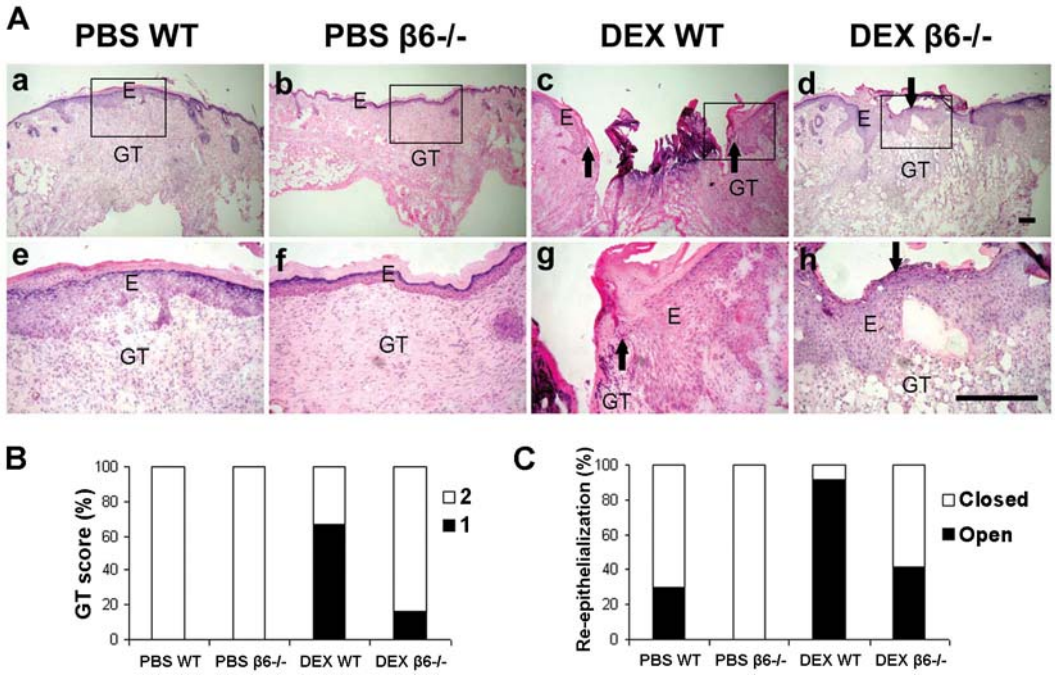
Figure 2.1 Wound healing rate in untreated or treated WT and β6-/- mice



Wounds in the dexamethasone-treated β6-/- mice close at a faster rate than in the similarly treated wild-type (WT) mice. (A) Representative clinical photographs from the WT and β6-/- mice with or without dexamethasone treatment at different time points after wounding. Wound area changes over time relative to the original wound area in the WT and β6-/- mice with or without dexamethasone treatment are shown in B, D, F and H. Data are expressed as mean ± SE (n = 40-47 wounds from 11-12 mice per group; * $p<0.05$; ** $p<0.01$; *** $p<0.001$). Number of wounds that showed a positive response to the H_2O_2 test over time expressed as a per cent of all the wounds in the WT and β6-/- mice with or without dexamethasone treatment are shown in C, E, G and I (n = 40-47 wounds from 11-12 mice per group; * $p<0.05$; ** $p<0.01$; *** $p<0.001$). PBS: control animals treated with phosphate buffered saline; DEX: dexamethasone-treated animals.

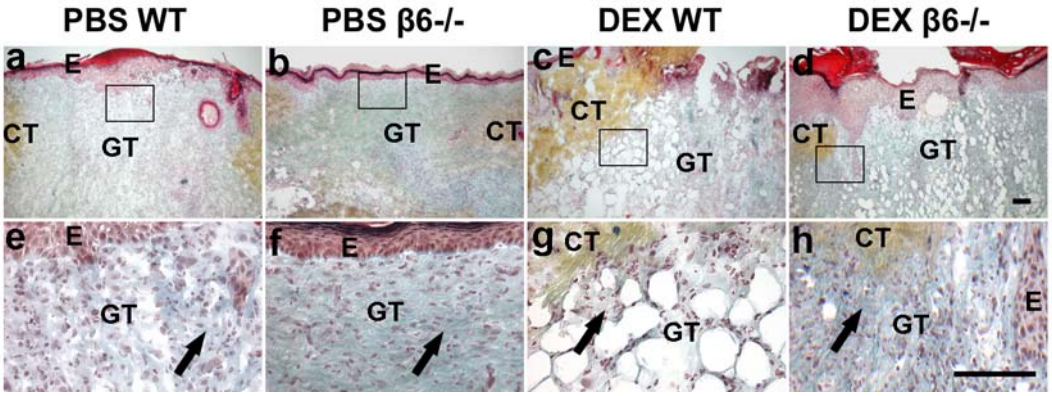
Figure 2.2 Evaluation of granulation tissue formation and re-epithelialization



The dexamethasone-treated β6-/- mice show accelerated granulation tissue formation and re-epithelialization compared to the corresponding wild-type (WT) controls. (A) Representative histological sections of the 10-day-old wounds from the WT and β6-/- mice with or without dexamethasone treatment are shown. The lower row (e-h) represents high magnification images of the boxed area shown in the upper low-magnification row (a-d). E: epithelium; GT: granulation tissue. Arrows indicate the migrating or fused wound epithelium. Scale bars = 200 μm. (B) Quantification of granulation tissue formation in 10-day-old wounds using histological sections from the WT and β6-/- mice with or without dexamethasone treatment. Score 1: granulation tissue

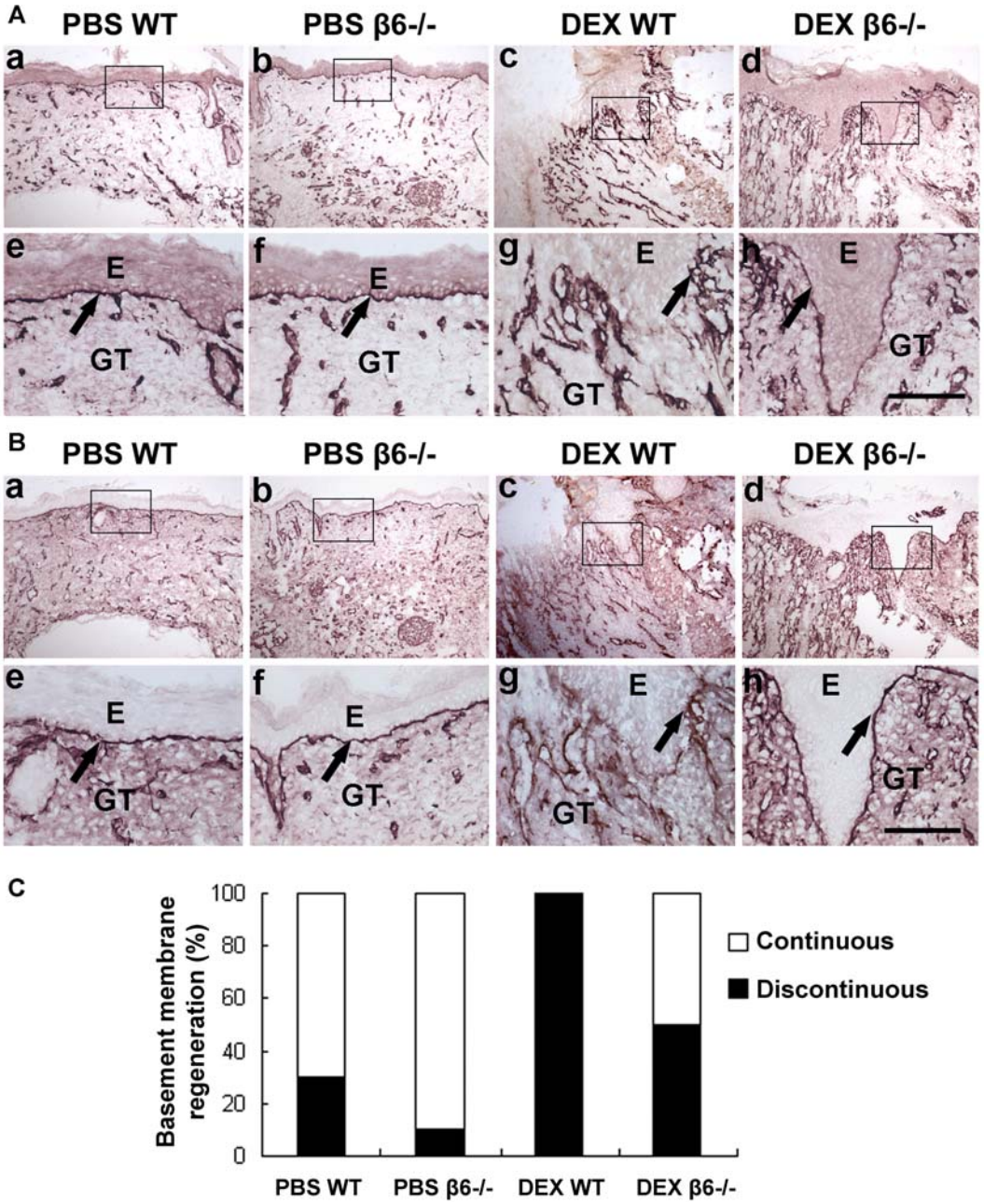
present but with a gap in the middle; Score 2: granulation tissue present through the section. (C) Quantification of wound re-epithelialization in 10-day-old wounds using histological sections from the WT and β6-/- mice with or without dexamethasone treatment. Open: epithelium does not cover the wound; Closed: epithelium cover the entire wound. A significant difference in granulation tissue formation and re-epithelialization was found between the dexamethasone-treated β6-/- and WT mice (n = 10-12 wounds from 10-12 mice per group; $p<0.05$). PBS: control animals treated with phosphate buffered saline; DEX: dexamethasone-treated animals.

Figure 2.3 Comparison of newly formed collagen organization



β6-/- mice show a higher density and a better organization of newly-formed collagen fiber bundles in the granulation tissue compared to the corresponding wild-type (WT) mice. Representative histological sections from the 10-day-old wounds from the WT and β6-/- mice with or without dexamethasone treatment stained with Movat's pentachrome stain are shown. Newly-formed blue-green collagen fiber bundles can be seen in the wound area while mature collagen in the unwounded connective tissue appears bright yellow. The lower row (e-h) represents high magnification images of the boxed area shown in the upper row (a-d). E: epithelium; GT: granulation tissue (blue-green); CT: connective tissue. PBS: control animals treated with phosphate buffered saline; DEX: dexamethasone-treated animals. Arrows indicate the newly-formed collagen fiber bundles. Scale bar = 100 μm.

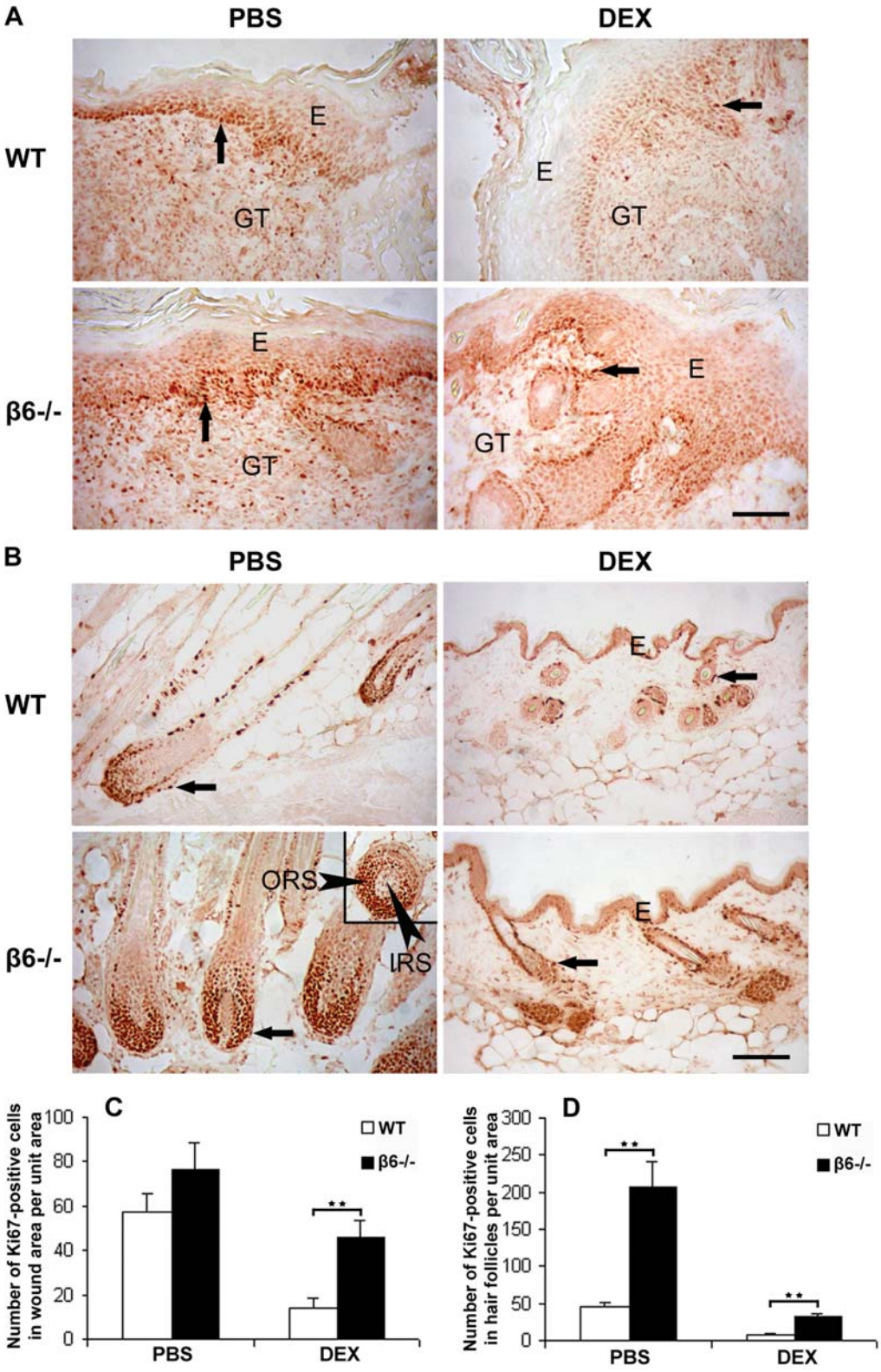
Figure 2.4 Assessment of basement membrane regeneration



Basement membrane regeneration proceeds at a faster rate in the dexamethasone-treated β6-/- mice compared to the corresponding wild-type (WT) mice. Immunolocalization of type IV collagen (A) and laminin-1 (B) in the 10-day-old wounds from the WT and β6-/- mice with or without dexamethasone treatment. The

