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Role of collagen VI in peripheral nerves and wound-induced hair regrowth

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Abstract

Collagen VI is an extracellular matrix (ECM) molecule dynamically expressed in a variety of tissues, including peripheral nerves and skin. However, the role of collagen VI in the peripheral nervous system (PNS) and hair follicle growth is yet unknown. The main focus of my PhD study was to investigate the role and the underlying mechanisms of collagen VI in peripheral nerve myelination and function, in PNS regeneration, as well as in wound-induced hair growth.

During the first year of my PhD, I focused on investigating the phenotype of peripheral nerve myelination and function in collagen VI null (*Col6a1*$^{-/-}$) mice. The data shows that Schwann cells, but not axons, contribute to collagen VI deposition in peripheral nerves. Lack of collagen VI in *Col6a1*$^{-/-}$ mice leads to hypermyelination via multiple signaling pathways, disorganized C-fibers in the PNS, impaired nerve conduction velocity, and sensorimotor dysfunction. These findings indicate that that collagen VI is a critical component of PNS contributing to the structural integrity and proper function of peripheral nerves.

The second part of my PhD work focused on investigating the role of collagen VI in PNS under pathological conditions using nerve crush injury models, and revealed a novel mechanism of this ECM protein in modulating macrophage function. The results show that collagen VI is critical for macrophage migration and polarization during peripheral nerve regeneration. Nerve injury induces a robust upregulation of collagen VI, whereas lack of collagen VI in *Col6a1*$^{-/-}$ mice delays peripheral nerve regeneration. *In vitro* studies demonstrated that collagen VI promotes macrophage migration and polarization via AKT and PKA pathways. *Col6a1*$^{-/-}$ macrophages exhibit impaired migration abilities and reduced anti-inflammatory (M2) phenotype polarization, but are prone to skewing towards pro-inflammatory (M1) phenotype. *In*

vivo, macrophage recruitment and M2 polarization are impaired in *Col6a1*$^{-/-}$ mice after nerve injury. The delayed nerve regeneration of *Col6a1*$^{-/-}$ mice is induced by macrophage deficits and rejuvenated by transplantation of wild-type bone marrow cells. These results identify collagen VI as a novel regulator for peripheral nerve regeneration by modulating macrophage function.

In the last year of my PhD I moved my focus to skin homeostasis and investigated the role of collagen VI in wound-induced hair regrowth. The data shows that collagen VI is strongly deposited in hair follicles, and it is dramatically upregulated by skin wounding. Lack of collagen VI in *Col6a1*$^{-/-}$ mice promotes wound-induced hair regrowth, but does not affect skin regeneration. Conversely, addition of purified collagen VI rescues the abnormal wound-induced hair regrowth in *Col6a1*$^{-/-}$ mice. Mechanistic studies revealed that the increased wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice is triggered by upregulation of Keratin 79 and activation of the Wnt/β-catenin signaling pathway, and is abolished by inhibition of the Wnt/β-catenin pathway. These findings highlight the essential relationships between ECM and hair follicle regeneration, and point at collagen VI as a potential therapeutic target for hair loss.

Altogether, the data I obtained during my PhD studies strongly support a key role of collagen VI in peripheral nerves and wound-induced hair follicle growth, thus paving the way for future studies on ECM molecules in PNS and skin under physiological and pathological conditions.

Riassunto

Il collagene VI è una proteina della matrice extracellulare (MEC), espressa in un'ampia varietà di tessuti, inclusi i nervi periferici e la pelle. La funzione del collagene VI nel sistema nervoso periferico (SNP) e nel follicolo pilifero rimane tuttavia ancora sconosciuta. L'obiettivo principale del mio studio di dottorato è stato quindi, quello di indagare il ruolo del collagene VI nella mielinizzazione dei nervi periferici ed i meccanismi molecolari con cui ne regola la funzione e la rigenerazione in seguito a danno, così come nella rigenerazione pilifera indotta da ferita.

Durante il primo anno di dottorato, mi sono concentrato sullo studio del processo di mielinizzazione e sulla funzione dei nervi periferici in topi *Col6a1*$^{-/-}$, privi di collagene VI. I dati dimostrano che le cellule di Schwann, ma non il comparto neuronale, contribuiscono alla deposizione del collagene VI nei nervi periferici. In assenza della proteina, si osservano ipermielinizzazione, causata dalla dis-regolazione di diversi meccanismi di segnalazione molecolare, disorganizzazione delle fibre di tipo C, deficits nella velocità di conduzione nervosa e nelle funzioni sensoriali e motorie. Questi risultati indicano che il collagene VI è un componente critico nel SNP, che contribuisce alla integrità strutturale e al corretto funzionamento dei nervi periferici.

La seconda parte del mio lavoro di dottorato è incentrata sullo studio del ruolo del collagene VI nel SNP in condizioni patologiche, sfruttando un modello di lesione nervosa. Ciò ha rivelato un nuovo ruolo di questa proteina della MEC nel modulare la funzione dei macrofagi. I risultati mostrano che il collagene VI è fondamentale per la migrazione e la polarizzazione dei macrofagi durante la rigenerazione dei nervi periferici. La lesione del nervo induce una notevole over-espressione del collagene VI, mentre in assenza della proteina, nei topi *Col6a1*$^{-/-}$ si osserva un ritardo nella rigenerazione. Studi *in vitro* hanno dimostrato che il collagene VI promuove la migrazione e la polarizzazione dei macrofagi per mezzo di AKT e PKA. Macrofagi derivati da topi *Col6a1*$^{-/-}$ presentano ridotte capacità di migrazione e di polarizzazione verso il fenotipo anti-infiammatorio (M2), mentre risultano inclini al fenotipo pro-infiammatorio (M1). Anche *in vivo*, il reclutamento dei macrofagi e la polarizzazione in senso M2 appaiono compromesse in topi *Col6a1*$^{-/-}$ post-lesione. La rigenerazione dei nervi periferici è ritardata nei topi *Col6a1*$^{-/-}$, a causa dei deficit a

carico dei macrofagi, ma è recuperata in seguito a trapianto di cellule wild-type del midollo osseo. Questi risultati identificano il collagene VI come componente fondamentale nella regolazione della rigenerazione del nervo periferico modulando la funzione dei macrofagi.

Nell'ultimo anno del mio dottorato la mia attenzione si è focalizzata sull'omostasi della pelle, studiando il ruolo del collagene VI nella ricrescita del pelo in seguito a lesione. I dati mostrano che il collagene VI è depositato ampiamente nei follicoli piliferi, ed è drammaticamente up-regolato in seguito a lesione della pelle. In assenza di collagene VI, in topi *Col6a1*$^{-/-}$, la ricrescita del pelo appare favorita in seguito a lesione, senza che vi sia alcuna influenza sulla rigenerazione della pelle. Inoltre l'iniezione in loco di collagene VI purificato, riduce l'anomala ricrescita del pelo post-lesione in topi *Col6a1*$^{-/-}$. Studi meccanicistici hanno rivelato che l'aumento della ricrescita del pelo in assenza di collagene VI è innescato dall'up-regolazione della cheratina-79 e dall'attivazione della via di segnalazione di Wnt/β-catenina, e l'inibizione esercitata dal collagne VI purificato, agisce sulla stessa via Wnt/β-catenina. Questi risultati evidenziano il rapporto essenziale tra la MEC e la rigenerazione del follicolo pilifero, e puntano al collagene VI come un potenziale bersaglio terapeutico per la perdita dei capelli.

Complessivamente, i dati che ho ottenuto durante gli studi di dottorato sostengono con forza un ruolo chiave del collagene VI nei nervi periferici e nella rigenerazione del follicolo pilifero in seguito a lesione, aprendo così la strada a futuri studi su altri componenti della MEC nel SNP e nella pelle in condizioni fisiologiche e patologiche.

Chapter 1: Literature review

1.1 Collagens

Collagens are a large family of extracellular matrix (ECM) proteins in animals that are widely distributed throughout the body (Kadler *et al.*, 2007). So far, 28 collagen types have been identified in vertebrates (Exposito *et al.*, 2010; Ferreira *et al.*, 2012). According to the different structures and functions, collagens can be classified into distinct subgroups, including fibril-forming collagens (types I, II, III, V, XI, XXIV and XXVII) (Exposito *et al.*, 2010), networking collagens (types IV, VI, VIII and X) (Gonzalez-Perez *et al.*, 2013; Ricard-Blum and Ruggiero, 2005), fibril associated collagens with interrupted triple helices (types IX, XII, XIV, XVI, XIX, XX, XXI, XXII and XXVI) (Ricard-Blum and Ruggiero, 2005), transmembrane collagens (types XIII, XVII, XXIII and XXV) (Gonzalez-Perez *et al.*, 2013; Ricard-Blum and Ruggiero, 2005; Franzke *et al.*, 2005), and other collagens (types VII, XV, XVIII and XXVIII). Fibril-forming collagens are the most abundant and conserved types and they are produced by connective tissue cells, such as fibroblasts, osteoblasts and chondrocytes (Exposito *et al.*, 2010). These collagens are typically made of α-chains containing a large triple helical domain with about 1,000 amino acids, flanked by N- and C-terminal domains (Chernousov *et al.*, 2008; Exposito *et al.*, 2010). Fibril associated collagens interact with other ECM molecules, and play an essential role for the stabilization and integrity of ECM (Ricard-Blum and Ruggiero, 2005). Networking collagens are grouped due to their ability to form networks in the extracellular space, and they represent major basement membrane components integrating laminins, nidogen, perlacan and other ECM molecules into stable superstructural aggregates (Koopmans *et al.*, 2009; Hudson *et al.*, 1993). Transmembrane collagens contain a transmembrane domain, which allows them to

participate in the formation of cell-matrix interactions and in ECM remodeling (Franzke *et al.*, 2005).

Despite high structure and function diversities among different collagen types, all members of collagen family share some common characteristics (Koopmans *et al.*, 2009; Kadler *et al.*, 2007). For instance, all collagens are made of three α-chains capable to form triple helical conformations of variable lengths (Koopmans *et al.*, 2009; Kadler *et al.*, 2007). Each chain is characterized by the presence of a collagenous domain containing repeated Gly-Xaa-Yaa amino acid triplets, where Xaa and Yaa are frequently proline and 4-hydroxyproline, respectively (Koopmans *et al.*, 2009; Kadler *et al.*, 2007). Collagens can assemble as homotrimers containing three identical α-chains, or as heterotrimers containing two or even three different α-chains (Kadler et al., 2007). A large body of studies have shown that collagens exhibit a broad range of functions, including cell adhesion, migration and proliferation; angiogenesis, cancer development and progression; as well as tissue scaffolding, morphogenesis and repair (Kadler et al., 2007).

1.2 Collagens and peripheral nerve myelination

Myelination is an important physiological process in peripheral nerves, where it is provided by Schwann cells. Derived from the embryonic neural crest, Schwann cells differentiate into myelinating or non-myelinated cells (Jessen and Mirsky, 2005; Kidd *et al.*, 2013). In the adult peripheral nerves, myelin is produced by myelinating Schwann cells, which envelop larger axons at a 1:1 ratio, whereas non-myelinating Schwann cells are linked to C-fibers (Chen *et al.*, 2014; Murinson *et al.*, 2005; Faroni *et al.*, 2014). Abnormal myelination in the peripheral nervous system is related to a number of neurological disorders, such as hereditary neuropathy with liability to pressure palsies, Charcot-Marie-Tooth disease, Dejerine-Sottas syndrome, congenital

hypomyelinating neuropathy and adrenomyeloneuropathy (Pujol *et al.*, 2002; Chance, 1999; Sander *et al.*, 1998; Warner *et al.*, 1996; Timmerman *et al.*, 2014). Thus proper myelination is critical for peripheral nerves and is tightly regulated by multiple signals, including ECM (Pereira *et al.*, 2012; Kidd *et al.*, 2013). Collagens are an essential component of Schwann cell ECM and play an important role in ECM assembly and peripheral nerve regeneration (Gao *et al.*, 2013; Cerri *et al.*, 2014). *In vitro* studies using primary cultures of Schwann cells and Schwann cell/dorsal root ganglion (DRG) co-cultures have demonstrated that the secretion of native collagens and the assembly of fibrillar and basement membrane ECM structures fail when cells are cultured without ascorbic acid, an essential factor for collagen post-translational modifications. Furthermore, addition of ascorbic acid promotes the deposition of ECM in Schwann cell plasma membrane (Chernousov *et al.*, 1998; Chernousov *et al.*, 2008) and is essential for *in vitro* myelination in Schwann cell/DRG co-cultures (Eldridge *et al.*, 1987; Olsen and Bunge, 1986). Sodium-dependent vitamin C transporter 2 (SVCT2) is necessary for the transport of ascorbic acid into Schwann cells (Gess *et al.*, 2010) and into the brain (Angelow *et al.*, 2003). Deficiency of SVCT2 leads to hypomyelination, as well as to impairments of nerve conduction velocities (NCVs) and sensorimotor function, by decreasing the deposition of ECM components such as collagen types IV, V and XXVIII (Gess *et al.*, 2011). These findings highlight the contribution of collagens in peripheral nerve myelination and function, which is also supported by the evidence that collagens can stimulate signal transduction in Schwann cells. Schwann cells not only express and secrete multiple collagen molecules both in cell culture and *in vivo* (Chernousov *et al.*, 2008; Chen *et al.*, 2014; Chernousov *et al.*, 1999; Veit *et al.*, 2006), but also express several types of collagen receptors (Chernousov *et al.*, 2008; Chernousov *et al.*, 1999; Erdman *et al.*, 2002; Rothblum *et al.*, 2004; Chernousov *et al.*, 2006; Veit *et al.*, 2006), and migrate on and adhere to

collagen substrates (Chernousov *et al.*, 2008; Chernousov *et al.*, 2001). By means of binding to their receptors on the cell surface, collagens play an important role in the regulation of Schwann cell function and myelination (Chernousov *et al.*, 2008; Milner *et al.*, 1997; Stewart *et al.*, 1997; Pereira *et al.*, 2012; Feltri *et al.*, 2002).

A number of studies demonstrated that several types of collagens (such as IV, V and XV) play an important role in regulating Schwann cell function, myelination and peripheral nerve function. For example, collagen IV can promote the attachment and spreading of Schwann cells through a mechanism that is mediated by $\alpha1\beta1$ and $\alpha2\beta1$ integrins (Detrait *et al.*, 1999), as well as enhance Schwann cell proliferation *in vitro* (Dreesmann *et al.*, 2009). Moreover, *in vivo* findings obtained from Trembler-J mice, an animal model for Charcot-Marie-Tooth disease, demonstrate that these mice display alterations in Schwann cell structure and ECM organization of peripheral nerves. Interestingly, this animal model exhibits high levels of macrophage-derived matrix metalloproteinases, thus inducing the decrease of collagen IV deposition in nerves (Misko *et al.*, 2002). In addition, clinical findings show that the expression of collagen IV is enhanced in the peripheral nerves of patients who were diagnosed as having myelin-related neuropathies, such as multiple sclerosis (Acar *et al.*, 2004), Charcot-Marie-Tooth type 1 (Palumbo *et al.*, 2002) and diabetic polyneuropathy (Bradley *et al.*, 2000). Collectively, these findings suggest that collagen IV may function as an important regulator involved in modulating myelination, and may contribute to the onset of myelin-related peripheral neuropathies.

Collagen V is a minor component of collagen fibrils that is composed of three polypeptide chains, $\alpha1(V)$, $\alpha2(V)$ and $\alpha3(V)$ (Niyibizi and Eyre, 1994; Mizuno and Hayashi, 1996). Some studies identified a collagen-like adhesive protein of 200 KDa (called p200) that binds with high affinity to the cell surface heparan sulfate

proteoglycan syndecan-3 and that is only found in the ECM surrounding Schwann cells-axon units of developing sciatic nerves (Chernousov *et al.*, 1996; Chernousov *et al.*, 1999). Subsequent work recognized p200 as a novel isoform of collagen V, named α4(V) collagen (Chernousov *et al.*, 2000), however further studies focused on the cloning of the same gene from mouse and human demonstrated that p200 corresponds to α3(V) collagen (Chernousov *et al.*, 2008; Imamura *et al.*, 2000). α3(V) collagen can promote the adhesion and spreading of Schwann cells (Chernousov *et al.*, 1996), increase the migration of premyelinating Schwann cells and inhibit the outgrowth of axons from DRG (Chernousov *et al.*, 2001). In mature myelinating Schwann cell-axon units, α3(V) collagen and its receptor syndecan-3 are highly concentrated at the nodes of Ranvier, which are structures strongly regulated by myelinating glia (Melendez-Vasquez *et al.*, 2005). Genetic studies also show that siRNA mediated suppression of α3(V) collagen significantly inhibits Schwann cell myelination *in vitro* (Chernousov *et al.*, 2006). Altogether, these findings not only provide direct evidence supporting the role of α3(V) collagen in Schwann cell adhesion, spreading and migration, but also suggest the potential role of this protein in regulating peripheral nerve myelination.

Collagen XV is widely distributed in the basement membranes of different tissues (Clementz and Harris, 2013; Li *et al.*, 2000). Immunostaining studies demonstrated that collagen XV is deposited in the endoneurium and perineurium of adult peripheral nerves (Muona *et al.*, 2002), and in the extrasynaptic and Schwann cell basement membranes of neuromuscular junction (Muona *et al.*, 2002), suggesting a potential role for collagen XV in the development and functional properties of peripheral nerves. Indeed, genetic studies in *Col15a1*$^{-/-}$ mice showed that lack of collagen XV causes polyaxonal myelination, loosely packed axons in C-fibers and less electron

dense cytoplasm in Schwann cells, suggesting an impairment of peripheral nerve maturation and C-fiber formation (Rasi *et al.*, 2010). Moreover, ablation of collagen XV leads to defective basement membrane assembly (Rasi *et al.*, 2010). Electrophysiological studies demonstrated that deficiency of collagen XV induces a decrease in NCVs, suggesting an impairment of sensory function in *Col15a1*$^{-/-}$ mice (Rasi *et al.*, 2010). Laminin-411, another component of the basement membrane of peripheral nerves, was shown to be involved in axon segregation and myelination in peripheral nerves (Wallquist *et al.*, 2005; Yang *et al.*, 2005). Lack of Laminin-411 in *Lama4*$^{-/-}$ mice causes an impairment of motor and tactile sensory functions, which is exacerbated by the simultaneous deficiency of collagen XV, although the sole ablation of collagen XV does not affect the motor function in mice (Rasi *et al.*, 2010). Taken together, these findings point at collagen XV as an essential factor regulating peripheral nerve maturation and C-fiber formation, as well as contributing to the modulation of another ECM molecule, laminin-411, that is involved in motor and sensory functions of peripheral nerves.

1.3 Collagens and peripheral nerve regeneration

Unlike the central nervous system, axons in the PNS have the ability to regenerate even after severe injury. The successful peripheral nerve regeneration is a process that requires the concerted interplay of glial cells, trophic factors, cell adhesion molecules and ECM molecules, as well as macrophage recruitment (Gonzalez-Perez *et al.*, 2013; Horie *et al.*, 2004). Among them, the switch of Schwann cell to a progenitor-like state, secretion of trophic factors, and production of ECM molecules are the key elements. ECM not only itself creates an environment for axon regeneration, but also exhibits trophic roles during the nerve regeneration (Gonzalez-Perez *et al.*, 2013).

Collagens are among the prominent molecules abundantly deposited in the ECM of peripheral nerves, where they play a key role in the development of peripheral nerves and in the maintenance of normal nerve function during the adult life (Gonzalez-Perez *et al.*, 2013; Hubert *et al.*, 2009). Upon injury, peripheral nerves first initiate nerve degeneration, a process where the debris and myelin sheaths are removed by glial cells. However, the original basal lamina is not degraded within the columns of collapsed Schwann cells, termed bands of Bungner, which form regeneration tracks (Koopmans *et al.*, 2009). During nerve regeneration, the regenerating axons grow within the bands of Bungner, and there is a long-lasting expression of collagens I, III and IV in the proximal nerve stumps. Axon reinnervation enhances the expression of collagen IV but does not affect collagens I and III (Siironen *et al.*, 1992b; Siironen *et al.*, 1992a; Seyer *et al.*, 1977; Nath *et al.*, 1997), suggesting different roles for distinct collagens in peripheral nerve regeneration. In consistent with this hypothesis, studies have shown that collagens I and III are able to provide mechanical support for axon regeneration (Koopmans *et al.*, 2009), a finding that is also supported by experiments using bio-composite P(LLA-CL)/collagen I/collagen III scaffolds that promote peripheral nerve regeneration (Kijenska *et al.*, 2012). Conversely, collagen IV exhibits an opposite effect, since inhibition of collagen IV deposition promotes axon regeneration (Stichel *et al.*, 1999). Further studies demonstrated that excessive collagen synthesis can function as a mechanical barrier for regeneration, which in turn inhibits peripheral nerve regeneration (Koopmans *et al.*, 2009). Although these findings indicate that collagens have an important function in peripheral nerve regeneration, additional studies will be needed to characterize the role of specific types of collagens in PNS regeneration.

1.4 Collagens and hair follicle growth

Mammalian skin is a complex organ consisting of three epithelial compartments, including the interfollicular epidemis, sebaceous glands and hair follicles (Stenn and Paus, 2001). Hair follicle is the most prominent miniorgan in the skin and it is composed of concentric layers or sheaths of keratinocytes surrounding the hair shaft (Osorio *et al.*, 2008). This miniorgan has a regenerative potential due to the fact that the outer root sheath contains hair follicle stem cells in bulge regions. Bulge cells regenerate the proliferating matrix progenitor cells, and then further differentiate into inner layers of the hair follicle and hair shaft (Osorio *et al.*, 2008) (Figure 1A). Each hair follicle in adult mammals goes through cycles of anagen (growth), catagen (regression) and telogen (quiescence) phases that are synchronously orchestrated in mouse skin during youth and take about three weeks to complete (Osorio *et al.*, 2008; Muller-Rover et al., 2001) (Figure 1B).

Literature studies point at collagens as key ECM molecules providing a functional niche for hair follicle stem cells. For example, several genes encoding for collagen proteins, such as collagens I, IV, V, VI and XVIII, display higher expression levels in bulge cells than that in differentiated keratinocytes (Fujiwara *et al.*, 2011). Immunohistochemical analysis showed that collagen I and III are deposited in the dermal sheath and papilla during all hair cycles, and collagen IV is expressed at the ECM of the dermal papilla and outer root sheath basement membranes of anagen and catagen follicles (Messenger *et al.*, 1991). These findings suggest a potential role of collagens in modulation of hair follicle activities. Indeed, it has been shown that collagen XVII, a hemidesmosomal transmembrane protein, is highly expressed in hair follicle stem cells and is required for the maintenance of hair follicle stem cells and melanocyte stem cells. Moreover, lack of collagen XVII in *Col17a1* null mice causes deficient stemness of hair follicle stem cells, premature hair graying and hair loss, as well as premature melanocyte stem cell differentiation with diminished TGF-β

signaling, and these defects can be rescued by the forced *Col17a1* expression in basal keratinocytes, including hair follicle stem cells (Tanimura *et al.*, 2011). Patients with collagen XXVII deficiency exhibit a characteristic premature hair loss with hair follicle atrophy (Darling *et al.*, 1997; Hintner and Wolff, 1982), suggesting that this ECM molecule plays an essential role in hair follicle homeostasis and provides a functional niche for melanocyte stem cell. Although these findings support an important role of collagens in hair follicle homeostasis and activities, several open questions remain regarding the role of specific collagen types in this process and the underlying molecular mechanisms, as well as the role of collagens in hair follicle growth under pathological conditions, such as wound-induced hair regrowth.

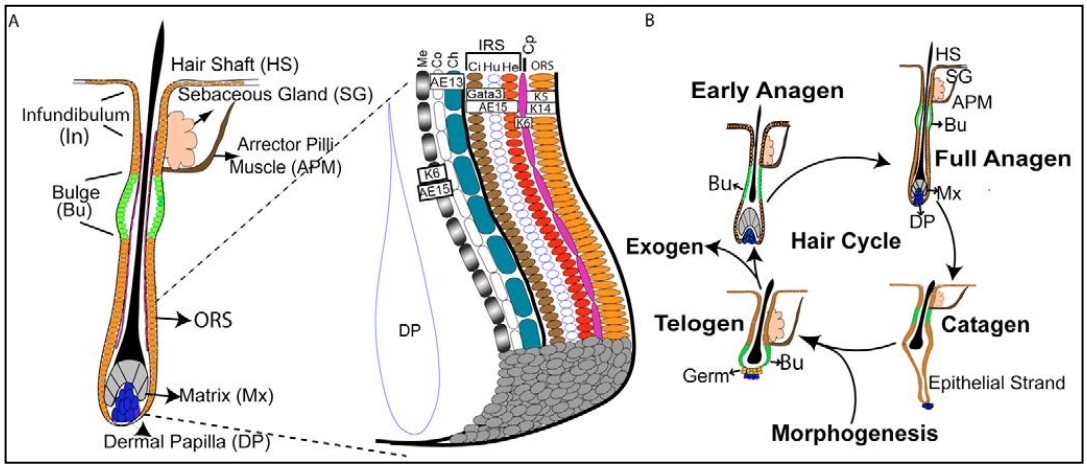

Figure 1. Hair follicle organization (A) and the hair cycle (B). The follicle cell layers are depicted in color and their respective protein markers are boxed. Stem cells are in the bulge and progenitor cells are in the matrix. Differentiated hair lineages: Ch, cuticle of hair shaft; Ci, cuticle of IRS; Co, cortex of hair shaft; Cp, companion cell layer; He, Henle's layer; Hu, Huxley's layer; IRS, inner root sheath; Me, medulla. Exogen is hair shaft loss. (Modified from Osorio *et al.*, 2008).

1.5 Collagen VI and its properties

Collagen VI is an ECM protein composed of three major polypeptide chains – α1(VI),

α2(VI) and α3(VI) – encoded by distinct genes (*COL6A1*, *COL6A2* and *COL6A3*, respectively). The α1(VI) and α2(VI) chains have molecular weights of 140–150 kDa and extend for nearly 1,000 amino acids, whereas the α3(VI) chain is three times larger (250–300 kDa) with several alternatively spliced variants ranging between 2,500 and 3,100 amino acids. Each chain is characterized by a short triple helical region flanked by large N- and C-terminal globular regions, which are composed of 200 amino acid motifs sharing similarity with the von Willebrand factor type A(vWF-A) module (Bernardi and Bonaldo, 2008). The α1(VI) and α2(VI) chains have one N-terminal- (N1) and two C-terminal (C1 and C2) vWF-A modules, whereas the α3(VI) chain displays larger N- and C-terminal globular regions made of twelve vWF-A modules (N1 to N10, C1, C2) and three distinct C-terminal domains (C3-C5) (Bernardi and Bonaldo, 2008; Bonaldo *et al.*, 1990). Interestingly, some studies indicate that α3(VI) undergoes proteolytic processing, leading to the release of the most C-terminal (C5) domain after collagen VI secretion (Aigner *et al.*, 2002) (Figure 2). Recent studies led to the identification of three other collagen VI subunits – α4(VI), α5(VI) and α6(VI) – encoded by separate genes. These chains contain one N-terminal region made of seven vWF-A modules, a collagen triple helical region that is similar to that of α3(VI), and a C-terminal region containing two or three vWF-A modules as well as one or two unique sequences (Fitzgerald *et al.*, 2008; Gara *et al.*, 2008; Sabatelli *et al.*, 2011; Sabatelli *et al.*, 2012) (Figure 2).

The synthesis and secretion of collagen VI requires the association of α1(VI), α2(VI) and α3(VI) in equimolar ratios. At difference from other collagens, collagen VI has a peculiar multistep pathway of intracellular assembly, which also involves extensive disulfide bond interactions. Association of the distinct α-chains allows the formation of triple-helical “monomers” (3 chains), followed by the assembly into “dimers" (6

chains) and "tetramers" (12 chains) before secretion (Bernardi and Bonaldo, 2008). Such tetramers are very large (more than 2,000 kDa) and once secreted they associate by non-covalent interactions, giving rise to characteristic "beaded" microfilaments that are deposited in the ECM (Bernardi and Bonaldo, 2008; Furthmayr *et al.*, 1983). Recent studies suggested that the α4(VI), α5(VI) or α6(VI) chains may substitute for α3(VI) during the synthesis and assembly of collagen VI, forming α1(VI)α2(VI)α4(VI), α1(VI)α2(VI)α5(VI) and α1(VI)α2(VI)α6(VI) isoforms (Fitzgerald *et al.*, 2008; Gara *et al.*, 2008). After secretion, collagen VI regulates the properties of local microenvironment by forming a distinct network of beaded microfilaments (Chen *et al.*, 2013; Bernardi and Bonaldo, 2008), that are able to interact with a number of cell surface receptors as well as with several ECM components. Collagen VI microfilaments not only provides structural support for cells and connective tissues, by fine-tuning the local stiffness (Urciuolo *et al.*, 2013; Alexopoulos *et al.*, 2009) and the size of collagen I fibrils (Izu *et al.*, 2011), but also regulate important functions in different cell types, such as apoptosis in muscle fibers and neurons (Irwin *et al.*, 2003; Cheng *et al.*, 2011; Cheng *et al.*, 2009), proliferation in cancer cells (Iyengar *et al.*, 2005), as well as angiogenesis and inflammation in endothelial cells and leukocytes, respectively (Park and Scherer, 2012).

Collagen VI is widely distributed in several tissues, including peripheral nerves and skin (Allen *et al.*, 2009; Bernardi and Bonaldo, 2008). In the PNS, collagen VI is produced by Schwann cells and endo-/perineurial cells (Allen *et al.*, 2009; Vitale *et al.*, 2001). Further evidence indicates that the *Col6a1* gene is not expressed in immature Schwann cells, but expressed in mature Schwann cells which have undertaken myelination (Vitale *et al.*, 2001). Interestingly, although activation of *Col6a1* transcription in the peripheral nerves is part of the differentiation program of

Schwann cells from the neural crest cells stimulated by neuregulin, once the Schwann cell precursors have acquired the competence of transcribing the *Col6a1* gene, transcriptional regulation becomes independent from neuregulins (Vitale *et al.*, 2001). These findings indicate that activation of *Col6a1* transcription is accounted for the molecular program underlying Schwann cell differentiation and is regulated by axonal signals. Furthermore, activation of the *Col6a1* gene in sciatic nerves after birth is associated with the time of withdrawal of immature Schwann cells from the cell cycle, when they start to differentiate into myelinating Schwann cells (Vitale *et al.*, 2001). Altogether, these findings highlight the contribution and underlying mechanisms of collagen VI expression in Schwann cell differentiation. *In vivo* evidence shows that collagen VI may interact with other ECM molecules to modulate peripheral nerve structure and function. For example, von Willebrand A domain-related protein (WARP) is an ECM molecule that interacts with collagen VI in peripheral nerves. Ablation of WARP severely reduces collagen VI deposition in peripheral nerves, which not only leads to compromised peripheral nerve structure, such as the fusion of adjacent Schwann cells basement membranes, but also causes an impairment of both motor and sensory functions (Allen *et al.*, 2009). Altogether, these findings point at a role for collagen VI in PNS. However, further studies are still needed to provide the direct evidence showing the role and underlying molecular mechanisms of collagen VI in peripheral nerve myelination and function, as well as in peripheral nerve regeneration. In the skin, the expression levels of *Col6a1* and *Col6a2* in bulge cells are higher than in differentiated keratinocytes (Fujiwara *et al.*, 2011), suggesting a potential role of collagen VI in regulating hair follicle stem cells. However, it is still completely unknown whether collagen VI contributes to hair follicle growth under both physiological and pathological conditions (such as wound-induced hair regrowth).

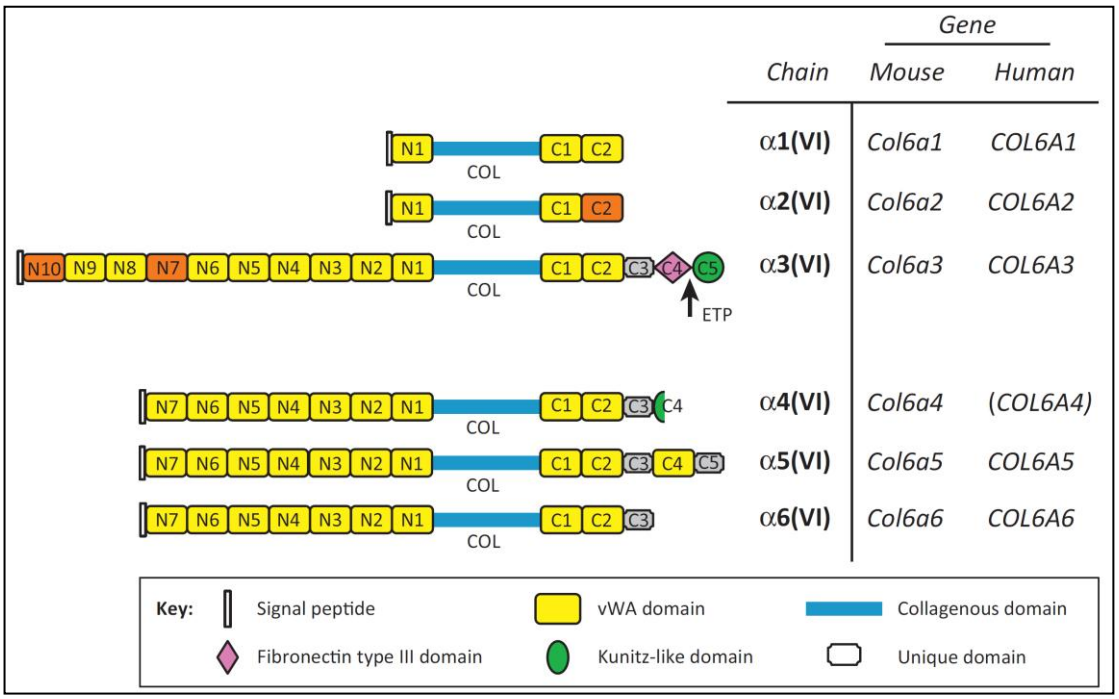

Figure 2. Schematic diagram of the primary structure of collagen VI chain. The most abundant form of collagen VI contains α1(VI), α2(VI), and α3(VI) chains. Each chain is made of a short collagenous (COL) region flanked by a variable number of vWF-A modules. The α3(VI) chain contains three additional domains at the carboxyl-terminal end; the arrow marks the cleavage site that releases the most C-terminal domain, giving rise to the soluble ETP peptide. The vWF-A modules depicted in orange were shown to undergo alternative splicing. The lower part of the diagram shows the domain structure of the three novel collagen VI chains described recently, which share a high degree of similarity with α3(VI). In humans, the *COL6A4* gene, coding for the α4(VI) chain, is split into two pieces due to a large chromosome inversion and represents a non-processed pseudogene.

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Chapter 2

Collagen VI regulates peripheral nerve myelination and function

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Collagen VI regulates peripheral nerve myelination and function

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ABSTRACT **Collagen VI is an extracellular matrix protein with broad distribution in several tissues. Although *Col6a1* is expressed by Schwann cells, the role of collagen VI in the peripheral nervous system (PNS) is yet unknown. Here we show that Schwann cells, but not axons, contribute to collagen VI deposition in peripheral nerves. By using *Col6a1*-null mice, in which collagen VI deposition is compromised, we demonstrate that lack of collagen VI leads to increased myelin thickness ($P<0.001$) along with 60–130% up-regulation in myelin-associated proteins and disorganized C fibers in the PNS. The hypermyelination of PNS in *Col6a1*$^{-/-}$ mice is supported by alterations of signaling pathways involved in myelination, including increase of P-FAK, P-AKT, P-ERK1, P-ERK2, and P-p38 (4.15, 1.67, 2.47, 3.34, and 2.60-fold, respectively) and reduction of vimentin (0.49-fold), P-JNK (0.74-fold), and P-c-Jun (0.50-fold). Pathologically, *Col6a1*$^{-/-}$ mice display an impairment of nerve conduction velocity and motor coordination ($P<0.05$), as well as a delayed response to acute pain stimuli ($P<0.001$), indicating that lack of collagen VI causes functional defects of peripheral nerves. Altogether, these results indicate that collagen VI is a critical component of PNS contributing to the structural integrity and proper function of peripheral nerves.—Chen, P., Cescon, M., Megighian, A., Bonaldo, P. Collagen VI regulates peripheral nerve myelination and function. *FASEB J.* 28, 1145–1156 (2014). www.fasebj.org**

Key Words: extracellular matrix · peripheral nervous system · Schwann cells

In the peripheral nervous system (PNS), myelin is produced by Schwann cells and plays a crucial role for the transmission of electric impulse. In the adult organism, myelinating Schwann cells envelop larger axons in a myelin sheath at a 1:1 ratio (1, 2), whereas nonmyelinating Schwann cells ensheath multiple smaller axons, known as C fibers (3). Altered myelination in the PNS is related to a variety of neurological disorders, such as hypomyelination in hereditary neuropathy with liability to pressure palsies (4), Charcot-Marie-Tooth disease (5), Dejerine-Sottas syndrome and congenital hypomyelination (6), and hypermyelination in adrenomyeloneuropathy (7).

Myelination in the PNS is regulated by extracellular matrix (ECM) molecules in the basement membrane that assembles at the surface of Schwann cell/axon units (8, 9). Coculture of Schwann cells and neurons demonstrated that basement membrane assembly is required for Schwann cells myelination *in vitro* (10). Genetic studies showed that basement membrane molecules and their receptors, such as β1 integrin (11) and laminins (1, 12, 13), are essential for proper myelination and/or motor function in the PNS. Collagens, the prominent components of ECM, also appear to be involved in myelination. Genetic studies demonstrated that lack of the α4(V) collagen subunit significantly inhibits Schwann cell myelination *in vitro* (14), and ablation of collagen XV in mice causes polyaxonal myelination and hypomyelination in the PNS (8).

Collagen VI is a peculiar component of the collagen superfamily made of 3 genetically distinct chains and abundantly deposited in the basement membrane of a variety of tissues, such as skeletal muscles (15, 16), skin (17), and peripheral nerves (18). Collagen VI exerts a neuroprotective activity in the central nervous system, where it has been shown to inhibit the neurotoxicity of Aβ peptides (19) and of ultraviolet irradiation (20). Although our previous studies demonstrated that *Col6a1* is expressed by Schwann cells (21), the role of collagen VI in the PNS remains largely unknown. Here, we utilized *Col6a1*$^{-/-}$ mice, where a targeted inactivation of the gene coding for the α1(VI) chain prevents the assembly and secretion of collagen VI in the ECM (22, 23) to demonstrate that collagen VI is crucial for

Abbreviations: CMAP, compound muscle action potential; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglion; ECM, extracellular matrix; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; NCV, nerve conduction velocity; NGFR p75, nerve growth factor receptor p75; PFA, paraformaldehyde; PNS, peripheral nervous system; TUNEL, terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling

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the structural integrity of PNS and for peripheral nerve function.

MATERIALS AND METHODS

Animals

Col6a1$^{+/+}$ (wild-type) and *Col6a1*$^{-/-}$ mice in the C57BL/6 background (22, 23) were used in this study. Pregnant Sprague-Dawley rats were purchased from Harlan Laboratories (Udine, Italy). Native collagen VI was purified from newborn mice as described previously (23). Animal procedures were approved by the Ethics Committee of the University of Padova and authorized by the Italian Ministry of Health.

Cell cultures

The RT4-D6P2T Schwann cell line was purchased from American Type Culture Collection (LGC Standards S.r.l., Sesto San Giovanni, Italy) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) containing 10% fetal bovine serum (Gibco), 0.2 M L-glutamine (Invitrogen, Carlsbad, CA, USA), and 1:100 penicillin-streptomycin (Invitrogen). RT4-D6P2T cells were treated with purified collagen VI to analyze protein expression by Western blot. Primary Schwann cell cultures were isolated from newborn rat sciatic nerves as described previously (24, 25) and grown for 48 h in DMEM/F12 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (Gibco) and 1:100 penicillin-streptomycin (Invitrogen). Cytosine arabinoside (Ara-C; 10 μM) was added, and the treatment was prolonged for further 48 h, resulting in Schwann cell cultures that were 99% pure and were used for further studies.

Histology

After mice were perfused with 4% paraformaldehyde (PFA), sciatic nerves were dissected and postfixed in 2% glutaraldehyde for 24 h at 4°C. Samples were osmicated in 2% osmium tetroxide for 2 h at room temperature, dehydrated in ascending acetone, and embedded in Epon E812 resin (Sigma, St. Louis, MO, USA). Semithin sections (0.5 μm) were cut using an Ultracut 200 microtome (Leica, Wetzlar, Germany), and stained with alkaline toluidine blue. Axon numbers were analyzed on 8 sections/sciatic nerve, and the g ratio of all axons was measured using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA) as described previously (8). For electron microscopy, ultrathin sections (80 nm) were cut, mounted on copper grids, and stained with uranyl citrate and lead citrate. Grids were observed and photographed on a FEI Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR, USA). For the internodal length measurement, sciatic nerves were dissected, fixed in 2% glutaraldehyde for 1 h at room temperature, washed in 0.12 M sodium phosphate buffer 3 times, incubated in 1% osmium for 2 h at room temperature, transferred into 66% glycerol overnight at room temperature, and then transferred into 100% glycerol at 4°C. Nerves were teased using acupuncture needles, and the images were obtained by light microscope (Leica). The internodal length was measured using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL)

TUNEL assay was performed using the Dead End Fluorometric *In Situ* Apoptosis Detection System (Promega, Madison, WI, USA). Cross-sections of sciatic nerves were permeabilized in 100% methanol for 10 min at −20°C. Slides were dried, washed in PBS 3 times, and incubated in proteinase K for 5 min at room temperature. After being washed 3 times in PBS, samples were incubated with equilibration buffer for 10 min and then incubated with a buffer containing fluorescent nucleotides, terminal deoxynucleotidyl transferase (TdT), and Hoechst for 1 h at 37°C. The reaction was then blocked with SSC solution (300 mM NaCl, 30 mM sodium citrate). After being washed 3 times in PBS, slides were mounted using 80% glycerol. TUNEL-positive cells were analyzed on 8 sections/sciatic nerve using ImageJ software.

Immunofluorescence

After mice were perfused with 4% PFA, sciatic nerves were removed and postfixed for 4 h at 4°C. Tissues were then transferred into 30% sucrose overnight for cryoprotection. Cross and longitudinal sections of sciatic nerves at 10 μm were cut in a cryostat (Leica). For nodal integrity analysis, sciatic nerves were dissected and fixed in 4% PFA for 4 h at 4°C, washed in several changes of PBS, and then teased on 3-aminopropyltriethoxysilane (TESPA; Sigma)-coated slides. After blocking with 10% goat serum for 1 h, sections were incubated with primary antibodies (1:200) for 2 h at room temperature or overnight at 4°C. Primary antibodies against the following proteins were used: CD45, CD68 (rat monoclonal; AbD Serotec, Raleigh, NC, USA); cleaved caspase-3 (rabbit polyclonal; Cell Signaling, Danvers, MA, USA); collagen VI (rabbit polyclonal; Fitzgerald, Acton, MA, USA); contactin-associated protein (Caspr; mouse monoclonal, a kind gift of Elior Peles, Weizmann Institute of Science, Rehovot, Israel); ezrin (rabbit polyclonal; Upstate, Lake Placid, NY, USA); Kv1.1 (rabbit polyclonal; Alomone Labs, Jerusalem, Israel.); myelin basic protein (MBP, rat monoclonal; Abcam, Cambridge, MA, USA); Nav1.6 (rabbit polyclonal, a kind gift of James S. Trimmer, University of California, Davis, CA, USA); nerve growth factor receptor p75 (NGFR p75, rabbit polyclonal; Millipore; Billerica, MA); neurofilament (mouse monoclonal; Covance, Chantilly, VA, USA); peripherin (rabbit polyclonal; Novus, St. Charles, MO, USA); S100 (rabbit polyclonal; Abcam). Guinea pig polyclonal α3(VI) collagen antibody (26) and rabbit polyclonal AS-72 specific for murine collagen VI antibody (27) were kindly provided by Raimund Wagener (Universität zu Köln, Cologne, Germany) and Alfonso Colombatti (Centro di Riferimento Oncologico di Aviano, Aviano, Italy), respectively. The sections were then transferred to secondary antibodies (1:200) and Hoechst 33258 (Sigma) for 1 h at room temperature. The following secondary antibodies were used: anti-mouse CY3, anti-rat CY3, anti-rabbit CY2, anti-rabbit CY3, anti-guinea-pig CY2 (Jackson Immunoresearch, West Grove, PA, USA); and anti-rabbit IRIS5 (Cyanine Technologies, Turin, Italy). After being washed 3 times in PBS, slides were mounted using 80% glycerol.

Western blotting

Mice were euthanized by cervical dislocation and sciatic nerves were removed and frozen in nitrogen immediately. The tissues were homogenized in lysis buffer (Millipore) with phosphatases (Sigma) and proteases inhibitors (Roche, Basel, Switzerland), using a dounce homogenizer (Sigma). Protein concentration was determined by BCA assay (Thermo Sciem-

tific, Rockford, IL, USA). Samples of 20 μg protein were applied to SDS-PAGE gels (Invitrogen) and blotted onto PDVF membrane (Millipore). Membranes were incubated with primary antibodies (1:1000) overnight at 4°C. Primary antibodies against the following proteins were used for Western blot: collagen VI (rabbit polyclonal; Fitzgerald); β-III tubulin, β-actin (mouse monoclonal; Sigma); neurofilament (mouse monoclonal; Abcam); phospho-neurofilament (mouse monoclonal; Covance); Bax, FAK, JNK, vimentin (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-JNK (mouse monoclonal; Santa Cruz); peripherin (rabbit polyclonal; Novus); AKT, caspase-9, phospho-c-Jun, CNPase, phospho-FAK (rabbit polyclonal; Cell Signaling); MBP (rat monoclonal; Abcam); phospho-AKT, Bcl-2, Bcl-X_L, c-Jun, ERK, phospho-ERK, myelin-associated glycoprotein (MAG), p38, and phospho-p38 (rabbit monoclonal; Cell Signaling). After being washed 3 times with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000; Amersham Bioscience, Dübendorf, Switzerland) for 1 h at room temperature. Detection was by chemiluminescence (Pierce, Rockford, IL, USA). Densitometric quantification was performed by Image-Pro Plus 6.0 software (Media Cybernetics).

Electrophysiology

Electrophysiological measurements were performed as described previously (28–30). Briefly, mice were anesthetized with ketamine (100 mg/kg body weight) and xylazine (8 mg/kg body weight). The body temperature of mice was adjusted to 37°C using a heating pad, which was continuously monitored by the thermal probe of a Peltier warming system (NPI, Tamm, Germany). Monopolar stainless steel needle electrodes (28 gauge; Grass-Astromed, West Warick, RI, USA) were used both as stimulating and recording electrodes. For stimulation, a pair of stimulating electrodes was placed along the nerve at the sciatic notch nerve (proximal stimulation), and a second pair of electrodes was placed along the tibial nerve above the ankle (distal stimulation). A recording electrode was inserted in muscles in the middle of the paw, while the reference electrode was subcutaneously inserted between the first and second digits. Square pulses of fixed duration (0.5 ms), delivered by a stimulator (Grass S88; Grass-Astromed) through a stimulus isolator unit (SIU 5; Grass-Astromed), were used to stimulate the nerve. The intensity of stimuli was adjusted to obtain supramaximal nerve stimulation. The compound muscle action potential (CMAP), recorded from the paw muscles using electrodes, was amplified using an extracellular amplifier (Grass P6; Grass-Astromed), displayed on a digital oscilloscope (Tektronix, Beaverton, OR, USA) for online view, and fed to a PC with an A/D interface (Norton Instruments), for storage and offline analysis, using appropriate software (pClamp 9, Axon; WinEDR, University of Strathclyde, Glasgow, Scotland). Nerve conduction velocities (NCVs) were calculated as the distance divided by the difference between the proximal and distal stimulation latencies.

Behavioral tests

The rotarod test was performed placing mice on a rotating rod (Ugo Basile, Comerio, Italy) at a fixed speed of 30 rpm. After three 2-min training trails for 2 d, the latency to fall off the rotating rod was recorded. The ledged beam test was performed by putting mice on a suspended 1 m long runway with a 6 cm width at the starting point, then gradually narrower to 0.5 cm. The distance from the start to the point where first foot fault occurred was measured, and the total number of hind foot faults was recorded. For the footprint test, gait was examined by applying nontoxic paint to hindpaws (green) and forepaws (black). Mice were placed on white paper, and stride width, stride difference, and angles were recorded. The stride difference was calculated by subtracting the shortest stride from the longest stride, and angles were measured between foot steps and walking direction. The hotplate test was performed by placing mouse in a heated hotplate chamber (55°C; Thermomix 1419; B. Braun Melsungen AG, Melsungen, Germany). The latency to distressful behavior, such as paw licking and squeaking, was recorded for each mouse. The von Frey filament test was performed by placing mouse in suspended plastic chambers with a perforated metal sheet for 0.5 h before testing. A series of calibrated von Frey hairs (North Coast Medical, Morgan Hill, CA, USA) was applied perpendicularly to the plantar surface of the hindpaw. The pain threshold was determined as the lower force that evoked a withdrawal response to 3 out of the 5 stimuli. The withdrawal threshold of each mouse was expressed as the average threshold of left and right hindpaws.

Statistical analysis

Data are presented as means ± SE. Statistical analysis of data was carried out using the Student's t test, except for the analysis of H reflex, where the χ^2 test was used. Values of $P < 0.05$ were considered significant.

RESULTS

Schwann cells contribute to collagen VI deposition in sciatic nerve

To analyze the deposition of collagen VI in peripheral nerves, we labeled murine sciatic nerves with specific antibodies against collagen VI. Immunofluorescence showed that collagen VI was abundant in the endoneurium of sciatic nerve and located outside of myelin as demonstrated by labeling for MBP, a major constituent of Schwann cell myelin sheaths (Supplemental Fig. S1*A*). Collagen VI was not colocalized with neurofilaments but partially colocalized with S100 (Schwann cell marker) and NGFR p75 (nonmyelinating Schwann cell marker), suggesting that collagen VI is produced by Schwann cells but not by axons (**Fig. 1*A–C***), as also confirmed by collagen VI labeling in teased fiber (Supplemental Fig. S1*B*). Immunofluorescence for CD68, a marker for macrophages, the major type of resident immune cells in the PNS, showed partial colocalization with collagen VI (Fig. 1*D*), suggesting that macrophages may also produce collagen VI in sciatic nerve. Blood-derived macrophages are known to express and secrete collagen VI depending on their activation status and differentiation stage (31). However, the restricted number of macrophages present in sciatic nerve is not activated under physiological conditions, suggesting that macrophages have a limited contribution on collagen VI deposition in sciatic nerve. We then further examined the *in vitro* deposition of collagen VI by Schwann cells. Both immunofluorescence and Western blot showed that collagen VI was abundantly produced by RT4-D6P2T Schwann cells (Fig. 1*E*,

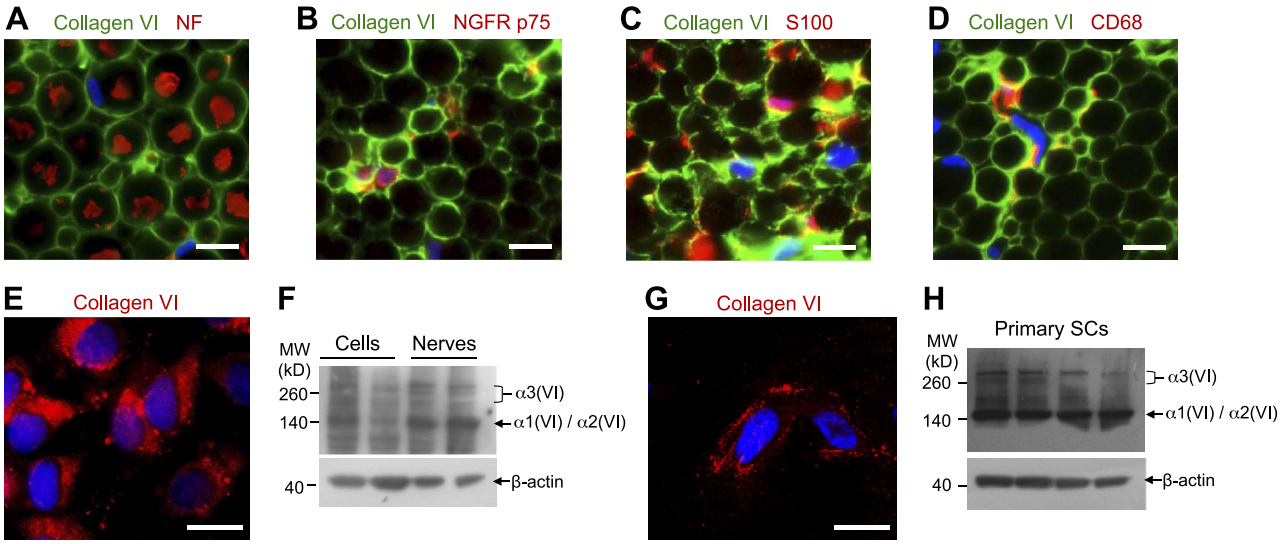

Figure 1. Schwann cells contribute to collagen VI deposition in sciatic nerve. *A–D*) Coimmunofluorescence labeling of collagen VI (green) with neurofilament (red; *A*), NGFR p75 (red; *B*), S100 (red; *C*), or CD68 (red; *D*) in wild-type mouse sciatic nerve. *E*) Immunofluorescence labeling of collagen VI (red) in RT4-D6P2T Schwann cells (SCs). *F*) Western blot for collagen VI in 2 replicate samples of RT4-D6P2T cells and sciatic nerves. *G*) Immunofluorescence labeling of collagen VI (red) in primary Schwann cells. (*H*) Western blot for collagen VI in 4 replicate samples of primary Schwann cells. Nuclei were stained with Hoechst (blue). Scale bars = 10 μm (*A–D*); 20 μm (*E*, *G*). NF, neurofilament; MW, molecular weight.

F) and primary Schwann cells (Fig. 1*G*, *H*). Taken together, these data indicate that Schwann cells contribute to collagen VI deposition in the sciatic nerve.

Lack of collagen VI leads to axon hypermyelination in the sciatic nerve

To assess the function of collagen VI in peripheral nerves, we utilized *Col6a1*$^{-/-}$ mice. *Col6a1*$^{-/-}$ sciatic nerves did not show any labeling for collagen VI (Supplemental Fig. S2), indicating that ablation of the α1(VI) chain leads to loss of collagen VI in peripheral nerves.

Light microscopy of sciatic nerves of 6- to 7-mo-old mice after toluidine blue staining showed that the total number of fibers (**Fig. 2*A*, *B***) and the distribution of various fiber types (Fig. 2*A*, *C*) were not affected by lack of collagen VI. The myelin sheath of most collagen VI-null fibers appeared thicker than that of control fibers (Fig. 2*A*), and the mean g-ratio (*i.e.*, the ratio between axon diameter and the diameter of fiber including myelin) of collagen VI-null fibers was significantly decreased when compared with controls (Fig. *2D*). Interestingly, the g ratio of *Col6a1*$^{-/-}$ nerves was decreased in all classes of fibers as indicated by the analysis of g-ratio within classes of fibers binned for their axonal diameter (Fig. *2E*). The increased myelination of *Col6a1*$^{-/-}$ sciatic nerves was also supported by electron microscopy (Fig. 2*F*, *G*) and by analyzing myelin associated proteins. Indeed, immunofluorescence and Western blot showed that MBP levels were significantly increased in *Col6a1*$^{-/-}$ nerves compared with controls (Fig. 2*H*, *I*). Moreover, Western blot for MAG and CNPase and immunofluorescence for S100 showed that the levels of all these proteins were markedly increased in *Col6a1*$^{-/-}$ nerves compared with controls (Fig. 2*J* and Supplemental Fig. S3*A*, *B*). Electron microscopy analysis of longitudinal section of sciatic nerves showed that the myelin sheaths are uniformly hypermyelinated in *Col6a1*$^{-/-}$ mice (Fig. 2*G*). To further examine whether collagen VI regulates myelination *in vitro*, we cultured RT4-D6P2T Schwann cells in the absence or in the presence of purified native collagen VI, used as a coating substrate in the culture dishes. Western blot showed that addition of collagen VI reduced MAG expression in Schwann cells (Supplemental Fig. S3*C*). These data indicate that lack of collagen VI leads to increased myelination and provide the first evidence that collagen VI is a crucial factor in peripheral nerve myelination.

We then examined whether the increased myelination of *Col6a1*$^{-/-}$ nerves was present at earlier time points, during postnatal development. However, analysis of 2- to 3-mo-old mice did not reveal significant differences in myelin thickness between wild-type and *Col6a1*$^{-/-}$ sciatic nerves (Supplemental Fig. S4). Therefore, we chose 6- to 7-mo-old mice for following studies. We next evaluated whether lack of collagen VI induces any compensatory change in axons. Western blot showed comparable β-III tubulin, neurofilament, and phospho-neurofilament levels in wild-type and *Col6a1*$^{-/-}$ sciatic nerves (**Fig. 3*A*, *B***). β-III tubulin immunofluorescence did not show any noticeable difference between wild-type and *Col6a1*$^{-/-}$ sciatic nerve axons (Fig. 3*C*). When peripheral nerves are injured, inflammatory cells, such as macrophages, are rapidly recruited to the damage site (32). To further confirm that axons were not damaged in *Col6a1*$^{-/-}$ mice, we assessed the amount of inflammatory cells in sciatic nerves. Immunofluorescence for markers of inflamma-

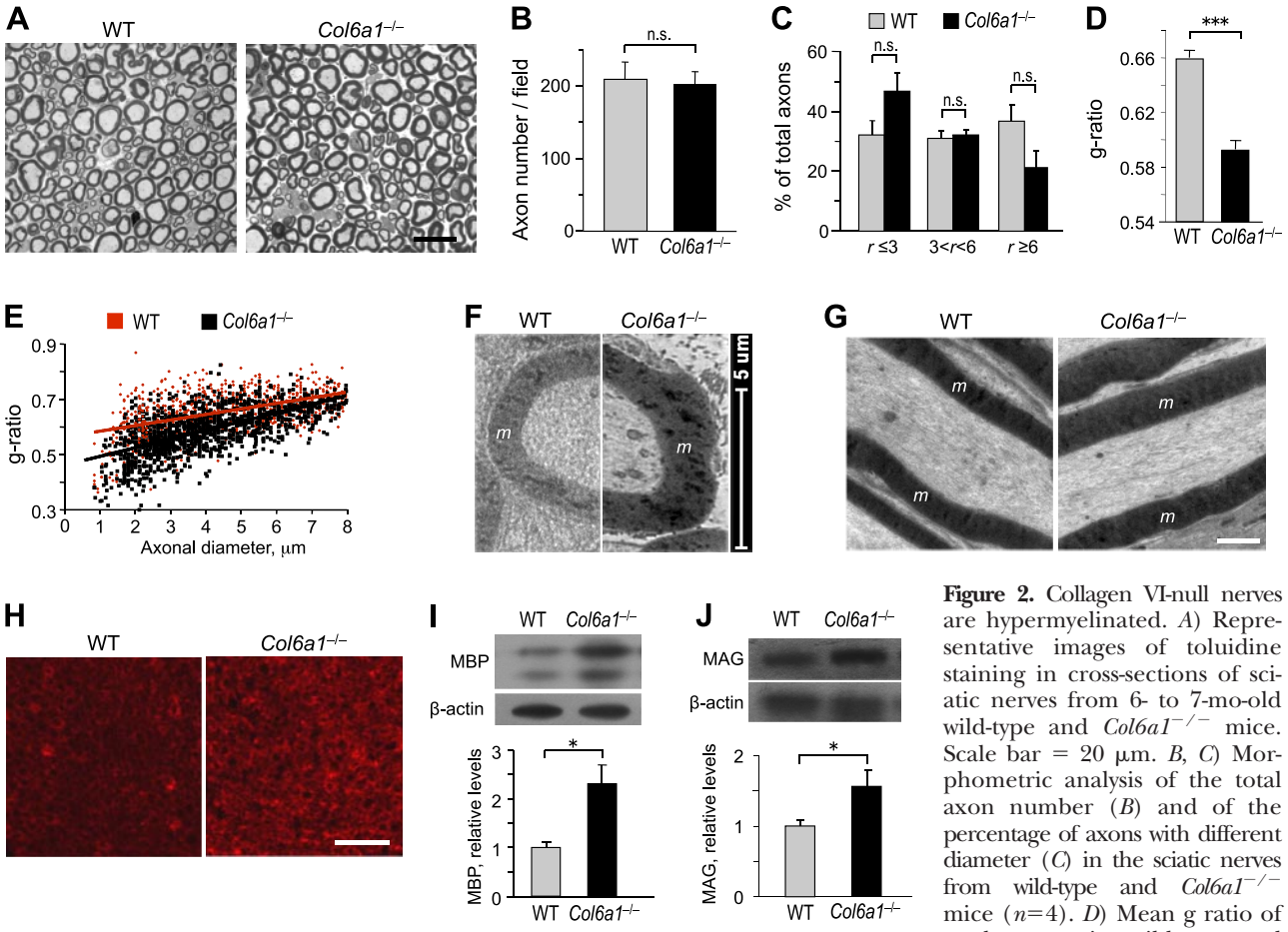

Figure 2. Collagen VI-null nerves are hypermyelinated. *A*) Representative images of toluidine staining in cross-sections of sciatic nerves from 6- to 7-mo-old wild-type and *Col6a1*$^{-/-}$ mice. Scale bar = 20 μm. *B*, *C*) Morphometric analysis of the total axon number (*B*) and of the percentage of axons with different diameter (*C*) in the sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice (n=4). *D*) Mean g ratio of total axons in wild-type and *Col6a1*$^{-/-}$ mice. ***$P < 0.001$; n = 3–4. *E*) Scatter plot indicating the g ratios of all fibers as a function of axon diameter (n=938 axons from 3 wild-type mice; n=1269 axons from 4 *Col6a1*$^{-/-}$ mice). *F*) Representative electron micrographs of cross sections of wild-type and *Col6a1*$^{-/-}$ sciatic nerves, showing a single fiber. *G*) Representative electron micrographs of longitudinal sections of wild-type and *Col6a1*$^{-/-}$ sciatic nerves. Scale bar = 2 μm. *H*) Immunofluorescence labeling for MBP in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice. Scale bar = 50 μm. *I*) Top panel: Western blot for MBP in wild-type and *Col6a1*$^{-/-}$ sciatic nerves. Bottom panel: densitometric quantification of MBP *vs.* actin, as determined by 3 independent Western blot experiments. Values for wild-type nerve were arbitrarily set to 1. *$P < 0.05$; n = 3. *J*) Top panel: Western blot for MAG in wild-type and *Col6a1*$^{-/-}$ sciatic nerves. Bottom panel: densitometric quantification of MAG *vs.* actin, as determined by 3 independent Western blot experiments. Values for wild-type nerve were arbitrarily set to 1. *$P < 0.05$; n = 5. WT, wild-type; m. myelin sheath; n.s., not significant.

tory cells showed that the number of CD68$^+$ macrophages and CD45$^+$ inflammatory cells was not significantly different between wild-type and *Col6a1*$^{-/-}$ sciatic nerves (Fig. 3*D*, *E*), thus confirming that axons remain intact in *Col6a1*$^{-/-}$ sciatic nerves.

Our previous studies indicated that lack of collagen VI causes spontaneous apoptosis in muscles (23). We therefore investigated apoptosis in the sciatic nerve of both genotypes. Although the amounts of procaspase-9 and cleaved caspase-9 were increased, the protein levels for Bcl-2, an antiapoptotic factor, were also increased in *Col6a1*$^{-/-}$ sciatic nerve when compared with controls (**Fig. 4*A*, *B***). Interestingly, Bcl-X_L and Bax protein levels were not significantly affected by collagen VI ablation (Fig. 4*C*, *D*). Moreover, immunofluorescence for cleaved caspase-3 and TUNEL assay did not show any significant difference between control and *Col6a1*$^{-/-}$ nerves (Fig. 4*E*, *F*). Altogether, these findings indicate that lack of collagen VI induces increased axon myelination but does not lead to spontaneous apoptosis in the sciatic nerve.

Molecular signals involved in PNS myelination are altered in *Col6a1*$^{-/-}$ nerves

To characterize the molecular basis of the abnormal myelination of peripheral nerves caused by collagen VI deficiency, we analyzed signaling pathways involved in the regulation of PNS myelination and function. Activation of FAK (33), AKT (34–36), ERK (37, 38), and p38 MAP kinase (39, 40) is required for axon myelination in the PNS. In agreement with the increased myelination of *Col6a1*$^{-/-}$ sciatic nerves, collagen VI deficiency was accompanied by higher phosphorylation levels of FAK, AKT, ERK, and p38 (**Fig. 5*A*–*D***). On the other hand, myelination is negatively regulated by vimentin (34), as well as by the JNK and c-Jun pathways (41). Western blot for vimentin, phospho-JNK, and

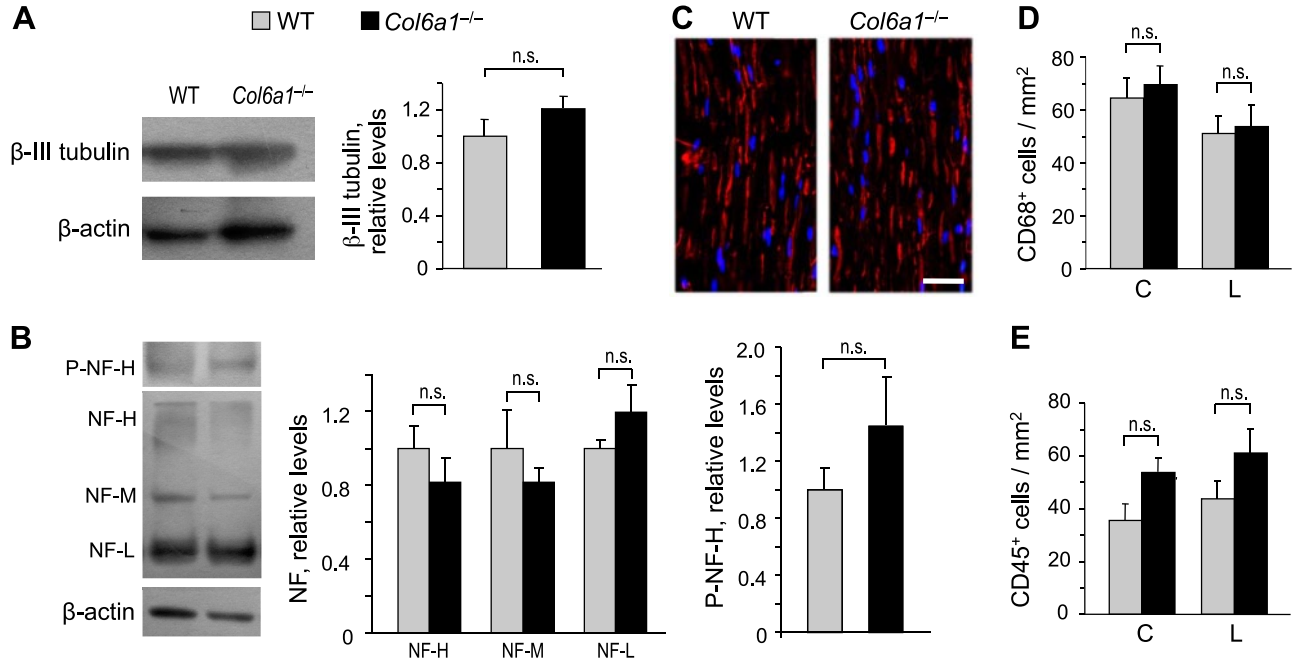

Figure 3. Loss of collagen VI does not cause axon damage or inflammation in sciatic nerve. *A*) Left panel: Western blot for β-III tubulin in wild-type and *Col6a1*$^{-/-}$ sciatic nerve. Actin was used as a loading control. Right panel: Densitometric quantification of β-III tubulin *vs.* actin, as determined by 3 independent Western blot experiments, showing that ablation of collagen VI does not affect β-III tubulin levels in sciatic nerve. Values for wild-type nerve were arbitrarily set to 1 (n=4). *B*) Left panel: Western blot for neurofilament and phospho-neurofilament in wild-type and *Col6a1*$^{-/-}$ sciatic nerve. Actin was used as a loading control. Middle and right panels: densitometric quantification of neurofilament *vs.* actin, phospho-neurofilament-H *vs.* neurofilament-H as determined by 3 independent Western blot experiments, showing that ablation of collagen VI does not affect neurofilament and phospho-neurofilament levels in sciatic nerve. Values for wild-type nerve were arbitrarily set to 1 (n=3–4). *C*) Immunofluorescence labeling for β-III tubulin in longitudinal sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice. Nuclei were stained with Hoechst (blue). Scale bar = 50 μm. *D, E*) Quantification of CD68^{+} macrophages (*D*) and CD45^{+} inflammatory cells (*E*) in cross- and longitudinal sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice (n=3–4). C, cross-section; L, longitudinal section; NF-H, neurofilament heavy chain; NF-L, neurofilament light chain; NF-M, neurofilament middle chain.

phospho-c-Jun showed that they were significantly decreased in *Col6a1*$^{-/-}$ nerves (Fig. 5*E–G*). To further examine whether collagen VI impinges on Schwann cell signals involved in the regulation of myelination, we treated RT4-D6P2T Schwann cells with exogenous collagen VI. Western blot showed that addition of collagen VI rapidly promotes c-Jun phosphorylation in Schwann cells (Fig. 5*H*), in agreement with the *in vivo* finding of decreased levels of phospho-c-Jun in *Col6a1*$^{-/-}$ nerves. These findings support the hypermyelinated phenotype of collagen VI-deficient nerves, indicating abnormal regulation of molecular pathways involved in the control of myelination.