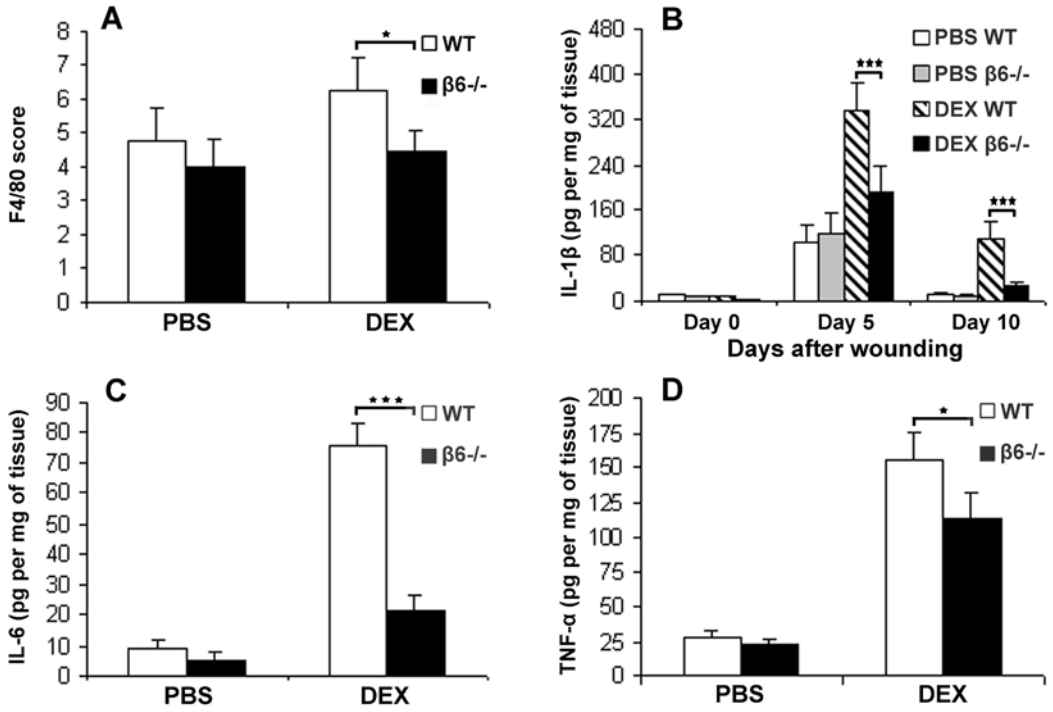
lower row (e-h) represents high magnification images of the boxed area shown in the upper low-magnification row (a-d). E: epithelium; GT: granulation tissue. Arrows indicate the basement membrane zone. Scale bars = 100 μm. (C) Quantification of wounds showing continuous or discontinuous regeneration of basement membrane in 10-day-old wounds from the WT and β6-/- mice with or without dexamethasone treatment assessed from immunostained histological sections. A significant difference in the basement membrane regeneration was found between the dexamethasone-treated β6-/- and WT mice (n = 10-12 wounds from 10-12 mice per group; $p<0.05$). PBS: control animals treated with phosphate buffered saline; DEX: dexamethasone-treated animals.

Figure 2.5 Keratinocyte proliferation in the neo-epidermis and in the hair follicles



The dexamethasone-treated β6-/- mice show significantly increased keratinocyte proliferation in the basal layer of the neo-epidermis (A) and in the hair follicles (B) compared to the corresponding wild-type (WT) animals. Keratinocyte proliferation in the 10-day-old wounds was evaluated by immunohistochemical staining of the proliferating cell marker Ki67. In the epidermis, Ki67-positive staining was mainly localized in the cell nuclei of the basal and one to two suprabasal layers of keratinocytes along the wound edges (A). In the hair follicles, Ki67-positive staining was localized in the cell nuclei of the outer root sheath keratinocytes (B). A cross section of the hair follicles in an untreated β6-/- mouse demonstrates that the increase in Ki67 positive cells in the hair follicles was localized in the outer root sheath (an example is shown in the inset of the PBS-treated β6-/- group in B). E: Epithelium; GT: granulation tissue; ORS: outer root sheath; IRS: inner root sheath. Arrows indicate Ki67 positively stained cell nuclei. Scale bars = 100 μm. Quantification of Ki67-positive keratincytes in the wound area (C) or in the hair follicles surrounding the wounded skin (D) in the 10-day-old wounds from the WT and β6-/- mice with or without dexamethasone treatment. The hair follicles surrounding the wounded skin in both the untreated and dexamethasone-treated β6-/- wounds showed a significant increase in the number of Ki67 positive proliferating keratinocytes compared to the corresponding WT controls. Data are expressed as mean ± SD (n = 6 mice per group; * $p<0.05$; ** $p<0.01$). PBS: control animals treated with phosphate buffered saline; DEX: dexamethasone-treated animals.

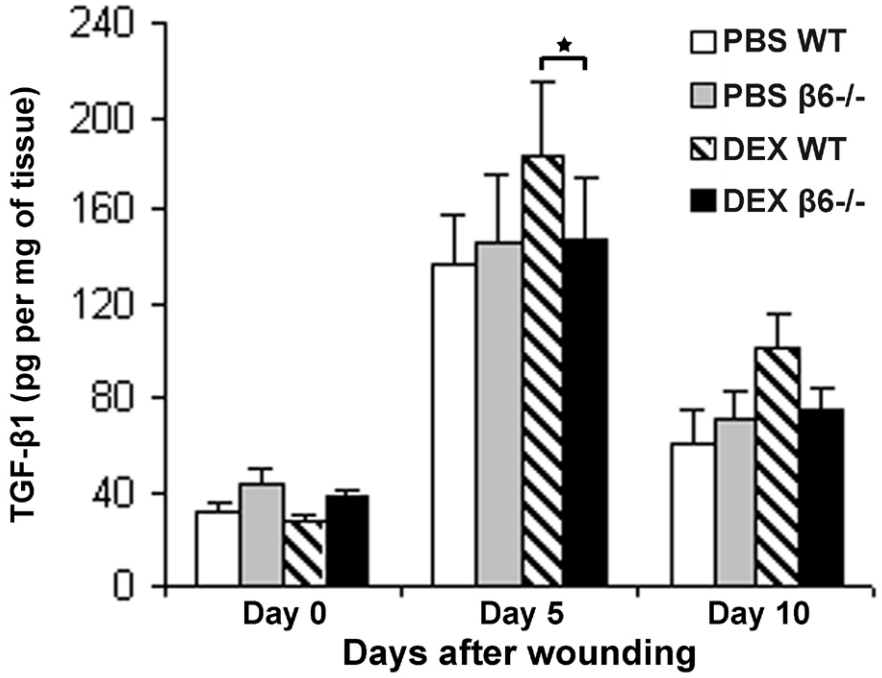
Figure 2.6 Evaluation of inflammatory response in the untreated or treated WT and β6-/- mice



A significant decrease in the number of F4/80-positive macrophages and in the production of the pro-inflammatory cytokines in the dexamethasone-treated β6-/- wounds compared to the treated wild-type (WT) wounds. (A) Semi-quantitative analysis of the immunohistochemical staining of the macrophage-specific F4/80-positive cells in the 5-day-old wounds from the WT and β6-/- mice with or without dexamethasone treatment. Data are expressed as mean±SD (n = 4 mice per group; * $p<0.05$). (B, C and D) Cytokine ELISA of wound tissues. The results showed a significant decrease in the local levels of IL-1β in the dexamethasone-treated β6-/-

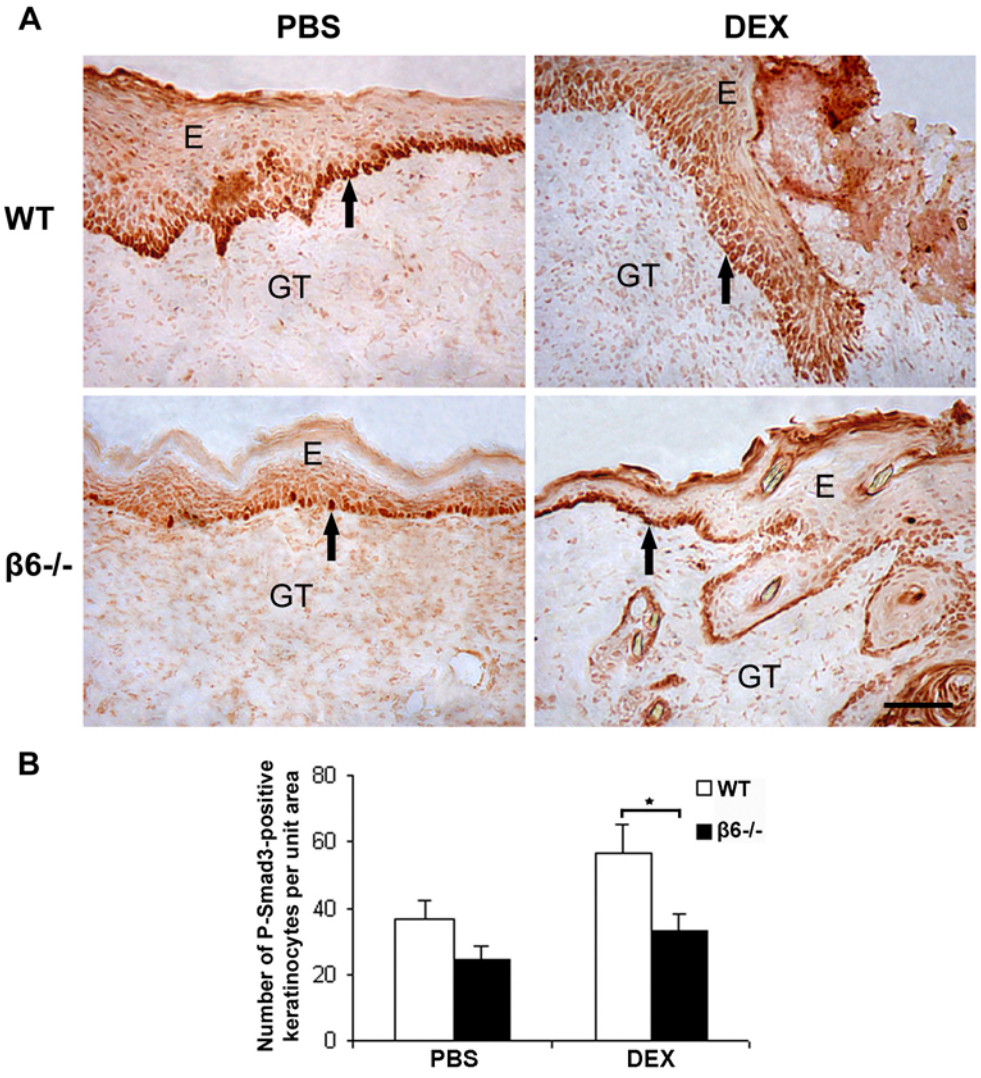
wounds compared to the treated WT wounds at both the 5-day and 10-day time points (B). The dexamethasone-treated β6-/- mice demonstrated significantly reduced levels of IL-6 (C) and TNF-α (D) in the 5-day-old wounds compared to the treated WT wounds. Data are expressed as mean ± SD (n = 7-8 wounds from 7-8 mice per group; * $p<0.05$; ** $p<0.01$; *** $p<0.001$). PBS: control animals treated with phosphate buffered saline; DEX: dexamethasone-treated animals.

Figure 2.7 Determination of the levels of TGF-β1 expression in the non-wounded and wounded skin of untreated or treated WT mice and β6-/- mice



A significantly reduced production of total TGF-β1 in the 5-day-old wounds from the dexamethasone-treated β6-/- mice compared to the treated wild-type (WT) mice. Production of TGF-β1 in the wound tissue was measured using ELISA. Data are expressed as mean ± SD (n = 7-8 wounds from 7-8 mice per group; * $p<0.05$). PBS: control animals treated with phosphate buffered saline; DEX: dexamethasone-treated animals.

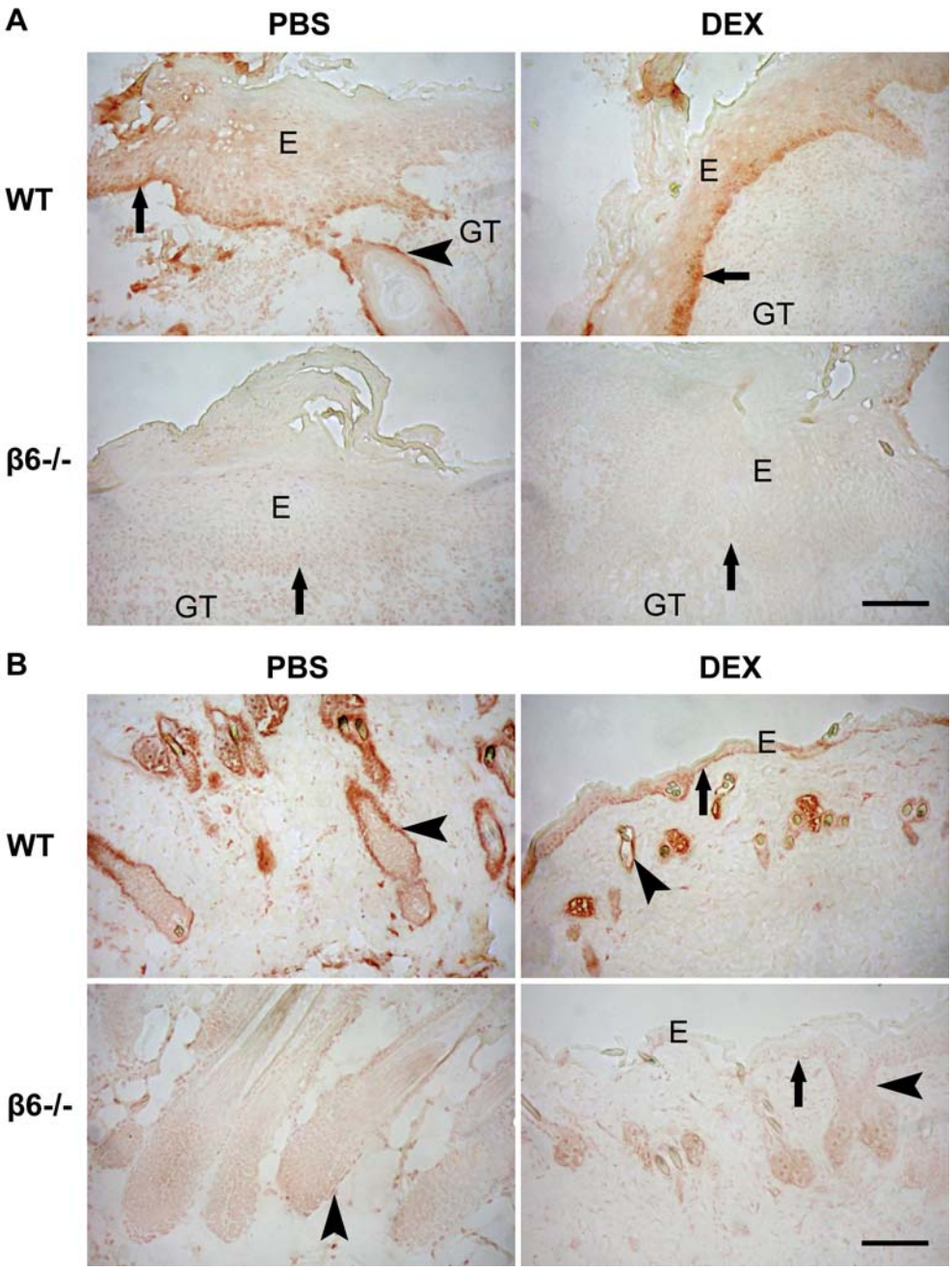
Figure 2.8 Determination of the levels of phospho-Smad3 in the untreated or treated WT and β6-/- wounds



The dexamethasone-treated β6-/- mice show significantly decreased TGF-β1 activation in the basal layer of the neo-epidermis (A) compared to the corresponding wild-type (WT) animals. TGF-β1 activation in the 10-day-old wounds was evaluated indirectly by immunohistochemical staining of phospho-Smad3. In the wound epithelium,

phospho-Smad3-positive staining was mainly localized in the cell nuclei of the basal and one to two suprabasal cell layers (A). E: Epithelium; GT: granulation tissue. Arrows indicate the cell nuclei with positive phospho-Smad3 staining. Scale bar = 100 μm. (B) Quantification of phospho-Smad3-positive keratinocytes in the wound epithelium in the 10-day-old wounds from the WT and β6-/- mice with or without dexamethasone treatment. The results showed that the level of phospho-Smad3 in the treated β6-/- wounds was significantly reduced compared to the treated WT wounds (* $p<0.05$). Data are expressed as mean $\pm$ SD (n = 6 mice per group). PBS: control animals treated with phosphate buffered saline; DEX: dexamethasone-treated animals.