Col6a1$^{-/-}$ mice display defective nerve conduction velocity and impaired motor coordination

To get insight into the functional significance of the structural PNS defects observed in *Col6a1*$^{-/-}$ mice, we performed electrophysiology experiments in sciatic nerves. Representative traces of wild-type and *Col6a1*$^{-/-}$ mice are shown in **Fig. 6*A***. CMAP amplitudes were slightly (albeit not significantly) reduced in *Col6a1*$^{-/-}$ mice with respect to controls, both after proximal and distal stimulation (Fig. 6*B*). Notably, *Col6a1*$^{-/-}$ mice displayed a significant decrease of NCVs when compared with wild-type mice (Fig. 6*C*). NCVs are influenced by Schwann cell internodal length and nodal integrity (42, 43). Thus, we examined whether internodal length and nodal integrity were changed in collagen VI-null nerves. In agreement with the decreased NCVs, we found that the internodal length was dramatically decreased in *Col6a1*$^{-/-}$ mice when compared with controls (Fig. 6*D*). However, the nodal integrity was not affected by the deficiency of collagen VI (Fig. 6*E*). These results indicate that the abnormal myelination of *Col6a1*$^{-/-}$ peripheral nerves is matched by reduced NCVs and decreased internodal length, pointing at functional deficits in collagen VI-deficient mice.

Next, we analyzed the motor function of collagen VI-deficient mice. Wild-type and *Col6a1*$^{-/-}$ mice showed similar performances in the rotarod test (**Fig. 7*A***), suggesting no major impairment of the general motor function in collagen VI-deficient mice. Conversely, when subjected to the ledged beam-walking test, the distance of first footslip was significantly decreased in *Col6a1*$^{-/-}$ mice when compared with wild-type mice (Fig. 7*B*). Moreover, the total number of hind footslips was dramatically increased in *Col6a1*$^{-/-}$ mice (Fig. 7*C*),

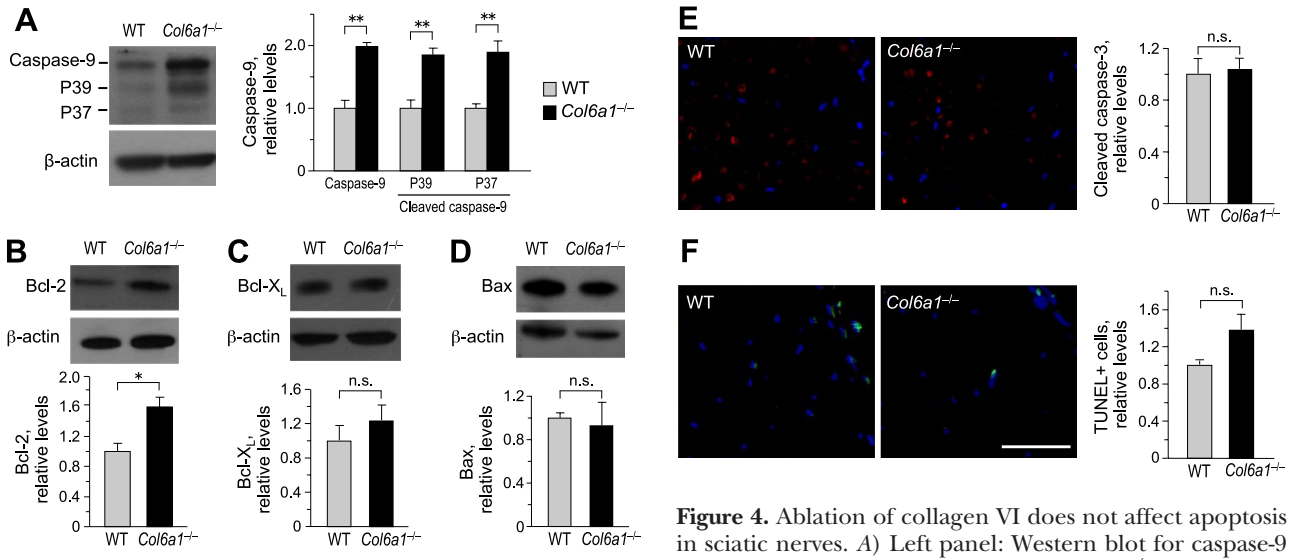

Figure 4. Ablation of collagen VI does not affect apoptosis in sciatic nerves. *A*) Left panel: Western blot for caspase-9 in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice. Right panel: relative protein levels, as determined by densitometric quantifications of 3 independent Western blot experiments. Values for wild-type nerve were arbitrarily set to 1. $^{**}P < 0.01$; $n = 3$. *B–D*) Top panels: Western blot for Bcl-2 (*B*), Bcl-X_L (*C*), and Bax (*D*) in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice. Bottom panels: relative protein levels, as determined by densitometric quantifications of 3 independent Western blot experiments. Values for wild-type nerve were arbitrarily set to 1. $^{*}P < 0.05$; $n = 3$. *E*) Left panels: immunofluorescence labeling for cleaved caspase-3 (red) in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice. Scale bar = 50 μm. Right panel: relative quantification of cells with cleaved caspase-3 (n=3). *F*) Left panels: TUNEL analysis in sciatic nerve sections from wild-type and *Col6a1*$^{-/-}$ mice. Scale bar = 50 μm. Right panel: relative quantification of TUNEL-positive cells (n=3).

suggesting that motor coordination and balance are impaired in collagen VI-deficient mice. Footprint analysis showed no significant difference in the stride width between wild-type and *Col6a1*$^{-/-}$ mice (Fig. 7*D, E*). Nevertheless, the stride length variability of hindlimbs was increased in *Col6a1*$^{-/-}$ mice (Fig. 7*D, F*), indicating increased gait instability in collagen VI-deficient mice. Furthermore, the angle between foot steps and walking direction of hindlimbs was wider in *Col6a1*$^{-/-}$ mice compared with wild-type mice (Fig. 7*D, G*), pointing at an impairment of motor coordination in *Col6a1*$^{-/-}$ mice. Taken together, these data demonstrate that collagen VI deficiency leads to the impairment of motor coordination and balance in mice.

Collagen VI-deficient mice display defective sensory function

As shown by the electrophysiological analysis, we found that a clear H reflex was elicited following both proxi-

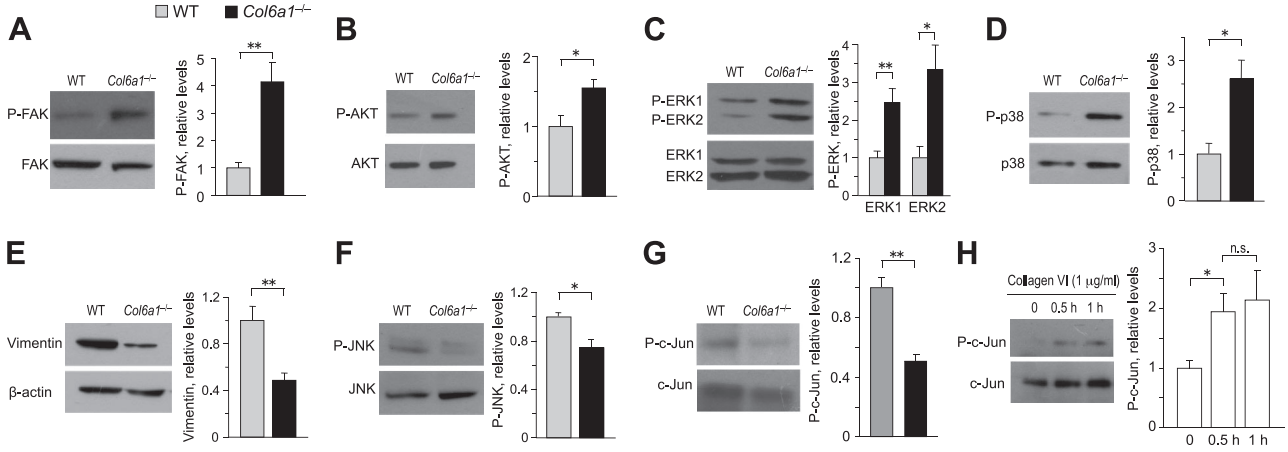

Figure 5. Ablation of collagen VI alters myelination-related signaling pathways. *A–G*) Left panel: Western blot for total and phosphorylated FAK (*A*), AKT (*B*), ERK1/ERK2 (*C*), p38 (*D*), JNK (*F*), and c-Jun (*G*), and for vimentin (*E*), in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice. *H*) Left panel: Western blot for total and phosphorylated c-Jun in RT4-D6P2T cells following treatment with collagen VI (1 μg/ml) for indicated times. Right panel: densitometric quantifications, as determined by 3 independent Western blot experiments, expressed as the ratio of phospho-FAK *vs.* total FAK, phospho-AKT *vs.* total AKT, phospho-ERK1/ERK2 *vs.* total ERK1/ERK2, phospho-p38 *vs.* total p38, phospho-JNK *vs.* total JNK, phospho-c-Jun *vs.* total c-Jun and vimentin *vs.* actin. Values for wild-type nerve were arbitrarily set to 1. $^{*}P < 0.05$; $^{**}P < 0.01$; $n = 3$–5.

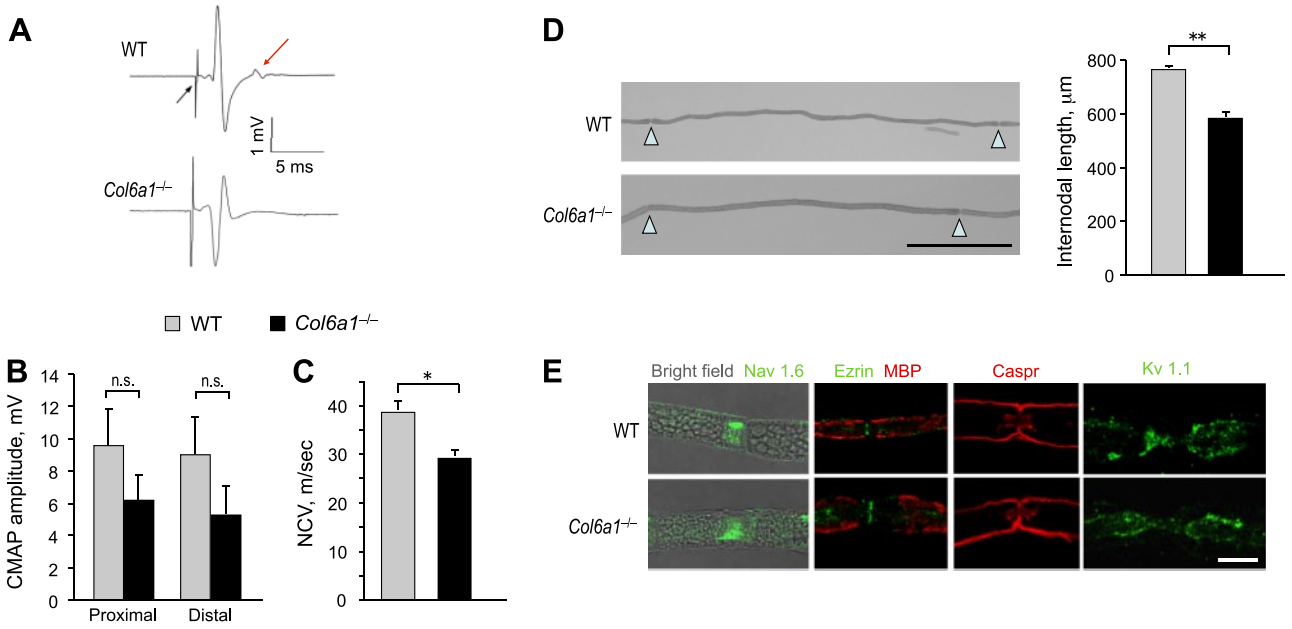

Figure 6. Ablation of collagen VI reduces nerve conduction velocity and Schwann cell internodal length. *A*) Representative traces of electrophysiological recordings of CMAP in paw muscles of wild-type and *Col6a1*$^{-/-}$ mice. Arrows indicate the artifact following nerve stimulation (black arrow) and the elicited H reflex (red arrow). Calibration bar is the same for both traces. *B*) Analysis of the CMAP amplitudes following proximal and distal stimulation (n=8). *C*) Analysis of NCV in wild-type and *Col6a1*$^{-/-}$ sciatic nerves. *$P < 0.01$; $n = 8$. *D*) Left panel: Representative images of teased nerve fiber of wild-type and *Col6a1*$^{-/-}$ sciatic nerves. Arrowheads point at nodes of Ranvier. Scale bar = 200 μm. Right panel: Quantification of intermodal length of wild-type and *Col6a1*$^{-/-}$ sciatic nerves. ***$P < 0.001$; n = 25–35 fibers/mouse, 4 mice/genotype. *E*) Immunofluorescence staining of the nodal (Nav1.6 and ezrin), paranodal (Caspr), and juxtaparanodal (Kv1.1) regions of teased fibers isolated from 6- to 7-mo-old mice. Scale bar = 10 μm.

mal and distal stimulation in all 8 wild-type mice but only in 1 out of 8 *Col6a1*$^{-/-}$ mice (Fig. 6*A* and **Table 1**), indicating a sensory deficit in *Col6a1*$^{-/-}$ mice. Moreover, the sole *Col6a1*$^{-/-}$ mouse with H reflex response showed enhanced latency when compared with controls (Table 1).

Nonmyelinating Schwann cells always engulf C fibers, which are high-threshold nociceptors. Thermal hot pain is predominantly transduced by C fibers (44), which transmit impulses from the periphery, through dorsal root ganglia (DRGs), the primary sensory neurons, into the dorsal horn of spinal cord (45). Therefore we investigated the role of collagen VI on C-fiber organization by transmission electron microscopy. C fibers in *Col6a1*$^{-/-}$ nerves were disorganized, displaying larger axons than in wild-type nerves (**Fig. 8*A***), thus suggesting altered sensory function in *Col6a1*$^{-/-}$ mice. Next, we evaluated the expression of peripherin, a nociceptive neuron marker, in *Col6a1*$^{-/-}$ mice. Western blot and immunofluorescence revealed that the levels of peripherin were markedly reduced in the sciatic nerves, in the DRGs, and in the superficial layers of spinal cord of *Col6a1*$^{-/-}$ mice when compared with the corresponding wild-type samples (Fig. 8*B–D*), indicating that nociceptive signals are decreased in *Col6a1*$^{-/-}$ mice. We then investigated the sensory function in collagen VI-null mice using thermal and mechanical stimuli as measured by hotplate and von Frey filament test, respectively. The sensitivity to both thermal and mechanical stimuli was significantly decreased in *Col6a1*$^{-/-}$ mice compared with wild-type mice (Fig. 8*E*, *F*), indicating a delay in nociception. These results suggest that collagen VI is also required for peripheral nerve function in stressful conditions.

DISCUSSION

In this study, we describe for the first time that collagen VI contributes to the structural integrity and physiological functions of peripheral nerve. We provide evidence for histopathological and cellular phenotypes with increased myelin thickness in the peripheral nerve of *Col6a1*$^{-/-}$ mice, leading to a functional phenotype with impairment of NCVs, motor coordination, and sensory transduction. Furthermore, we show that collagen VI regulates myelin thickness by modulating myelination-related signaling pathways.

A proper thickness of myelin is required for the correct transmission of electrical impulses along the axons and for preservation of axonal integrity in PNS. The increased myelination detected in *Col6a1*$^{-/-}$ mice provides the first evidence that collagen VI is a crucial factor in peripheral nerve myelination *in vivo*. Collagen VI is an abundant ECM protein of peripheral nerves (18, 46). Previous studies indicated that *Col6* genes are abundantly expressed by Schwann cells in peripheral nerves (21, 47). In the current study, we provided both *in vivo* and *in vitro* evidence that collagen VI is produced by Schwann cells and macrophages but not

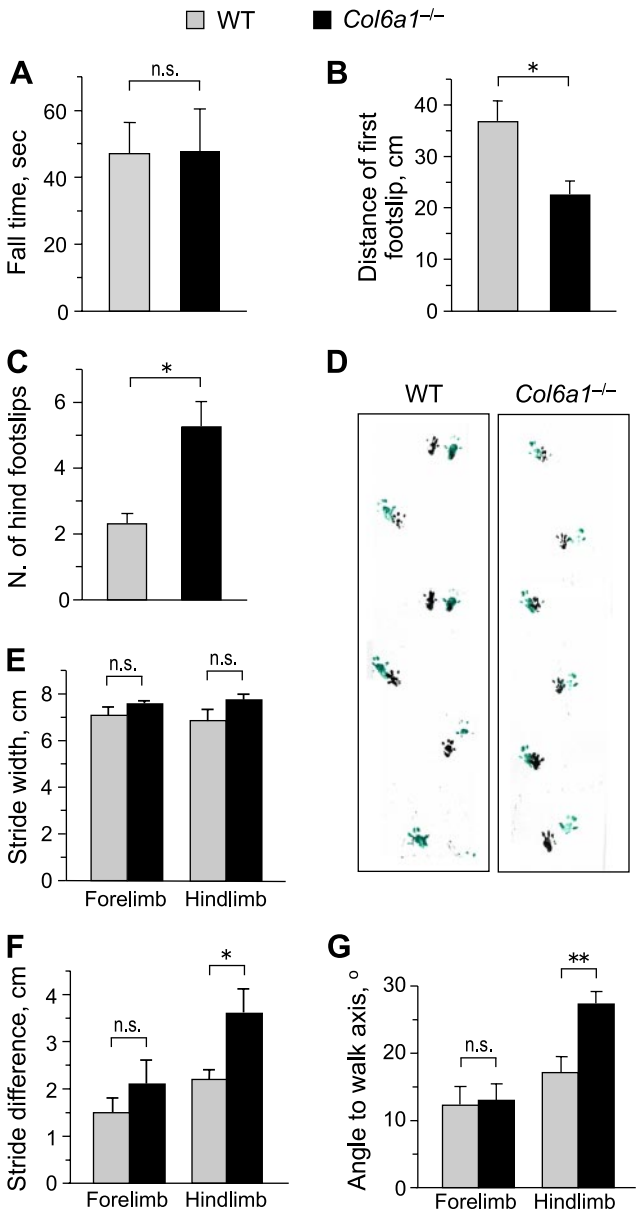

Figure 7. Collagen VI-null mice show deficits in motor coordination. *A*) Analysis of the average latency on the rotarod of wild-type and *Col6a1*$^{-/-}$ mice (n=6–9). *B, C*) Analysis of the average distance before the first footslip (*B*) and of the number of total hind footslips (*C*) in wild-type and *Col6a1*$^{-/-}$ mice. *$P < 0.05$; $n = 4$. *D–G*) Footprint test, showing representative walking tracks of wild-type and *Col6a1*$^{-/-}$ mice (*D*; black, forepaws; green, hindpaws), quantification of the stride width of forelimbs and hindlimbs (*E*), quantification of stride variability of forelimbs and hindlimbs (*F*), and measurement of foot-to-walk axis in wild-type and *Col6a1*$^{-/-}$ mice (*G*). **$P < 0.01$; *$P < 0.05$; $n = 4$.

deposited by axons of sciatic nerves. Furthermore, ablation of collagen VI does not induce any significant change in axon and macrophage density in sciatic nerves. These data suggest that the hypermyelination of *Col6a1*$^{-/-}$ nerves is a Schwann cell-related defect and point at collagen VI as an ECM molecule involved in the regulation of Schwann cells myelination *in vivo*. On the other hand, collagen VI is considered as a cell prosurvival factor, since collagen VI deficiency induces apoptosis in muscle (23) and enhances neuron death on toxic treatments in the central nervous system (19, 20). Nevertheless, we did not find any difference between wild-type and *Col6a1*$^{-/-}$ mice in the incidence of spontaneous apoptosis of peripheral nerves. This may be explained considering that collagen VI forms different structures in different tissues (17), thus exerting potentially distinct functions. The underlying mechanisms for the different prosurvival effects exerted by collagen VI in different tissues will require further investigations in the future.

Understanding the molecular machinery that regulates myelination is crucial for developing new potential strategies to control the proper myelination. Vimentin is an intermediate filament protein that is expressed in both Schwann cells and neurons and functions as a negative regulator of peripheral nerve myelination through the AKT pathway (34). In our study, we observed increased AKT phosphorylation and decreased vimentin expression in *Col6a1*$^{-/-}$ nerves. Hence, the increased myelination induced by collagen VI deficiency is partly modulated by activation of AKT signal pathway. C-jun and FAK are 2 important signals in Schwann cells, which exert distinct roles in the inhibition (41) or promotion (33) of myelination in peripheral nerves, respectively. Interestingly, we found that in *Col6a1*$^{-/-}$ nerves phospho-JNK is reduced, whereas phospho-FAK is increased. These results highlight the involvement of both positive and negative myelination regulatory pathways in mediating collagen VI deficiency-induced hypermyelination in peripheral nerves. The function of ERK (32, 37, 38, 48) and p38 (39, 40, 49) signals in peripheral myelination is still controversial. Our results show that ERK and p38 signals are activated in *Col6a1*$^{-/-}$ nerves, which support the concept that both signals positively regulate myelination. The observed altered signaling pathways in sciatic nerves of *Col6a1*$^{-/-}$ mice provide mechanistic insight into the role of collagen VI in axonal myelination. Our findings in collagen VI-deficient mice, together with the fact that collagen VI is produced by Schwann cells, suggest that the hypermyelination of *Col6a1*$^{-/-}$ nerves is Schwann cell autonomous. Further investigations using *in vivo* models with Schwann cell-specific conditional deficiency of collagen VI will allow addressing such mechanistic aspects in the future.

Abnormal myelin thickness is closely related with motor dysfunction in animals and peripheral neuropa-

TABLE 1. *Summary of H reflex presence and its stimulation latency in wild-type and* Col6a1$^{-/-}$ *mice*

Parameter	Wild-type	*Col6a1*$^{-/-}$
H reflex	8/8	1/8**
Latency		
Proximal	5.1 ± 0.3	6.9
Distal	5.2 ± 0.2	9.5

See Fig. 8. **P < 0.01; n=8.

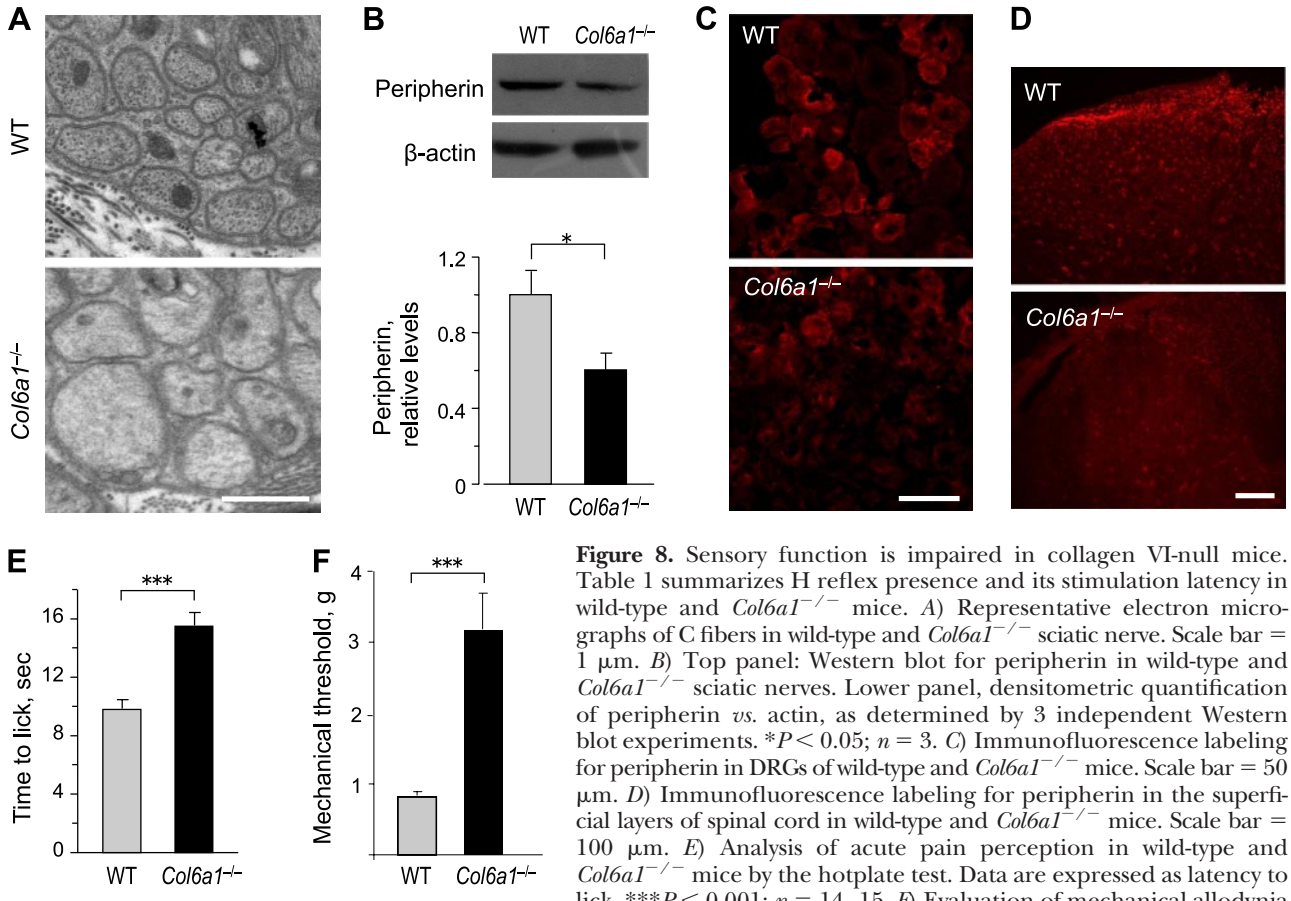

Figure 8. Sensory function is impaired in collagen VI-null mice. Table 1 summarizes H reflex presence and its stimulation latency in wild-type and *Col6a1*$^{-/-}$ mice. *A*) Representative electron micrographs of C fibers in wild-type and *Col6a1*$^{-/-}$ sciatic nerve. Scale bar = 1 μm. *B*) Top panel: Western blot for peripherin in wild-type and *Col6a1*$^{-/-}$ sciatic nerves. Lower panel, densitometric quantification of peripherin *vs.* actin, as determined by 3 independent Western blot experiments. *$P < 0.05$; $n = 3$. *C*) Immunofluorescence labeling for peripherin in DRGs of wild-type and *Col6a1*$^{-/-}$ mice. Scale bar = 50 μm. *D*) Immunofluorescence labeling for peripherin in the superficial layers of spinal cord in wild-type and *Col6a1*$^{-/-}$ mice. Scale bar = 100 μm. *E*) Analysis of acute pain perception in wild-type and *Col6a1*$^{-/-}$ mice by the hotplate test. Data are expressed as latency to lick. ***$P < 0.001$; $n = 14–15$. *F*) Evaluation of mechanical allodynia in the hind limb of wild-type and *Col6a1*$^{-/-}$ mice by the von Frey filament test. Data are expressed as withdrawal threshold to mechanical stimuli. ***$P < 0.001$; n=12–14.

thies in human (4–7). Our results show that ablation of collagen VI induces increased PNS myelination *in vivo* with an impairment of motor coordination. This may seem to be in contradiction with the general opinion that myelin is required for the transmission of electric impulse and that PNS demyelination is related to the impairment of motor functions in animals (50) and humans (4–6). However, myelin thickness needs to be properly maintained and axonal hypermyelination can increase the proportion of the surrounding wire volume, thus altering biophysical properties and axonal functions (51). Indeed, hypermyelination was found to be associated with defective axonal function in several murine models. In a mouse model of adrenomyeloneuropathy with inactivation of the *X-ALD* gene, focal hypermyelination of sciatic nerve is accompanied by impaired motor coordination activity and electrophysiological alterations in nerve conduction (7). Similarly, ablation of the ZPR1 zinc finger protein induces focal hypermyelination in peripheral nerves and motor function defects in mice (52). Furthermore, clinical evidence showed that laminin α2 chain deficiency-induced neuropathy is also related to nerve hypermyelination (53). These findings strongly support our results that the increased myelination of collagen VI-deficient sciatic nerves is accompanied by motor function alterations. Moreover, nerve electrophysiology studies showed that the NCVs are significantly reduced in *Col6a1*$^{-/-}$ mice, which on the one hand supports the impairment of motor function, whereas on the other hand may suggest a possible axon damage induced by the increased myelination. However, our data indicate that there is no evidence for axon damage or inflammation in collagen VI-null nerves, thus excluding their contribution in the decreased NCVs of *Col6a1*$^{-/-}$ mice. NCVs are also influenced by Schwann cell internodal length (42, 43). In agreement with the decreased NVC, we found that the internodal length is dramatically decreased in *Col6a1*$^{-/-}$ sciatic nerves. Together, these results not only point at collagen VI as an important factor for axonal myelination and motor function but also support the concept that proper myelination is required for the function of peripheral nerves.

The defective PNS function in *Col6a1*$^{-/-}$ mice is also apparent in stressful conditions, such as in the hotplate and von Frey filament tests, indicating that the acute nociceptive response is delayed in collagen VI-deficient mice. Our ultrastructural analysis shows that C fibers are disorganized in *Col6a1*$^{-/-}$ sciatic nerve, a defect paralleled by decreased expression of the C-fiber marker peripherin in the sciatic nerves, DRGs and superficial layers of spinal cord, as well as delayed pain response in *Col6a1*$^{-/-}$ mice. These data suggest that

collagen VI is also required for the peripheral nerve function in stressful conditions. Our findings of decreased motor and sensory functions in collagen VI null mice indicate that collagen VI plays a critical role in the PNS. Similar motor and sensory deficits were described in mice lacking von Willebrand A domain-related protein (18), as well as in *Col15a1*$^{-/-}$;*Lama4*$^{-/-}$ double-knockout mice (8). Altogether, our findings support collagen VI as a key regulator in peripheral nerve function.

In summary, we found that lack of collagen VI causes structural abnormalities in peripheral nerves, leading to functional defects in NCVs, motor coordination, and nociception. This is the first evidence demonstrating that collagen VI plays a pivotal role in axonal myelination, C-fiber organization, and peripheral nerve function. Until now, collagen VI was linked to different forms of genetic muscle diseases, such as Ullrich congenital muscular dystrophy, Bethlem myopathy, and congenital myosclerosis (15). Interestingly, a possible clinical implication for collagen VI in the nervous compartment was indicated by the evidence that collagen VI is significantly increased in the endoneurium and perineurium of peripheral nerves in diabetic neuropathy patients (54, 55) and by a very recent study where a mutation of the *COL6A2* gene was found in a consanguineous family affected by progressive myoclonus epilepsy (56). Our results in the collagen VI-deficient mouse model open the possibility that collagen VI may be involved in the onset and/or progression of human peripheral neuropathies and that it may represent a novel target for the development of therapeutic approaches for peripheral neuropathies. FJ

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Chapter 2

Collagen VI regulates peripheral nerve myelination and function

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Supplemental Figures S1 to S4

Supplemental Figure S1

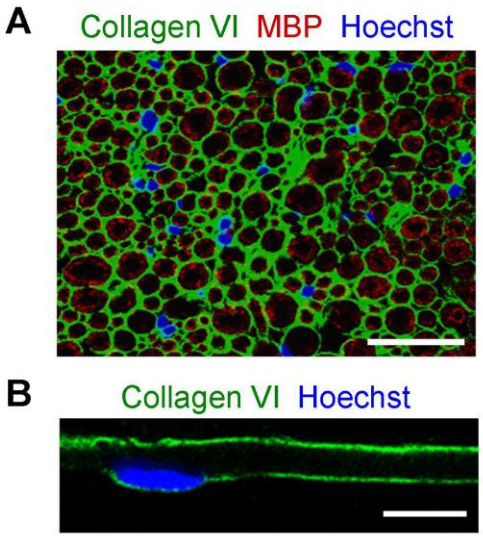

Figure S1. Collagen VI labeling in sciatic nerve and teased fiber. Immunofluorescence for collagen VI (green) and MBP (red) in wild-type mouse sciatic nerve. (B) Immunofluorescence for collagen VI (green) in teased fiber. Nuclei were stained with Hoechst (blue). Scale bar, 30 μm (A) or 10 μm (B).

Supplemental Figure S2

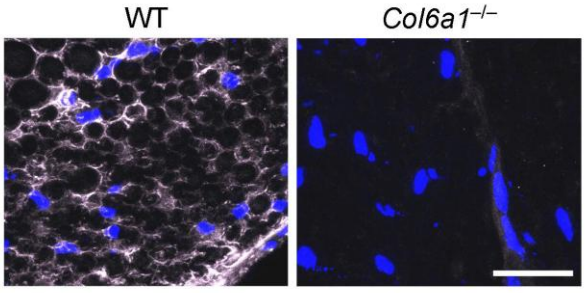

Figure S2. Collagen VI is expressed in wild-type nerves, but not in *Col6a1*$^{-/-}$ nerves. Immunofluorescence for collagen VI in wild-type and *Col6a1*$^{-/-}$ sciatic nerves. Nuclei were stained with Hoechst (blue). Scale bar, 20 μm. WT, wild-type.

Supplemental Figure S3

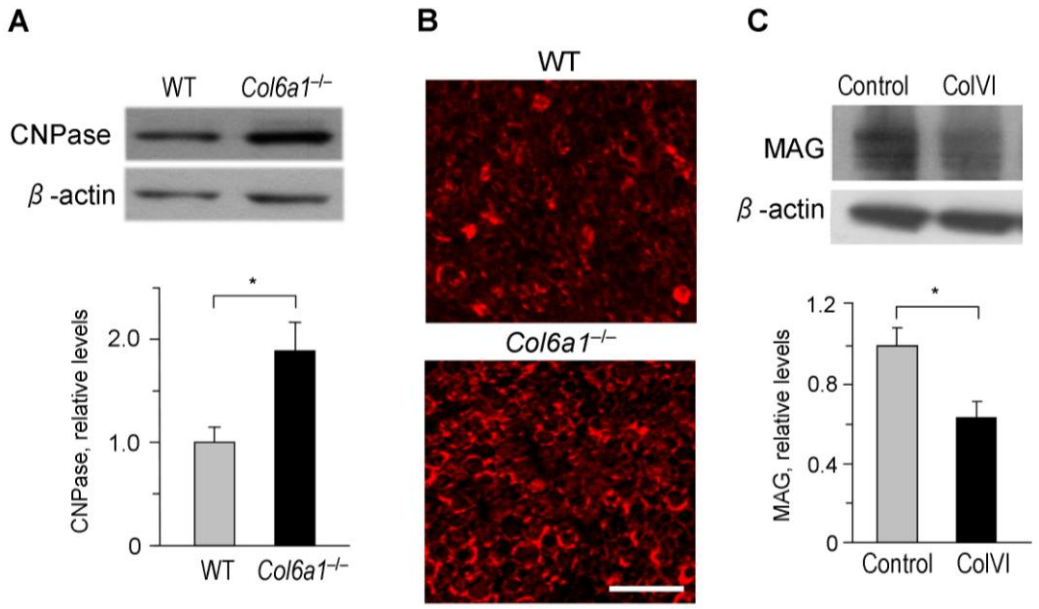

Figure S3. Collagen VI regulates CNPase, S100 and MAG expression in sciatic nerve and Schwann cells. (**A**) Upper panel, western blot for CNPase in wild-type and *Col6a1*$^{-/-}$ sciatic nerves. Actin was used as a loading control. Lower panel, densitometric quantification of CNPase vs actin as determined by three independent western blot experiments. Values for wild-type nerve were arbitrarily set to 1 (*, $P < 0.05$; $n = 3$). (**B**) Immunofluorescence labeling for S100 in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice. (**C**) Upper panel, western blot for MAG in RT4-D6P2T Schwann cells following 24 h culture in the absence (Control) or in the presence (ColVI) of purified native collagen VI (5 μg/cm^2) used as a coating substrate in the culture dishes. Actin was used as a loading control. Lower panel, densitometric quantification of MAG vs actin as determined by three independent western blot experiments. Values for wild-type nerve were arbitrarily set to 1 (*, $P < 0.05$; $n = 3$). Scale bar, 50 μm. WT, wild-type.

Supplemental Figure S4

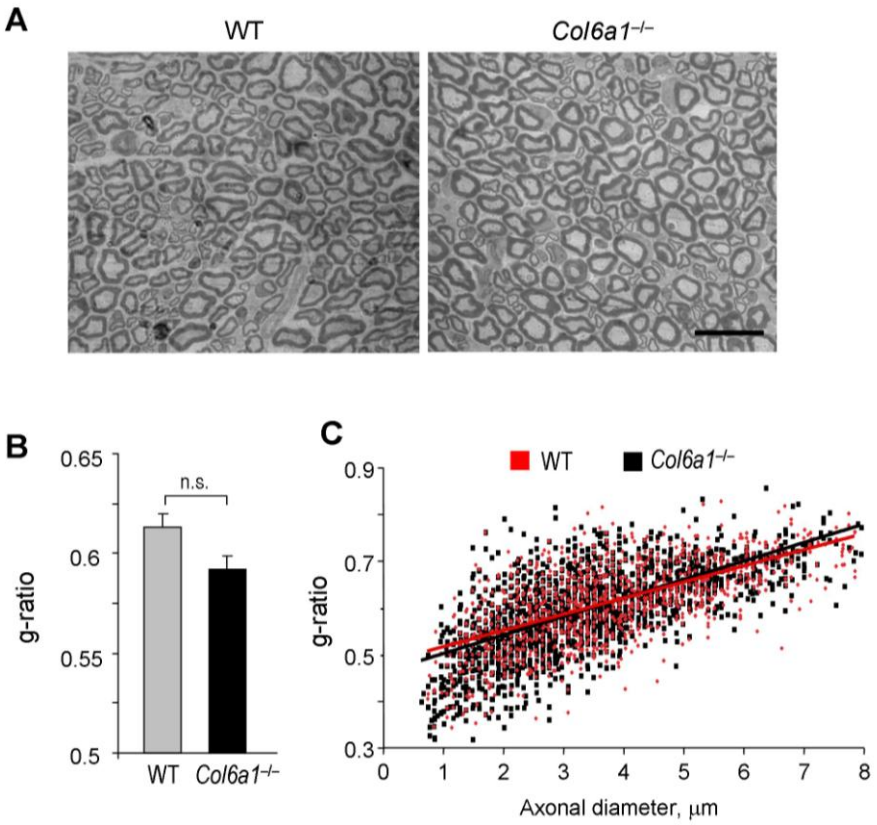

Figure S4. Ablation of collagen VI does not affect myelin thickness in young mice. (**A**) Representative images of toluidine staining in cross-sections of sciatic nerves from 2-3 month-old wild-type and *Col6a1*$^{-/-}$ mice. Scale bar, 20 μm. (**B**) Mean g-ratio of total axons in sciatic nerves of 2-3 month-old wild-type and *Col6a1*$^{-/-}$ mice (n.s., not significant; *n* =3-4). (**C**) Scatter plot indicating the g-ratios of all fibers as a function of axon diameter in sciatic nerves from 2-3 month-old mice (*n* = 1161 axons from 3 wild-type mice; *n* = 1844 axons from 4 *Col6a1*$^{-/-}$ mice). WT, wild-type.

Chapter 3

Collagen VI regulates peripheral nerve regeneration by modulating macrophage recruitment and polarization

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ORIGINAL PAPER

Collagen VI regulates peripheral nerve regeneration by modulating macrophage recruitment and polarization

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Abstract Macrophages contribute to peripheral nerve regeneration and produce collagen VI, an extracellular matrix protein involved in nerve function. Here, we show that collagen VI is critical for macrophage migration and polarization during peripheral nerve regeneration. Nerve injury induces a robust upregulation of collagen VI, whereas lack of collagen VI in *Col6a1*$^{-/-}$ mice delays peripheral nerve regeneration. In vitro studies demonstrated that collagen VI promotes macrophage migration and polarization via AKT and PKA pathways. *Col6a1*$^{-/-}$ macrophages exhibit impaired migration abilities and reduced antiinflammatory (M2) phenotype polarization, but are prone to skewing toward the proinflammatory (M1) phenotype. In vivo, macrophage recruitment and M2 polarization are impaired in *Col6a1*$^{-/-}$ mice after nerve injury. The delayed nerve regeneration of *Col6a1*$^{-/-}$ mice is induced by macrophage deficits and rejuvenated by transplantation of wild-type bone marrow cells. These results identify collagen VI as a novel regulator for peripheral nerve regeneration by modulating macrophage function.

Keywords Collagen VI · Nerve regeneration · Macrophage · Migration · Polarization · Peripheral nerve

Introduction

Unlike the central nervous system, axons in the peripheral nervous system (PNS) have the ability to regenerate even after severe injury. Successful peripheral nerve regeneration is a process that requires the concerted interplay of glial cells, growth factors, cell adhesion molecules and extracellular matrix (ECM) proteins, as well as the recruitment of macrophages [21]. Macrophages are critical for the inflammatory response, a process that needs to be tightly controlled to avoid

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excessive tissue damage after injury [31]. Following PNS injury, macrophages not only contribute to debris clearance, growth factor production and ECM remodeling in the distal nerve, but also stimulate regeneration near the axotomized neuronal cell bodies [20, 32, 34]. Leukemia inhibitory factor (LIF), interleukin (IL)-1α, IL-1β and monocyte chemoattractant protein-1 (MCP-1) have been identified as the major regulators for macrophage recruitment after peripheral nerve injury [36, 41, 45]. However, how these factors are modulated during macrophage recruitment remains elusive. Furthermore, additional factors for regulating macrophage migration after peripheral nerve injury need to be identified.

Macrophages exhibit remarkable plasticity and adopt pro- and antiinflammatory phenotypes (M1 and M2, respectively) in response to the stimulation of environmental signals [4, 5, 19]. Indeed, M1 and M2 macrophages exhibit distinct functions, where M1 macrophages stimulate an immune response, and M2 macrophages are immunosuppressive cells promoting tissue repair and remodeling [4, 6, 18, 29]. Interestingly, macrophages can undergo dynamic changes between M1 and M2 phenotypes, a process known as polarization skewing [32]. For example, when macrophages are stimulated with lipopolysaccharides (LPS) or interferon (IFN)-γ, they skew to an M1 phenotype characterized by high expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2. Conversely, macrophages are polarized to an M2 phenotype upon stimulation with IL-4, IL-10 or IL-13, a condition characterized by high expression of mannose receptor C type 1 (MRC1/CD206), arginase I (Arg-1) and peroxisomal proliferator activated receptor gamma (PPARγ) [4]. Polarization of macrophages toward the M2 phenotype in injury sites by local delivery of IL-4 promotes peripheral nerve regeneration [30]. However, the precise mechanisms governing macrophage polarization, especially in the peripheral nerve injury model, are still incompletely understood.

Collagen VI is a large ECM molecule made of three major genetically distinct chains, α1(VI), α2(VI) and α3(VI), which are encoded by *Col6a1*, *Col6a2* and *Col6a3* genes, respectively [5]. Although our previous studies demonstrated that collagen VI is an essential component of peripheral nerves required for proper nerve myelination and function [7], the role of collagen VI in peripheral nerve regeneration is completely unknown. M2 macrophages produce higher amounts of collagen VI than M1 macrophages [40]. Moreover, collagen VI enhances the adhesion of monocytes [40]. These findings raise the question whether collagen VI is required for macrophage activities, such as migration and polarization. Here, we show that collagen VI is critical for macrophage migration and M2 polarization via AKT and PKA pathways. As a result, peripheral nerve regeneration is strikingly impaired in collagen VI null ($Col6a1^{-/-}$) mice, where a targeted inactivation of the *Col6a1* gene blocks the assembly and secretion of collagen VI [2, 23]. These findings provide novel mechanistic data for macrophage activity and plasticity and demonstrate that collagen VI is a key regulator of PNS regeneration through modulation of macrophage function.

Materials and methods

Animals

$Col6a1^{+/+}$ (wild-type) and $Col6a1^{-/-}$ mice in the C57BL/6 background were used in this study [2, 23]. All in vivo experiments were performed in 6–7-month-old mice. Native collagen VI protein was purified from newborn mice as previously described [23]. Animal procedures were approved by the Ethics Committee of the University of Padua and authorized by the Italian Ministry of Health.

Surgical procedures

Mice were anesthetized with ketamine (100 mg/kg body weight) and xylazine (8 mg/kg body weight), and the right sciatic nerve was exposed and crushed with a liquid nitrogen-cooled Dumont forceps for 20 s, stopped for a 10 s interval and then subjected to a second crush at the same site. The crush site was about 45 mm from the tip of the third digit, which was labeled with India ink.

Macrophage depletion

Macrophages were depleted by intraperitoneal injection of clodronate liposome (ClodronateLiposomes.com) in mice as reported previously [16]. Briefly, clodronate liposome (200 μl/mouse) was injected at 1, 3, 5 and 8 days post-crush to obtain macrophage-depleted mice. Control mice received an equal volume of PBS liposome at the same time points.

Bone marrow transplantation

The bone marrow transplantation was performed as described previously [12]. Briefly, bone marrow was harvested from 6- to 8-week-old wild-type mice by flushing the femurs and tibias with 2 % fetal bovine serum in phosphate-buffered saline. Cells (2×10^6) were intravenously injected through the tail vein into lethally irradiated (10 Gy) 4-month-old wild-type and $Col6a1^{-/-}$ mice. Sciatic nerve injury was performed 6 weeks after the transplantation.

Functional tests

Prior to and after crush, nontoxic paint was applied to the hindpaws, and the mice were allowed to walk on a white

paper. From the footprint gait, the parameters of print length (the distance between the heel and the third toe, abbreviated as PL) and toe spread (the distance from the first toe to the fifth toe, abbreviated as TS) from both the normal side (N) and experimental side (E) were recorded. The sciatic functional index (SFI) was calculated with the following formula: SFI = 118.9 [(ETS−NTS)/NTS] −51.2 [(EPL−NPL)/NPL] −7.5 as previously reported [22, 52]. Analysis of the toe spread reflex and toe pinch was carried out as previously described [43].

Histology

Prior to injury and at 7, 21 and 29 days following crush injury, 3 mice of each group were perfused with 4 % paraformaldehyde, the 3-mm distal portion of sciatic nerves was dissected into 1-mm segments and postfixed in 2 % glutaraldehyde for 24 h at 4 °C. Samples (3 mm distal to the site of injury) were osmicated in 2 % osmium tetroxide for 2 h at room temperature, dehydrated in ascending acetone and embedded in Epon E812 resin (Sigma). Semithin sections (0.5 μm) were cut using an Ultracut 200 microtome (Leica) and stained with alkaline toluidine blue. Myelinated axon numbers were analyzed on eight sections per sciatic nerve. For electron microscopy, ultrathin sections (80 nm) were cut, mounted on copper grids, and stained with uranyl citrate and lead citrate. Grids were observed and photographed on an FEI Tecnai 12 transmission electron microscope. Phagocytic macrophages were identified by the "foamy" morphology, which is induced by the presence of end products of myelin/lipid degradation, as described in previous studies [26].

Matrigel plug assay

A total of 500 μl growth factor-reduced Matrigel (Gibco) supplemented with PBS, 2 % FBS, purified collagen VI (500 ng/ml), purified collagen I (Sigma, 500 ng/ml) or MCP-1 (ImmunoTools, 10 ng/ml) was injected subcutaneously into wild-type and *Col6a1*$^{-/-}$ mice. After 7 days, the Matrigel plug was harvested and processed for immunofluorescence.

Cell cultures

The J774 macrophage cell line was purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10 % fetal bovine serum (FBS, Gibco), 0.2 M L-glutamine (Invitrogen) and 1:100 penicillin-streptomycin (Invitrogen). J774 cells were treated with purified native collagen VI for further studies. Primary bone marrow-derived macrophages (BMDMs) and peritoneal macrophage (PMs) were isolated and cultured as previously described with minor modifications [8, 25, 52]. Briefly, for BMDM isolation and culture, 2–3-month-old mice were killed, and bone marrow cells collected by flushing both femurs and tibias with culture medium. Red blood cells were removed using a lysis buffer (150 mM NH_4Cl, 0.1 mM Na_2EDTA and 1 mM $KHCO_3$, pH 7.2) for 10 min at room temperature. The remaining cells were then differentiated with 30 ng/ml M-CSF (ImmunoTools) in DMEM containing 20 % FBS, 0.2 M L-glutamine and 1:100 penicillin-streptomycin for 1 week until the cells reached confluence. For PM isolation and culture, 3 % thioglycolate broth (Sigma) was injected intraperitoneally to induce peritonitis in 2–3-month-old mice. Three days later, peritoneal cells were collected and cultured in DMEM containing 10 % FBS, 0.2 M L-glutamine and 1:100 penicillin-streptomycin. Two hours later, nonadherent cells were removed by washing with PBS, and adherent macrophages were used for further studies. BMDMs and PMs were differentiated into the M2 or M1 phenotype with 20 ng/ml IL-4 (ImmunoTools) or 5 ng/ml LPS (Sigma), respectively, for 24 h.

Migration assay

Macrophage migration was assessed using transwell inserts with 5-μm pores (Millipore). Briefly, J774 macrophages (2×10^4 cells per well) were seeded into the upper chamber of a transwell filter with DMEM. The same culture medium and purified collagen VI (0.5 or 1 μg/ml), purified collagen I (1 μg/ml) or MCP-1 (10 ng/ml) were added to the lower chamber. When indicated, cells were treated with AKTi (Sigma, 10 μM) or H89 (Sigma, 30 μM). Cells were allowed to migrate for 8 h at 37 °C and 5 % CO_2. After being fixed and stained with ethanol and 0.05 % crystal violet (Sigma), the migrated cells were counted in eight different areas under a light microscope.

Scratch assay

A wound was made in confluent monolayers of J774 cells grown on six-well cell culture plates by scraping with a sterile 200-μl pipette tip. The cells were gently rinsed with PBS and further cultured in the presence or absence of purified collagen VI (1 μg/ml), purified collagen I (1 μg/ml), MCP-1 (10 ng/ml), AKTi (10 μM) or H89 (30 μM). Images of the cultures were taken immediately after scratching and after 8 h. The migration distances of the macrophages were measured and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics).

RNA isolation and real-time RT-PCR

Total RNA from mouse sciatic nerves (3 mm distal to injury site) was isolated using Trizol reagent (Life Technologies)

following the manufacturer's instructions; 200 ng of total RNA was used to make cDNA using the Superscript II kit (Invitrogen). Quantitative PCR was carried out using the LightCycler 480 system (Roche). The expression level of each gene was calculated by comparing with the *Gapdh* housekeeping gene. Primers used in this study are shown in Supplementary Table 1.

Immunofluorescence

After mice had been perfused with 4 % paraformaldehyde, a 3-mm length of sciatic nerve distal to the crush site was removed and postfixed for 4 h at 4 °C. Tissues or Matrigel plugs were then transferred into 30 % sucrose overnight for cryoprotection. Samples of 10 μm were cut in a cryostat (Leica). After blocking with 10 % goat serum for 1 h, sections were incubated with primary antibodies (1:200 dilution) for 2 h at room temperature or overnight at 4 °C. Primary antibodies against the following proteins were used: α3(VI) collagen (guinea pig polyclonal, a gift of Raimund Wagener, Cologne, Germany) [15]; β-III tubulin (mouse monoclonal, Sigma); CD68, F4/80 (rat monoclonal, AbD Serotec); CD206 (rabbit polyclonal, Abcam); MAG (rabbit monoclonal, Cell Signaling). The samples were then transferred to secondary antibodies (1:200 dilution) and Hoechst 33258 (Sigma) for 1 h at room temperature. The following secondary antibodies were used: anti-rat CY3, anti-rabbit CY3 and anti-guinea-pig CY2 (Jackson Immunoresearch). After washing three times in PBS, slides were mounted using 80 % glycerol.

Western blotting

Mice were killed by cervical dislocation, and sciatic nerves (3 mm distal to the crush site) were removed and frozen in nitrogen immediately. The tissues or cells were homogenized in lysis buffer (Millipore) with phosphatase inhibitors (Sigma) and protease inhibitors (Roche, Basel, Switzerland). The protein concentration was determined by BCA assay (Thermo Sciemtific). Samples of 20 μg protein were applied to SDS-PAGE gels (Invitrogen) and blotted onto a PDVF membrane (Millipore). Membranes were incubated with primary antibodies (1:1,000 dilution) overnight at 4 °C. Primary antibodies against the following proteins were used for Western blot analysis: α1(VI) collagen, Arg-1, CD206, iNOS (rabbit polyclonal, Santa Cruz Biotechnology); β-actin (mouse monoclonal, Sigma); AKT, phospho-PKA, PKA (rabbit polyclonal, Cell Signaling); phospho-AKT, COX-2, MAG, PPARγ (rabbit monoclonal, Cell Signaling); CD16 (rabbit monoclonal, Abcam); CD206 (rabbit polyclonal, Abcam); CD68 (rat monoclonal, AbD Serotec). After washing three times with TBST, membranes were incubated with HRP-conjugated secondary antibodies (1:1,000 dilution; Amersham Bioscience) for 1 h at room temperature. Detection was by chemiluminescence (Pierce). The panels show representative images of two separate protein extracts derived from two different mice. Densitometric quantification was performed by Image-Pro Plus 6.0 software (Media Cybernetics).

Statistical analysis

Data are represented as mean ± SEM. Statistical analysis of data was carried out using Student's *t* test, except for the analysis of the toe spread reflex in PBS- and clodronate-liposome-treated mice, where the chi-square test was used, and the analysis of collagen VI mRNA expression after injury, where one-way ANOVA followed by post hoc tests was used. $P < 0.05$ was considered as a significant difference.

Results

Expression of collagen VI is increased after sciatic nerve crush injury

To explore the role of collagen VI in PNS regeneration, we first examined whether collagen VI expression is upregulated upon sciatic nerve crush injury in adult mice. Real-time RT-PCR showed that the levels of *Col6a1* and *Col6a3* transcripts were increased at 7 and 14 days post-injury, whereas the levels of *Col6a2* transcripts started to increase within 3 days after sciatic nerve crush, and the expression of all three mRNAs reached a peak between 3 and 7 days post-injury (Fig. 1a–c). Western blot analysis for α1(VI) and α2(VI) chains (Fig. 1d) and immunofluorescence for α3(VI) chain (Fig. 1e, f) showed that the protein levels for collagen VI were also increased between 3 and 7 days post-injury. Taken together, these data indicate that crush injury of the sciatic nerve induces a robust upregulation of both mRNA and protein levels of collagen VI, pointing to a potential role for this molecule during PNS regeneration.

Collagen VI is required for peripheral nerve regeneration

Wallerian degeneration is a process that includes the breakdown of axons and phagocytosis of damaged axons and myelin debris after injury, which is strictly required for axon regeneration [36]. We therefore first examined Wallerian degeneration in collagen VI-deficient mice. Toluidine blue staining and electron microscopy showed that at 7 days post-injury sciatic nerves from wild-type mice had advanced signs of myelin breakdown and a high incidence of phagocytic macrophages. However, both of these features were noticeably lower in *Col6a1*$^{-/-}$ mice

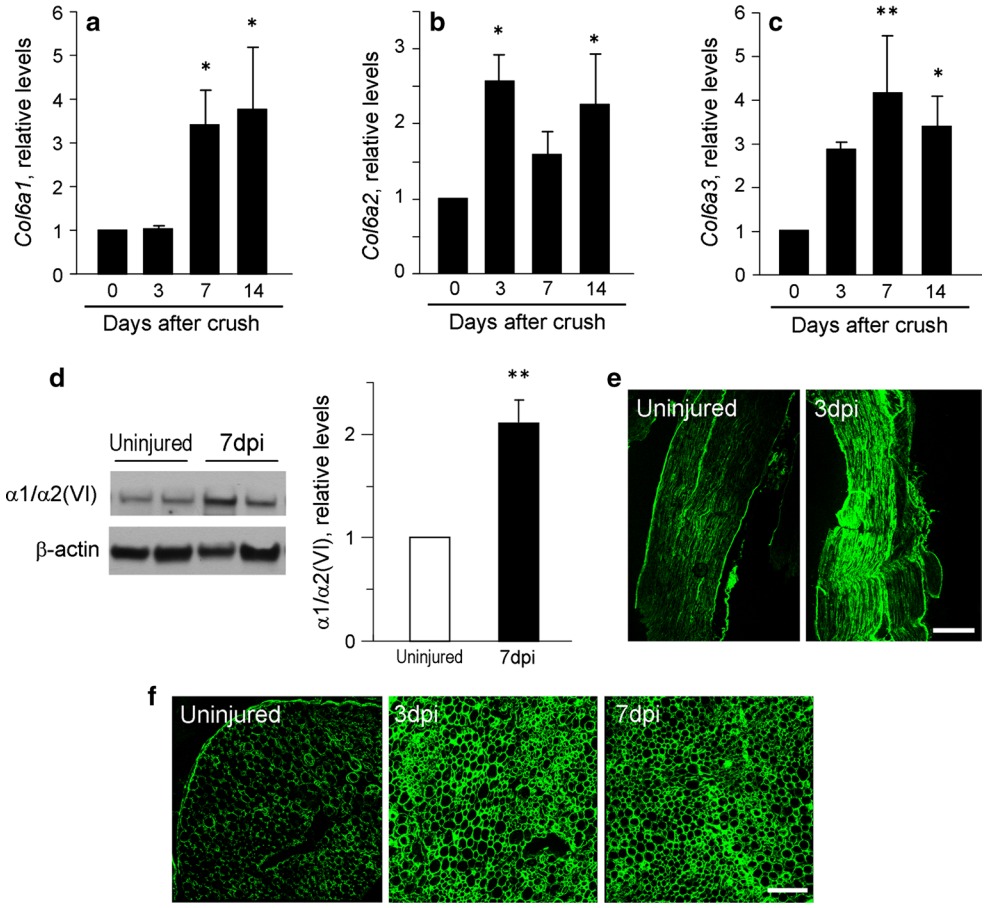


Fig. 1 Collagen VI expression is enhanced upon peripheral nerve crush. Real-time RT-PCR for *Col6a1* (**a**), *Col6a2* (**b**) and *Col6a3* (**c**) in uninjured (0) or injured sciatic nerves at 3, 7 and 14 days post-crush ($n = 3$–5). There was a statistically significant difference among groups as determined by one-way ANOVA analysis of *Col6a1* [$F(3,13) = 3.739$, $P = 0.039$], *Col6a2* [$F(3,13) = 3.506$, $P = 0.046$] and *Col6a3* [$F(3,13) = 4.600$, $P = 0.021$]. A post hoc test revealed that the relative levels of *Col6a1* or *Col6a3* were statistically significantly increased in injured nerves at 7 dpi (3.46 ± 0.77, $P = 0.035$, or 4.15 ± 1.29, $P = 0.004$) and 14 dpi (3.76 ± 1.44, $P = 0.018$, or 3.38 ± 0.68, $P = 0.033$), but not at 3 dpi (1.04 ± 0.05, $P = 0.973$, or 2.85 ± 0.17, $P = 0.087$), when compared to uninjured nerves, and the relative levels of *Col6a2* were statistically significantly increased in injured nerves at 3 dpi (2.55 ± 0.35, $P = 0.014$) and 14 dpi (2.24 ± 0.68, $P = 0.028$), but not at 7 dpi (1.59 ± 029, $P = 0.271$), when compared to uninjured nerves. There was no statistically significant difference among the 3-, 7- and 14-dpi groups for *Col6a2* ($P = 0.415$) and *Col6a3* ($P = 0.639$). *Col6a1* was significantly increased in injured nerves at 14 dpi compared to 3 dpi ($P = 0.043$), but no significant differences were seen between the 7- and 14-dpi ($P = 0.758$) and the 3- and 7-dpi ($P = 0.072$) groups. **d** *Left panel* Western blot analysis for α1/α2(VI) in uninjured sciatic nerves or injured nerves at 7 days post-crush. *Right panel* Densitometric quantification of α1/α2(VI) vs. actin as determined by three independent Western blot experiments ($n = 4$; $^{**}P < 0.01$). **e** Immunofluorescence for α3(VI) in longitudinal sections of uninjured sciatic nerves and injured nerves at 3 days post-crush. *Scale bar* 250 μm. **f** Immunofluorescence for α3(VI) in cross sections of uninjured sciatic nerves and injured nerves at 3 and 7 days post-crush. *Scale bar* 50 μm. *dpi* days post-injury

(Supplementary Fig. S1a, d). Quantitative analysis confirmed that *Col6a1*$^{-/-}$ nerves had more myelinated axons and fewer phagocytic macrophages than wild-type nerves at 7 days post-injury (Supplementary Fig. S1b, c). In keeping with the concept that clearance of myelin debris from injured nerves is necessary for PNS regeneration [14, 47], more myelin was present in *Col6a1*$^{-/-}$ nerves than in wild-type nerves at 7 days post-injury (Supplementary Fig. S1a, d). Axonal growth inhibitors, such as myelin-associated glycoprotein (MAG), are usually present in myelin debris after nerve injury [39, 51]. Immunofluorescence and Western blot analysis revealed that MAG reactivity was higher in *Col6a1*$^{-/-}$ nerves than wild-type nerves at 7 days post-injury (Supplementary Fig. S1e, f). These data support the potential role of collagen VI in Wallerian degeneration following injury.

We then performed experiments to assess whether the inhibited Wallerian degeneration of *Col6a1*$^{-/-}$ mice influences PNS regeneration. First, we measured the sciatic functional index to evaluate the recovery of sensory motor coordination [22] in mice of both genotypes. As shown in Fig. 2a, the sciatic functional index score was not different

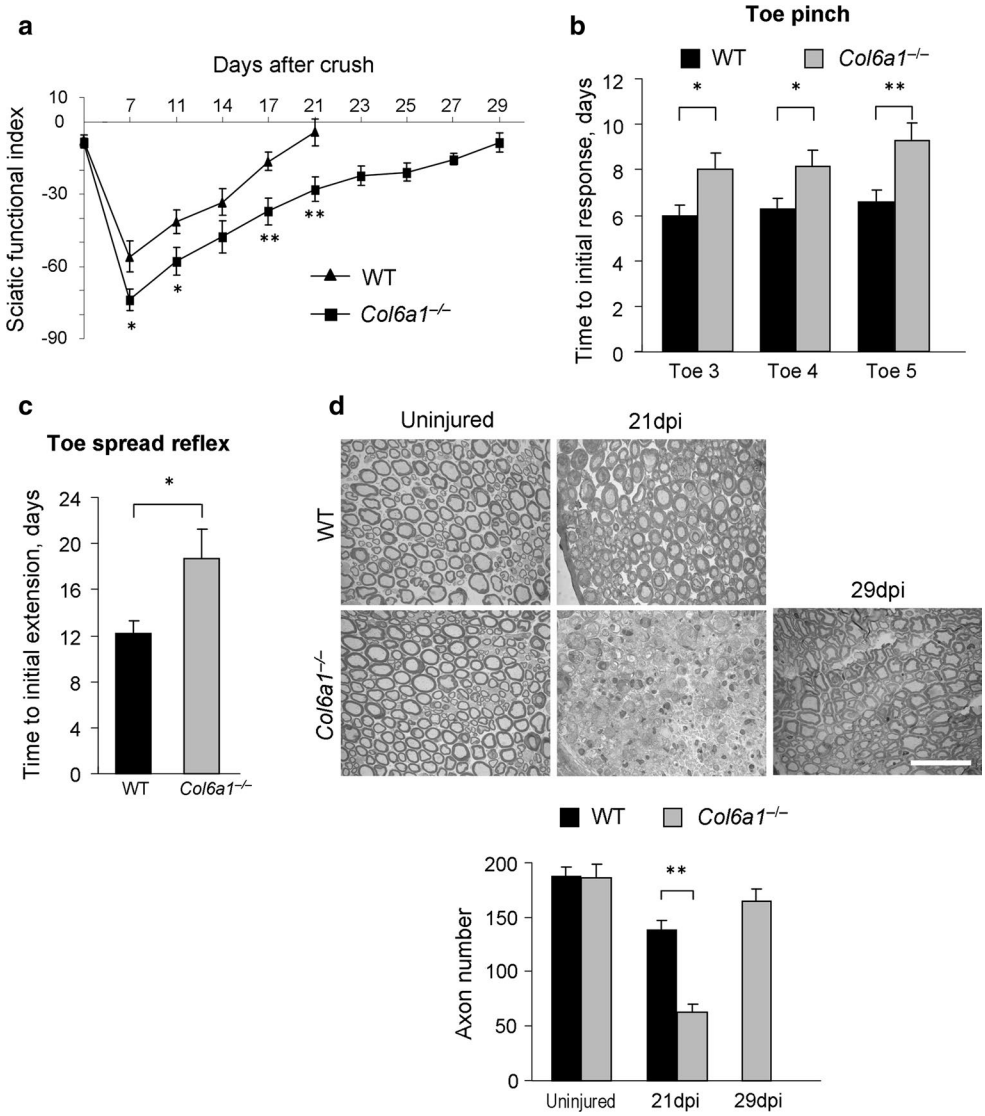


Fig. 2 Lack of collagen VI impairs peripheral nerve regeneration. **a** Quantification of sensory-motor function of wild-type and *Col6a1*$^{-/-}$ mice by analyzing the sciatic functional index from the footprint track before crush and at 7, 11, 14, 17, 21 and 29 days post-crush ($n = 7$; $^{*}P < 0.05$; $^{**}P < 0.01$). **b** Quantification of sensory function of wild-type and *Col6a1*$^{-/-}$ mice after sciatic nerve crush by recording the initial response time (day post-injury) to the pinch using forceps in the digits 3, 4 and 5 ($n = 7$; $^{*}P < 0.05$; $^{**}P < 0.01$). **c** Quantification of motor function of wild-type and *Col6a1*$^{-/-}$ mice after sciatic nerve crush by recording the initial extension time (day post-injury) to toe spreading reflex ($n = 7$; $^{*}P < 0.05$). **d** Representative images of toluidine blue staining and morphometric analysis of the myelinated axon number in cross sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 21 and 29 days post-crush ($n = 3$; $^{**}P < 0.01$). *Scale bar* 40 μm. *dpi* days post-injury, *WT* wild type

between wild-type and *Col6a1*$^{-/-}$ mice before nerve crush. In contrast, the sciatic functional index of *Col6a1*$^{-/-}$ mice was significantly lower than that of wild-type mice at 7 days after injury. This parameter remained significantly lower in collagen VI-deficient mice during the following time points, and a complete functional recovery was observed at 21 and 29 days post-injury in wild-type and *Col6a1*$^{-/-}$ mice, respectively (Fig. 2a). To measure sensory functions, we recorded the response to toe pinch in digits 3, 4 and 5 of the crushed hindlimb because they are the main digits innervated by nerves for sensory functions [26]. The time to initial response to the stimuli after sciatic nerve crush was significantly longer in *Col6a1*$^{-/-}$ mice than wild-type mice (Fig. 2b). Next, we utilized the toe spread reflex to evaluate motor function and found that the time to initial toe extension after nerve injury was significantly increased in *Col6a1*$^{-/-}$ mice when compared to wild-type mice (Fig. 2c). Toluidine blue staining showed that the number of myelinated axons was significantly lower in *Col6a1*$^{-/-}$ nerves than in wild-type nerves at 21 days post-injury, whereas there was no difference between the two genotypes in uninjured conditions (Fig. 2d). In agreement with the sciatic functional index, the number of myelinated axons was almost completely restored at 21 days post-injury in wild-type mice, while this required 29 days in *Col6a1*$^{-/-}$ mice (Fig. 2d). Altogether, these findings indicate that lack of collagen VI delays peripheral nerve regeneration after injury.

Collagen VI stimulates macrophage migration in vitro and in vivo

In the PNS, macrophages are critical for the removal of debris and contribute to nerve regeneration [13, 51]. To determine whether collagen VI is critical for macrophage activities, we performed in vitro and in vivo experiments to analyze macrophage migration. Transwell assay showed that

addition of purified collagen VI to the culture medium in the lower chambers at the concentration of 1 μg/ml significantly increased the number of macrophages that had migrated (Fig. 3a and Supplementary Fig. S2a). Scratch assay revealed that collagen VI promotes macrophage motility after scratching, as demonstrated by the markedly enhanced migration distance when macrophages were treated with collagen VI (Fig. 3b and Supplementary Fig. S2b). To investigate the in vivo chemoattractant ability of collagen VI, we used a Matrigel plug assay to examine macrophage density in the Matrigel implanted subcutaneously into wild-type mice. Immunofluorescence for CD68 and F4/80 showed that both these macrophage markers were markedly increased in wild-type mice treated with Matrigel plugs supplemented with purified collagen VI compared to mice treated with PBS-supplemented Matrigel plugs (Fig. 3c and Supplementary Fig. S2c). In addition, we utilized collagen I and MCP-1 as negative and positive controls, respectively, and found that MCP-1, but not collagen I, significantly enhanced macrophage migration in transwell, scratch and Matrigel plug assays (Fig. 3a–c and Supplementary Fig. S2a, b). Next, we used a different experimental setting, where Matrigel plugs supplemented with 2 % FBS were subcutaneously injected in wild-type and *Col6a1*$^{-/-}$ mice. Immunofluorescence for the F4/80 marker showed that the macrophage migration capability was dramatically impaired in *Col6a1*$^{-/-}$ mice compared to wild-type animals (Fig. 3d), suggesting that in addition to as a chemokine itself, collagen VI is required for other factors inducing macrophage migration.

It has been demonstrated that the AKT and PKA pathways are necessary for macrophage migration [10, 11]. We thus investigated whether collagen VI-induced macrophage migration is regulated by these signals. Western blot analysis showed that the addition of collagen VI to in vitro macrophages promoted AKT and PKA phosphorylation (Supplementary Fig. S3a, b). Transwell assay demonstrated that the collagen VI-induced increase in the number of migrated macrophages was inhibited by pretreatment with AKT inhibitor (AKTi) or H89, a PKA inhibitor (Fig. 3a and Supplementary Fig. S2a). Moreover, the scratch assay revealed that pretreatment with AKTi or H89 decreased the collagen VI-induced macrophage migration distance (Fig. 3b and Supplementary Fig. S2b). Taken together, these data indicate that collagen VI promotes macrophage migration by regulating the AKT and PKA pathways.