Figure 2.9 Expression pattern of β6 integrin in the epidermis and hair follicles



Immunolocalization of β6 integrin in the epidermis and hair follicles. (A) Representative staining of β6 integrin in the 10-day-old wounds from wild-type (WT) and β6-/- mice with or without dexamethasone treatment are shown. β6-/- mice showed a complete lack of β6 integrin staining in all specimens examined. In the WT mice, the

expression of β6 integrin was localized in the cell membrane of basal and one to two suprabasal cell layers in the epidermis along the wound edges. (B) Representative staining of β6 integrin in the hair follicles from the skin surrounding the 10-day-old wounds in the WT and β6-/- mice with or without dexamethasone treatment. β6-/- mice showed a complete lack of β6 integrin staining in all specimens examined. In the WT mice, the expression of β6 integrin was localized in the cell membrane of the outer root sheath keratinocytes of the hair follicles. E: Epithelium; GT: granulation tissue. Arrows indicate the basal layer of the epidermis. Arrowheads indicate hair follicles. PBS: control animals treated with phosphate buffered saline; DEX: dexamethasone-treated animals. Scale bars = 100 μm.

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Chapter 3: Loss of αvβ6 integrin causes enhanced keratinocyte proliferation and retarded hair follicle regression*

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3.1 Introduction

Hair follicles are mini-organs that constantly renew themselves throughout adult life, and are an important model for organ regeneration (Yu *et al.* 2008). Hair follicles continuously cycle through anagen (growth), catagen (involution) to telogen (resting) phases. This process depends on epidermal stem cells contained in hair follicles, residing in a region of the outer root sheath (ORS) termed the "bulge" (Cotsarelis *et al.* 1990). Accumulated evidence has confirmed that the bulge is the repository of multipotent epidermal stem cells in skin**.** Epidermal stem cells maintain the capability of self-renewal and multi-lineage differentiation, which is regulated by the surrounding microenvironment, or niche (Blanpain *et al.* 2004; Moore KA 2006). Therefore, it is critical to understand how epidermal stem cell activity is regulated in order to manipulate its functions during hair follicle and skin regeneration.

The anatomical location of the bulge region has been defined in the ORS between the sebaceous gland and the insertion point of the arrector pili muscle level (Ohyama *et al.* 2006). Epidermal stem cell populations residing in the bulge area are slow-cycling and express elevated transcripts encoding cell cycle regulatory proteins, and particularly, keratinocyte growth inhibitors involved in transforming growth factor-β (TGF-β) signaling. Among them, LTBP-1, is required for latent TGF-β activation and is selectively and strongly localized to the bulge (Tumbar *et al.* 2004). Activated TGF-β/Smad target transcripts, protein, and downstream interacting proteins were also

up-regulated in the hair follicle bulge. One of these, a more prevalent phospho-Smad2 immunoreactivity was observed in the bulge, suggesting a higher level of TGF-β activation in bulge stem cells than their progeny. Similarly, β6 integrin was strongly and specifically upregulated in the bulge cells relative to other progeny cells (Tumbar *et al.* 2004).

TGF-β1 is a well-established inhibitor of epithelial cell proliferation (Massague and Weinberg 1992). Loss of TGF-β1 in skin and skin tumors is associated with a basal cell hyperproliferation phenotype (Glick *et al.* 1993). Transgenic mice expressing a dominant-negative TGF-β type II receptor show epidermal hyperproliferation (Wang *et al.* 1997). Over-expression of TGF-β1 in the epidermis of transgenic mice leads to the inhibition of normal skin development and suppression of epithelial cell proliferation (Sellheyer *et al.* 1993). TGF-β inhibits cell growth through transcriptional repression of c-Myc (Frederick *et al.* 2004) and upregulation of the cyclin-dependent kinase inhibitors p15, p21 and p27 (Reynisdottir *et al.* 1995; Massague *et al.* 2000; Moustakas *et al.* 2002; Frederick *et al.* 2004). Additional evidence has shown that Smad3 functions to inhibit reepithelialization after wounding, specifically via effects on keratinocyte proliferation (Ashcroft *et al.* 1999). Moreover, TGF-β1 has been implicated as an endogenous inducer of hair follicle regression *in vivo* (Foitzik *et al.* 1999; Foitzik *et al.* 2000; Soma *et al.* 2003) through the inhibition of keratinocyte proliferation and induction of apoptosis. The regulation of TGF-β1 activity in hair follicles, however, is unknown.

Integrins are heterodimeric transmembrane receptors that mediate cell-substratum

adhesion. Integrin αvβ6 is unique in that it is an epithelial cell-specific receptor, and β6 partners only with αv forming a single heterodimer. Integrin αvβ6 recognizes a tripeptide amino acid sequence arginine–glycine–aspartate acid (RGD-motif) in its ligands, which include fibronectin, tenascin-C, vitronectin and the latency-associated peptide (LAP) of TGF-β1 and TGF-β3 (Busk *et al.* 1992; Huang *et al.* 1998; Annes *et al.* 2004). In addition to mediating cellular adhesion to these extracellular matrix proteins, αvβ6 integrin binds and activates latent TGF-β1 (Munger *et al.* 1999; Annes *et al.* 2004). Integrin-mediated activation of latent TGF-β1 plays an important role *in vivo*, as the absence of integrin-mediated TGF-β1 activation recapitulates the abnormalities of TGF-β1-null mice (Yang *et al.* 2007). Similarly, mice that lack activity of integrins αvβ6 and αvβ8 reproduce the phenotype of TGF-β1 and TGF-β3-null mice (Aluwihare *et al.* 2009). Specifically αvβ6 integrin-dependent activation of TGF-β1 has been confirmed to be pivotal in mouse models of TGF-β1-dependent fibrosis in various epithelial organs, including lung (Horan *et al.* 2008; Puthawala *et al.* 2008), kidney (Ma *et al.* 2003; Hahm *et al.* 2007) and liver (Wang *et al.* 2007; Patsenker *et al.* 2008; Popov *et al.* 2008), suggesting that this mechanism of TGF-β activation may be of general importance in tissues that express αvβ6 integrin.

The αvβ6 integrin is expressed at high levels in the multiple developing epithelial organs including hair follicle during embryogenesis and its expression is downregulated in adult epithelia (Breuss *et al.* 1995). More recently, αvβ6 integrin expression has been identified in adult hair follicles in humans (Hakkinen *et al.* 2004), mice (Tumbar *et al.*

2004; Bandyopadhyay and Raghavan 2009), sheep (Brown *et al.* 2006) and pigs (our unpublished observations). We have previously reported that β6 integrin knock-out (β6-/-) mice exhibited an accelerated wound repair and increased numbers of proliferating keratinocytes in both the wound epithelium and especially in the hair follicles surrounding the wounded skin compared to wild-type (WT) controls in a compromised wound healing model (Xie *et al.* 2009). It is well known that hair follicle stem cells contribute to cutaneous wound repair (Taylor *et al.* 2000; Levy *et al.* 2007). After wounding, stem cells from the hair follicle bulge were activated, exited their niche, migrated and proliferated to repopulate the interfollicular epidermis (Ito *et al.* 2005). Our data circumstantially suggested that αvβ6 integrin-mediated regulation of TGF-β1 activity play a specific role in hair regeneration and cycling. As a potent endogenous activator of TGF-β1, the function of αvβ6 integrin in hair follicles remains, however, unknown.

We hypothesize that αvβ6 integrin regulates TGF-β1 signaling in hair regeneration and the hair follicle involution process. To this end, we used a well-established depilation-induced hair cycling model (Paus *et al.* 1994; Muller-Rover *et al.* 2001) to trigger hair regeneration in both WT and β6-/- mice. Our data shows that loss of αvβ6 integrin leads to enhanced keratinocyte proliferation and retarded hair follicle regression associated with reduced TGF-β1 expression and activation. Moreover, our data indicates that a high level of β6-integrin expression presents only in early anagen and a remarkably decreased level occurs in the bulge region during catagen and telogen stages, which

marks αvβ6 integrin potentially as a specific cell surface marker for the activation and migration of bulge stem cells. The constitutive expression of β6 integrin at a low level in the bulge area of the telogen follicle in normal skin suggests that a small number of epidermal stem cells always keep their wound response status. As a result, although generally quiescent, bulge cells can promptly respond to wound stimuli upon injury.

3.2 Materials and methods

3.2.1 Animals

All animal studies were conducted in compliance with Canadian Council on Animal Care guidelines and approved by The University of British Columbia Animal Care Committee. Sixty-three 7-week-old female WT FVB mice and sixty-three age- and sex-matched β6-/- mice with the same genetic background were used in this study (generous gift from Dr. Dean Sheppard, University of California, San Francisco). Mice were housed in community cages with 12 h light periods and had free access to water and food.

3.2.2 Hair cycle induction

Hair cycling was induced by depilation in the back skin of WT and β6-/- adolescent mice with all dorsal skin HFs in telogen stage of the hair cycle as described (Paus *et al.* 1990; Muller-Rover *et al.* 2001). Mice were euthanized by CO_2 inhalation. The depilated, full-thickness skin samples, including the panniculus carnosus muscle in the subcutis, were harvested immediately (day 0), and at days 1, 2, 3, 5, 8, 10, 12, 17, 18, 19, 20, 21, 22 and 25 after depilation. Full-thickness of normal skin samples in the same area of mouse back were collected as the control group. Three to five mice were studied at each time point per group. The skin biopsies were snap frozen on dry ice for frozen sectioning or in liquid nitrogen for western-blotting and kept at -80°C until used.

3.2.3 Histology

Frozen longitudinal hair follicle sections (7 μm) were prepared and stored at -80°C**.** Sections were routinely stained by Harris' hematoxylin and eosin (H&E). The time point that anagen HFs regress spontaneously and enter catagen was determined, following precisely a described technique of quantitative histomorphometry of catagen development (Muller-Rover *et al.* 2001). The thickness of epidermis and dermis in WT and β6-/- mice was measured at days 0 and 3, respectively. Fifteen such measurements were performed from three animals per group at each time point. Hair follicle density was analyzed by using transverse sections cut at the level of the permanent hair follicle at days 0 and 8. The number of hair follicles was counted per high-power fields (HPFs, ×200 magnification). A total of five HPFs in each section were counted with three different mice in each group.

3.2.4 Histomorphometry

The percentage of hair follicles in the different hair growth cycle stages was assessed and calculated at day 18 after depilation in WT and β6-/- mice. All evaluations were performed on the basis of accepted morphological criteria for HF classification (Botchkarev *et al.* 1998; Paus and Cotsarelis 1999). Only every tenth cryosection was used for analysis to exclude the repetitive evaluation of the same HF, and 2 to 3 sections were assessed per animal. All together, 100 longitudinal HFs derived from five mice (20

longitudinal follicles per animal) were analyzed and compared to that of a corresponding number of HFs from the WT controls.

3.2.5 Immunohistochemistry

Frozen sections were fixed with ice-cold acetone at room temperature for 5 minutes. For Ki67 staining, samples were fixed with 10% buffered neutral formalin at room temperature for 10 minutes. Sections were rinsed in PBS containing bovine serum albumin (BSA, 1 mg ml^{-1}) and incubated with normal blocking serum (Vector Laboratories, Burlingame, CA). Sections were then incubated with the primary antibody raised against β6 integrin (β6B1; a generous gift from Dr. Dean Sheppard, University of California–San Francisco; 1:10), Ki67 (ab15580; Abcam, Cambridge, MA; 1:1000), collagen IV (PS057; Cedarlane Laboratories, Hornby, ON, Canada; 1:200), laminin-1 (L9393; Sigma-Aldrich, St. Louis, MO; 1:100) or phospho-Smad2 (AB3849; Millipore; Billerica, MA; 1:50) diluted in PBS/BSA in a humidified chamber at 4°C overnight. After washing with PBS/BSA, species-appropriate secondary antibodies were incubated at room temperature for 60 minutes, followed by the Vectastain ABC reagent (Vector Laboratories) according to the manufacturer's instructions. The reaction was visualized with either a VIP or a DAB substrate kit (Vector Laboratories). As negative controls, sections were treated with PBS instead of the primary or secondary antibodies to rule out non-specific immunostaining. In all cases, they showed no positive staining, except some

non-specific staining was seen in sebaceous glands probably as a result of the sticky oil residue in the sebaceous glands non-specifically picking up the antibodies.

To determine any difference in keratinocyte proliferation during early anagen development, the numbers of Ki67-positive keratinocyte nuclei were counted in the epidermis and hair follicles in WT and β6-/- mice at an identical anagen growth cycle stage. A total of five representative high-power fields (HPFs, ×200 magnification) per sample were counted with three different mice in each group. All the data was expressed as the mean of positive cells per HPF ± standard deviation of the mean. To assess TGF-β1 activation, the number of phospho-Smad2-positive cells was quantified using the same method as described above.

3.2.6 Immunofluorescence staining

Frozen sections were thawed, air dried, fixed with ice-cold acetone at room temperature for 5 minutes, rehydrated with phosphate buffered saline (PBS) and blocked with PBS containing bovine serum albumin (BSA; 10mg/ml) and Triton X-100 (0.01%) for 30 minutes at room temperature. TGF-β1 antibody (NB100-91995; Novus Biologicals, Littleton, CO; 1:200) diluted in PBS containing BSA (1 mg/ml) and Triton X-100 (0.01%) was incubated on tissue sections at 4°C overnight. The tissue sections were then washed and incubated with Alexa-conjugated secondary antibody (Alexa 488; Molecular Probes Inc., Eugene, OR) for one hour at room temperature followed by mounting using

Immuno-mount solution (Thermo Shadon, Pittsburrgh, PA). Control immunostainings performed by using appropriate non-immune serum instead of the primary antibody or by omitting the primary antibody incubation step gave negative staining results (data not shown). For DAPI staining, the samples of days 0 and 3 were incubated with DAPI (10 μM) at room temperature for 5 minutes in order to stain nuclei.

To quantify the difference in the expression level of TGF-β1 between groups, the positive reaction of TGF-β1 immunodetection was scored using the following scale according to the intensity of the staining: 0, 1+, 2+, and 3+. The area populated by TGF-β1-positive cells out of the total area of epidermis and hair follicles was assessed using the following scale: 0, 1(Only basal layer), 2 (Basal layer + less than 50% ORS) and 3 (Basal layer + more than 50% ORS). The sum of the intensity and the percentage scores was used as the final staining score as described previously (Gao *et al.* 2006). 15 such measurements were performed in three mice per mutant and WT group at each time point.

The DAPI-positive cell nuclei were counted by using ImageJ software (http://rsb.info.nih.gov/ij/). Standardized images from five representative microscopic fields per sample were captured using a ×20 objective with standardized digital camera setting for all samples. 15 such counts were performed in three mice per mutant and WT group at each time point.

3.2.7 Western Blotting

The whole skin samples were snap-frozen in liquid nitrogen and stored at -80°C until used. For protein extraction, the tissue samples were weighed and homogenized in the presence of a fixed weight-per-volume ratio of homogenization buffer containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolia, IN) and a Tissue Extraction Reagent I (Invitrogen, Camarillo, CA) by disposable hard-tissue Omini-tips (OMNI International, Marietta, GA) attached to the rotor-stator homogenizer (Hakkinen *et al.* 2004). The homogenate was cleared of tissue debris by centrifugation at 12,000 RPM at 4°C for 5 minutes. The resulting supernatant was collected. The total protein concentration was determined using a protein-assay reagent (Bio-Rad Laboratories, Hercules, CA). The protein extract was aliquoted and stored at -80°C until required. Equal amounts of proteins from skin samples were separated in SDS-Polyacrylamide Gel Electrophoresis (4%/10%). After electrophoresis, the proteins were transferred onto a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Quebec, PQ, Canada) at 4°C overnight. The nonspecific protein-binding sites were blocked by incubating the membranes with TBS containing 0.1% Tween-20 (TBS-T) and 5% non-fat powdered milk at room temperature for 60 minutes. The membranes were then incubated with the primary antibody raised against TGF-β1 (NB100-91995; Novus Biologicals, Littleton, CO), total-Smad2 (#3103; Cell Signaling; Danvers, MA) or phospho-Smad2 (#3101; Cell Signaling) in TBS containing 3% non-fat powdered milk at 4°C overnight. After washing,

the membranes were incubated with species-appropriate peroxidate-conjugated secondary antibodies in TBS containing 3% non-fat powdered milk at room temperature for 60 minutes. After washing, the blots were reacted with enhanced chemiluminescence detection reagent (Amersham Biosciences) and exposed to Kodak Biomax X-ray film (Eastman Kodak Company, Rochester, NY). The band intensities were quantified using NIH ImageJ software. In order to normalize the band intensities, the same membranes were immunobloted with a polyclonal antibody against β-actin (Abcam; Cambridge, MA; 1:5000) followed by incubation with a peroxidase-conjugated anti-rabbit secondary antibody. The experiments were performed three times with similar results from three mice in each group.

3.2.8 Statistical analysis

The statistical differences at each time point between WT and β6-/- mice were determined by Student's *t*-test. The statistical analyses were done by SPSS 11.5 software for Windows and $p < 0.05$ was considered statistically significant.

3.3 Result

3.3.1 Hair follicle regeneration was triggered after depilation

Mouse model of depilation-induced hair cycling can be regarded as a "wound healing" response of the hair follicles to the plucking trauma (Argyris 1968; Muller-Rover *et al.* 2000). This microwounding-induced hair cycle is fully synchronized over the entire area of depilation. Hair follicle morphogenesis and consecutive stages of the hair cycle develop following a predictable time-scale. Figure 3.1 shows the dynamic morphological changes of the regenerating hair follicles throughout the three stages of the hair cycling in WT mice. At day 8 after depilation, hair follicles in WT mice entered anagen VI. Around day 18 after depilation, anagen hair follicles in WT mice regressed spontaneously and entered catagen thereafter.

3.3.2 The abundance of β6 integrin was strongly up-regulated during hair regeneration in WT follicles and its expression was hair cycle stage dependent

To delineate the spatial and temporal expression of β6 integrin in regenerating follicles, β6 integrin was detected at different time points before and after depilation. The β6-/- mice showed a complete lack of staining for β6 integrin in all specimens examined (data not shown). In the WT mice, keratinocytes in the intact epidermis did not express β6

integrin before depilation. Some expression in the hair follicles was, however, noted (Fig. 3.1A), localized on the cell membrane of ORS keratinocytes below the sebaceous gland. After depilation, the expression of β6-integrin was induced in WT mice. β6 integrin-positive staining was detected in both the basal layer of the epidermis and the ORS of the hair follicle. A relatively higher expression of β6 integrin in the hair follicle than the basal layer of the epidermis was noted. The level of β6 integrin was rapidly up-regulated during early anagen development. At day 8, although extended to the lower ORS, a relatively higher intensity of β6 integrin staining was noted in the ORS below sebaceous gland compared to other regions of the ORS (Fig. 3.2B). The level of β6 integrin gradually decreased thereafter. And the expression was restricted to the bulge area again in the following catagen and telogen stage. Thus, our results indicate that the abundance of αvβ6 integrin was strongly up-regulated during hair regeneration and its expression was hair cycle dependent.

3.3.3 β6-/- mice exhibited significantly accelerated skin thickness increase and hair regeneration during the early anagen development

To test whether β6 integrin plays a role during hair regeneration after depilation, the hair cycle stages were assessed between WT and β6-/- mice. Before depilation, both WT and β6-/- follicles stayed in the telogen stage of the hair cycle (data not shown). In early anagen (days 3, 5), β6-/- mice showed increased skin thickness and accelerated hair

regeneration compared to WT controls (Fig. 3.3). Quantitative analysis indicated that the epidermis and dermis in β6-/- mice were significantly thicker than those in WT mice at day 3 after depilation ($p<0.05$). In addition, DAPI staining showed significantly higher numbers of DAPI-positive cell nuclei in β6-/- skin compared to WT controls at day 3 after depilation (Fig. 3.4). Hair follicle density was analyzed at days 0 and 8. Quantification of hair density, however, showed that there was no significant difference between WT and β6-/- mice (data not shown). Type IV collagen and laminin-1 were detected at different time-points after depilation by immunohistochemistry to assess basement membrane regeneration. At day 8, both WT and β6-/- mice showed continuous, linear and well-defined type IV collagen and laminin-1 staining at the basement membrane zone of the epidermis and in the hyaline membrane zone of the hair follicles (data not shown). Our results indicate no significant difference in basement membrane regeneration between WT and β6-/- mice.

3.3.4 Deletion of αvβ6 integrin causes the retardation of hair regression

To determine whether the knockout of β6 integrin exerts an effect on the hair regression process, catagen development was examined by quantitative histomorphometry at day 18 after depilation in β6-/- mice and WT controls. At day 18 after depilation, 44% WT follicles were in early catagen (catagen II-III) and 40% in mid catagen (catagen IV-V), while 54% β6-/- follicles remained in an anagen stage (anagen VI) and only 30% in early

catagen (Fig. 3.5). Our results indicated a significantly delayed hair regression in β6-/- mice compared to WT controls. At day 20, most hair follicles in β6-/- mice had entered a catagen stage, whereas hair follicles of WT mice had already progressed through late catagen into the subsequent telogen phase (Fig. 3.3A).

3.3.5 β6-/- mice contained significantly higher numbers of proliferating keratinocytes in the interfollicular epidermis and hair follicles than WT controls at an identical hair growth cycle stage

Having established an accelerated skin thickness increase and significantly higher numbers of DAPI-positive cell nuclei in the β6-/- skin, we investigated whether the accelerated regeneration process in β6-/- mice is due to a difference in cell proliferation. Keratinocyte proliferation during hair regeneration was assessed by immunohistochemistry for the proliferating cell marker Ki67 in WT and β6-/- follicles at different time points after depilation. Before depilation, there were no significant differences in the numbers of Ki67-positive cells between WT and β6-/- mice. After depilation, a notable increase in proliferating keratinocytes labeled by Ki67-positive immunoreaction were present in both WT and β6-/- skin compared to the day-0 normal skin, mainly localized in the cell nuclei of the basal keratinocytes of the interfollicular epidermis and keratinocytes in the ORS and hair matrix of the regenerating follicles (Fig. 3.6A).

During early anagen development (days 3 and 5), β6-/- mice displayed significantly higher numbers of Ki67-positive keratinocytes in hair follicles and the basal layer of the epidermis than comparable WT follicles (Fig. 3.6B). In epidermis, the hyperproliferative phenotype in β6-/- skin was characterized by containing several layers of proliferating keratinocytes in not only the basal layer but also the suprabasal layers (Fig. 3.6). These findings indicate that a β6 integrin-deficiency in the skin potentially enhances the keratinocyte activation and proliferation after depilation-induced hair regeneration.

3.3.6 Deletion of αvβ6 integrin leads to reduced TGF-β1 levels during hair regeneration

To determine whether the hyperproliferative phenotype and delayed hair regression in β6-/- skin was associated with an altered TGF-β1 expression, the level of total TGF-β1 was measured at different time points after depilation in the WT and β6-/- mice by immunofluorescence staining (Fig. 3.7A & B). Before depilation, TGF-β1 was detected in the cell membrane of the basal keratinocytes of the epidermis but not in the telogen hair follicles. The expression and distribution of TGF-β1 showed no significant difference at day 0 between WT and β6-/- mice. After depilation, the expression of TGF-β1 was remarkably elevated in both WT and β6-/- skin compared to the day-0 normal skin. During anagen development, TGF-β1 was detected in the basal layer of the epidermis and the upper ORS that was immediately continuous with the basal layer in

WT mice. β6-/- mice showed a lower intensity of TGF-β1 expression in the epidermis and occasional distribution in the upper ORS. Its maximum expression was detected around day 18 in WT mice, with extended distribution from epidermis to ORS of hair follicle. In contrast, TGF-β1 was still restricted in the basal layer in β6-/- mice. Quantitative analysis of immunofluorescence showed significantly reduced levels of TGF-β1 during early anagen and the anagen-catagen transition in β6-/- mice compared to WT controls. The expression of TGF-β1 was also analyzed by western blot (Fig. 3.7C & D). The results indicated that the level of TGF-β1 was notably up-regulated in both WT and β6-/- skin after depilation. However, WT mice showed a significantly higher level of TGF-β1 expression at days 3, 8 and 18 after depilation compared to β6-/- mice. Our data showed that there were two expression peaks of TGF-β1 during hair regeneration in WT mice. The first peak appeared at the beginning of hair regeneration (day 3). The second one appeared when WT follicles were at the anagen-catagen transition (day 18). The expression of TGF-β1 reached the maximum level at the second peak with the extended distribution from epidermis to ORS of the hair follicle. β6-/- mice, however, displayed a loss of the second peak during the anagen-catagen transition.

3.3.7 TGF-β1 activation is reduced in β6-/- skin during hair regeneration

To determine whether the level of TGF-β1 activation is altered in β6-/- skin after depilation, we detected phospho-Smad2 as an indirect measurement of TGF-β1 activation

(Massague 1998). The positive staining for phospho-Smad2 was detected in both the WT and β6-/- mice, indicating that the β6 integrin independent mechanisms for the activation of TGF-β1 also exist in the depilated skin of the β6-/- mice. Phospho-Smad2-positive staining was detected in the cell nuclei in both the epidermis and hair follicles (Fig. 3.8). Before depilation, immunodetection of phospho-Smad2 showed no significant difference between WT and β6-/- skin. After depilation, β6-/- mice displayed significantly reduced levels of phospho-Smad2 in hair follicles and the epidermis during early anagen (day 3) and the anagen-catagen transition compared to WT controls (day 18). Consistent with the immunohistochemistry data, western blotting results showed that the level of phospho-Smad2, total-Smad2 was up-regulated in both WT and β6-/- skin after depilation (Fig. 3.8C, D & E). The expression of phospho-Smad2 and total-Smad2 peaked at the anagen-catagen transition (day 18) in WT mice. In contrast, β6-/- mice showed a significantly lower level of phospho-Smad2 expression at days 3, 8 and 18 after depilation compared to WT mice. The total-Smad2 showed a similar expression trend in WT and β6-/- mice, but with a lower relative expression between WT and β6-/- mice.

3.4 Discussion

In the present study, we showed that loss of αvβ6 integrin led to an enhanced keratinocyte proliferation and retarded hair follicle regression in a standardized mouse model of depilation-induced hair regeneration. This is a novel and significant finding because although the role of αvβ6 integrin in multiple epithelial organs has been described, this work is the first to demonstrate that αvβ6 integrin is an important functional component in hair follicle cycling probably via the modulation of the epidermal stem cell behavior.

These observations are in agreement with our previous studies that indicated an accelerated wound repair and increased numbers of proliferating keratinocytes in the hair follicles surrounding the wounded skin in the β6-/- mice compared to WT controls in a compromised wound healing model (Xie *et al.* 2009). Using a β6 integrin-specific antibody, we identified intense membrane staining in keratinocytes, which is strongly and specifically enhanced in the bulge region of the early anagen hair follicle. Consistent with our data, gene profiling analysis indicated that β6 integrin transcripts were among the genes involved in TGF-β pathway that were strongly and selectively upregulated in the bulge cells (Tumbar *et al.* 2004). Similarly, a recent study has identified the αvβ6 integrin-positive stem cells in human oral squamous cell carcinoma (SCC). αvβ6 integrin is co-localized with the human embryonic stem cell marker TRA-1-60 in the oral SCC biopsy specimens and is an important regulator of pluripotential cell behavior (Dang and

Ramos 2009).

Our study demonstrates that the expression and distribution of αvβ6 integrin was not only different between epidermis and HFs but also distinct within HFs themselves (Fig. 3.2C). In the intact epidermis of nonwounded skin in WT mice, keratinocytes did not express αvβ6 integrin, which is consistent with previous studies (Breuss *et al.* 1993). The *de novo* expression of αvβ6 integrin is induced in the epidermis upon wounding (Haapasalmi *et al.* 1996). Although at a very low level, αvβ6 integrin, however, was constitutively expressed and confined to the keratinocytes in the ORS below the sebaceous gland in the HFs of the nonwounded skin in the WT mice (Fig. 3.2A). In our previous mouse cutaneous wound model, 4 full-thickness 4mm excisional wounds were created in the back skin of WT mice (Xie *et al.* 2009). After wounding, the expression of β6 integrin was induced in the basal layer of the wound epithelium, localized to the basal layer along the wound edges (Larjava *et al.* 1993; Haapasalmi *et al.* 1996; Hakkinen *et al.* 2000; Hakkinen *et al.* 2004). We also observed a remarkable up-regulation of β6 integrin in the hair follicles surrounding the wound area. A relatively higher intensity of αvβ6 integrin expression in HFs than wound epithelium was noted.

In the depilation-induced hair regeneration model, hair plucking generated numerous “micro-wounds” in the hair follicles of the depilated area. After wounding, a rapid up-regulation of β6 integrin expression was observed in early anagen hair follicles, and was specifically enhanced in the bulge area. In contrast, a relatively weaker intensity of β6 integrin expression was noted in the basal layer of the epidermis. Our data also

indicate that the expression of β6 integrin is only up-regulated at a high level in the rapidly growing early anagen hair follicle. In the catagen and telogen stages, the expression of β6 integrin exhibited a remarkable decrease and was restricted to the bulge area again. Interestingly, during embryogenesis αvβ6 integrin is expressed at high levels in the ORS of the developing hair follicles and its expression is downregulated in adult follicles (Breuss *et al.* 1995). Moreover, tenascin-C has been shown to be present in several stem cell niches and may be an important functional component of stem cell niches (von Holst 2008). β6 integrin uses tenascin-C as a ligand. Since both of them are activated and *de novo* expressed at a high level during skin wounding and tumorigenesis as well as co-expressed and significantly upregulated in the bulge region (Morris *et al.* 2004; Tumbar *et al.* 2004; Kloepper *et al.* 2008), αvβ6 integrin may constitute a functionally important component of the bulge stem cell niche of hair follicles. This may also suggest αvβ6 integrin is potentially a useful marker for the activation stage of bulge stem cells.