Ablation of collagen VI leads to impaired macrophage recruitment to the injured nerve

Given the robust chemoattractant activity of collagen VI for macrophages, we further investigated whether the delayed myelin clearance and PNS regeneration are the result of impaired macrophage recruitment in *Col6a1*$^{-/-}$ injured nerves. Immunofluorescence showed that more CD68- and F4/80-positive macrophages were present in the injured nerves of wild-type mice than *Col6a1*$^{-/-}$ mice (Fig. 4a–c). Western blot analysis confirmed that although CD68 was enhanced in both genotypes at 7 days post-injury, the CD68 levels of injured *Col6a1*$^{-/-}$ nerves were significantly lower than those of injured wild-type nerves (Fig. 4d). These data indicate that lack of collagen VI impairs macrophage accumulation in injured nerves.

Chemokines and cytokines are important mediators of the immune response. Among them, IL-1β and MCP-1 are two prominent regulators of macrophage recruitment in injured peripheral nerves [36, 41, 45]. We therefore examined whether the impaired macrophage recruitment in *Col6a1*$^{-/-}$ mice after injury was paralleled by a lower abundance of these two inflammatory regulators. Real-time RT-PCR revealed that although the expression of IL-1β and MCP-1 mRNA was upregulated in both wild-type and *Col6a1*$^{-/-}$ nerves at 1 day after crush, the levels of the two transcripts were significantly lower in injured *Col6a1*$^{-/-}$ nerves compared to injured wild-type nerves (Supplementary Fig. S4a, b). These results suggest that in addition to its chemoattractant activity for macrophage migration, collagen VI also affects other inflammatory mediators in injured peripheral nerves.

Collagen VI is critical for macrophage polarization

Peripheral nerve regeneration not only depends on macrophage density, but also requires macrophage polarization toward the M2 phenotype [30]. To investigate the potential role of collagen VI in macrophage polarization, we isolated primary BMDMs and PMs from wild-type and *Col6a1*$^{-/-}$ mice and differentiated them toward the M2 and M1 phenotypes with IL-4 and LPS, respectively. Western blot analysis for M2 markers showed that the protein levels of Arg-1, CD206 and PPARγ were increased in wild-type, but not in *Col6a1*$^{-/-}$ BMDMs upon stimulation with IL-4 (Fig. 5a). Similar results were found in PMs, where Arg-1 and PPARγ levels were upregulated in wild-type cells upon stimulation of IL-4, whereas this enhancement was prevented in *Col6a1*$^{-/-}$ cells (Fig. 5b). Furthermore, the defective response of *Col6a1*$^{-/-}$ PMs was reversed when cells were cultured in the presence of purified collagen VI (Fig. 5b). These results indicate that collagen VI is required for macrophage M2 polarization.

Since collagen VI expression is reduced when macrophages are subjected to M1 stimuli [40], we hypothesized that this reduction may be essential for M1 polarization. Upon LPS stimulation, *Col6a1*$^{-/-}$ PMs displayed a marked enhancement of COX-2, a M1 marker, when compared to wild-type PMs (Fig. 5c). A similar response was found in BMDMs, since upon LPS stimulation *Col6a1*$^{-/-}$ cells exhibited higher enhancement of several M1 markers,

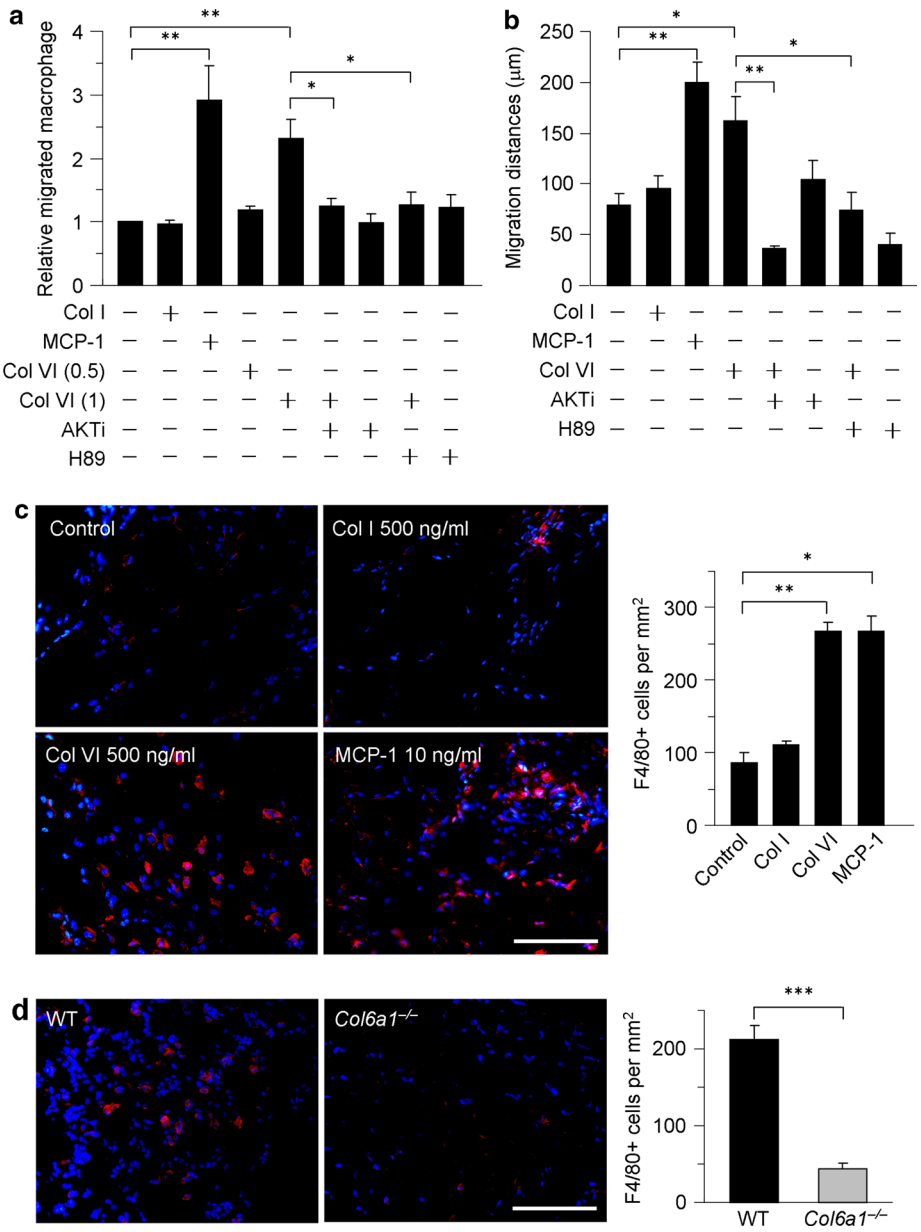


Fig. 3 Collagen VI promotes in vitro and in vivo macrophage migration. **a** Quantification of migrated J774 macrophages in transwell migration assays upon treatment with PBS (control), collagen VI (0.5 or 1 μg/ml), collagen I or MCP-1. AKTi or H89 was added where indicated ($n = 3$–4; $^*P < 0.05$; $^{**}P < 0.01$). **b** Quantification of mean migration distances of J774 macrophages upon treatment with PBS (control), collagen VI (1 μg/ml), collagen I or MCP-1 and subjected to a scratch assay. AKTi or H89 was added where indicated. Cells were analyzed 8 h after the scratch ($n = 3$–4; $^*P < 0.05$; $^{**}P < 0.01$). **c** *Left and middle panels* Representative images of immunofluorescence for F4/80 in growth factor-reduced Matrigel plugs supplemented with PBS, collagen VI, collagen I or MCP-1 subcutaneously injected into wild-type mice. *Scale bar* 100 μm. *Right panel* Quantitative analysis of migrated F4/80-positive macrophages in Matrigel plugs ($n = 3$; $^*P < 0.05$; $^{**}P < 0.01$). **d** *Left and middle panels* Representative images of immunofluorescence for F4/80 in growth factor-reduced Matrigel plugs supplemented with 2 % FBS subcutaneously injected into wild-type (*left*) and *Col6a1*$^{-/-}$ (*middle*) mice. *Scale bar* 100 μm. *Right panel* Quantitative analysis of migrated F4/80-positive macrophages in Matrigel plugs ($n = 3$; $^{***}P < 0.001$). *AKTi* AKT inhibitor, *Col I* Collagen I, *Col VI* collagen VI, *WT* wild type

such as iNOS, CD16 and COX-2, when compared to wild-type cells (Fig. 5d, e). Of note, these enhancements in *Col6a1*$^{-/-}$ cells were partially rescued when cells were cultured in the presence of purified collagen VI (Fig. 5d, e). These results indicate that collagen VI inhibits macrophage M1 polarization.

To further confirm the effect of collagen VI in macrophage polarization, we cultured J774 macrophages and

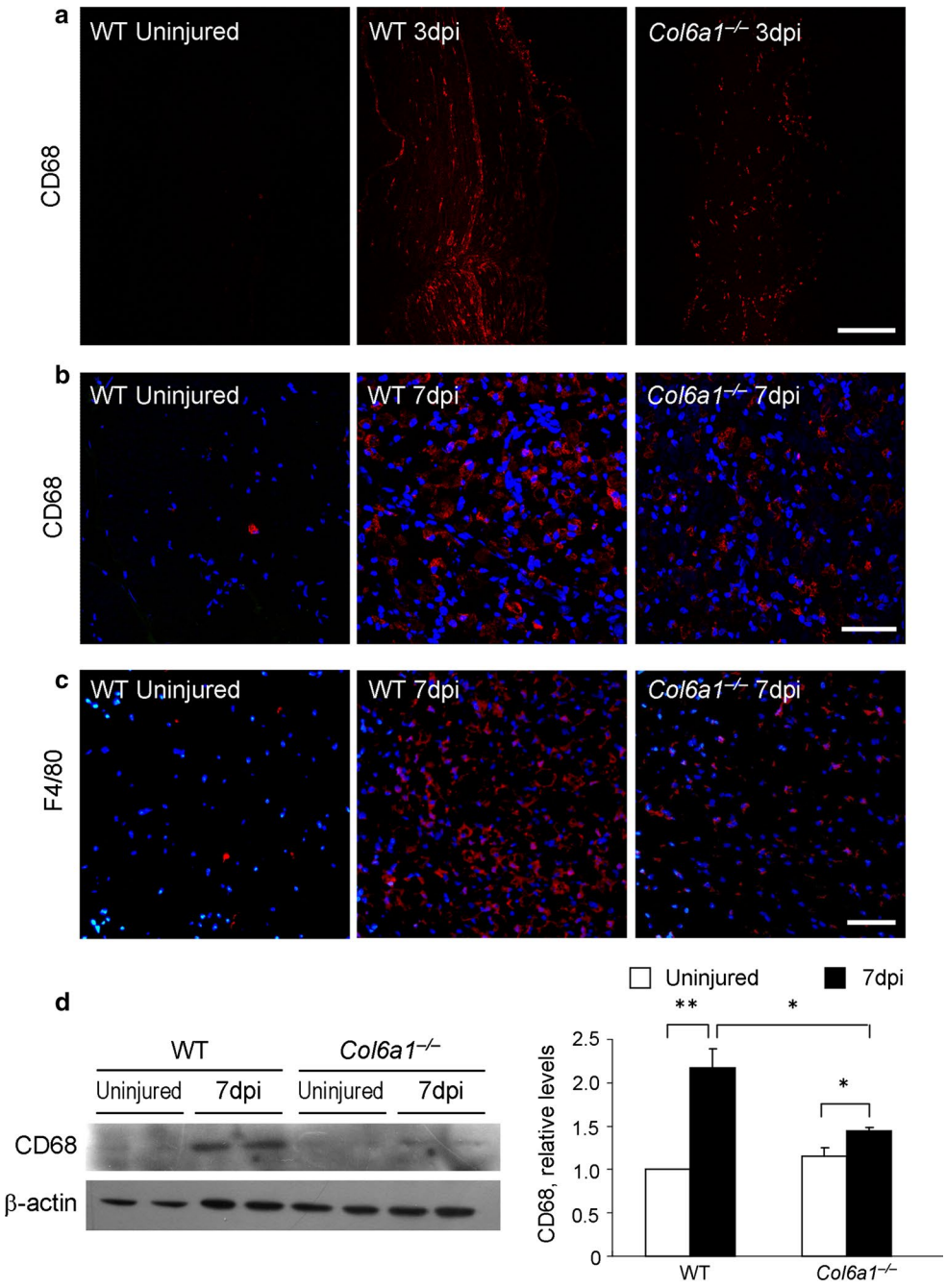
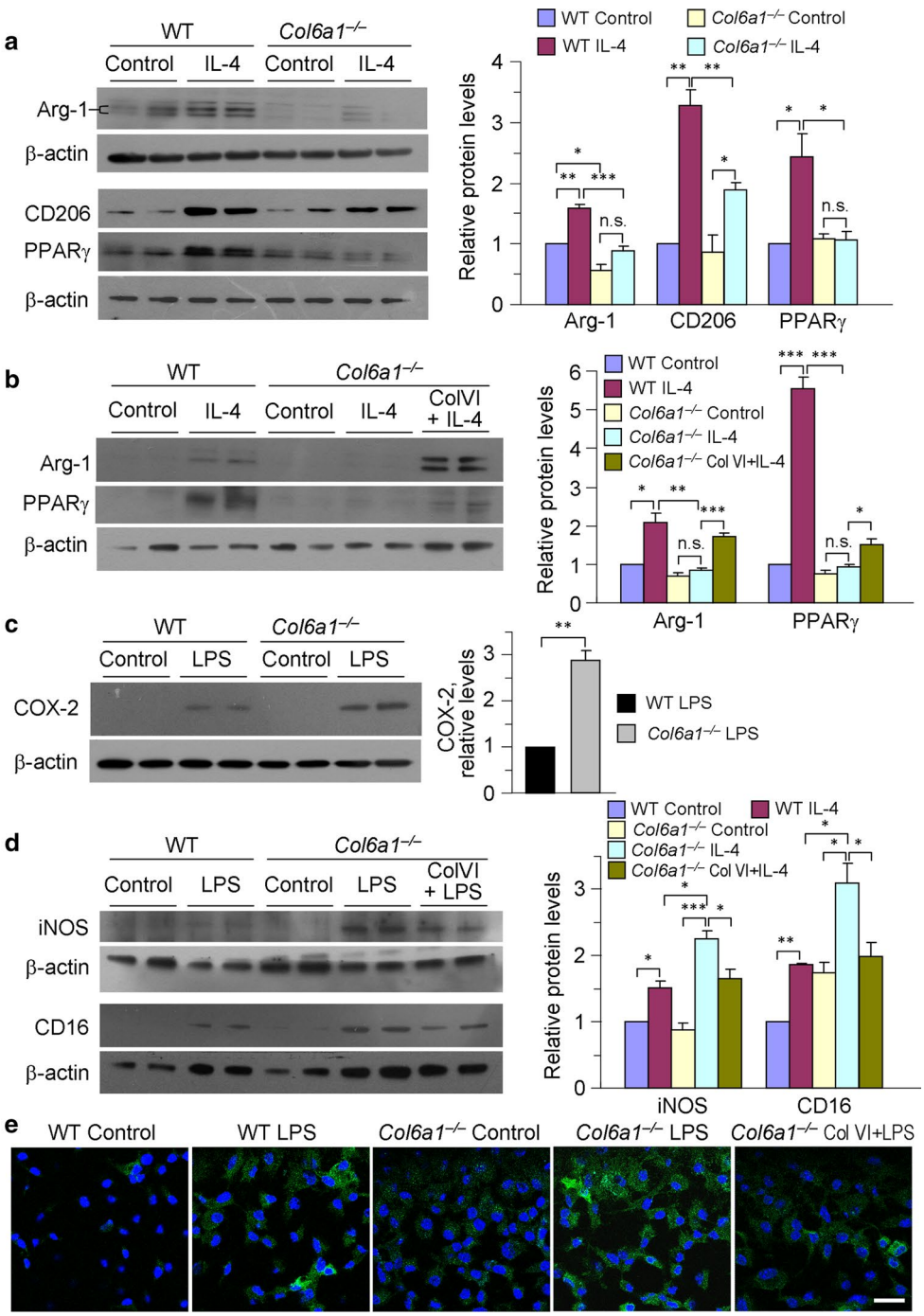


Fig. 4 Lack of collagen VI leads to impaired of macrophage recruitment into injured nerves. **a** Immunofluorescence for CD68 in longitudinal sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 3 days post-crush. *Scale bar* 250 μm. **b** Immunofluorescence for CD68 in cross sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. *Scale bar* 50 μm. **c** Immunofluorescence for F4/80 in cross sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. *Scale bar* 50 μm. **d** *Left panel* Western blot analysis for CD68 in sciatic nerves of wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. *Right panel* Densitometric quantification of CD68 vs. actin as determined by three independent Western blot experiments. Values for uninjured wild-type nerves were arbitrarily set to 1 ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$). *dpi* days post-injury, *WT* wild type

wild-type PMs in the absence or presence of purified collagen VI. Addition of collagen VI enhanced Arg-1 levels and reduced the levels of CD16 and iNOS in J774 macrophages (Supplementary Fig. S5a–c). Moreover, Western blot analysis for Arg-1 and PPARγ in PMs showed that both of these M2 markers were significantly increased upon treatment with purified collagen VI (Supplementary Fig. S5d, e). Taken together, these findings indicate that collagen VI promotes macrophage M2 polarization and exhibits an inhibitory effect on macrophage M1 polarization.

AKT and PKA are two key mediators of signaling pathways involved in macrophage polarization [3, 27, 38]. We thus evaluated whether collagen VI-induced macrophage polarization involves the activation of AKT and PKA signals. Western blot analysis showed that the phosphorylation of both AKT and PKA was increased upon stimulation with IL-4 in wild-type, but not in *Col6a1*$^{-/-}$ PMs (Supplementary Fig. S6a). Addition of collagen VI to BMDMs enhanced CD206 levels, which were inhibited by AKTi and H89 (Supplementary Fig. S6b). Moreover, immunofluorescence on J774 macrophages showed that collagen



VI-promoted PPARγ expression was attenuated by AKTi and H89 (Supplementary Fig. S6c). These data support a role for the AKT and PKA pathways in modulating collagen VI-related macrophage M2 polarization.

Lack of collagen VI impairs macrophage M2 polarization in vivo after peripheral nerve injury

Next, we investigated the in vivo role of collagen VI in macrophage polarization in injured nerves. CD16 protein levels were higher in *Col6a1*$^{-/-}$ nerves than in wild-type nerves at 7 days post-crush (Fig. 6a). Furthermore, the expression of M2 marker genes *Arg1* and *Mrc1* was upregulated in wild-type nerves, but not in *Col6a1*$^{-/-}$ nerves at 7 days after crush (Fig. 6b, c). Immunofluorescence for CD206 showed that the amount of M2 macrophages in *Col6a1*$^{-/-}$ nerves was dramatically lower than in wild-type nerves at 7 days post-injury (Fig. 6d). Western blot analysis confirmed that the levels of CD206 were significantly increased in wild-type nerves, but not in *Col6a1*$^{-/-}$ nerves,

◀ **Fig. 5** Effect of collagen VI on macrophage polarization. **a** *Left panel* Western blot analysis for Arg-1, CD206 and PPARγ in wild-type and *Col6a1*$^{-/-}$ BMDMs under control conditions or following induction with IL-4. *Right panel* Densitometric quantification of Arg-1 vs. actin, CD206 vs. actin and PPARγ vs. actin, as determined by three independent Western blot experiments. Values for the wild-type control group were arbitrarily set to 1 ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; *n.s.* not significant). **b** *Left panel* Western blot analysis for Arg-1 and PPARγ in wild-type, *Col6a1*$^{-/-}$ and collagen VI-coated (5 μg/cm^2) *Col6a1*$^{-/-}$ PMs under control conditions or following induction with IL-4. *Right panel* Densitometric quantification of Arg-1 vs. actin and PPARγ vs. actin, as determined by three independent Western blot experiments. Values for the wild-type control group were arbitrarily set to 1 ($n = 3$–4; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; *n.s.* not significant). **c** *Left panel* Western blot analysis for COX-2 in wild-type and *Col6a1*$^{-/-}$ PMs under control conditions or following induction with LPS. *Right panel* Densitometric quantification of COX-2 vs. actin, as determined by three independent Western blot experiments. Only LPS-induced COX-2 levels were calculated because of the extremely low COX-2 levels at baseline. Values for the wild-type LPS group were arbitrarily set to 1 ($n = 3$; $^{**}P < 0.01$). **d** *Left panel* Western blot analysis for iNOS and CD16 in wild-type, *Col6a1*$^{-/-}$ and collagen VI-coated (5 μg/cm^2) *Col6a1*$^{-/-}$ BMDMs under control conditions or following induction with LPS. *Right panel* Densitometric quantification of iNOS vs. actin and CD16 vs. actin, as determined by three independent Western blot experiments. Values for the wild-type control group were arbitrarily set to 1 ($n = 3$–4; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). **e** Immunofluorescence for COX-2 in wild-type, *Col6a1*$^{-/-}$ and collagen VI-coated (5 μg/cm^2) *Col6a1*$^{-/-}$ BMDMs under control conditions or following induction with LPS. *Scale bar* 25 μm. *Col VI* collagen VI, *WT* wild type

at 7 days after crush when compared to uninjured nerves (Fig. 6e). Finally, we normalized CD206 levels to CD68 levels and found that the relative amounts of CD206-positive M2 macrophages at 7 days post-injury were significantly lower in *Col6a1*$^{-/-}$ nerves than in wild-type nerves (Fig. 6f). Taken together, these data point to an impairment of macrophage M2 polarization in injured *Col6a1*$^{-/-}$ nerves.

To assess the in vivo role of AKT and PKA pathways in modulating collagen VI-mediated macrophage M2 polarization, we analyzed the activation of these two signals in injured wild-type and *Col6a1*$^{-/-}$ nerves. At 7 days post-crush, phosphorylation of both AKT and PKA was markedly enhanced in wild-type nerves, but not in *Col6a1*$^{-/-}$ nerves (Supplementary Fig. S7a, b). These findings support a role for the AKT and PKA pathways in the impaired macrophage M2 polarization and PNS regeneration of *Col6a1*$^{-/-}$ mice.

Collagen VI-regulated macrophage function contributes to PNS regeneration

To further confirm that the modulation of macrophage activities by collagen VI is crucial for PNS regeneration, we used macrophage-depleted in vivo models by injection of clodronate liposomes in wild-type and *Col6a1*$^{-/-}$ mice. Immunofluorescence for CD68 and F4/80 showed that injection of clodronate liposomes effectively depleted macrophages in sciatic nerves after crush (Supplementary Fig. S8). In the control group treated with PBS liposomes, the sciatic functional index was lower in *Col6a1*$^{-/-}$ mice than in wild-type mice, as expected (Fig. 7a). Following macrophage depletion by clodronate liposomes, the sciatic functional index was similarly reduced in wild-type and *Col6a1*$^{-/-}$ mice, thus indicating that the difference between the two genotypes was abolished after macrophage depletion (Fig. 7a). Moreover, the response to toe pinching was similar between wild-type and *Col6a1*$^{-/-}$ mice after macrophage depletion with clodronate liposomes, but was delayed when compared to control treatment with PBS liposomes (Fig. 7b). Similarly, analysis of the toe spreading reflex showed that depletion of macrophages significantly delayed the toe extension in both wild-type and *Col6a1*$^{-/-}$ mice, and it abolished the difference between the two genotypes (Supplementary Table S2). Toluidine blue staining of sciatic nerve cross sections showed that in mice treated with PBS liposomes the number of myelinated axons was significant higher in wild-type nerves than in *Col6a1*$^{-/-}$ nerves at 21 days post-injury, as expected (Fig. 7c). However, when mice were treated with clodronate liposomes, the difference in myelinated axon number between the two genotypes was completely abolished (Fig. 7c). These results indicate that defective macrophage recruitment is the main cause for the delayed PNS regeneration of collagen VI-deficient mice.

To directly investigate whether the delayed PNS regeneration of *Col6a1*$^{-/-}$ mice is due to the defects of collagen VI-regulated macrophage activities, we transplanted wild-type bone marrow cells into lethally irradiated wild-type mice (WT-WT) or collagen VI-deficient mice (WT-*Col6a1*$^{-/-}$). Functional studies showed that there were no significant differences between WT-WT and WT-*Col6a1*$^{-/-}$ mice in the sciatic functional index score (Fig. 8a), time to initial response to toe pinch in digits 3, 4 and 5 (Fig. 8b) and time to initial toe extension (Fig. 8c), indicating that the delayed PNS regeneration in *Col6a1*$^{-/-}$ mice is rescued by transplantation of wild-type bone marrow cells. Next, we investigated whether the transplanted wild-type cells were able to rescue the decreased macrophage recruitment and M2 polarization of *Col6a1*$^{-/-}$ mice after nerve crush injury. Immunofluorescence for CD68 and F4/80 showed comparable CD68- and F4/80-positive macrophages in sciatic nerves of WT-WT and WT-*Col6a1*$^{-/-}$ mice at 7 days post-injury (Fig. 8d). Western blot analysis showed that CD206 levels were similar between WT-WT and WT-*Col6a1*$^{-/-}$ mice at 7 days post-injury (Fig. 8e). Taken together, these findings provide evidence demonstrating that the delayed PNS regeneration in *Col6a1*$^{-/-}$ mice is induced by the deficits in macrophage migration and M2 polarization.

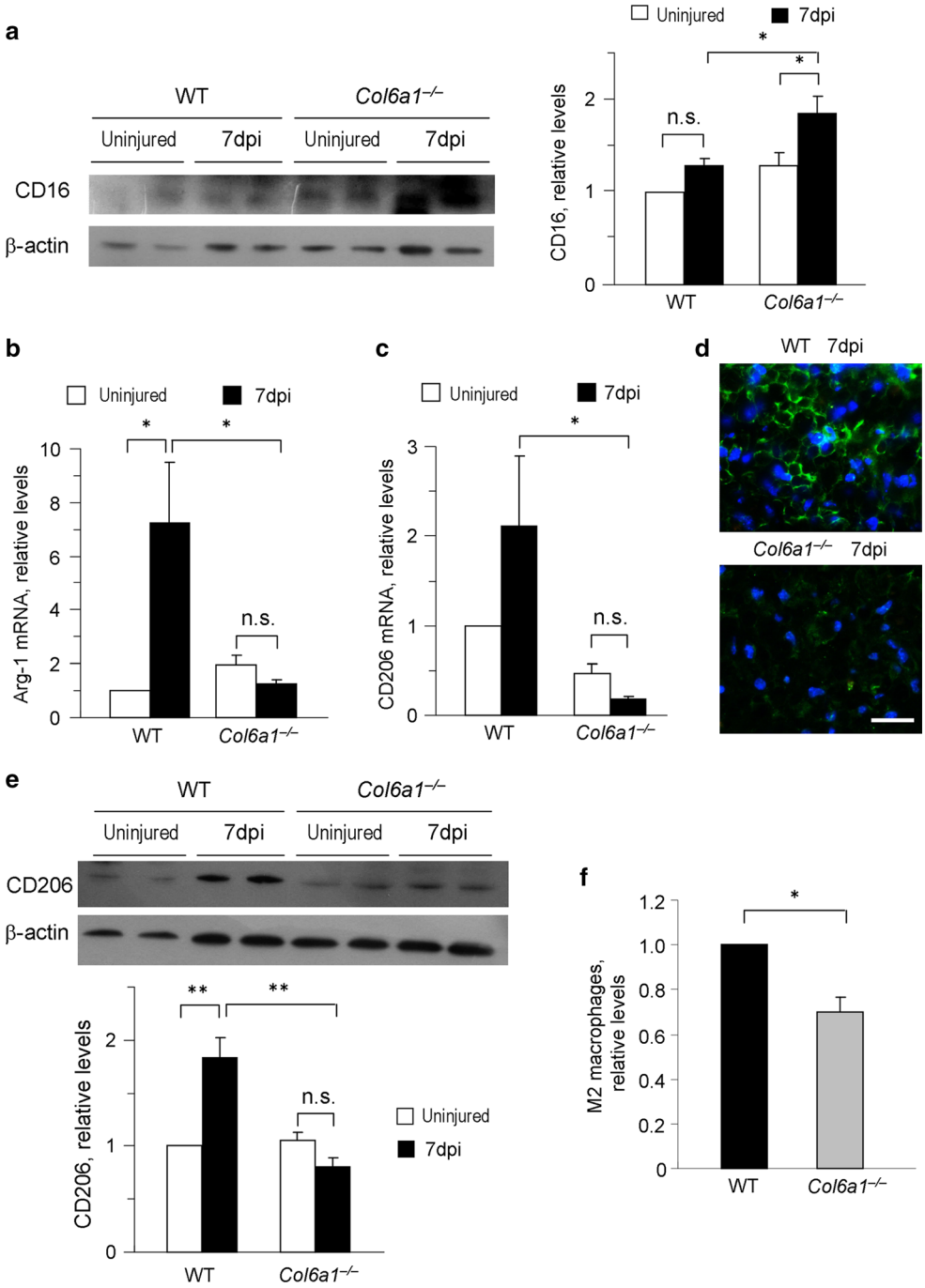


Fig. 6 Ablation of collagen VI decreases macrophage M2 polarization after nerve injury. **a** *Left panel* Western blot analysis for CD16 in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. *Right panel* Densitometric quantification of CD16 vs. actin, as determined by three independent Western blot experiments. Values for uninjured wild-type nerves were arbitrarily set to 1 ($n = 4$; $^{*}P < 0.05$; *n.s.* not significant). Real-time RT-PCR analysis for Arg-1 (**b**) and CD206 (**c**) mRNA in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. Values for uninjured wild-type nerves were arbitrarily set to 1. GAPDH was used as a reference gene ($n = 3$–5; $^{*}P < 0.05$; *n.s.* not significant). **d** Immunofluorescence for CD206 in cross sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice at 7 days post-crush. *Scale bar* 25 μm. **e** *Top panel* Western blot analysis for CD206 in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. *Bottom panel* Densitometric quantification of CD206 vs. actin as determined by three independent Western blot experiments. Values for uninjured wild-type nerves were arbitrarily set to 1 ($n = 4$; $^{**}P < 0.01$; *n.s.* not significant). **f** Quantification of CD206 vs. CD68 in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice at 7 days post-crush as determined by three independent Western blot experiments. Values for uninjured wild-type nerves were arbitrarily set to 1 ($n = 4$; $^{**}P < 0.05$). *dpi* days post-injury, *WT* wild type

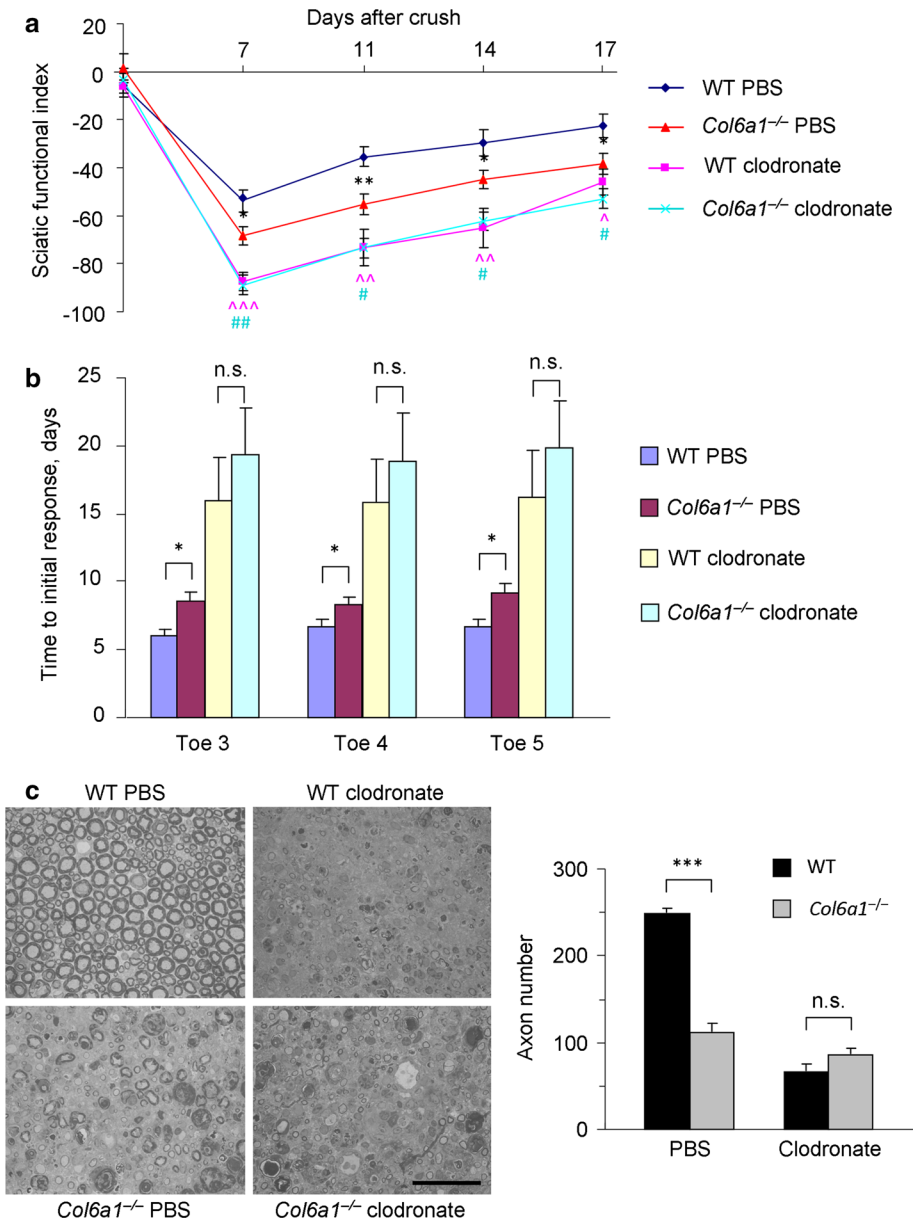


Fig. 7 Macrophage depletion leads to similar regenerative responses in wild-type and collagen VI-deficient peripheral nerves. **a** Quantification of the sensory-motor function of wild-type and *Col6a1*$^{-/-}$ mice under control conditions (PBS liposomes) and after macrophage depletion (clodronate liposomes) by analyzing the sciatic functional index from footprint tracks before crush and at 7, 11, 14 and 17 days post-crush ($n = 5$–7; $^{*}P < 0.05$ and $^{**}P < 0.01$, *Col6a1*$^{-/-}$ PBS vs. wild-type PBS; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$ and $^{\wedge\wedge\wedge}P < 0.001$, wild-type clodronate vs. wild-type PBS; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, *Col6a1*$^{-/-}$ clodronate vs. *Col6a1*$^{-/-}$ PBS). **b** Quantification of the sensory function of wild-type and *Col6a1*$^{-/-}$ mice under control conditions (PBS liposomes) and after macrophage depletion (clodronate liposomes) by recording the initial response time to the pinch using forceps in the digits 3, 4 and 5 after sciatic nerve crush ($n = 5$–7; $^{*}P < 0.05$; *n.s.* not significant). **c** Representative images of toluidine blue staining and morphometric analysis of the myelinated axon number in cross sections of injured sciatic nerves at 21 days post-crush from wild-type and *Col6a1*$^{-/-}$ mice that received PBS liposomes or clodronate liposomes. *Scale bar* 40 μm ($n = 3$; $^{***}P < 0.001$; *n.s.* not significant). *dpi* days post-injury, *WT* wild type