Although it is generally believed that epidermal stem cells are restricted to both the ORS of the hair follicle bulge and basal layer of the interfollicular epidermis, substantial evidence supports the idea that stem cells in the interfollicular epidermis are less potent than bulge stem cells (Alonso and Fuchs 2003). In addition, only a small fraction of stem cells were discovered in the basal layer of the interfollicular epidermis, while the majority of the stem cells in the skin reside in the hair follicle bulge (Cotsarelis *et al.* 1990; Morris and Potten 1994). Thus, the constitutive expression of β6 integrin at a low level in the

bulge area of telogen follicles in normal skin suggests that a small number of epidermal stem cells retain an active status. As a result, although generally quiescent, bulge cells can promptly respond to wound stimuli upon injury. As a reservoir of epidermal stem cells, hair follicles always present a higher level of β6 integrin than the basal layer of the interfollicular epidermis in response to wounding. The higher levels of β6 integrin expression in rapidly growing early anagen hair follicles suggest higher activation levels of hair follicle stem cells that change their biochemistry, exit the bulge, migrate, and proliferate to regenerate hair follicles and repopulate the interfollicular epidermis.

Our results suggest that the accelerated hair regeneration in the β6-/- mice resulted from an enhanced proliferation of keratinocytes in the epidermis and hair follicles. This is likely linked to a reduced anti-proliferative effect of TGF-β1 on keratinocytes as αvβ6 integrin is a potent activator of latent TGF-β1 (Munger *et al.* 1999; Annes *et al.* 2004). TGF-β1 has been confirmed to be an inhibitor of keratinocytes and to delay wound re-epithelialization (Glick *et al.* 1993). Inhibition of epithelial cell proliferation by TGF-β1 involves down regulation of c-Myc leading to upregulation of cyclin-dependent kinase inhibitors p15, p21 and p27, which inhibit the CDK4/6-cyclin D and CDK2-cyclin E-mediated phosphorylation of the retinoblastoma protein (Reynisdottir *et al.* 1995; Robson *et al.* 1999; Massague *et al.* 2000; Moustakas *et al.* 2002; Ten Dijke *et al.* 2002; Frederick *et al.* 2004). Consistent with our findings in the β6-/- mice, TGF-β1-/- mice display a delayed catagen development and increased Ki67-positive cells in hair follicles than comparable WT follicles. In contrast, TGF-β1 treatment into the back skin of mice

induces premature catagen development and the number of proliferating follicle keratinocytes is reduced in TGF-β1-treated mice compared to similarly treated controls (Foitzik *et al.* 2000). Similarly, expression of αvβ6 integrin in β6-transfected cells plated on tenascin-C completely fails to proliferate whereas expression of α9β1 integrin induces proliferation (Yokosaki *et al.* 1996).

Keratinocyte activation occurs after epidermal injury. In the β6-/- epidermis, we observed notably enhanced keratinocyte activation during early anagen of the hair regeneration, characterized by a hyperproliferative phenotype in both the basal layer and several suprabasal layers. In contrast, proliferating keratinocytes were mainly detected in the basal layer of the epidermis in WT mice.

Our data indicate a reduced TGF-β1 expression and activation in β6-/- follicles. In WT mice, there were two expression peaks of TGF-β1 during hair regeneration. The expression of TGF-β1 reached the maximum level at the second peak with extended distribution from epidermis to the ORS of the hair follicles. This result is consistent with a previous study that showed TGF-β1 acted as an endogenous inducer of hair follicle regression (Foitzik *et al.* 2000). β6-/- mice, however, displayed a loss of the second peak during the anagen-catagen transition. As a negative regulator of keratinocyte proliferation, transcripts involved in the TGF-β signaling were significantly upregulated in bulge cells (Tumbar *et al.* 2004), suggesting TGF-β signaling participates in the epidermal stem cell activities in the bulge niche. Thus, loss of β6 integrin may influence epidermal stem cell behavior after injury due to the altered TGF-β signaling in β6-/- mice.

The *de novo,* transient expression of αvβ6 integrin by keratinocytes after injury suggests that the primary role of αvβ6 integrin in cutaneous wound healing is to control the activation of TGF-β1, the key player in wound repair. Activation of latent TGF-β1 by αvβ6 integrin is a highly localized process and likely occurs under strict spatio-temporal regulation. We used immunohistochemistry to examine the level of phospho-Smad2 as an indirect measurement of TGF-β1 activation. The results suggest that the presence of αvβ6 integrin contributes to the activation of TGF-β1 in the hair follicles and interfollicular epidermis. The altered surrounding microenvironment of the bulge niche in the β6-/- follicle may lead to altered epidermal stem cell behavior and might be associated with the enhanced keratinocyte proliferation and subsequently retarded hair cycle regression. Similarly, the constitutive expression of αvβ6 integrin in the basal layer of the epidermis of the transgenic mice leads to elevated TGF-β1 activation and the development of spontaneous chronic ulcers with severe fibrosis (Hakkinen *et al.* 2004). Therefore, our data suggest a possible link between TGF-β1 and the observed effects, although other mechanisms of TGF-β1 activation might also play a role during the hair regeneration process.

Some initial work has implicated the integrins as one class of epidermal stem cell surface markers. The integrin family of transmembrane receptors mediates the attachment of the basal layer of the interfollicular epidermis to the underlying basement membrane. Epidermal stem cells are confined to the basal layer of the epidermis or the hair follicle bulge, which suggest that they require strong adherence to the basement membrane to

maintain their position or their stem cell properties in the stem cell niche. Therefore, cell surface molecules that facilitate their adhesion to the basement memrane are theoretically possible stem cell markers (Watt 2002; Alonso and Fuchs 2003). The two members in this family that most frequently appear on the list of the candidate markers are β1 and α6 integrins. None of them, however, are specific due to their extensive expression in epidermis and hair follicles. In the present study, our data indicate that the expression of β6-integrin is restricted in the bulge region in normal skin. After wounding, although β6-integrin presence extended to the lower ORS, its expression was specifically and strongly enhanced in the bulge area compared to the lower ORS. Moreover, its expression was rapidly up-regulated at a high level in the faster growing early anagen hair follicles but decreased in late anagen and in the following catagen and telogen stage hair follicles, suggesting that αvβ6 integrin constitutes a functionally important component of the bulge stem cell niche. Also, the significantly enhanced expression in the bulge region marks αvβ6 integrin potentially as a specific cell surface marker for the activation stage of epidermal stem cells. This specific marker may allow isolation of live epithelial cells with stem and progenitor cell characteristics, potentially providing a tool for the study of gene therapy, carcinogen target cells, and tissue engineering applications.

Taken together, our study indicates that αvβ6 integrin plays an important inhibitory role in keratinocyte proliferation in both the hair follicles and interfollicular epidermis after depilation-induced hair regeneration. The downregulated TGF-β1 signaling in β6-/- mice may impact bulge stem cell behavior via modulating the surrounding microenvironment

of the bulge niche after injury, which suggests a possible manipulation target in the functions of epidermal stem cells. Elucidation of the molecular mechanism of the synergistic interaction of αvβ6 integrin and TGF-β1 in regulating the proliferation and differentiation of the epidermal stem cells will require further investigation.

3.5 Acknowledgments

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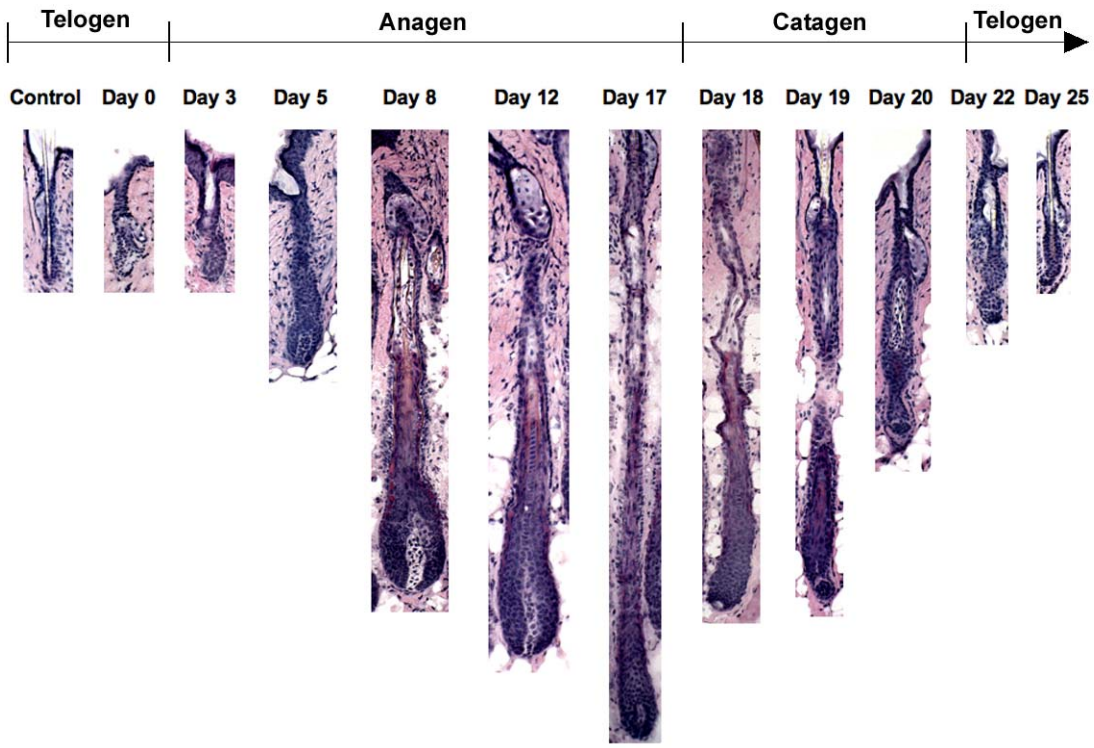
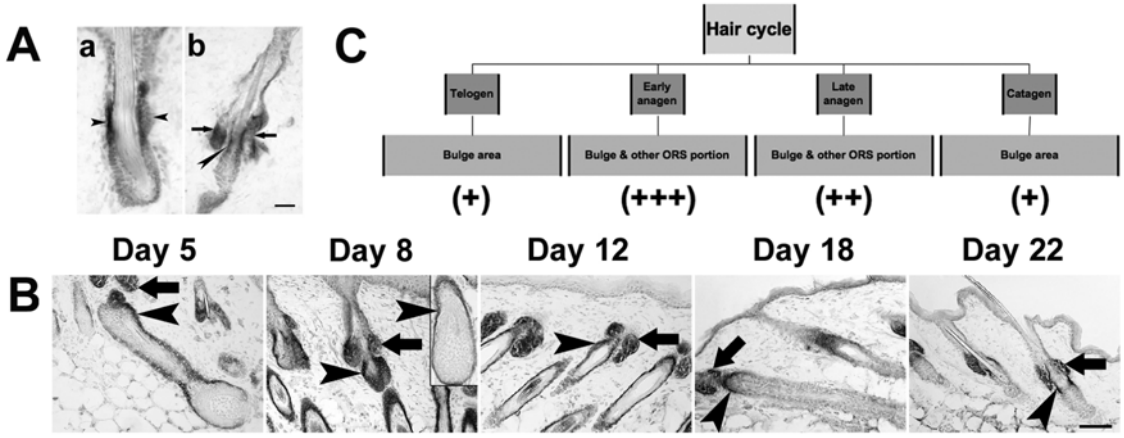
Figure 3.1 Hair growth cycling triggered after depilation in WT FVB mice



The dynamic morphological changes of hair follicles through the complete hair growth cycle in WT mice. Hair follicle regeneration was triggered after depilation, as a response of the hair follicles to the plucking trauma. The hair cycle stages were assessed at different time points by morphological classification criteria. Control: Hair follicle in normal back skin of 7-week-old female WT mice before depilation. Day 0: Immediately after hair shaft was removed. Days 3-25 present representative regenerating hair follicles at different time points after depilation. Days 0, 25 = telogen; Days 3–17 = anagen; Day 18-22 = catagen. Around days 18 after depilation, anagen hair follicles in WT mice regressed spontaneously and entered catagen thereafter.

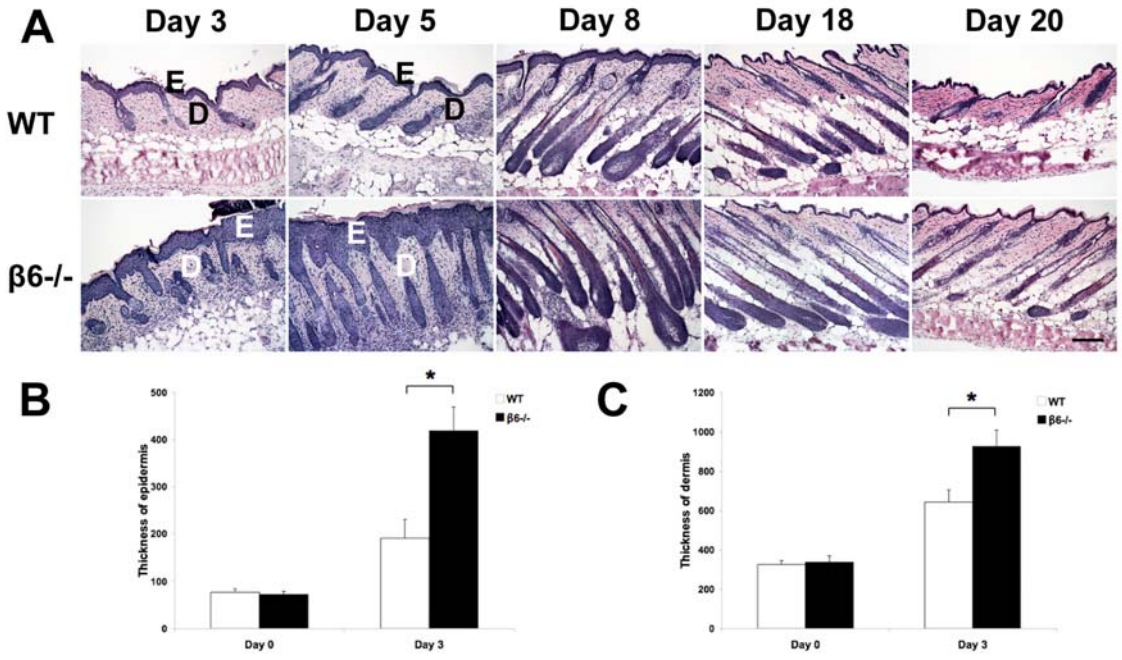
Figure 3.2 Spatio-temporal expression patterns of αvβ6 integrin in different stages of hair growth cycle



The abundance of αvβ6 integrin was strongly up-regulated during the hair regeneration in WT follicles and its expression was hair cycle dependent. (A) Immunolocalization of αvβ6 integrin in nonwounded hair follicles of WT mice (a & b). Although absent from intact epidermis, αvβ6 integrin was constitutively expressed in the hair follicle in the day-0 nonwounded skin. In intact skin, hair follicles stayed at the telogen stage of the hair cycle. αvβ6 integrin-positive staining was detected in the cell membrane of the outer root sheath (ORS) keratinocytes, restricted in the area below sebaceous gland (b). Scale bar = 20 μm. (B) Expression of αvβ6 integrin was studied in regenerating hair follicles by immunohistochemistry in WT mice. Frozen sections of mouse back skin at defined stages of the hair cycle were stained to detect the spatio-temporal expression of αvβ6 integrin. The abundance of αvβ6 integrin was rapidly

up-regulated during early anagen development (day 5). The maximum expression of αvβ6 integrin was observed at day 8 after depilation. Although extended to the lower ORS (an example of hair bulb is shown in the inset of day 8 in B), a relatively higher intensity of αvβ6 integrin expression in the ORS below sebaceous gland than the other portion of the ORS was noted (day 8). Its expression was gradually decreased thereafter (day 12) and restricted in the bulge area again in the following catagen and telogen stage (days 18, 22). Note that the staining of sebaceous glands is non-specific. Arrows indicate the sebaceous glands (non-specific immunostaining). Arrowheads indicate the αvβ6 integrin-positive keratinocytes in the ORS of the hair follicles. Scale bar = 100 μm. (C) A schema shows the spatial and temporal expression pattern of αvβ6 integrin during depilation-induced hair cycle.

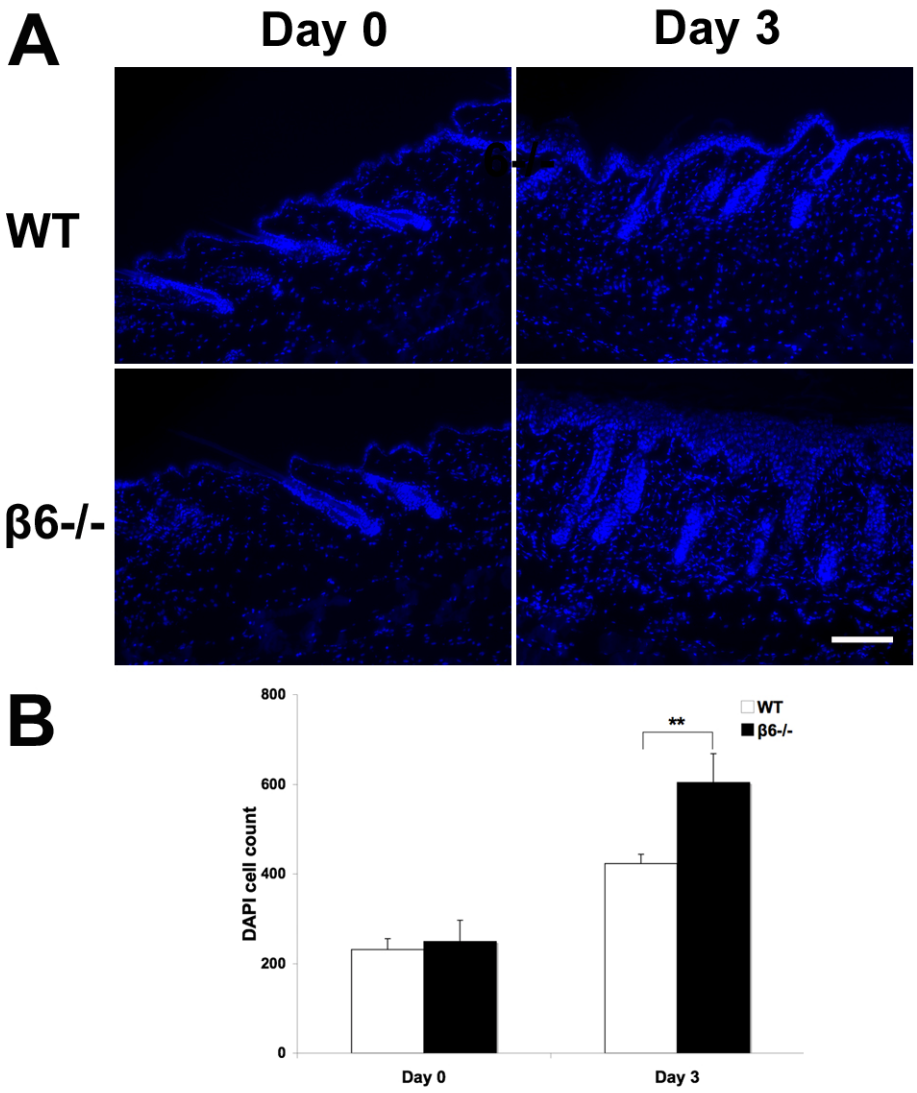
Figure 3.3 Regenerating hair follicles and interfollicular epidermis in the WT and β6-/- mice



β6-/- mice showed significantly increased skin thickness and accelerated hair regeneration during the early anagen development. (A) Frozen sections of WT and β6-/- mouse back skin at different time points after depilation were stained with H&E. Hair follicles were assessed by morphological classification criteria. In early anagen (days 3, 5), a remarkably accelerated skin thickness increase was observed in β6-/- mice compared to WT controls. At day 8 after depilation, both WT and β6-/- follicles reached anagen VI. At day18, most WT follicles had already entered catagen, while most β6-/- hair follicles remained in anagen. At day 20, most β6-/- HFs had entered a catagen stage, whereas HFs of WT mice had already progressed through late catagen into the

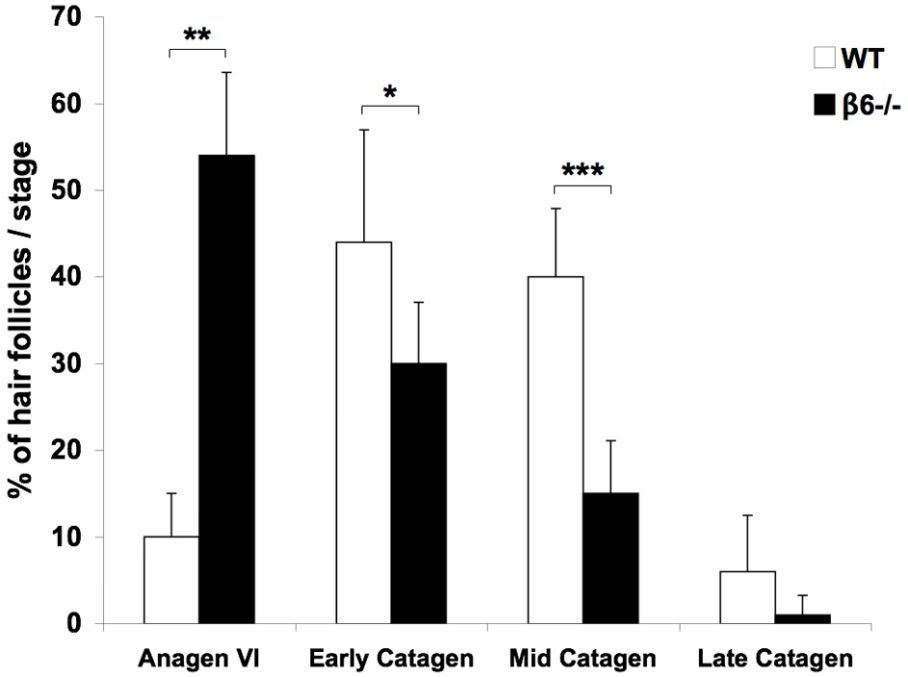
subsequent telogen phase. E: Epidermis; D: Dermis. Scale bars = 200 μm. (B) Quantification of the thickness of the epidermis in WT and β6-/- mice at day 0 and day 3. (C) Quantification of the thickness of the dermis in WT and β6-/- mice at day 0 and day 3 (n = 3 mice per group; * $p<0.05$).

Figure 3.4 DAPI cell count in WT and β6-/- skin before and 3 days after depilation



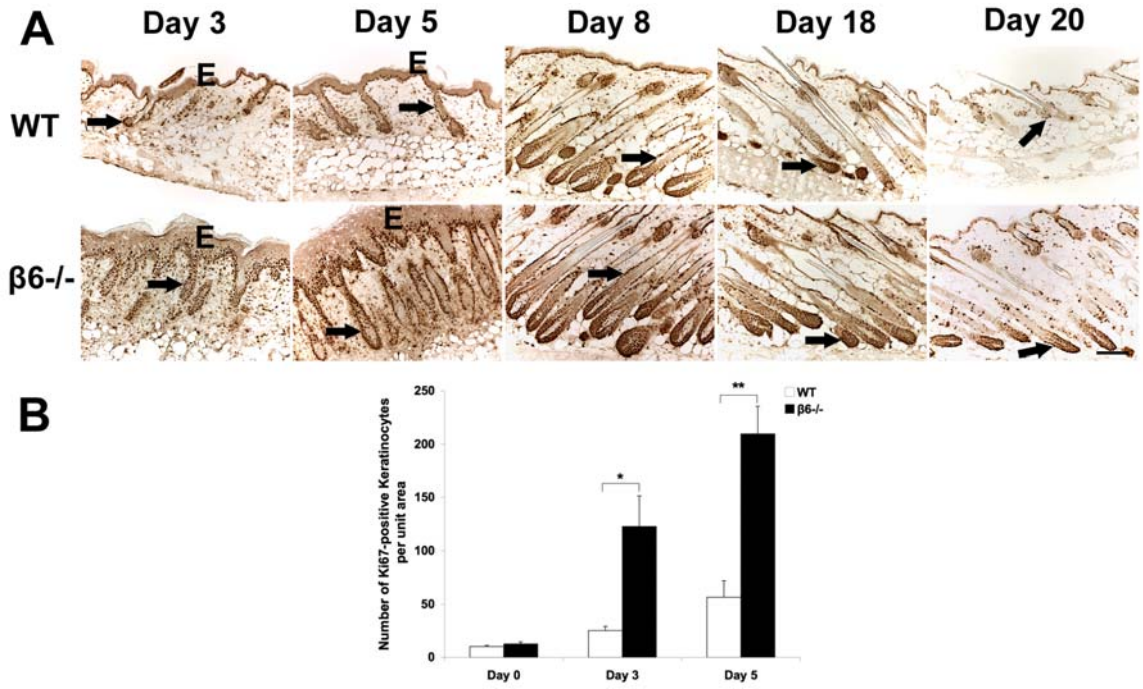
β6-/- skin showed a significantly higher number of cell nuclei in early anagen. (A) DAPI staining indicated increased numbers of 4′, 6′- diamidino-2-phenylindole (DAPI)-positive cell nuclei (blue) in β6-/- skin compared to WT skin at day 3 after depilation. Scale bars = 200 μm. (B) Quantification of DAPI-positive cell nuclei in WT and β6-/- mice at day 0 and day 3 (n = 3 mice per group; ** $p<0.01$).

Figure 3.5 Assessment of catagen development in the WT and β6-/- mice by histomorphometry



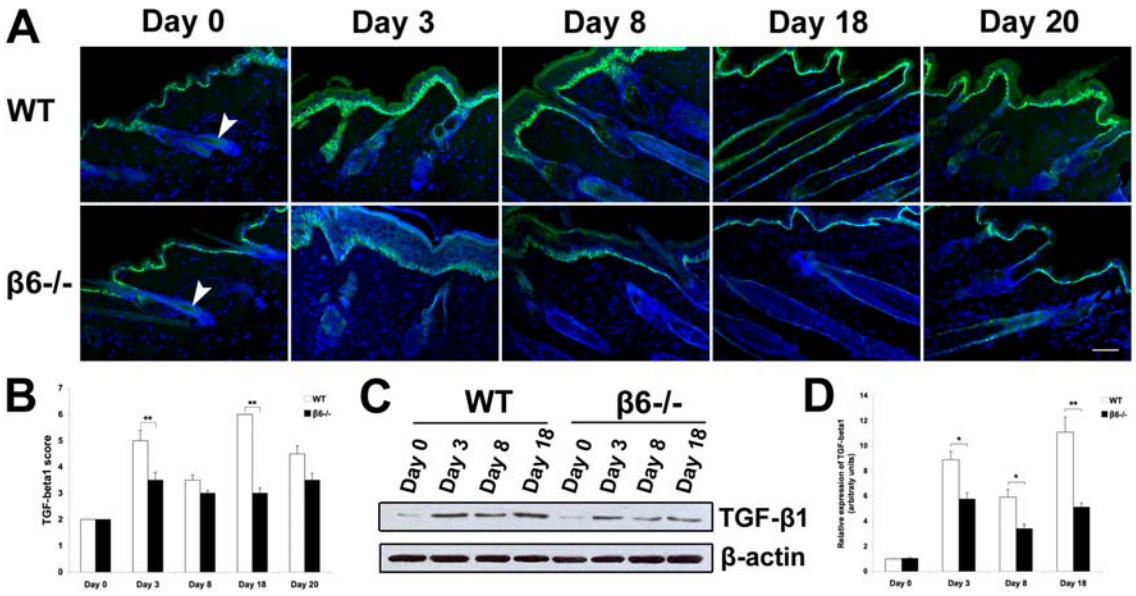
Deletion of αvβ6 integrin causes the retardation of hair regression. Frozen sections of WT and β6-/- mouse back skin at day18 post-depilation were stained with H&E and hair follicles were assessed by morphological classification criteria. Catagen development was compared in the WT and β6-/- mice by quantitative histomorphometry. The results indicated a significant delayed catagen development in β6-/- follicles compared to WT controls (n = 5 mice per group; * $p<0.05$; ** $p<0.01$; *** $p<0.001$). Graph shows the percentage of hair follicles/hair cycle stage at day 18 post-depilation in β6-/- mice compared to WT controls. X axis: Early catagen = catagen II-III; Mid catagen = catagen IV-V; late catagen = catagen VI-VIII.

Figure 3.6 Keratinocyte proliferation in the WT and β6-/- mice after depilation



β6-/- mice contained significantly higher numbers of proliferating keratinocytes in interfollicular epidermis and hair follicles than WT controls at an identical hair growth cycle stage. (A) Keratinocyte proliferation was assessed by immunohistochemistry of the proliferating cell marker Ki67 at different time points in WT and β6-/- mice. In the epidermis, Ki67-positive staining was mainly localized in the cell nuclei of the basal keratinocytes. In the hair follicles, Ki67-positive staining was localized in the outer root sheath keratinocytes and hair matrix. Note that some non-specific staining was seen in sebaceous glands. E: Epithelium. Arrows indicate the Ki67 positively stained cell nuclei. Scale bar = 200 μm. (B) Quantification of the Ki-67-positive keratinocytes showed a significant increase in β6-/- follicles and epidermis compared to comparable WT controls during early anagen development (days 3, 5; n = 3 mice per group; * $p<0.05$; ** $p<0.01$).