Discussion

Our previous work demonstrated that collagen VI is required for muscle regeneration [46], suggesting a role in tissue repair. The results presented in this study show that collagen VI promotes PNS regeneration by regulating macrophage recruitment and polarization. Lack of collagen VI in *Col6a1*$^{-/-}$ mice prevents macrophage recruitment and phenotypic transition after sciatic nerve crush, which in turn inhibits PNS regeneration. Previous studies showed that macrophages play a pivotal function in Wallerian degeneration by clearing myelin debris and in axonal regeneration by secreting a variety of soluble factors [33]. However, the molecular mechanisms underlying macrophage recruitment into injured nerves are not well understood. It is well accepted that soluble factors secreted by the disrupted axon/Schwann cell nerve unit are responsible for macrophage recruitment following nerve injury [33, 45]. Among these factors, IL-1β and MCP-1 are two major macrophage chemoattractants in injured peripheral nerves [36, 41, 45]. However, blockade of IL-1β and MCP-1 with function-blocking antibodies does not completely inhibit macrophage recruitment into injured peripheral nerves in vivo [36]. Similar effects were also displayed by in vitro experiments, where addition of MCP-1 neutralizing antibodies to conditioned media from Schwann cell cultures and nerve segments does not completely block macrophage migration [45]. These findings indicate that other chemoattractants are also secreted by the injured peripheral nerves. In the current study, we found that collagen VI promotes

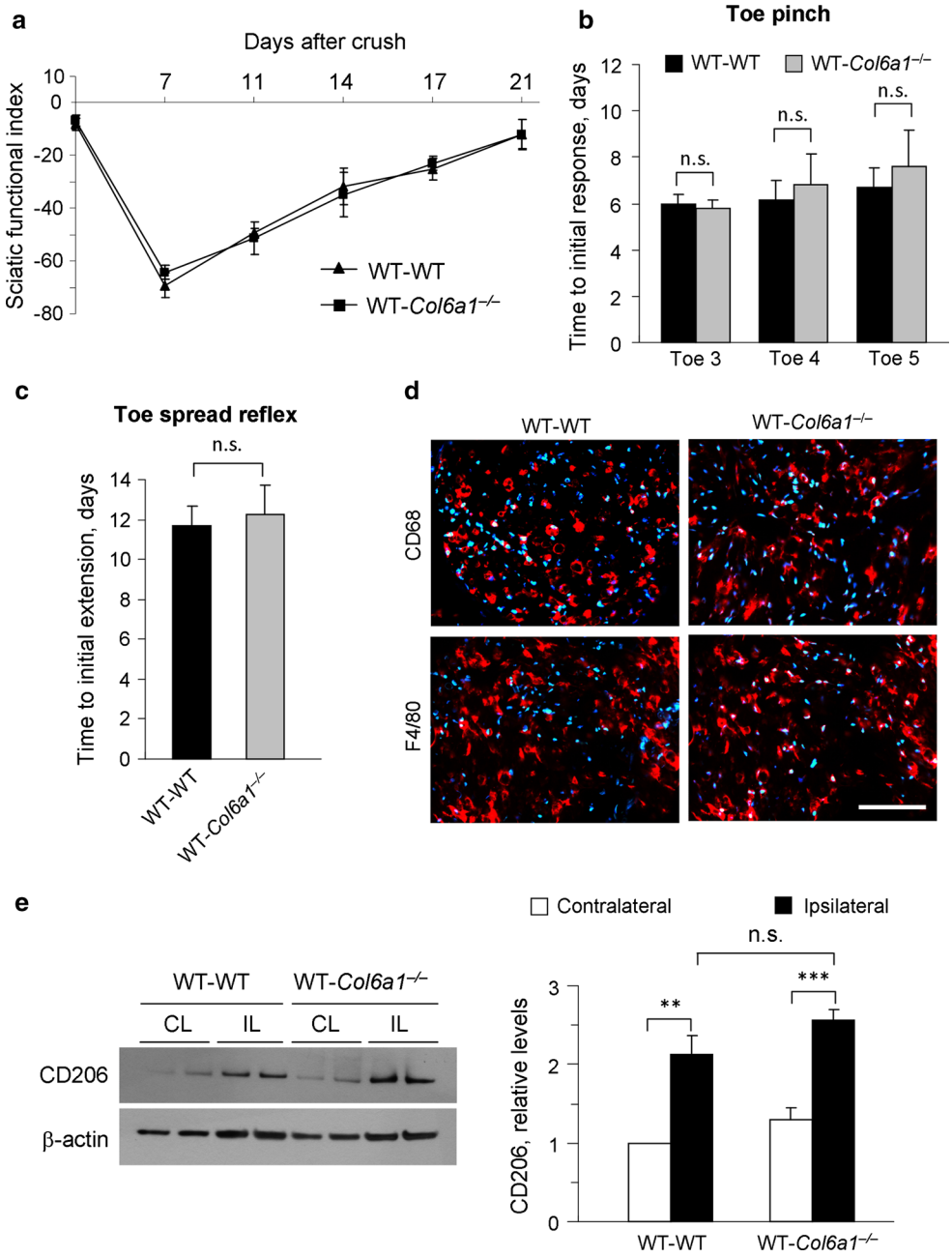


Fig. 8 Transplantation of wild-type bone marrow cells into *Col6a1*$^{-/-}$ host mice rejuvenates regeneration and macrophage activities after nerve injury. **a** Quantification of sensory-motor function of wild-type bone marrow cells transplanted into wild-type mice (WT-WT) and of wild-type bone marrow cells transplanted into *Col6a1*$^{-/-}$ mice (WT-*Col6a1*$^{-/-}$) by analyzing the sciatic functional index from footprint tracks before crush and at 7, 11, 14, 17 and 21 days post-crush (n = 5–7). **b** Quantification of sensory function of WT-WT and WT-*Col6a1*$^{-/-}$ mice after sciatic nerve crush by recording the initial response time (day post-injury) to the pinch using forceps in the digits 3, 4 and 5 (n = 5–7; *n.s.* not significant). **c** Quantification of motor function of WT-WT and WT-*Col6a1*$^{-/-}$ mice after sciatic nerve crush by recording the initial extension time (day post-injury) to the toe spreading reflex (n = 4–6; *n.s.* not significant). **d** Immunofluorescence for CD68 and F4/80 in cross sections of injured sciatic nerves from WT-WT and WT-*Col6a1*$^{-/-}$ mice at 7 days post-crush. *Scale bar* 50 μm. **e** *Left panel* Western blot analysis for CD206 in sciatic nerves from WT-WT and WT-*Col6a1*$^{-/-}$ mice at 7 days post-crush. *Right panel* Densitometric quantification of CD206 vs. actin as determined by three independent Western blot experiments. Values for uninjured WT-WT contralateral nerves were arbitrarily set to 1 (n = 4; $^{**}P < 0.01$; $^{***}P < 0.001$; *n.s.* not significant). *CL* contralateral, *IL* ipsilateral, *WT-Col6a1*$^{-/-}$ wild-type bone marrow cells transplanted into *Col6a1*$^{-/-}$ mice, *WT-WT* wild-type bone marrow cells transplanted into wild-type mice

macrophage migration both in vitro and in vivo and that *Col6a1*$^{-/-}$ macrophages exhibit a reduced migration capability in the Matrigel plug assay. In the sciatic nerve crush model, macrophage recruitment was markedly impaired in *Col6a1*$^{-/-}$ nerves. Thus, we identified collagen VI as a novel chemoattractant that triggers macrophage recruitment into injured nerves.

Our recent work demonstrated that in peripheral nerves collagen VI is abundantly deposited by Schwann cells and macrophages, but not by axons [7]. Upon injury in the PNS, Schwann cells dedifferentiate to a progenitor/stem cell-like state [35], expressing high levels of collagen VI [48]. In this study, we found that the expression of collagen VI is significantly upregulated upon sciatic nerve injury. On the one hand, this enhancement is likely contributed by the dedifferentiated Schwann cells; on the other hand, it is related to the increased number of macrophages after injury. In this regard, it is plausible that at the initial stage the dedifferentiated Schwann cells are responsible for increasing collagen VI deposition in injured nerves, which in turn promotes macrophage recruitment in a paracrine manner. Thereafter, both paracrine and autocrine effects may exist for the collagen VI contribution to macrophage recruitment. Our findings demonstrate that the impaired macrophage recruitment in injured *Col6a1*$^{-/-}$ nerves is rescued by transplantation of wild-type bone marrow cells, highlighting the autocrine effect of collagen VI in macrophage recruitment after peripheral nerve injury. Our data indicate that collagen VI acts as a chemoattractant for macrophages, a finding that is fully consistent with previous studies showing that certain ECM proteins, such as fibronectin, laminin and collagen IV, exhibit specific chemoattractant activities for different cells [1, 24, 49]. In addition to directly exhibiting chemotactic activity, collagen VI also influence the expression of other chemoattractants. For example, we found in this study that the upregulation of IL-1β and MCP-1 induced by sciatic nerve crush injury is significantly impaired in *Col6a1*$^{-/-}$ mice, suggesting that collagen VI is able to promote the recruitment of macrophages into the injured nerves through a variety of molecular mechanisms.

The function of macrophages in PNS regeneration is also related to their phenotype, where M2 macrophages stimulate regeneration [30]. Therefore, macrophage polarization from the M1 to M2 phenotype is crucial for successful PNS regeneration. It has been shown that acute peripheral nerve injury elicits an M2 macrophage response [50]. However, the mechanisms that trigger and modulate macrophage polarization are not well understood. To date, it is known that macrophage polarization is largely controlled by a small group of signals and factors, such as nuclear factor κB (NF-κB), mammalian target of rapamycin (mTOR), signal transducer and activator of transcription 6 (STAT6), PPARγ, Kruppel-like factor 4 (KLF4), AKT and PKA [4, 25, 27, 38]. In this study, we identified collagen VI as a novel factor regulating macrophage polarization. We found that addition of collagen VI promotes the J774 macrophage polarized toward the M2 phenotype. In the light of these findings, we isolated primary BMDMs and PMs from wild-type and *Col6a1*$^{-/-}$ mice and stimulated their polarization into M1 and M2 phenotypes with LPS and IL-4, respectively. Consistent with our hypothesis, deficiency of collagen VI impairs macrophage M2 polarization and promotes macrophage M1 polarization, which can be reversed by addition of purified native collagen VI.

Our findings indicate that collagen VI plays a pivotal role in macrophage polarization. Although one recent in vitro work showed that some specific ECM components, namely collagen I and fibronectin, are not needed for macrophage polarization [37], previous evidence suggests that ECM plays a key role in this process. For example, ECM-derived biologic scaffolds induce an in vivo constructive tissue remodeling by promoting an M2 macrophage response [42]. In a myocardial infarction model, deficiency of matrix metalloproteinase 28 (MMP-28) was found to attenuate macrophage M2 polarization and reduce the expression of several ECM genes [28]. Among the different ECM molecules, collagen VI seemed to be the best candidate for regulating macrophage polarization. M2 macrophages produce higher levels of collagen VI than M1 macrophages [40]. Moreover, macrophages in the adipose tissue of insulin-resistant subjects are associated with collagen VI deposition and exhibit M2 phenotype [44]. These indirect observations prompted us to investigate the role of this ECM molecule for macrophage polarization. In addition to in vitro data, we obtained direct in vivo evidence showing that collagen VI regulates macrophage polarization during PNS regeneration. We found that at 7 days post-injury, the levels of M2 macrophages were decreased in *Col6a1*$^{-/-}$ nerves, indicating an impairment of macrophage skewing, which in turn inhibits PNS regeneration.

Notably, the different nerve regeneration response of wild-type and *Col6a1*$^{-/-}$ mice is abolished by in vivo macrophage depletion. Moreover, our data demonstrate that the defective PNS regeneration of *Col6a1*$^{-/-}$ mice, and the defective injury-induced macrophage migration and polarization, are rescued by transplantation of wild-type bone marrow cells. Together, these findings highlight the mechanistic insight of collagen VI regulation of macrophage activities as a critical player for PNS regeneration. In addition, we provide evidence showing that the AKT and PKA pathways contribute to collagen VI-regulated macrophage function. Addition of collagen VI to cultured macrophages promotes the activation of AKT and PKA, whereas collagen VI ablation abolishes IL-4-induced activation of both signals. Blockade of AKT and PKA by their inhibitors abrogates collagen VI-induced

macrophage migration and polarization. Furthermore, our in vivo data indicate that the increased activation of AKT and PKA in injured nerves is completely inhibited by collagen VI ablation. Our results on the one hand support the concept that AKT and PKA pathways are necessary for macrophage migration and polarization [3, 10, 11, 27, 38] and axonal regeneration [9, 17]; on the other hand, they provide insights into the downstream targets of collagen VI-regulated macrophage function in PNS regeneration.

In summary, we demonstrate in this study that collagen VI is a pivotal factor for macrophage function. In this context, besides providing novel molecular understanding for macrophage migration and polarization, our study points out potentially broad implications for collagen VI in inflammatory diseases. These data also provide evidence for a beneficial impact of collagen VI on peripheral nerve regeneration via modulation of macrophage activities. In addition to contributing to the understanding of the roles of collagen VI in the experimental setting of PNS regeneration, our findings might have useful implications for clinical study. For example, it is reasonable that application of collagen VI as a coating substrate for the artificial nerve guide conduits may be beneficial for improving the peripheral nerve functional recovery in patients. Future studies will allow testing this possibility and evaluating the effectiveness of such regenerative approaches.

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Conflict of interest The authors declare no potential conflicts of interest.

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Chapter 3

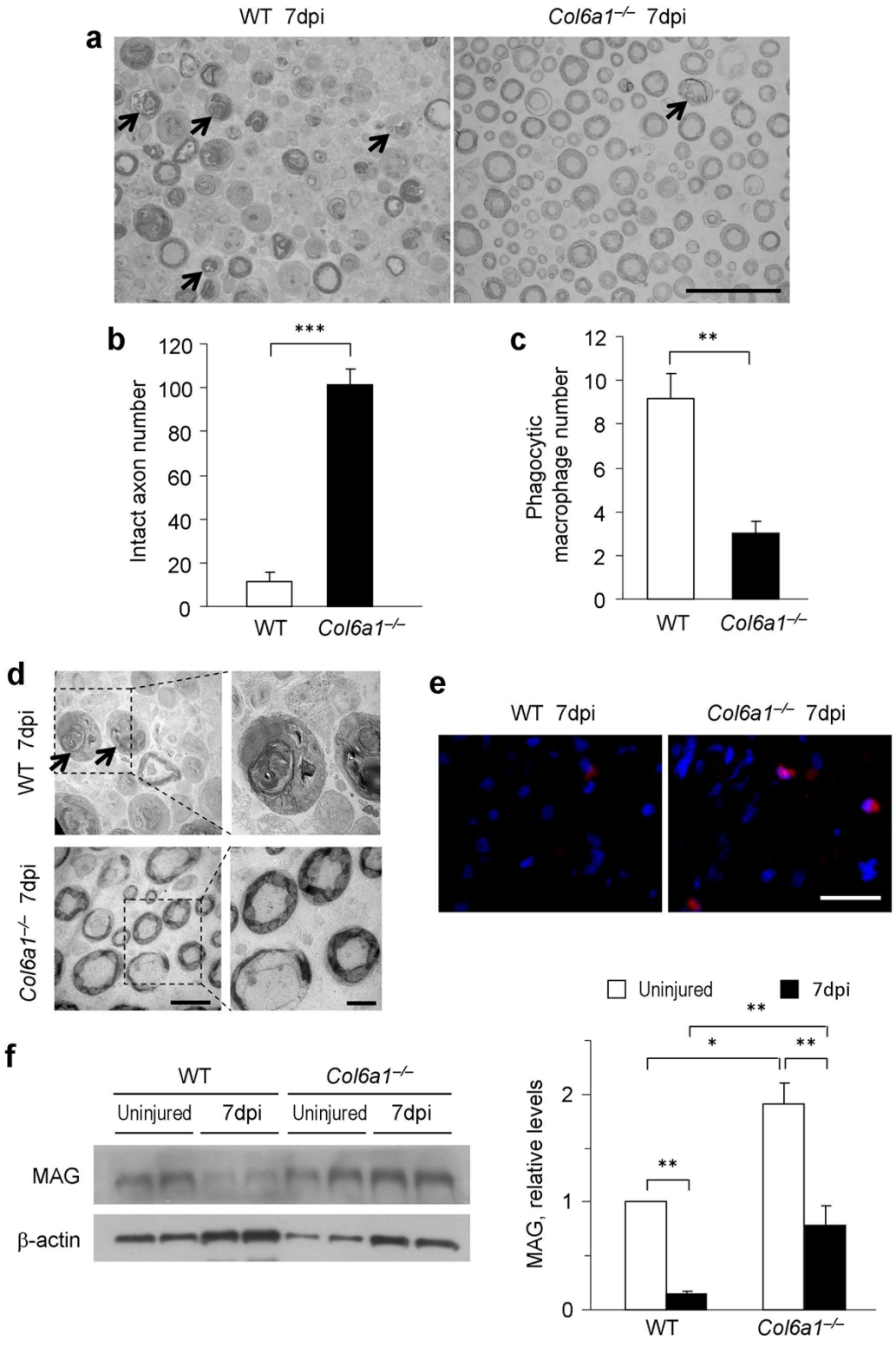
Chen P, Cescon M, Zuccolotto G, Nobbio L, Colombelli C, Filaferro M, Vitale G, Feltri ML, Bonaldo P. Collagen VI regulates peripheral nerve regeneration by modulating macrophage recruitment and polarization. **Acta Neuropathol**. 2015 Jan; 129(1):97-113.

Supplemental information contains:

Supplementary Figures S1 to S8

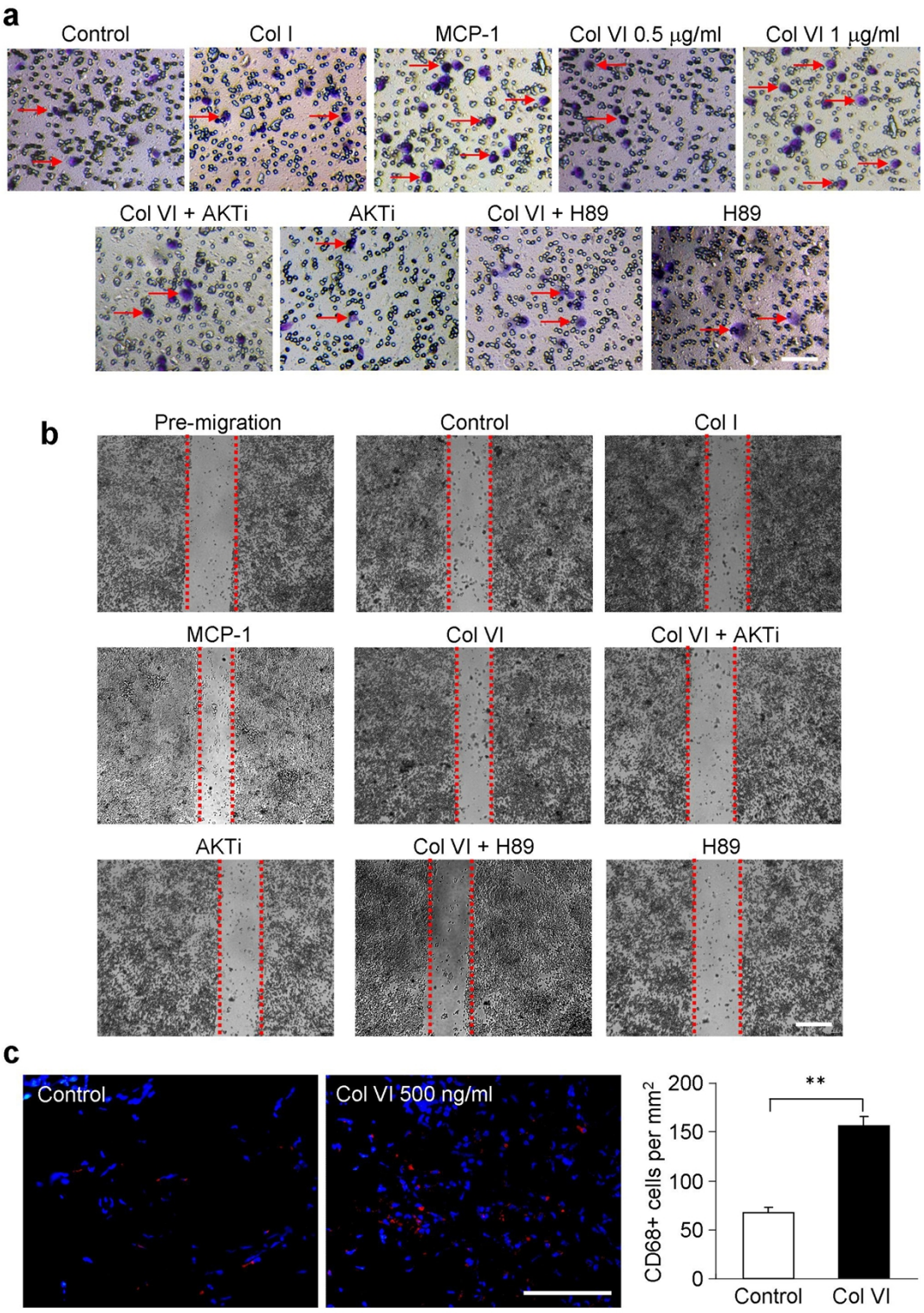
Supplementary Tables S1,S2

Supplementary Figure S1



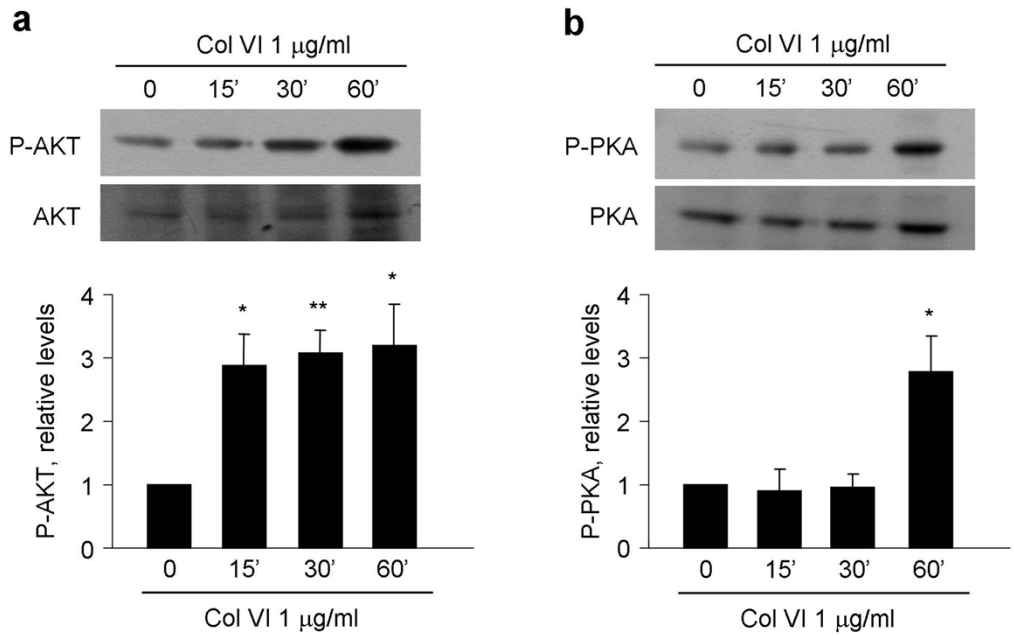
Supplementary Figure S1. Wallerian degeneration is inhibited in injured *Col6a1*$^{-/-}$ nerves. (**a**) Representative images of toluidine blue staining in cross-sections of injured sciatic nerves at 7 days post-crush from wild-type and *Col6a1*$^{-/-}$ mice. Arrows indicate phagocytic macrophages. Scale bar, 40 μm. (**b,c**) Quantification of the number of fibers with intact myelin sheaths (**b**) and of phagocytic macrophages (**b**) in cross-sections of sciatic nerves at 7 days post-crush from wild-type and *Col6a1*$^{-/-}$ mice. (n = 4; **, $P < 0.01$; ***, $P < 0.001$). (**d**) Left panels, representative electron micrographs of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice at 7 days post-crush. Right panels, higher magnification images showing the areas marked in left panels. Arrows indicate phagocytic macrophages. Scale bar, 10 μm (left panels) or 4 μm (right panels). (**e**) Immunofluorescence for MAG in cross-sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice at 7 days post-crush. Scale bar, 25 μm. (**f**) Left panel, western blot for MAG in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. Right panel, densitometric quantification of MAG vs actin, as determined by three independent western blot experiments. Values for uninjured wild-type nerve were arbitrarily set to 1 (n = 4; *, $P < 0.05$; **, $P < 0.01$). dpi, days post-injury; WT, wild-type.

Supplementary Figure S2



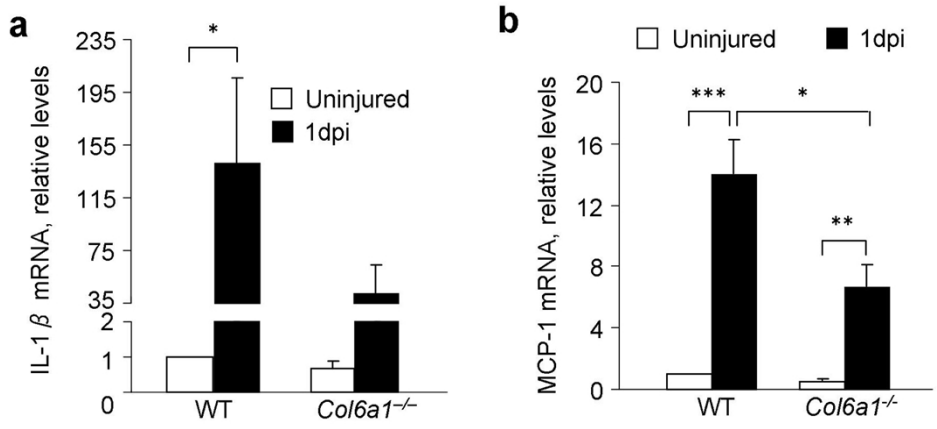
Supplementary Figure S2. Collagen VI promotes *in vitro* macrophage migration. (**a**) Representative images of migrated J774 macrophages in transwell migration assays upon stimulation with PBS (control), collagen I, MCP-1 or collagen VI in the absence or presence of AKTi or H89. The red arrows indicate the migrated macrophages. Scale bar, 50 μm. (**b**) Representative images of J774 macrophages migrating into the wounded area when treated for 8 h with PBS (control), collagen I, MCP-1 or collagen VI in the absence or presence of AKTi or H89, and subjected to a scratch assay. The red dotted lines indicate the migration front of macrophages. Scale bar, 500 μm. (**c**) Left and middle panels, representative images of immunofluorescence for CD68 in growth factor-reduced Matrigel plugs supplemented with PBS (left) or purified collagen VI at 0.5 μg/ml (middle) subcutaneously injected into wild-type mice. Scale bar, 100 μm. Right panel, quantitative analysis of migrated CD68-positive macrophages in Matrigel plugs (n = 3; ** $P < 0.01$). AKTi, AKT inhibitor; Col I, collagen I; Col VI, collagen VI.

Supplementary Figure S3



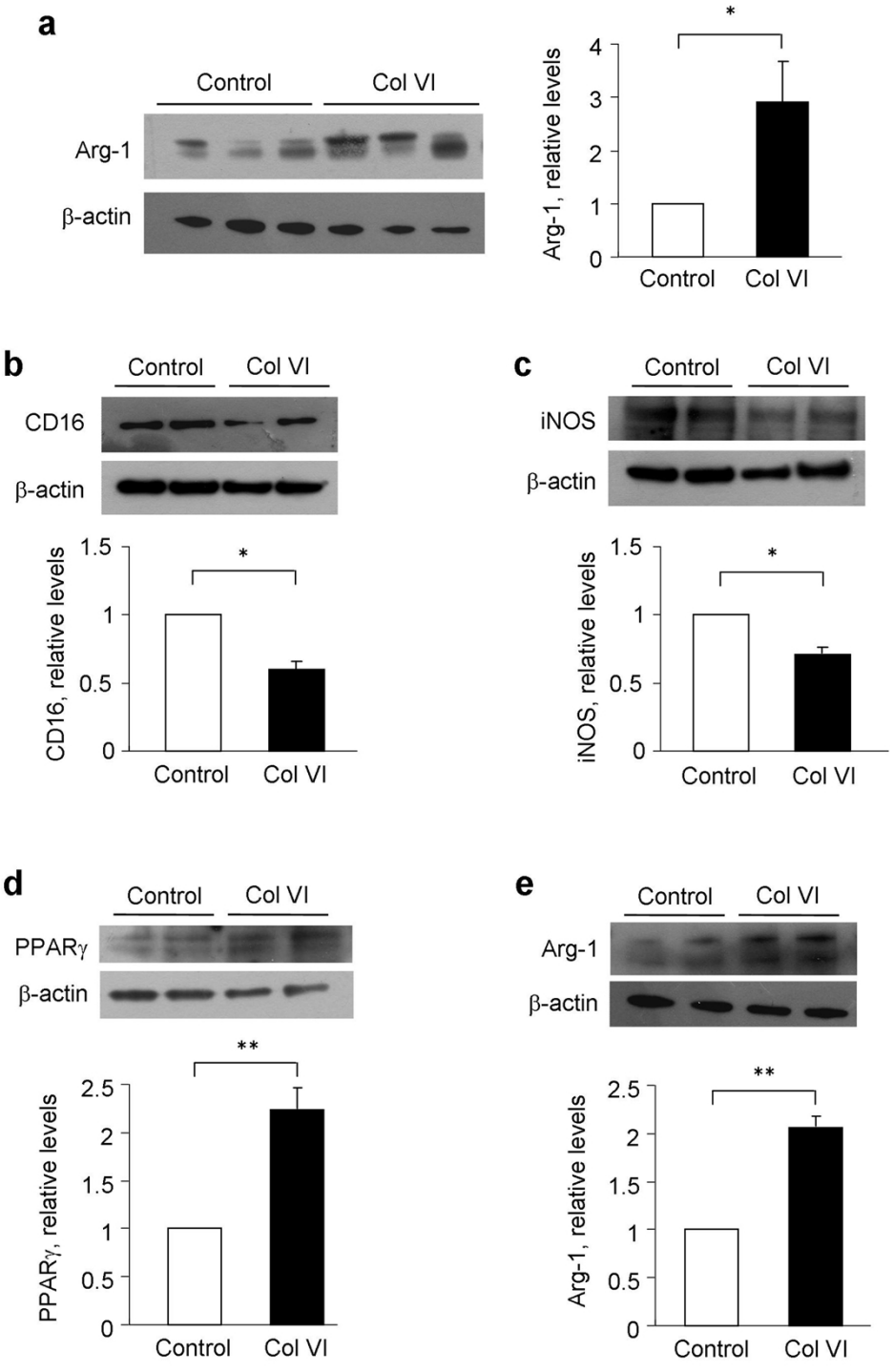
Supplementary Figure S3. Collagen VI activates AKT and PKA pathways in macrophages. (**a,b**) Top panels, western blot for total and phosphorylated AKT (**a**) and for total and phosphorylated PKA (**b**) in J774 macrophages following treatment with collagen VI (1 μg/ml) for the indicated times. Densitometric quantifications, as determined by three independent western blot experiments, are shown in bottom panels and expressed as the ratio of phospho-AKT vs total AKT or of phospho-PKA vs total PKA. Values for cells without collagen VI treatment were arbitrarily set to 1 ($n = 3$; *, $P < 0.05$; **, $P < 0.01$). Col VI, collagen VI.

Supplementary Figure S4



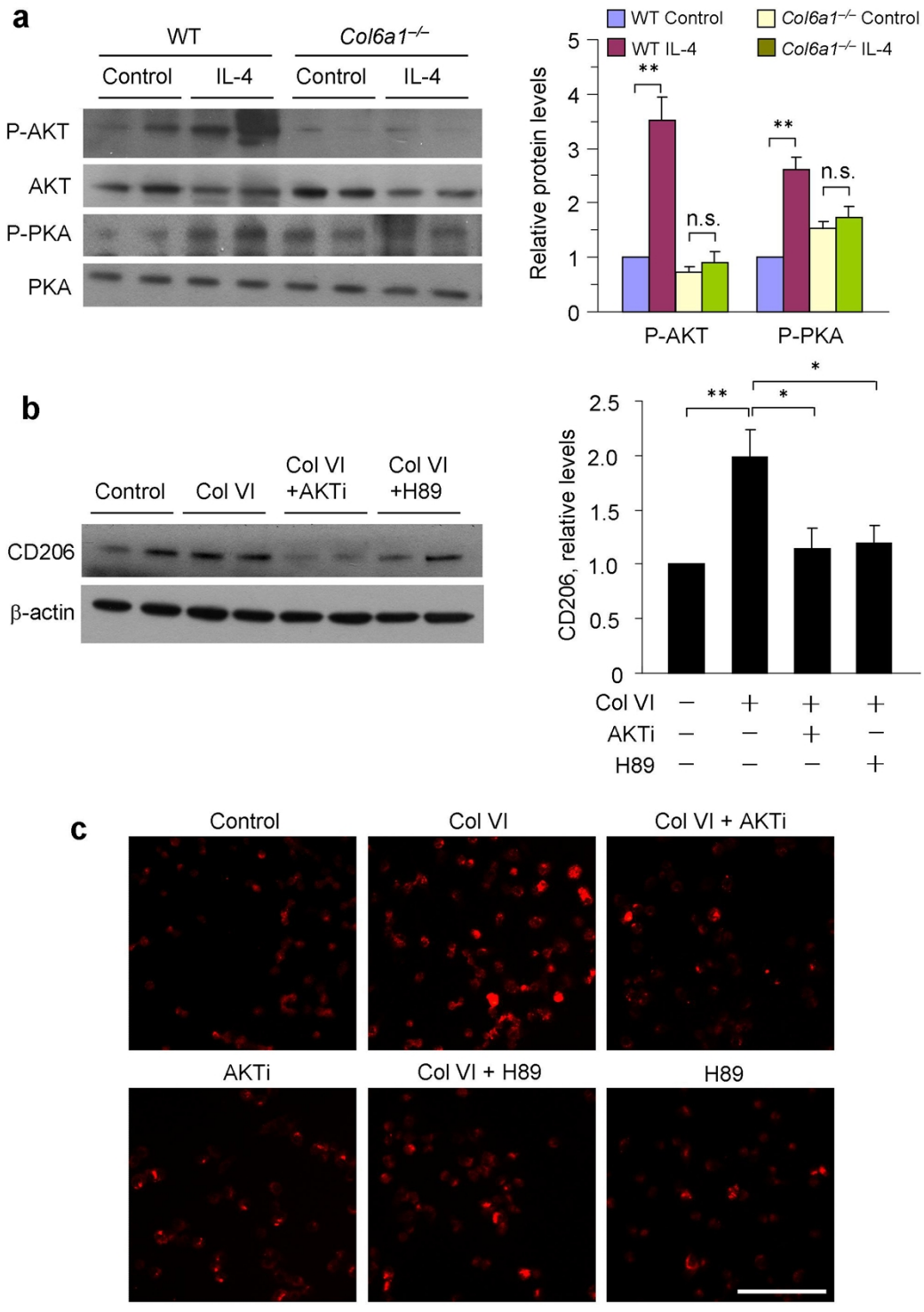
Supplementary Figure S4. Lack of collagen VI causes impaired cytokine production after injury. (**a**,**b**) Real-time RT-PCR analysis for IL-1β (**a**) and MCP-1 (**b**) mRNAs in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 1 day post-crash. Values for uninjured wild-type nerve were arbitrarily set to 1. GAPDH was used as a reference gene (n = 3-5; *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$). dpi, days post-injury; WT, wild-type.