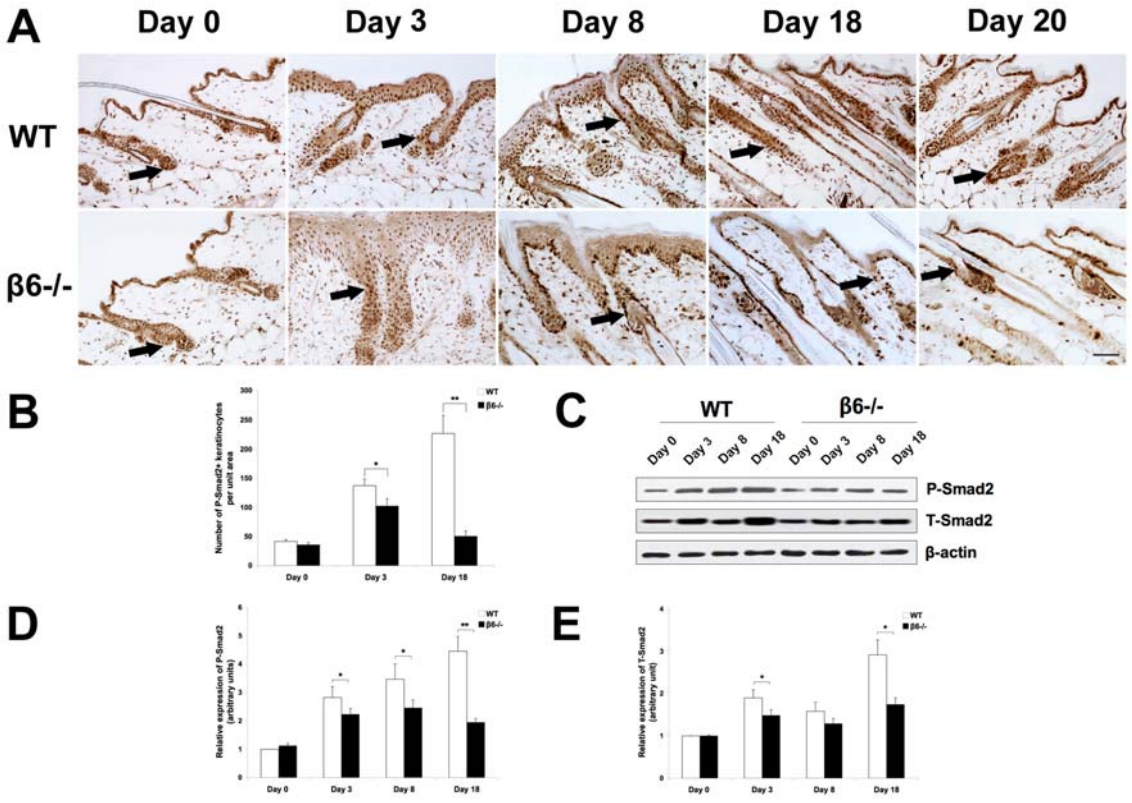
Figure 3.7 Spatio-temporal expression patterns of TGF-β1 in different stages of hair growth cycle in the WT and β6-/- mice



Deficiency of αvβ6 integrin leads to reduced TGF-β1 levels during hair regeneration. (A) The expression and distribution of TGF-β1 was studied by immunofluorescence staining in WT and β6-/- skin at different time points before and after depilation. Shown are immunofluorescence of TGF-β1 antibody (green) and 4′, 6′-diamidino-2-phenylindole (DAPI, blue). At day 0, TGF-β1 was detected in the cell membrane of the basal layer keratinocytes of the epidermis. During anagen development, TGF-β1 was detected in the basal layer of the epidermis and the upper portion of ORS in WT mice (days 3, 8). Its maximum expression was detected at day 18 in WT mice, with extended distribution from epidermis to ORS of hair follicle. In contrast, TGF-β1 was

still restricted in the basal layer in β6-/- mice at day 18. Arrowheads denote hair shaft autofluorescence. Scale bar = 100 μm. (B) TGF-β1 score showed significantly reduced levels of total TGF-β1 during the early anagen (day 3) and the anagen-catagen transition (day 18) in β6-/- mice compared to WT controls (n = 3 mice per group; ** $p<0.01$). (C) The expression of TGF-β1 was analyzed by western blot. (D) Quantification of the relative expression of TGF-β1 by western blot showed that the level of TGF-β1 was up-regulated in both WT and β6-/- skin after depilation. WT mice showed a significantly higher level of TGF-β1 expression in the depilated skin compared to β6-/- skin at days 3, 8 and 18. The maximum expression of TGF-β1 appeared at the anagen-catagen transition in WT skin whereas in β6-/- skin, it peaked at the beginning of hair regeneration (n = 3 mice per group; * $p<0.05$; ** $p<0.01$).

Figure 3.8 Determination of the levels of Smad2 phosphorization in different stages of hair growth cycle in the WT and β6-/- mice



TGF-β1 activation is reduced in β6-/- skin during hair regeneration. (A) phospho-Smad2 was detected by immunohistochemistry in WT and β6-/- skin at different time points before and after depilation. Phospho-Smad2-positive staining was mainly localized in the cell nuclei in both the epidermis and hair follicles. Note that some non-specific staining was seen in sebaceous glands. Arrows indicate the phospho-Smad2 positively stained cell nuclei. Scale bar = 100 μm. (B) Quantification of phospho-Smad2-positive keratinocytes showed a significantly reduced number in β6-/-

follicles and the epidermis at early anagen (day 3) and the anagen-catagen transition (day 18) compared to WT controls (n = 3 mice per group; * $p<0.05$; ** $p<0.01$). (C) The expression of phospho-Smad2 was analyzed by western blot. The results showed that the level of phospho-Smad2 was up-regulated in both WT and β6-/- skin after depilation. (D) Quantification of the relative expression of phospho-Smad2 indicated that β6-/- mice showed a significantly lower level of phospho-Smad2 expression in the depilated skin compared to WT skin at days 3, 8 and 18 (n = 3 mice per group; * $p<0.05$; ** $p<0.01$). (E) Quantification of the relative expression of total-Smad2 indicated that β6-/- mice showed a significantly lower level of total-Smad2 expression in the depilated skin compared to WT skin at days 3 and 18 (n = 3 mice per group; * $p<0.05$).

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Chapter 4: General discussion, conclusions and future directions

4.1 General discussion

Integrin-mediated activation of latent TGF-β1 plays an important role *in vivo*, as the absence of integrin-mediated TGF-β1 activation recapitulates the abnormalities of TGF-β1-null mice (Yang *et al.* 2007). Similarly, mice that lack activity of integrins αvβ6 and αvβ8 reproduce the phenotype of TGF-β1 and TGF-β3-null mice (Aluwihare *et al.* 2009). Specifically, αvβ6 integrin-dependent activation of TGF-β1 has been confirmed to be pivotal in mouse models of TGF-β1-dependent fibrosis in various epithelial organs, including lung (Horan *et al.* 2008; Puthawala *et al.* 2008), kidney (Ma *et al.* 2003; Hahm *et al.* 2007) and liver (Wang *et al.* 2007; Patsenker *et al.* 2008; Popov *et al.* 2008), suggesting that this mechanism of TGF-β activation may be of general importance in tissues that express αvβ6 integrin.

We have previously reported that chronic human wounds continue to express αvβ6 integrin and transgenic mice over-expressing αvβ6 integrin produce chronic hypertrophic wounds that contain high levels of TGF-β1 (Hakkinen *et al.* 2004). Therefore, we hypothesize that αvβ6 integrin-mediated regulation of TGF-β1 activity may play a role in the impaired wound healing process. To this end, a well-established dexamethasone-induced mouse impaired wound model was used in WT and β6-/- mice. Additionally, since we observed a constitutive expression of αvβ6 integrin in hair

follicles and TGF-β1 has been implicated as an endogenous inducer of hair follicle regression (Foitzik *et al.* 1999; Foitzik *et al.* 2000; Soma *et al.* 2003), we subsequently utilized a depilation-induced mouse hair cycling model to test whether αvβ6 integrin-mediated TGF-β1 signaling regulates hair regeneration and hair follicle involution process. Our study indicates that loss of αvβ6 integrin leads to enhanced keratinocyte proliferation in both epidermis and hair follicles, which might be associated with the down-regulated TGF-β1 signaling in β6-/- mice. Moreover, we delineated the spatio-temporal expression patterns of αvβ6 integrin in the non-wounded and wounded skin at different time points under the untreated and dexamethasone-treated conditions as well as in different stages of hair growth cycle. These observations indicated a characteristic expression of αvβ6 integrin in the bulge stem cells, which suggests that αvβ6 integrin is an important functional component in hair follicle bulge and potentially a specific cell surface marker for the activation stage of the bulge stem cells. Finally, our findings provided evidence for a potential mechanism of the quiescent bulge stem cells to respond rapidly to wound stimuli.

4.1.1 An inhibitory role of αvβ6 integrin in keratinocyte proliferation in both epidermis and hair follicles

In the impaired wound model, our results showed that wounds healed significantly faster in dexamethasone-treated β6-/- mice compared to similarly treated WT animals. In fact,

the healing rate in these treated β6-/- mice was remarkably close to the untreated animals. In contrast to the dexamethasone-treated animals, no significant difference was observed in the wound closing rates between untreated WT and β6-/- animals after 10 days of healing. This observation is in agreement with previous studies that examined younger and older, female and male β6-/- mice and reported no abnormalities in the healing response to experimental excisional wounding (Huang *et al.* 1996; AlDahlawi *et al.* 2006). In the hair regeneration model, loss of αvβ6 integrin led to an enhanced keratinocyte proliferation and retarded hair follicle regression. This is a novel finding and first to demonstrate that αvβ6 is an important functional component in hair follicle cycling probably via the modulation of the epidermal stem cell behavior. Taken together, mice that lack αvβ6 integrin in skin demonstrated an accelerated wound repair and increased numbers of proliferating keratinocytes in the interfollicular epidermis and hair follicles compared to WT controls in both impaired wound model and depilation-induced hair follicle regeneration model (Xie *et al.* 2009).

Our results suggest that the accelerated healing and hair regeneration in the β6-/- mice resulted from an enhanced proliferation of keratinocytes in the wound epithelium and hair follicles. This is likely linked to a reduced anti-proliferative effect of TGF-β1 on keratinocytes as αvβ6 integrin is a potent activator of latent TGF-β1 (Munger *et al.* 1999; Annes *et al.* 2004). TGF-β1 has been confirmed to be a negative regulator of epithelial cell proliferation in the basal layer of the epidermis and delay wound re-epithelialization (Glick *et al.* 1993). Inhibition of epithelial cell proliferation by TGF-β1 involves down

regulation of c-Myc leading to upregulation of cyclin-dependent kinase inhibitors p15, p21 and p27, which inhibit the CDK4/6-cyclin D and CDK2-cyclin E-mediated phosphorylation of the retinoblastoma protein (Reynisdottir *et al.* 1995; Robson *et al.* 1999; Massague *et al.* 2000; Moustakas *et al.* 2002; Ten Dijke *et al.* 2002; Frederick *et al.* 2004). Our findings in the dexamethasone-treated β6-/- mice are consistent with the transgenic mice overexpressing TGF-β1 in the epidermis. These mice show a decreased rate of re-epithelialization, due to reduced keratinocyte proliferation in the basal layer of the neo-epidermis (Yang *et al.* 2001). These observations are also in line with studies using Smad3-null mice that demonstrate disrupted signaling of TGF-β1 and an increased rate of re-epithelialization after incisional wounding (Ashcroft *et al.* 1999). In addition, a TGF-β1 antagonist accelerates wound re-epithelialization (Huang *et al.* 2002). All of this evidence suggests that reduced TGF-β1 signaling, including elimination of TGF-β1 activator αvβ6 integrin, speeds up the healing process through accelerated keratinocyte proliferation. In the impaired wound model, the largest difference in keratinocyte proliferation between the β6-/- and WT mice was observed in the hair follicles. The contribution of hair follicles to cutaneous wound repair has been recognized for decades (Argyris 1976; Taylor *et al.* 2000). In addition to the basal layer of the epidermis, hair follicles have been confirmed to be another source of epidermal stem cells, residing in a region of the outer root sheath called the bulge. Hair follicle stem cells can transform into epidermal stem cells in response to wounding and participate in the regeneration of the epidermis in wounded skin (Taylor *et al.* 2000; Ito *et al.* 2005; Levy *et al.* 2007). Studies

have shown that a significant portion of the newly-formed epidermis originates from the bulge cells of the hair follicles after excisional wounding (Ito *et al.* 2005). Our data demonstrated that, in contrast to the wound epithelium, a relatively higher expression of αvβ6 integrin was specifically localized to the outer root sheath of the hair follicles that fuses to the basal keratinocyte layer of the epidermis. Interestingly, a notable increase of Ki67-positive proliferating cells in the outer root sheath of the hair follicles surrounding the wounded skin in the β6-/- mice was observed. These findings suggest a cause-and-effect relationship between lacking β6 integrin and enhanced keratinocyte proliferation in the hair follicles after wounding.

A new research question was identified and raised based on the hyperproliferative phenotype of hair follicle keratinocytes in the β6-/- mice. We subsequently designed and conducted the hair regeneration project in a mouse model. Consistently, we observed accelerated hair regeneration and retarded hair regression in the β6-/- mice. Additionally, we observed notably enhanced keratinocyte activation during early anagen of the hair regeneration in the β6-/- epidermis, characterized by a hyperproliferative phenotype in both the basal layer and several suprabasal layers. In contrast, proliferating keratinocytes were mainly detected in the basal layer of the epidermis in WT mice. In line with our findings in the β6-/- mice, TGF-β1-/- mice display a delayed catagen development and more ki-67-positive cells in hair follicles than comparable WT follicles. In contrast, TGF-β1 treatment into the back skin of mice induces premature catagen development and the number of proliferating follicle keratinocytes is reduced in TGF-β1-treated mice

compared to similarly treated controls (Foitzik *et al.* 2000). Similarly, expression of αvβ6 integrin in β6-transfected cells plated on tenascin-C completely fails to proliferate (Yokosaki *et al.* 1996). It is also well know that dexamethasone is an exogenous inducer of hair follicle regression (Paus *et al.* 1994). The synergistic effect of dexamethasone and αvβ6 integrin (via TGF-β1) to suppress cells in the hair follicles probably explains why enhanced wound healing was observed mainly in the dexamethasone-treated β6-/- mice, and only at the early time points in the untreated β6-/- mice. Additionally, to evaluate the effect of αvβ6 integrin on keratinocyte migration with or without dexamethasone treatment, we used HaCaT (Human epidermal keratinocyte cell line) cells to perform scratch wound assays. Our results suggested that loss of αvβ6 integrin has little if any effect on the keratinocyte migration with or without dexamethasone treatment in vitro.

Taken together, our study indicates that αvβ6 integrin functions to reduce keratinocyte proliferation in both the wound epithelium and in the hair follicles. This reduction has only a mild effect on wound healing unless an additional inhibitory factor is introduced (dexamethasone) or the wounding happened to the hair follicles, where the enriched epidermal stem cells reside in.

4.1.2 The downregulated TGF-β1 signaling in β6-/- wound tissue

Activation of latent TGF-β1 by αvβ6 integrin is a highly localized process and likely occurs under strict spatio-temporal regulation. In the impaired wound model, we used

immunohistochemistry to examine the level of phospho-Smad3 as an indirect measurement of TGF-β1 activation. The results of the phospho-Smad3 immunostaining suggest that the presence of αvβ6 integrin contributes to the activation of TGF-β1 in the basal keratinocytes in the presence of dexamethasone, leading to reduced keratinocyte proliferation and subsequently slower migration and wound closure. Reduced TGF-β1 signaling may also reflect total TGF-β1 levels in these wounds.

In the hair regeneration model, our data indicate a reduced TGF-β1 expression and activation in β6-/- follicles. In WT mice, there were two expression peaks of TGF-β1 during hair regeneration. The expression of TGF-β1 reached the maximum level at the second peak with extended distribution from epidermis to the ORS of the hair follicles. This result is consistent with a previous study that showed TGF-β1 acted as an endogenous inducer of hair follicle regression (Foitzik *et al.* 2000). β6-/- mice, however, displayed a loss of the second peak during the anagen-catagen transition. As a negative regulator of keratinocyte proliferation, transcripts involved in the TGF-β signaling were significantly upregulated in bulge cells (Tumbar *et al.* 2004), suggesting TGF-β signaling participates in the epidermal stem cell activities in the bulge niche. Thus, loss of β6 integrin may influence epidermal stem cell behavior after injury due to the altered TGF-β signaling in β6-/- mice.