Supplementary Figure S5



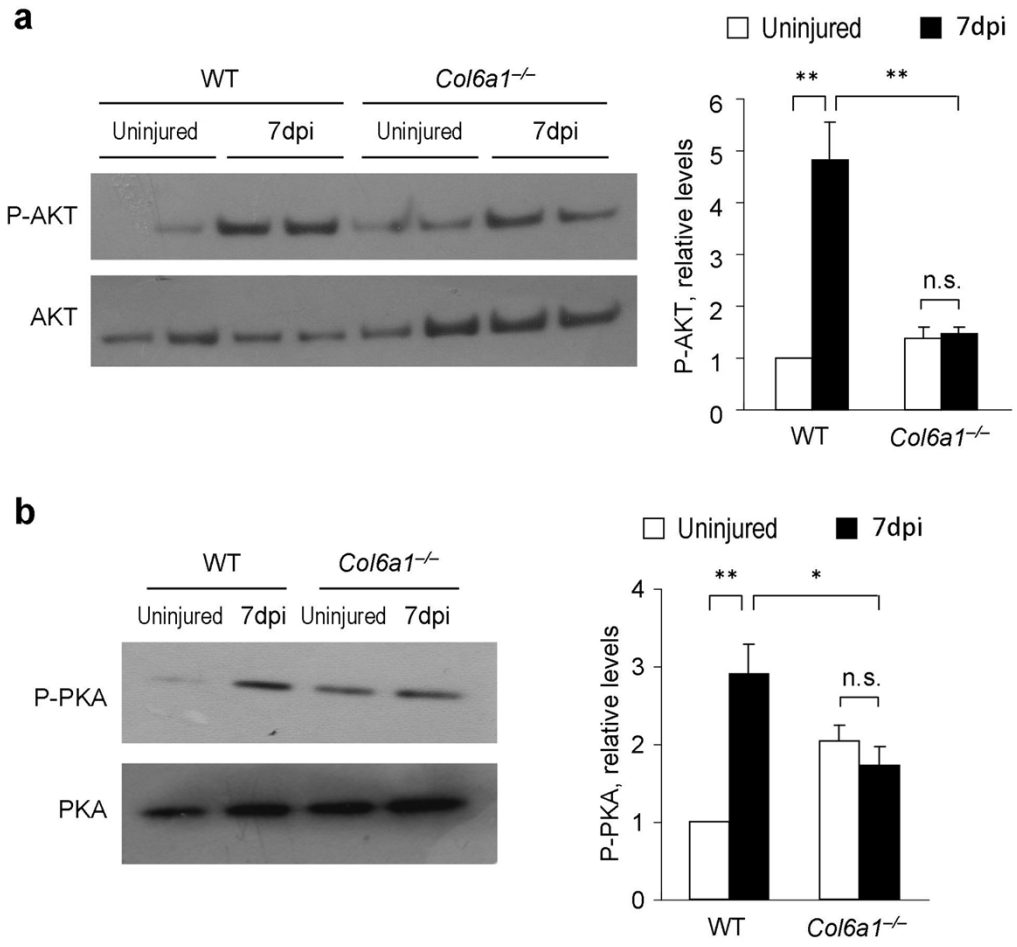
Supplementary Figure S5. Collagen VI promotes macrophage M2 polarization, but inhibits M1 polarization. (**a-c**) Western blot for Arg-1 (**a**), CD16 (**b**) and iNOS (**c**) in J774 macrophages upon treatment with BSA (control) or with purified collagen VI (1 μg/ml) for 24 h. (**d,e**) Western blot for PPAR□ (**d**) and Arg-1 **(e**) in PMs upon treatment with BSA (control) or with purified collagen VI (1 μg/ml) for 24 h. Densitometric quantifications, as determined by three independent western blot experiments, are shown on the right (**a**) or in the bottom (**b-e**) panels and expressed as the ratio of each protein vs actin. Values for control cells were arbitrarily set to 1 (n = 4; *, $P < 0.05$; **, $P < 0.01$). Col VI, collagen VI.

Supplementary Figure S6



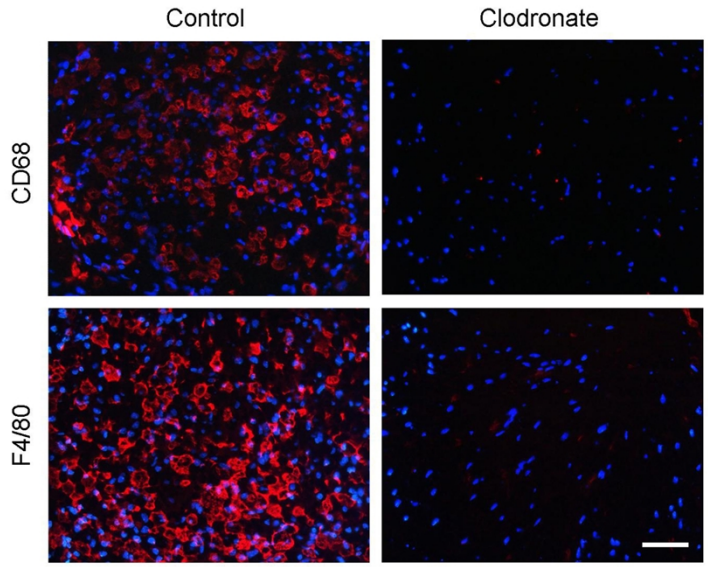
Supplementary Figure S6. Collagen VI promotes macrophage M2 polarization via AKT and PKA pathways. (**a**) Left panel, western blot for total and phosphorylated AKT, and for total and phosphorylated PKA in wild-type and *Col6a1*$^{-/-}$ PMs under control conditions or following induction with IL-4. Right panel, densitometric quantification of phospho-AKT vs total AKT or of phospho-PKA vs total PKA, as determined by three independent western blot experiments. Values for wild-type cells without IL-4 treatment were arbitrarily set to 1 (n = 3; **, $P < 0.01$; n.s., not significant). (**b**) Left panel, western blot for CD206 in BMDMs under control conditions or following treatment for 24 h with purified collagen VI (1 μg/ml), in the absence or presence of AKTi (10 μM) or H89 (30 μM). Right panel, densitometric quantification of CD206 vs actin, as determined by three independent western blot experiments. Values for cells without collagen VI treatment were arbitrarily set to 1 (n = 4; *, $P < 0.05$; **, $P < 0.01$). (**c**) Immunofluorescence for PPARγ in J774 macrophages under control conditions or following treatment for 24 h with purified collagen VI (as a coating substrate at 5 μg/cm^2), in the absence or presence of AKTi (10 μM) or H89 (30 μM). Scale bar, 100 μm. AKTi, AKT inhibitor; Col VI, collagen VI; WT, wild-type.

Supplementary Figure S7



Supplementary Figure S7. Lack of collagen VI impairs AKT and PKA activation upon sciatic nerve crush injury. (**a,b**) Left panels, western blot for total and phosphorylated AKT (**a**) and for total and phosphorylated PKA (**b**) in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 day post-crush. Right panels, densitometric quantification of phospho-AKT vs total AKT or of phospho-PKA vs total PKA, as determined by three independent western blot experiments. Values for uninjured wild-type nerves were arbitrarily set to 1 ($n = 4$; *, $P < 0.05$; **, $P < 0.01$; n.s., not significant). dpi, days post-injury; WT, wild-type.

Supplementary Figure S8



Supplementary Figure S8. Clodronate liposomes deplete macrophages in sciatic nerves after crush. Immunofluorescence for CD68 and F4/80 in cross-sections of sciatic nerves from wild-type mice at 7 days post-injury and receiving PBS liposomes or clodronate liposomes. Scale bar, 50 μm.

Supplementary Table S1. RT-PCR primer sequences.

Protein	Gene	Primer sequence
α1(VI)	*Col6a1*	Forward: 5'– TGCCCTGTGGATCTATTCTTCG –3' Reverse: 5'– CTGTCTCTCAGGTTGTCAATG –3'
α2(VI)	*Col6a2*	Forward: 5'– CTACTCACCCCAGGAGCAGGAA –3' Reverse: 5'– TCAACGTTGACTGGGCGATCGG –3'
α3(VI)	*Col6a3*	Forward: 5'– AACCCTCCACATACTGCTAATTC –3' Reverse: 5'– TCGTTGTCACTGDCTTCATT –3'
IL-1β	*Il1b*	Forward: 5'– ACCTGTGTCTTTCCCGTGGAC –3' Reverse: 5'– GGGAACGTCACACACCAGCA –3'
MCP-1	*Ccl2*	Forward: 5'– GAGAGCTACAAGAGGATCACCA –3' Reverse: 5'– GTATGTCTGGACCCATTCCTTC –3'
Arg-1	*Arg1*	Forward: 5'– GAACACGGCAGTGGCTTTAAC –3' Reverse: 5'– TGCTTAGCTCTGTCTGCTTTGC –3'
CD206	*Mrc1*	Forward: 5'– GGGCAATGCAAATGGAGCCG –3' Reverse: 5'– TCCACACCAGAGCCATCCGT –3'
GAPDH	*Gapdh*	Forward: 5'– GGGAAGCCCATCACCATCTT –3' Reverse: 5'– GCCTTCTCCATGGTGGTGAA –3'

Supplementary Table S2. Macrophage depletion leads to similar toe spread reflex responses in wild-type and collagen VI-deficient mice.

dpi / Groups	7	11	15	17
WT PBS	2/5	3/5	5/5	5/5
Col6a1$^{-/-}$ PBS	0/6	1/6	2/6 *	5/6
WT clodronate	0/5	0/5	1/5 ^^	2/5 ^
Col6a1$^{-/-}$ clodronate	0/6 n.s.	0/6 n.s.	0/6 n.s.	1/6 # n.s.

*, $P < 0.05$, *Col6a1*$^{-/-}$ PBS vs wild-type PBS; ^, $P < 0.05$ and ^^, $P < 0.01$, wild-type clodronate vs wild-type PBS; #, $P < 0.05$, *Col6a1*$^{-/-}$ clodronate vs *Col6a1*$^{-/-}$ PBS; n.s., not significant, *Col6a1*$^{-/-}$ clodronate vs wild-type clodronate. dpi, days post-injury; WT, wild-type.

Chapter 4

Lack of collagen VI promotes wound-induced hair growth

Abstract

Collagen VI is an extracellular matrix molecule abundantly expressed in the skin. However, the role of collagen VI in hair follicle growth is unknown. Here, we show that collagen VI is strongly deposited in hair follicles, and dramatically upregulated by skin wounding. Lack of collagen VI in *Col6a1*$^{-/-}$ mice promotes wound-induced hair regrowth, but not affects skin regeneration. Conversely, addition of purified collagen VI rescues the abnormal wound-induced hair regrowth in *Col6a1*$^{-/-}$ mice. Mechanistic studies revealed that the increased wounding-induced hair regrowth of *Col6a1*$^{-/-}$ mice is triggered by upregulation of Keratin 79 and activation of the Wnt/β-catenin signaling pathway, and is abolished by inhibition of the Wnt/β-catenin pathway. These findings highlight the essential relationships between extracellular matrix and hair follicle regeneration, and suggest that collagen VI could be a potential therapeutic target for hair loss and other skin-related diseases.

Introduction

Mammalian skin is a complex organ that contains three epithelial compartments, including the interfollicular epidemis, sebaceous glands and hair follicles (Stenn and Paus, 2001). Each hair follicle in adult mammals goes through cycles of anagen (growth), catagen (regression) and telogen (quiescence) phases, a process which rely on a group of stem cells, including bulge cells and secondary hair germ (Greco *et al.*, 2009; Myung and Ito, 2012). In addition, hair follicles play an important role in early epidermal repair following skin wounding (Ito *et al.*, 2005), where they regenerate *de novo* in adult mice in a manner similar to embryonic hair follicle development (Ito *et al.*, 2007). Remarkably, the expression pattern of epithelial stem cells in hair follicles around wound regions, and the signals coordinating the growth and activation of follicular epithelial cells, are similar to that in embryonic hair development (Ito and Kizawa, 2001; Millar, 2002). These findings suggest that characterization of the molecular signals governing the wound-induced hair regrowth may reveal the general understanding of hair growth. The central pathway that mediates wound-induced hair follicle regeneration is Wnt/β-catenin (Ito *et al.*, 2007; Myung *et al.*, 2013; Gay *et al.*, 2013). However, it is currently unclear how Wnt/β-catenin signaling is regulated during hair follicle regeneration.

It has been shown that the onset of epithelial stem cell development is triggered by environmental signals, defined as niche (Fuchs *et al.*, 2004). Extracellular matrix (ECM) is an important hair follicle stem cell niche, which regulates bulge cell behavior and hair development (Gattazzo *et al.*, 2014). Collagen VI is a major ECM component made of three genetically chains encoded by distinct genes (*Col6a1*, *Col6a2*, *Col6a3*) and abundantly deposited in a variety of tissues, including skin (Chen *et al.*, 2014b; Chen *et al.*, 2014a; Chen *et al.*, 2013). Our recent work demonstrated that collagen VI is a key component of adult muscle stem cell niche required for proper muscle regeneration after injury (Urciuolo *et al.*, 2013). These findings suggest that collagen VI may play critical functions in stem cell niches in tissues, thus affecting tissues homeostasis and regeneration. Microarray studies showed that the expression levels of *Col6a1* and *Col6a2* genes in mouse bulge cells are higher than in differentiated keratinocytes (Fujiwara *et al.*, 2011). However, it is

still completely unknown whether collagen VI contributes to hair follicle growth and regeneration.

Here we show that collagen VI is abundantly deposited in hair follicles and upregulated by skin wounding. By using collagen VI null (*Col6a1*$^{-/-}$) mice, where a targeted inactivation of the *Col6a1* gene prevents the assembly and secretion of collagen VI in the ECM (Chen *et al.*, 2014b; Bonaldo *et al.*, 1998; Irwin *et al.*, 2003), we demonstrate that lack of collagen VI promotes wound-induced hair regrowth by regulating Keratin (K) 79 and Wnt/β-catenin signaling pathway.

Materials and Methods

Animals. *Col6a1*$^{+/+}$ (wild-type) and *Col6a1*$^{-/-}$ mice in the C57BL/6 background (Bonaldo *et al.*, 1998; Irwin *et al.*, 2003) were used in this study. The *in vivo* experiments were performed in 2-3 month-old mice. Native collagen VI protein was purified from newborn mice as previously described (Irwin *et al.*, 2003). Animal procedures were authorized by the Ethics Committee of the University of Padova and by the Italian Ministry of Health.

Surgical procedure. Mice were anesthetized with xylazine (8 mg/kg body weight) and ketamine (100 mg/kg body weight), and the dorsal hair was shaved. Skin wounds were made by excising a 1 cm^2 square of full-thickness dorsal skin (utilized for investigating wound healing and hair regrowth) or by cutting three 6-mm wounds (1 midline and 2 on each side of the midline).

Histology. After perfusion with 4% paraformaldehyde, the dorsal skin of healthy and wounds from wild-type and *Col6a1*$^{-/-}$ mice was removed and postfixed for 4 h at 4 °C. Tissues were transferred into 30% sucrose overnight for cryoprotection, and then cut at 10 μm using cryostat (Leica). Samples were stained with H&E to determine the hair follicle length using Image-Pro Plus 6.0 software (Media Cybernetics).

RNA isolation and real-time RT-PCR. Total RNA from mouse dorsal skin of healthy and wounds from wild-type mice, was isolated using Trizol reagent (Life Technologies) following the manufacturer's instructions. 200 ng of total RNA was used to make cDNA using Superscript II kit (Invitrogen). Quantitative PCR was carried out using LightCycler 480 system (Roche). The expression level of each gene was calculated by comparing with the *Gapdh* housekeeping gene. Primers used in this study are shown in **Table 1**.

Immunofluorescence. Immunofluorescence was performed on frozen sections (10 μm) of healthy and wounds from wild-type and *Col6a1*$^{-/-}$ mice. After blocking with 10% goat serum for 1 h, sections were incubated with primary antibodies (1:200 dilution) overnight at 4 °C. Primary antibodies against the following proteins were used: β-catenin, Notch1 (rabbit monoclonal, Abcam); collagen VI (rabbit polyclonal; Fitzgerald); DKK-1, α1(VI) collagen (rabbit polyclonal, Santa Cruz Biotechnology); α3(VI) collagen (guinea pig polyclonal, a gift of Raimund Wagener, Cologne, Germany) (Lettmann *et al.*, 2014); K15 (mouse monoclonal, Thermo Scientific). The samples were then transferred to secondary antibodies (1:200 dilution) and Hoechst 33258 (Sigma) for 1 h at room temperature. The following secondary antibodies were used: anti-rabbit CY2, anti-guinea-pig CY2 and anti-mouse CY3 (Jackson Immunoresearch). After washing three times in PBS, slides were mounted using 80% glycerol.

Western blotting. Mice were sacrificed by cervical dislocation and dorsal skin of healthy and wounds from wild-type and *Col6a1*$^{-/-}$ mice were removed and frozen in nitrogen immediately. The tissues were homogenized in lysis buffer (Millipore) with protease inhibitors (Roche, Basel, Switzerland) and phosphatase inhibitors (Sigma). Protein concentration was determined by BCA assay (Thermo Scientific). Samples of

20 μg protein were applied to SDS-PAGE gels (Invitrogen) and blotted onto PDVF membrane (Millipore). Membranes were incubated with primary antibodies (1:1000 dilution) overnight at 4 °C. Primary antibodies against the following proteins were used for western blot: α1/α2 and α3 (VI) collagens; K79 (goat polyclonal, Santa Cruz Biotechnology); β-actin (mouse monoclonal, Sigma); β-catenin and Notch1. After washing three times with TBST, membranes were incubated with HRP-conjugated secondary antibodies (1:1000 dilution; Amersham Bioscience) for 1 h at room temperature. Detection was conducted using chemiluminescence (Pierce). Densitometric quantification was performed by Image-Pro Plus 6.0 software (Media Cybernetics).

Statistical analysis. Data are represented as mean ± SEM. Statistical analysis of data was carried out using the Student's t-test. A P value < 0.05 was considered as a significant difference.

Results

Collagen VI is expressed in hair follicles and regulated by skin wounding

To analyze the deposition of collagen VI in hair follicles, we labeled murine skin with different antibodies against collagen VI chains. Immunofluorescence analysis showed that collagen VI is abundantly deposited in different regions of the hair follicle, including bulge, sebaceous gland, hair germ and dermal papilla (**Figure 1a**). Double immunofluorescence showed partial colocalization of α1(VI) and α3(VI) chains with the bulge stem cell marker K15, suggesting that bulge stem cells may contribute to collagen VI deposition in the hair follicle (**Figure 1b** and **c**). Together with previous microarray data showing that the expression levels of *Col6a1* and *Col6a2* genes in

bulge stem cells are higher than in differentiated keratinocytes (Fujiwara *et al.*, 2011), these findings suggest a potential role for collagen VI in hair follicle.

To explore the role of collagen VI in skin regeneration and wound-induced hair regrowth, we first examined whether skin wounding impinges on collagen VI expression in adult mice. Real-time RT-PCR showed that the levels of *Col6a1*, *Col6a2* and *Col6a3* transcripts were increased at post-wound day 4 and 8 (**Figure 2a-c**). Western blot showed that the protein levels of α1/2(VI) were first decreased at post-wound day 2 and 4 and then increased at post-wound day 8, whereas α3(VI) levels were increased as early as at post-wound day 2 (**Figure 2c** and **d**). Taken together, these data indicate that the expression of collagen VI is strongly regulated upon skin wounding, pointing at a potential role for this molecule during skin regeneration and wound-induced hair regrowth.

Lack of collagen VI promotes wound-induced hair regrowth

Although collagen VI is highly expressed in hair follicles, we did not find any difference in hair growth between wild-type and *Col6a1*$^{-/-}$ mice at various ages under physiological conditions (**Figure 3**). To explore the role of collagen VI in wound healing, full-thickness incisional wounds (1 cm^2 square) were made on the shaved dorsal skin of wild-type and *Col6a1*$^{-/-}$ mice, and wound areas were monitored for two weeks. Surprisingly and unexpectedly, we found that although the wound healing was similar in two genotypes, the wound-induced hair regrowth was dramatically accelerated in *Col6a1*$^{-/-}$ mice (**Figure 4a** and **b**). To further confirm these results, we performed another skin injury model where mice were subjected to three 6-mm incisional wounds. The results demonstrated that the wound-induced hair regrowth in this injury model was also significantly promoted in *Col6a1*$^{-/-}$ mice when compared to wild-type mice (**Figure 4c**). Haematoxylin and eosin (H&E) staining showed that

the length of hair follicle in *Col6a1*$^{-/-}$ mice was significantly higher than that in wild-type mice at post-wound day 8 (**Figure 4d**). Altogether, these findings indicate that lack of collagen VI facilitates wound-induced hair regrowth.

Lack of collagen VI upregulates K79 and activates Wnt/β-catenin pathway

K79-positive cells are a population of migratory epithelial cells that initiate hair regeneration (Veniaminova *et al.*, 2013). We thus investigated whether wound-induced hair regrowth in *Col6a1*$^{-/-}$ animals is regulated by upregulation of K79. Western blot analysis showed that although ablation of collagen VI attenuated K79 expression under physiological conditions, K79 protein levels in skin wounds of *Col6a1*$^{-/-}$ mice were higher than those of wild-type mice at post-wound day 8 (**Figure 5a**), suggesting the contribution of K79-positive cells in the enhanced wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice.

Wnt/β-catenin signaling is a key signaling pathway in wound-induced hair regrowth (Ito *et al.*, 2007). To determine whether Wnt/β-catenin signaling contributes to the enhanced wound-induced hair regrowth of collagen VI deficient mice, we performed a thorough investigation of this pathway in skin wounds of wild-type and *Col6a1*$^{-/-}$ mice. Western blotting showed that although β-catenin levels were decreased upon skin wounding in both genotypes, β-catenin levels in skin wounds of *Col6a1*$^{-/-}$ mice were significantly higher than those of wild-type mice at post-wound day 8 (**Figure 5b**). Immunofluorescence analysis confirmed that β-catenin labeling in hair follicles was much stronger in *Col6a1*$^{-/-}$ mice than wild-type mice at post-wound day 8 (**Figure 5c**). Furthermore, immunofluorescence for the Wnt inhibitor dickkopf-related protein 1(DKK-1) at post-wound day 8 showed lower labeling in the hair follicles of *Col6a1*$^{-/-}$ mice than in those of wild-type mice (**Figure 5d**). These findings indicate

that Wnt/β-catenin signaling is involved in the enhanced wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice.

Notch signaling is involved in embryonic and postnatal hair homeostasis, whereas inactivation of Notch results in hair loss (Vauclair *et al.*, 2005). Thus, we investigated whether Notch signaling is involved in mediating the increased wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice. Western blot and immunofluorescence analysis at post-wound day 8 showed that the levels and reactivity of Notch1 in skin wounds of *Col6a1*$^{-/-}$ mice were similar to those of wild-type mice (**Figure 6a** and **b**). Taken together, these data indicate that upregulation of K79 and activation of Wnt/β-catenin pathway contributing to the enhanced wound-induced hair regrowth in *Col6a1*$^{-/-}$ mice.

The increased wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice is abolished by addition of collagen VI or by inhibition of Wnt/β-catenin pathway

To confirm the role of collagen VI in wound-induced hair regrowth and the underlying molecular mechanism involving modulation of the Wnt/β-catenin signaling pathway, we treated *Col6a1*$^{-/-}$ mice with purified native collagen VI or with the Wnt/β-catenin inhibitor ICG-001 after skin wounding. Notably, the enhanced hair regrowth of *Col6a1*$^{-/-}$ mice was rescued by addition of purified collagen VI protein (**Figure 7a**) and by treatment with ICG-001 (**Figure 7b**), suggesting that the inhibitory effect of collagen VI in wound-induced hair regrowth is regulated by the Wnt/β-catenin pathway. Furthermore, western blot showed that addition of purified collagen VI or of ICG-001 blocked the wound-induced upregulation of K79 and β-catenin in *Col6a1*$^{-/-}$ mice (**Figure 7c**). Altogether, these data indicate that the enhanced wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice is regulated by the activation of Wnt/β-catenin signaling pathway.

Discussion

Previous microarray studies showed that the expression levels of *Col6a1* and *Col6a2* in bulge stem cells are higher than in differentiated keratinocytes, suggesting that collagen VI may contribute to bulge function and hair development. However, a direct evidence for such a role was still lacking (Fujiwara *et al.*, 2011). In this study, we showed that collagen VI has a distinct distribution in hair follicles and responds to skin wounds, and demonstrated that lack of collagen VI in *Col6a1*$^{-/-}$ mice has a strong impact on wound-induced hair regrowth.

Skin is normally able to regenerate hair follicles during wound healing in rodents (Ito *et al.*, 2007; BREEDIS, 1954). However, wound healing sometimes results in inadequate tissue regeneration by fibrosis or scarring, especially for the cutaneous wounds in adult humans (Gay *et al.*, 2013). The underlying mechanisms controlling wound scarring and tissue regeneration are not yet fully understood (Nelson *et al.*, 2013). It has been well established that ECM molecules play an important role in skin wound healing (Olczyk *et al.*, 2014). Given our previous studies showing that collagen VI is essential for muscle regeneration by modulating satellite cell activities (Urciuolo *et al.*, 2013), and for peripheral nerve regeneration by modulating macrophage function (Chen *et al.*, 2014c), we originally predicted that the *Col6a1*$^{-/-}$ mice may have deficits in skin regeneration. However, we found that lack of collagen VI in *Col6a1*$^{-/-}$ mice does not affect skin wound healing (Lettmann *et al.*, 2014). Interestingly, a previous study demonstrated that lack of collagen VI in *Col6a1*$^{-/-}$ mice improves cardiac function, structure and remodeling after myocardial infarction (Luther *et al.*, 2012). These findings suggest that this ECM molecule plays different roles in distinct tissues during regeneration, which may be due to the specific context of each tissue. Further studies will be needed to understand the detailed underlying

mechanisms involved in the specific functions of collagen VI during regeneration in different organs.

Similarly to the previous unexpected finding that *Col6a1*$^{-/-}$ mice have improved cardiac function after myocardial infarction (Luther *et al.*, 2012), in this study we found that lack of collagen VI unexpectedly promotes wound-induced hair regrowth. We speculate that ablation of collagen VI does not alter the hair follicle activities *per se*, because we did not find any difference about hair growth between wild-type and *Col6a1*$^{-/-}$ mice under physiological conditions. These findings suggest that the wound-induced hair regrowth elicited by lack of collagen VI should be regulated by wounding-related cell components and/or signals.

K79-positive cells are a recently identified population of epithelial cells, which is essential for initiating hair canal morphogenesis and regeneration independently of Notch signaling pathway (Veniaminova *et al.*, 2013). Interestingly, our data show higher immunolabeling for K79, but not for Notch1, in skin wounds of *Col6a1*$^{-/-}$ mice than those of wild-type mice. These data suggest that regulation of K79-positive cells serves as a wound-induced mechanism governing the increased hair regrowth in *Col6a1*$^{-/-}$ mice independently of Notch signaling.

The Wnt/β-catenin pathway plays a central role in regulating embryonic and adult hair follicle growth under both physiological and pathological conditions. For example, it has been shown that activation of Wnt/β-catenin signaling is essential for the initiation of embryonic hair follicle development (Andl *et al.*, 2002; Zhang *et al.*, 2009; Huelsken *et al.*, 2001). Wnt/β-catenin signaling is also required for wound-induced hair neogenesis, where overexpression of Wnt7a in mouse epidermis enhances wound-induced hair follicle growth, while development of new hair follicles after wounding is blocked by overexpression of DKK-1 or by ablation of β-catenin

(Ito *et al.*, 2007). Interestingly, in this study we found that the enhanced wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice was associated with higher β-catenin levels at 8 days post-wound, whereas under physiological conditions β-catenin levels were slightly (albeit not significantly) lower in the skin of *Col6a1*$^{-/-}$ mice with respect to wild-type mice. These findings are consistent with previous studies which showed that collagen VI is able to stabilize and activate β-catenin in cancer cells (Iyengar *et al.*, 2005), pointing at a potential positive regulation between collagen VI and Wnt/β-catenin signaling. In this context, it must be underlined that Wnt/β-catenin signaling is dynamically regulated dependent on distinct microenvironments. For example, during the initiation of hair follicle development Wnt/β-catenin signaling is first enhanced uniformly in the upper dermis and then focally in both the underlying dermal condensate and the epithelial hair follicle placode (Zhang *et al.*, 2009; Chen *et al.*, 2012; Myung *et al.*, 2013). In agreement with this concept, previous studies showed that lack of collagen VI inhibits tumor growth by destabilizing and inactivating β-catenin signals (Iyengar *et al.*, 2005), whereas here we show that ablation of collagen VI promotes wound-induced hair regrowth by activation of the Wnt/β-catenin pathway. The contribution of Wnt/β-catenin signals in wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice is also supported by the pharmacological treatment with the Wnt/β-catenin inhibitor ICG-001. Our results show that ICG-001 blocks the increased wound-induced hair regrowth and enhanced K79 expression in *Col6a1*$^{-/-}$ mice, thus providing further evidence that the increased wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice relies upon activation of Wnt/β-catenin signals. Although further studies aimed elucidating in detail the link between collagen VI, skin wounds and Wnt/β-catenin signaling are needed to obtain a thorough understanding of the mechanisms governing hair follicle development and wound-induced hair regrowth,

the present findings reveal a novel and unanticipated role for collagen VI in wound-induced hair regrowth.

The reasons why hair growth is induced by wounding are not yet fully understood (Stenn and Paus, 2001). In the current study, we demonstrate that collagen VI is mainly deposited in hair follicles and is regulated by skin wounding, which in turn regulates hair regrowth by modulation of K79 and Wnt/β-catenin signaling. Interestingly, we found that the enhanced wound-induced hair regrowth in *Col6a1*$^{-/-}$ mice is abolished by treatment with purified collagen VI. Altogether, this study provides novel evidence on the role of specific ECM molecules in wound-induced hair regrowth, and sheds light on the potential therapeutic benefit in accelerating impaired hair growth by targeting collagen VI.

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Figure 1

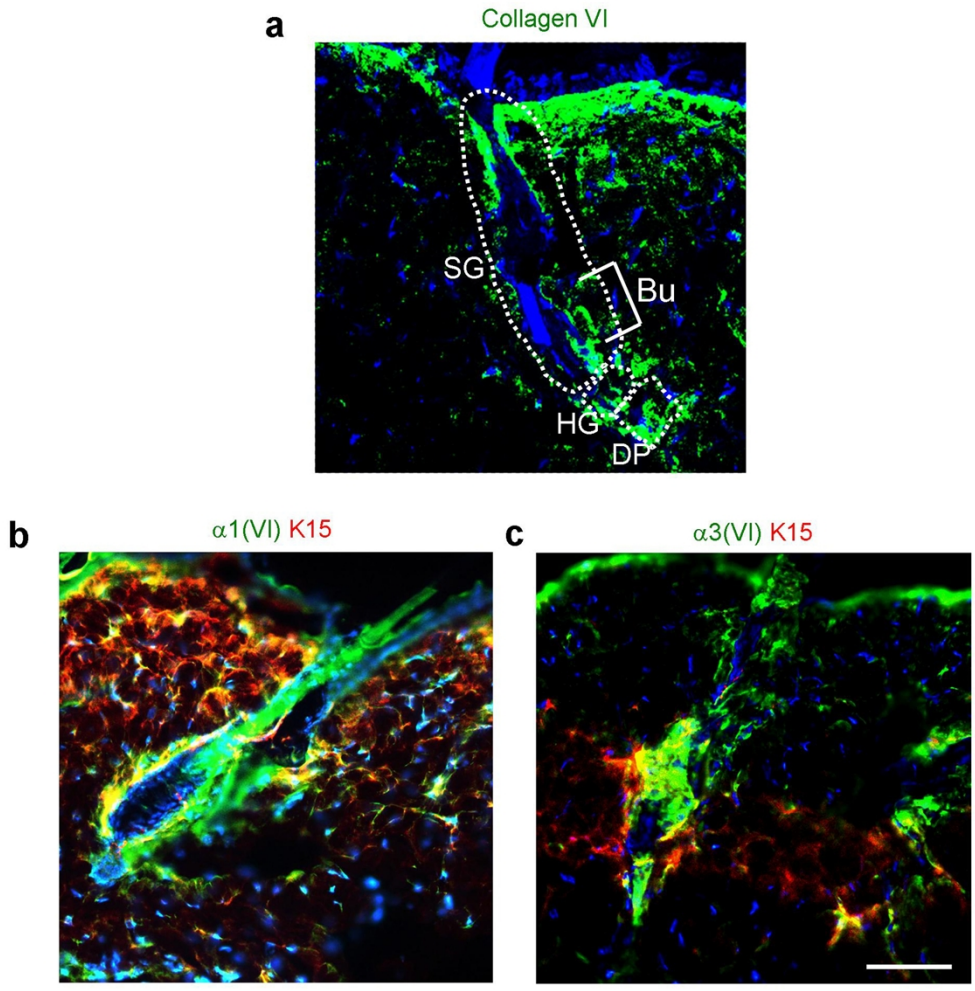

Figure 1. Collagen VI is deposited in hair follicles. (**a**) Immunofluorescence for collagen VI in hair follicles of wild-type adult mice. (**b** and **c**) Co-immunofluorescence labeling of K15 (red) with α1(VI) (green, **b**) and α3(VI) (green, **c**) in hair follicles of wild-type mouse. The dotted areas mark dermal papilla (DP), hair germ (HG) and sebaceous gland (SG) of hair follicle as indicated. Nuclei were stained with Hoechst (blue). Scale bar, 50 μm. Bu, bulge.