The *de novo,* transient expression of αvβ6 integrin by keratinocytes after injury suggests that the primary role of αvβ6 integrin in cutaneous wound healing is to control the activation of TGF-β1, the key player in wound repair. In the hair regeneration model, we

first immunohistochemically detected the level of phospho-Smad2. Consistent with our previous data, the results suggest that the presence of αvβ6 integrin contributes to the activation of TGF-β1 in the hair follicles and interfollicular epidermis. The altered surrounding microenvironment of the bulge niche in the β6-/- follicle may lead to altered epidermal stem cell behavior and might be associated with the enhanced keratinocyte proliferation and subsequently retarded hair cycle regression. Similarly, the constitutive expression of αvβ6 integrin in the basal layer of the epidermis of the transgenic mice leads to elevated TGF-β1 activation and the development of spontaneous chronic ulcers with severe fibrosis (Hakkinen *et al.* 2004). As suggested by our data from the hair regeneration model, the downregulated TGF-β1 signaling in β6-/- mice may exert an impact on epidermal stem cell behavior via modulating the surrounding microenvironment of the bulge niche after injury. Taken together, our data suggest a possible link between the downregulated TGF-β1 signaling and the observed effects in β6-/- mice, although other mechanisms of TGF-β1 activation might also play a role during the cutaneous wound repair and hair regeneration process.

4.1.3 A characteristic expression of αvβ6 integrin in hair follicle bulge

Our study demonstrates that the expression and distribution of αvβ6 integrin was not only different between epidermis and HFs (Figure 4.1), but also distinct within HFs themselves (Figure 4.2). In the intact epidermis of nonwounded skin in WT mice,

keratinocytes did not express αvβ6 integrin, which is consistent with previous studies (Breuss *et al.* 1993). The *de novo* expression of αvβ6 integrin is induced in the epidermis upon wounding (Haapasalmi *et al.* 1996). Although at a very low level, αvβ6 integrin, however, was constitutively expressed and confined to the keratinocytes in the ORS below the sebaceous gland in the HFs of nonwounded skin in the WT mice. In our mouse cutaneous wound model, 4 full-thickness 4mm excisional wounds were created in the back skin of WT mice (Xie *et al.* 2009). After wounding, the expression of β6 integrin was induced in the basal layer of the wound epithelium as well as a remarkable up-regulation of β6 integrin in the hair follicles adjacent to the wound area. In the epidermis, αvβ6 integrin–positive staining was localized to the basal layer along the wound edges and peaked at 7 days (Larjava *et al.* 1993; Haapasalmi *et al.* 1996; Hakkinen *et al.* 2000; Hakkinen *et al.* 2004). In HFs, αvβ6 integrin-positive staining was restricted in the ORS keratinocytes below the sebaceous gland. A relatively higher intensity of αvβ6 integrin expression in HFs than wound epithelium was noted.

In the depilation-induced hair regeneration model, hair plucking generated numerous “micro-wounds” in the hair follicles of the depilated area. After wounding, a rapid up-regulation of β6 integrin expression was observed in early anagen hair follicles, and was specifically enhanced in the bulge area. In contrast, a relatively weaker intensity of β6 integrin expression was noted in the basal layer of the epidermis. Our data also indicate that the expression of β6-integrin is only upregulated at a high level in the rapidly growing early anagen hair follicle. In the catagen and telogen stages, the

expression of β6 integrin exhibited a remarkable decrease and was restricted to the bulge area again.

Although the expression of αvβ6 integrin has been identified in hair follicles by several studies (Breuss *et al.* 1995; Hakkinen *et al.* 2004; Tumbar *et al.* 2004; Brown *et al.* 2006), to our knowledge, this is the first work to delineate the characteristic spatio-temporal expression patterns of αvβ6 integrin in hair follicle bulge throughout the hair growth cycle, which suggests αvβ6 integrin is an important functional component in hair follicle bulge and also marks this integrin as a specific cell surface candidate marker for the activation stage of bulge stem cells.

4.1.4 Integrin αvβ6 - a potential specific cell surface marker for the activation stage of bulge stem cells

Using a β6 integrin-specific antibody, we identified intense membrane staining in keratinocytes, which is strongly and specifically enhanced in the bulge region of the early anagen hair follicle. Consistent with our data, gene profiling analysis indicated that β6 integrin transcripts were among the genes involved in TGF-β pathway that were strongly and selectively upregulated in the bulge cells (Tumbar *et al.* 2004). Similarly, a recent study has identified the αvβ6 integrin-positive stem cells in human oral squamous cell carcinoma (SCC). αvβ6 integrin is co-localized with the human embryonic stem cell marker TRA-1-60 in the oral SCC biopsy specimens and is an important regulator of

pluripotential cell behavior (Dang and Ramos 2009).

During embryogenesis, αvβ6 integrin is expressed at high levels in the ORS of the developing hair follicles and its expression is downregulated in adult follicles (Breuss *et al.* 1995). β6 integrin uses tenascin-C as a ligand and tenascin-C has been shown to be present in several stem cell niches (von Holst 2008). Since both of them are activated and *de novo* expressed at a high level during skin wounding and tumorigenesis as well as co-expressed and significantly upregulated in the bulge region (Morris *et al.* 2004; Tumbar *et al.* 2004; Kloepper *et al.* 2008), αvβ6 integrin may constitute a functionally important component of the bulge stem cell niche of hair follicles.

Some initial work has implicated the integrins as one class of epidermal stem cell surface markers. The integrin family of transmembrane receptors mediates the attachment of the basal layer of the interfollicular epidermis to the underlying basement membrane. Epidermal stem cells are confined to the basal layer of the epidermis or the hair follicle bulge, which suggest that they require strong adherence to the basement membrane to maintain their position or their stem cell properties in the stem cell niche. Therefore, cell surface molecules that facilitate their adhesion to the basement memrane are theoretically possible stem cell markers (Watt 2002; Alonso and Fuchs 2003). The two members in this family that most frequently appear on the list of the candidate markers are β1 and α6 integrins. None of them, however, are specific due to their extensive expression in epidermis and hair follicles. In the dissertation research, our data indicate that the expression of β6 integrin is restricted in the bulge region in normal skin. After wounding,

although β6 integrin presence extended to the lower ORS, its expression was specifically and strongly enhanced in the bulge area compared to the lower ORS. Moreover, its expression was rapidly upregulated at a high level in the faster growing early anagen hair follicles but remarkably decreased in late anagen and in the following catagen and telogen stage hair follicles, suggesting that αvβ6 integrin constitutes a functionally important component of the bulge stem cell niche. Also, the significantly enhanced expression in the bulge region marks αvβ6 integrin potentially as a specific cell surface marker for the activation stage of epidermal stem cells. This specific marker may allow isolation of live epithelial cells with stem and progenitor cell characteristics, potentially providing a tool for the study of gene therapy, carcinogen target cells, and tissue engineering applications.

4.1.5 A potential mechanism of the quiescent bulge stem cells to respond promptly to wound stimuli

A key question for scientists in the stem cell field is how the generally quiescent stem cells contribute rapidly to the regeneration of a tissue upon injury. In the research field of hair follicle stem cells, studies have showed the slow-cycling properties of bulge stem cells in their niche, which also can be exemplified by the many upregulated transcriptss encoding cell-cycle inhibitory factors by gene profiling analysis. Little is known about the source and signals that promptly initiate bulge activation to regenerate hair follicles

and re-epithelialize epidermal wounds, although some pioneering studies have put forward different theories, for example, the theory of bi-compartmental organization of hair follicle niche in the hair germ and the bulge (Greco and Guo 2010). Unexpectedly, the findings in this dissertation research provide evidence for a potential mechanism for this intriguing question.

Although it is generally believed that epidermal stem cells are restricted to both the ORS of the hair follicle bulge and basal layer of the interfollicular epidermis, substantial evidence supports the idea that stem cells in the interfollicular epidermis are less potent than bulge stem cells (Alonso and Fuchs 2003). In addition, the majority of the stem cells in the skin reside in the bulge region of the hair follicle, with only a small fraction of stem cells in the basal layer of interfollicular epidermis (Cotsarelis *et al.* 1990; Morris and Potten 1994). As a reservoir of epidermal stem cells, hair follicles always present a higher level of β6 integrin than the basal layer of the interfollicular epidermis in response to wounding. The higher levels of β6 integrin expression in rapidly growing early anagen hair follicles suggest higher activation levels of hair follicle stem cells that change their biochemistry, exit their niche, migrate, and proliferate to regenerate hair follicles and repopulate the interfolliclular epidermis. Thus, the constitutive expression of β6 integrin at a low level in the bulge area of the telogen follicle in normal skin suggests that a small number of epidermal stem cells retain an active status. As a result, although generally quiescent, bulge cells can promptly respond to wound stimuli upon injury.

4.2 Conclusions

We conclude the overall contribution and significance of the dissertation research in light of current research in the field as follows:

1. We have revealed an important inhibitory role of αvβ6 integrin in keratinocyte proliferation in both the interfollicular epidermis and hair follicles and an involvement of αvβ6 integrin in the biological functions of epidermal stem cells. Our findings suggest that blocking the functions of αvβ6 integrin may lead to significantly enhanced keratinocyte proliferation in not only the epidermis but also hair follicles under certain wounding conditions, which may provide a useful tool for future therapeutic intervention in the cutaneous regenerative medicine.

2. We confirmed the αvβ6 integrin-mediated regulation of TGF-β1 activity plays a role in impaired wound healing induced by dexamethasone. The improved healing in impaied β6-/- wounds might be associated with the depressed anti-proliferative effects of TGF-β1. Also, our data showed that αvβ6 integrin-mediated TGF-β1 signaling regulates hair regeneration and hair follicle involution. The downregulated TGF-β1 signaling in β6-/- mice may exert an impact on epidermal stem cell behavior via modulating the surrounding microenvironment of the bulge niche after injury.

3. We demonstrated characteristic spatio-temporal expression patterns of αvβ6 integrin in hair follicle bulge throughout the hair growth cycle, which suggests αvβ6 integrin is an important functional component in hair follicle bulge.

4. We recognized αvβ6 Integrin as a potential specific cell surface marker for the activation stage of bulge stem cells. This specific marker may allow isolation of live epithelial cells with stem and progenitor cell characteristics, potentially providing a tool for the study of gene therapy, carcinogen target cells, and tissue engineering applications.

4.3 Future directions

There are several research directions that we may explore to further widen our understanding of the functions of αvβ6 integrin and its potential applications in the regenerative medicine and adult epidermal stem cell biology.

1. This dissertation work indicated an involvement of αvβ6 integrin in the biological functions of epidermal stem cells. Exploring the role of keratinocyte αvβ6 integrin in epidermal stem cells will facilitate to provide important insights into the stem cells biology in skin. To date, although our data indicates a link between the downregulated TGF-β1 signaling and the observed effects in β6-/- mice, however, further investigation will be required to elucidate the molecular mechanism of the synergistic interaction of αvβ6 integrin and TGF-β1 in regulating keratinocyte proliferation and differentiation. We propose that αvβ6 integrin plays a critical role in the regulation of keratinocyte proliferation through its capacity to activate latent TGF-β1 in the epidermal stem cells. To this end, we may need to utilize laser microdissection combined with cDNA microarray to compare the gene profiling of bulge stem cells in WT and β6-/- mice.

2. Our data suggested that blocking the functions of αvβ6 integrin lead to significantly enhanced keratinocyte proliferation in not only the epidermis but also hair follicles under certain wounding conditions. We would explore the applications whether αvβ6 integrin

can be used as a novel manipulation target to modulate the functions of epidermal stem cells in animal models. If proven, focal suppression of αvβ6 integrin functions by using specific antagonist, shRNA constructs, or together with neutralizing agent might provide new therapeutic tool for the study of gene therapy and tissue engineering applications.

3. This dissertation work have demonstrated the specifically expression of αvβ6 integrin in the hair follicle bulge, however, we still need further evidence to confirm whether this integrin is a novel cell surface marker for the activation stage of epidermal stem cells. To achieve this goal, we need to isolate the β6 integrin-positive bulge keratinocytes and test their proliferative potential by examining the clonogenicity of individual cells through serial passage or colony-forming efficacy in vitro. Moreover, *in vivo* lineage tracing analysis would be required to validate our findings.

Figure 4.1 The expression and distribution pattern of αvβ6 integrin in skin before and after wounding.

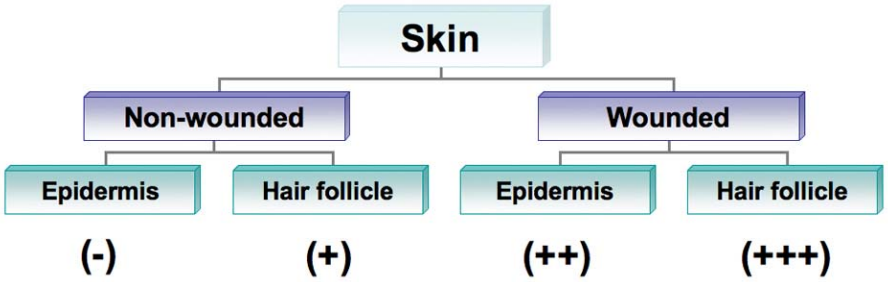
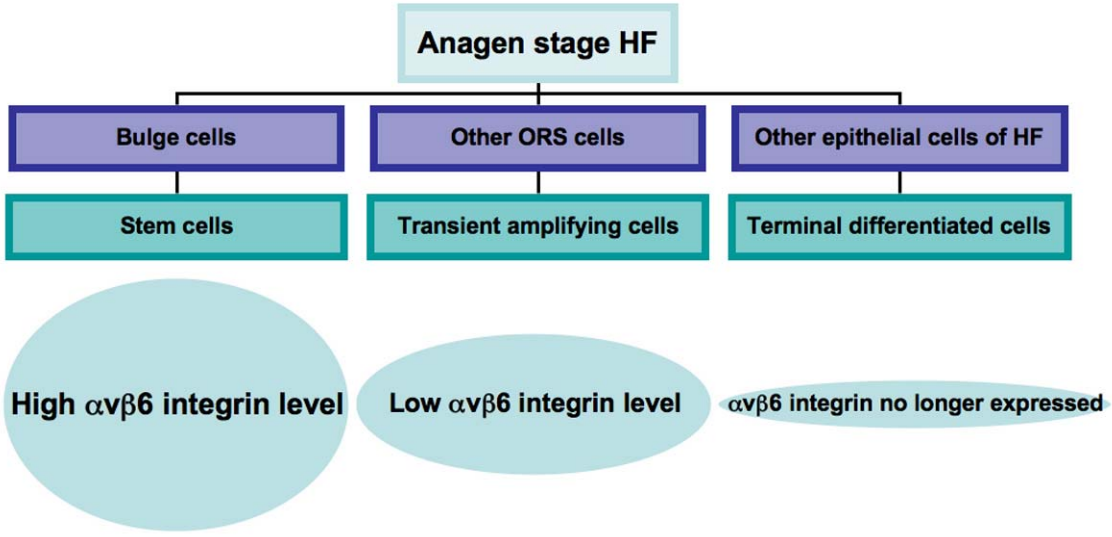


Figure 4.2 The expression and distribution pattern of αvβ6 integrin in hair follicle after wounding.



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