Figure 2

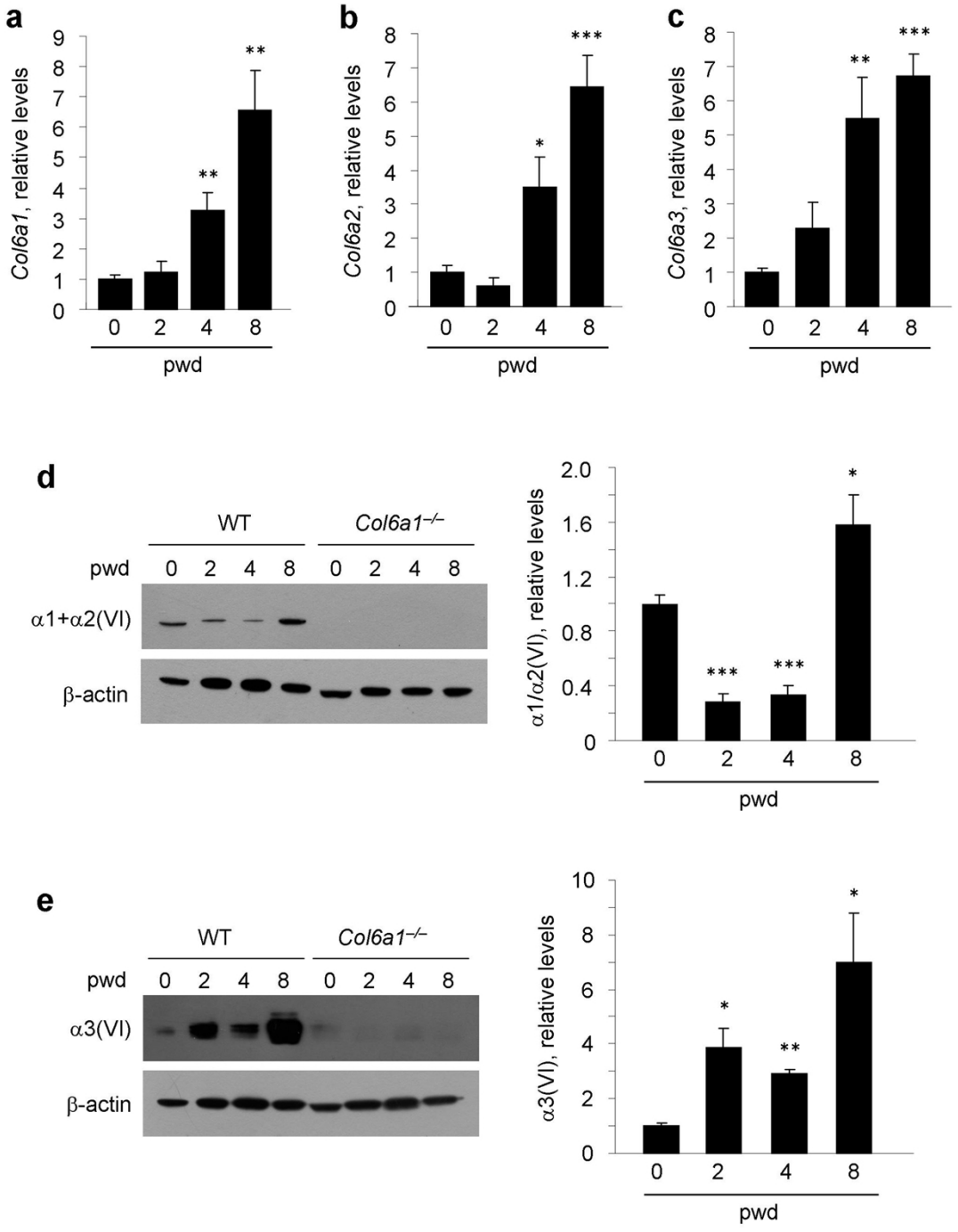
a
Col6a1, relative levels
b
Col6a2, relative levels
c
Col6a3, relative levels
pwd
d
WT
Col6a1−/−
α1+α2(VI)
β-actin
α1/α2(VI), relative levels
e
α3(VI)
α3(VI), relative levels

Figure 2. Skin injury regulates collagen VI expression in wounds. (**a-c**) Real-time RT-PCR for *Col6a1* (**a**), *Col6a2* (**b**) and *Col6a3* (**c**) transcripts in skin from wild-type mice under uninjured conditions (0) and at post-wound day 2, 4 and 8 (n = 5; *, P <0.05; **, P < 0.01; ***, P <0.001). (**d** and **e**) Left panels, western blot for α1/α2(VI) and α3(VI) in skin from wild-type mice under uninjured conditions (0) and at post-wound day 2, 4 and 8. Right panels, densitometric quantification of α1/α2(VI) vs actin (**d**) and α3(VI) vs actin (**e**), as determined by three independent western blot experiments (n = 4-5; *, P <0.05; **, P < 0.01; ***, P <0.001). pwd, post-wound day.

Figure 3

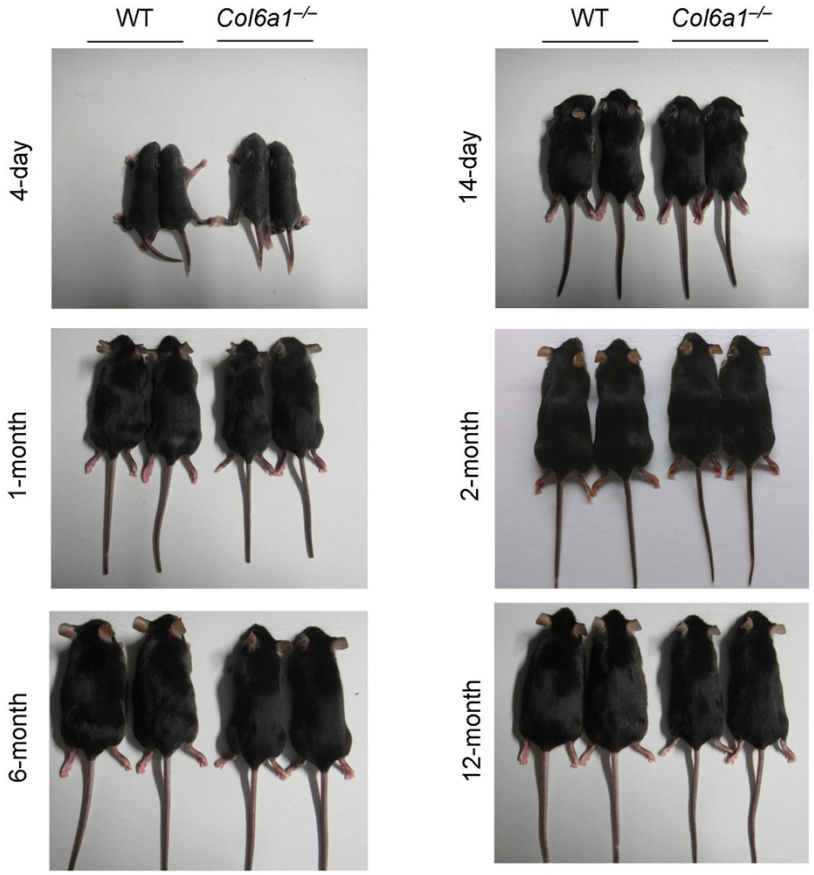

Figure 3. Ablation of collagen VI does not affect hair growth under physiological conditions. Representative images showing the hair growth in wild-type and *Col6a1*$^{-/-}$ mice under physiological conditions at different ages as indicated.

Figure 4

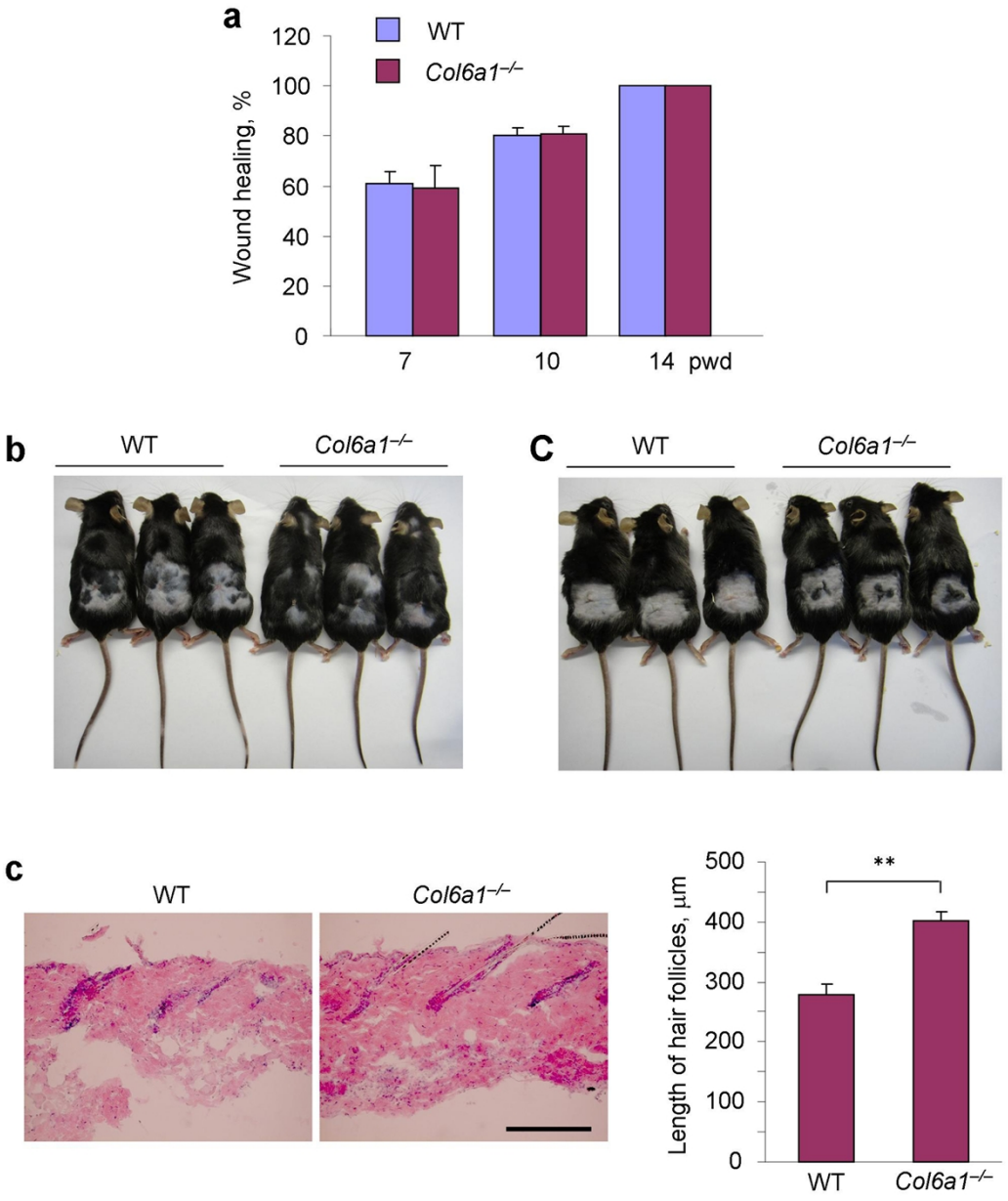
a
WT
Col6a1−/−
Wound healing, %
120
100
80
60
40
20
0
7
10
14 pwd
b
WT
Col6a1−/−
C
WT
Col6a1−/−
c
WT
Col6a1−/−
Length of hair follicles, µm
500
400
300
200
100
0
**
WT
Col6a1−/−

Figure 4. Lack of collagen VI does not affect wound healing, but promotes wound-induced hair regrowth. (**a**) Quantification of the percentage of initial wound areas in wild-type and *Col6a1*$^{-/-}$ mice after excising a 1 cm^2 square of full-thickness dorsal skin (n = 3). (**b** and **c**) Wound-induced hair regrowth in *Col6a1*$^{-/-}$ mice is faster than in wild-type mice in the injury models obtained by excising a 1 cm^2 square of full-thickness dorsal skin (**b**) or by excising three 6-mm wounds (**c**). Photographs were taken at post-wound day 14. (**d**) H&E staining in skin wounds from wild-type and *Col6a1*$^{-/-}$ mice at post-wound day 8. Scale bar, 200 μm. The quantification of hair follicle lengths is shown in the right panel (**, $P < 0.01$; n = 3-4). pwd, post-wound day; WT, wild-type.

Figure 5

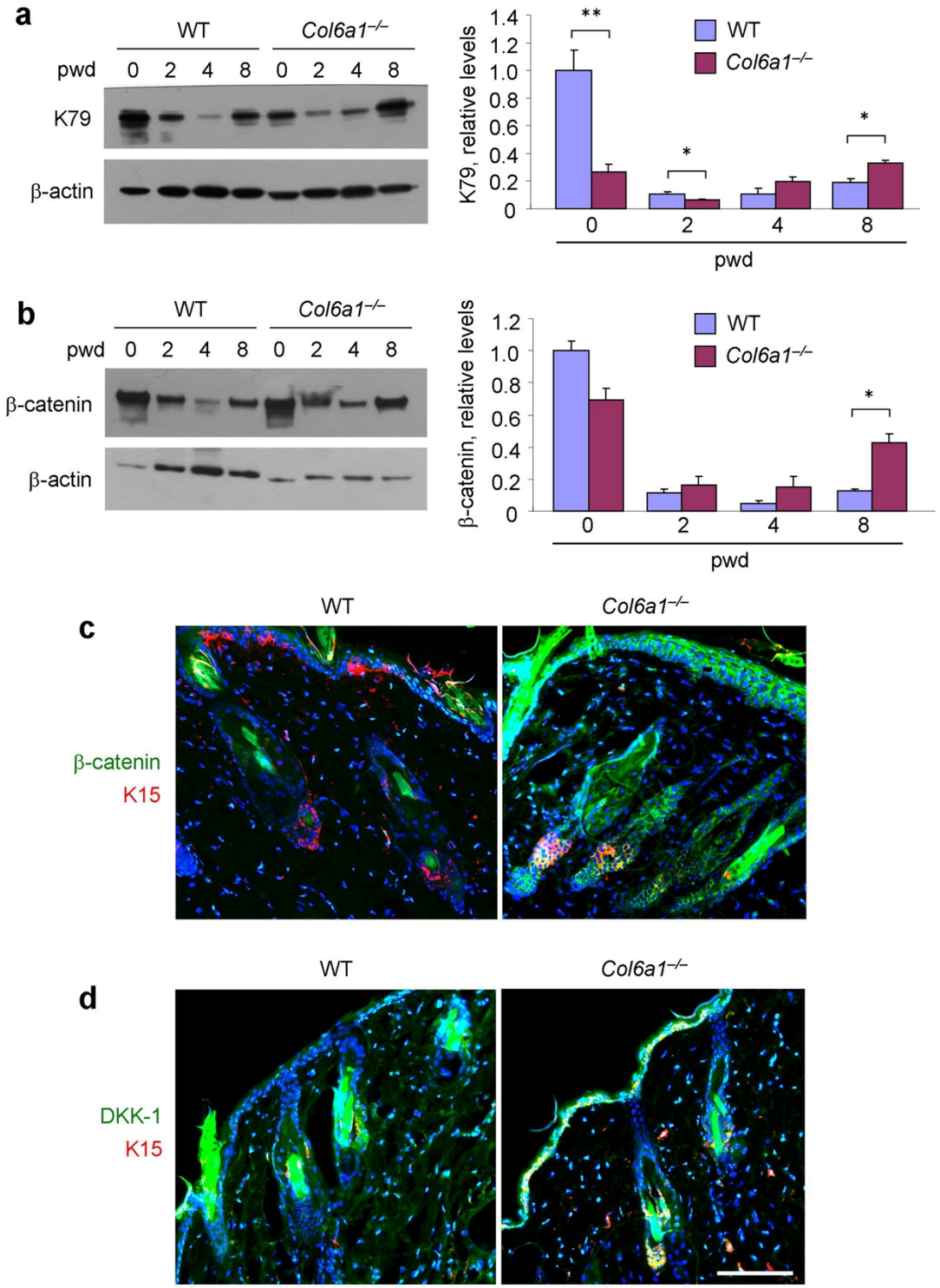
a
WT
Col6a1−/−
pwd 0 2 4 8 0 2 4 8
K79
β-actin
K79, relative levels
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
*
*
WT
Col6a1−/−
0 2 4 8
pwd
b
WT
Col6a1−/−
pwd 0 2 4 8 0 2 4 8
β-catenin
β-actin
β-catenin, relative levels
1.2
1.0
0.8
0.6
0.4
0.2
0
*
WT
Col6a1−/−
0 2 4 8
pwd
c
WT
Col6a1−/−
β-catenin
K15
d
WT
Col6a1−/−
DKK-1
K15

Figure 5. Ablation of collagen VI increases K79 expression and activates Wnt/β-catenin signaling after skin wounding. (**a** and **b**) Left panels, western blot for K79 (**a**) or β-catenin (**b**) in skin from wild-type mice and *Col6a1*$^{-/-}$ mice under uninjured conditions (0) and at post-wound day 2, 4 and 8. Right panels, densitometric quantification of K79 vs actin (**a**) or β-catenin vs actin (**b**), as determined by three independent experiments. Values for uninjured wild-type skin were arbitrarily set to 1 ($n = 4$; *, $P < 0.05$; **, $P < 0.01$). (**c** and **d**) Co-immunofluorescence labeling of K15 (red) with β-catenin (green, **c**) or DKK-1 (green, **d**) in skin from wild-type and *Col6a1*$^{-/-}$ mice at post-wound day 8. Nuclei were stained with Hoechst (blue). Scale bar, 100 μm. pwd, post-wound day; WT, wild-type.

Figure 6

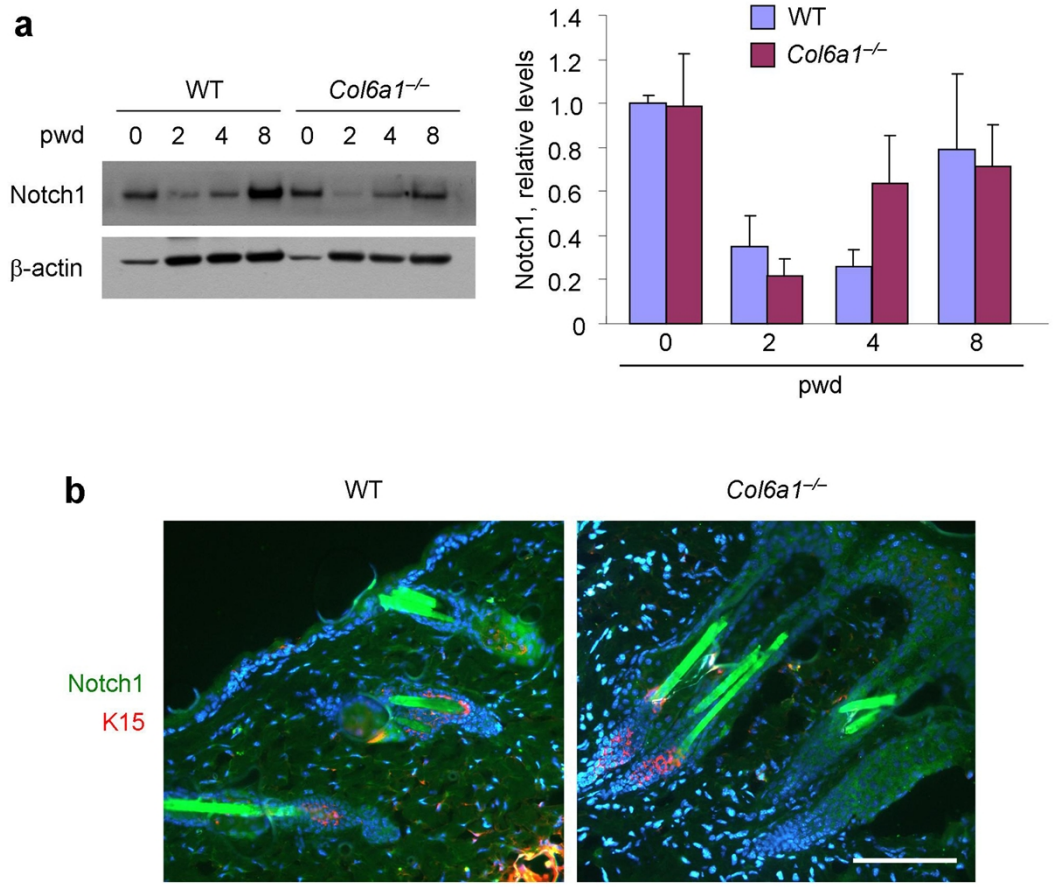

Figure 6. Lack of collagen VI does not affect Notch1 signaling after skin wounding. (**A**) Left panel, western blot for Notch1 in skin from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions or at post-wound day 2, 4 and 8. Right panel, densitometric quantification of Notch1 vs actin as determined by three independent western blot experiments. Values for uninjured wild-type skin were arbitrarily set to 1 (n = 3). (**B**) Co-immunofluorescence labeling of K15 (red) with Notch1 (green) in skin wounds from wild-type and *Col6a1*$^{-/-}$ mice at post-wound day 8. Nuclei were stained with Hoechst (blue). Scale bar, 100 μm. pwd, post-wound day; WT, wild-type.

Figure 7

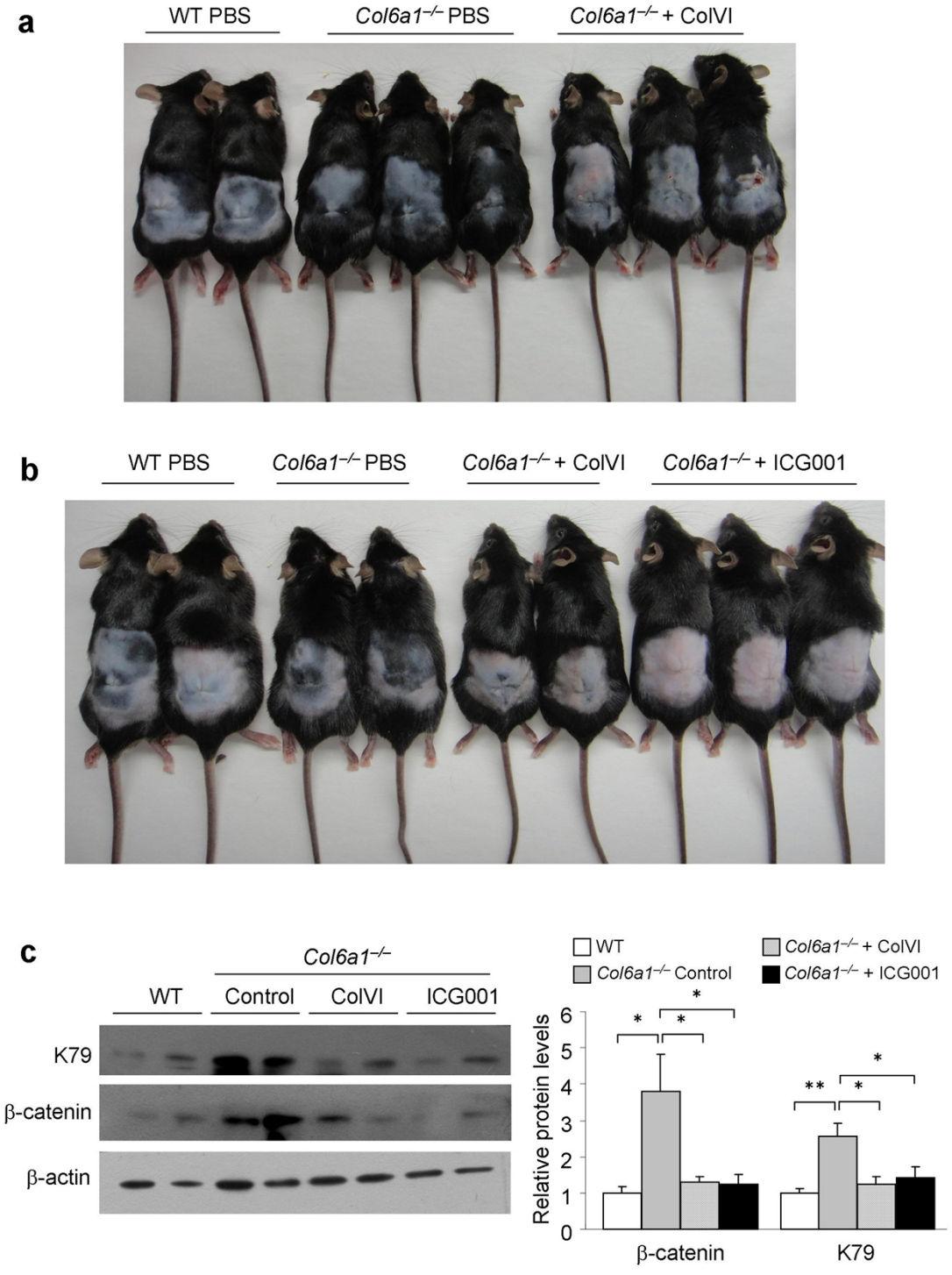
a
WT PBS
Col6a1−/− PBS
Col6a1−/− + ColVI
b
WT PBS
Col6a1−/− PBS
Col6a1−/− + ColVI
Col6a1−/− + ICG001
c
Col6a1−/−
WT
Control
ColVI
ICG001
K79
β-catenin
β-actin
WT
Col6a1−/− Control
Col6a1−/− + ColVI
Col6a1−/− + ICG001
Relative protein levels
0
1
2
3
4
5
6
*
*
*
**
*
*
β-catenin
K79

Figure 7. Treatment with purified collagen VI or Wnt/β-catenin inhibitor abolishes the increased wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice. (**a** and **b**) Representative images show the effect of purified collagen VI (3 mg/kg/day) or Wnt/β-catenin inhibitor ICG-001 (5 mg/kg/day) on wound-induced hair regrowth of *Col6a1*$^{-/-}$ mice at post-wound day 14. Drugs were administered near the wounds at post-wound day 4. (**c**) Left panel, western blot for K79 and β-catenin in skin wounds at post-wound day 8 from wild-type and from *Col6a1*$^{-/-}$ mice in the absence (Control) or in the presence of purified collagen VI (3 mg/kg/day) or ICG-001 (5 mg/kg/day) starting at post-wound day 4. Right panel, densitometric quantification of K79 vs actin and β-catenin vs actin, as determined by three independent experiments. Values for wild-type skin were arbitrarily set to 1 (n = 4; *, $P < 0.05$; **, $P < 0.01$). ColVI, purified collagen VI; PBS, phosphate buffered saline; WT, wild-type.

Table

Table 1. RT-PCR primer sequences.

Protein	Gene	Primer sequence
α1(VI)	*Col6a1*	Forward: 5'– TGCCCTGTGGATCTATTCTTCG –3' Reverse: 5'– CTGTCTCTCAGGTTGTCAATG –3'
α2(VI)	*Col6a2*	Forward: 5'– CTACTCACCCCAGGAGCAGGAA –3' Reverse: 5'– TCAACGTTGACTGGGCGATCGG –3'
α3(VI)	*Col6a3*	Forward: 5'– AACCCTCCACATACTGCTAATTC –3' Reverse: 5'– TCGTTGTCACTGDCTTCATT –3'
GAPDH	*Gapdh*	Forward: 5'– GGGAAGCCCATCACCATCTT –3' Reverse: 5'– GCCTTCTCCATGGTGGTGAA –3'

Chapter 5: Other publications (reviews)

5.1 **Chen P**, Cescon M, Bonaldo P. The role of collagens in peripheral nerve myelination and function. Mol Neurobiol. 2014 Aug 21 (in press).

5.2 **Chen P**, Cescon M, Bonaldo P. Collagen VI in cancer and its biological mechanisms. Trends Mol Med. 2013 Jul; 19(7):410-7.

5.3 **Chen P**, Cescon M, Bonaldo P. Autophagy-mediated regulation of macrophages and its applications for cancer. Autophagy. 2014 Feb; 10(2):192-200.

5.4 **Chen P**, Bonaldo P. Role of macrophage polarization in tumor angiogenesis and vessel normalization: implications for new anticancer therapies. Int Rev Cell Mol Biol. 2013; 301:1-35.

Mol Neurobiol
DOI 10.1007/s12035-014-8862-y

The Role of Collagens in Peripheral Nerve Myelination and Function

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Abstract In the peripheral nervous system, myelin is formed by Schwann cells, which are surrounded by a basal lamina. Extracellular matrix (ECM) molecules in the basal lamina play an important role in regulating Schwann cell functions, including adhesion, survival, spreading, and myelination, as well as in supporting neurite outgrowth. Collagens are a major component of ECM molecules, which include 28 types that differ in structure and function. A growing body of evidence suggests that collagens are key components of peripheral nerves, where they not only provide a structural support but also affect cell behavior by triggering intracellular signals. In this review, we will summarize the main properties of collagen family, discuss the role of extensively studied collagen types (collagens IV, V, VI, and XV) in Schwann cell function and myelination, and provide a detailed overview of the recent advances with respect to these collagens in peripheral nerve function.

Keywords Collagen · Extracellular matrix · Myelination · Peripheral nervous system · PNS function · Schwann cells

Introduction

Peripheral nerves are composed of three distinct layers or compartments, corresponding to epineurium, perineurium, and endoneurium. The epineurium is the outermost layer that surrounds a thin lamellated perineurium composed of flat perineurial cells and an outer layer of collagen fiber bundles [1, 2]. The endoneurium is surrounded by perineurium, which is made of Schwann cell-axon units, fibroblasts, and extracellular matrix (ECM) [1]. ECM molecules are pivotal for both providing structural support and triggering many intracellular signals in peripheral nerves [2, 3].

Collagens are a major component of ECM in peripheral nerves, which play a key role in the peripheral nerve development and in the maintenance of normal nerve function during adulthood [4, 5]. So far, 28 collagen types have been identified in vertebrates [6, 7]. According to their different structures and functions, collagens can be classified into distinct subgroups, including fibril-forming collagens (types I, II, III, V, XI, XXIV, and XXVII) [6], networking collagens (types IV, VI, VIII, and X) [2, 8], fibril-associated collagens with interrupted triple helices (types IX, XII, XIV, XVI, XIX, XX, XXI, XXII, and XXVI) [8], transmembrane collagens (types XIII, XVII, XXIII, and XXV) [2, 8, 9], and other collagens (types VII, XV, XVIII, and XXVIII). Fibril-forming collagens are the most abundant and conserved types, and they are produced by connective tissue cells, such as fibroblasts, osteoblasts, and chondrocytes [6]. These collagens are typically made of α-chains containing a large triple-helical domain, flanked by N- and C-terminal domains [3, 6]. Fibril-associated collagens interact with other ECM molecules and play an essential role for the stabilization and integrity of ECM [8]. Networking collagens are grouped due to their ability to form networks in the extracellular space and are the most important basement membrane components integrating laminins, nidogen, perlecan, and other ECM molecules into a stable superstructural aggregate [4, 10]. Transmembrane collagens contain a transmembrane domain, which allows them to participate in the formation of cell-matrix interactions and in ECM remodeling [9]. Despite high structure and function diversities among different collagen types, all members of collagen family share some common characteristics. For instance, all collagens are made of three α-chains capable to

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form triple-helical conformations of variable lengths. Each chain is characterized by the presence of a collagenous domain containing repeated Gly-Xaa-Yaa amino acid triplets, where Xaa and Yaa are frequently proline and 4-hydroxyproline, respectively [4, 11]. Collagens can assemble as homotrimers containing three identical α-chains or as heterotrimers containing two or even three different α-chains [11].

Myelination is an important physiological process in peripheral nerves that contributed by Schwann cells. Derived from the embryonic neural crest, Schwann cells differentiate into myelinating or nonmyelinating cells [12, 13]. In the adult peripheral nerves, myelin is produced by myelinating Schwann cells, which envelop larger axons at a 1:1 ratio, whereas nonmyelinating Schwann cells are linked to C fibers [14–16]. Abnormal myelination in the peripheral nervous system is related to a number of neurological disorders, such as hereditary neuropathy with liability to pressure palsies, Charcot-Marie-Tooth disease, Dejerine-Sottas syndrome, congenital hypomyelinating neuropathy, and adrenomyeloneuropathy [17–21]. Thus, the proper myelination is critical for peripheral nerves and is tightly regulated by multiple signals, including ECM [13, 22]. Collagens are an essential component of Schwann cell ECM and play an important role in ECM assembly and peripheral nerve regeneration [23, 24]. In vitro studies using primary cultures of Schwann cells and Schwann cell/dorsal root ganglion (DRG) co-cultures have demonstrated that the secretion of native collagens and the assembly of fibrillar and basement membrane ECM structures fail when cells cultured without ascorbic acid, an essential factor for collagen posttranslational modification. Furthermore, addition of ascorbic acid promotes the deposition of ECM in Schwann cell plasma membrane [3, 25] and is essential for in vitro myelination in Schwann cell/DRG co-cultures [26, 27]. Sodium-dependent vitamin C transporter 2 (SVCT2) is necessary for the transport of ascorbic acid into Schwann cells [28] and into the brain [29]. Deficiency of SVCT2 leads to hypomyelination, as well as to impairments of nerve conduction velocities (NCVs) and sensorimotor function by decreasing the deposition of ECM components including collagen types IV, V and XXVIII [30]. These findings highlight the contribution of collagens in peripheral nerve myelination and function, which is also supported by the evidence that collagens can stimulate signal transduction in Schwann cells. Schwann cells not only express and secrete multiple collagen molecules both in cell culture and in vivo [3, 14, 31, 32] but also express several types of collagen receptors [3, 31–35] and migrate on and adhere to collagen substrates [3, 36]. By means of binding to their receptors on the cell surface, collagens play an important role in the regulation of Schwann cell function and myelination [3, 22, 37–39]. To better understand the contribution of collagens in peripheral nerves, this review will summarize the main properties of collagen types IV, V, VI, and XV that have been extensively studied in peripheral nerves and discuss the research findings with respect to their roles and underlying molecular mechanisms in Schwann cell function and myelination, as well as in peripheral nerve function.

Collagen IV

Collagen IV is a major component of basement membranes, and it can be deposited in the ECM as distinct isoforms made of up to six chains, α1(IV), α2(IV), α3(IV) α4(IV), α5(IV), and α6(IV), encoded by genes *COL4A1* to *COL4A6*, respectively [40, 41]. These chains can assemble into three distinct protomers, $[\alpha1]_2\alpha2$(IV), α3α4α5(IV), and $[\alpha5\alpha]_2\alpha6$(IV) [42, 43]. The α1(IV) and α2(IV) are considered as the "classical" collagen IV chains, which were first described. Both chains are deposited in basement membranes of all tissues, while the other four chains are deposited in restricted tissues during the development. Mutations in the gene encoding for either α1(IV) or α2(IV) lead to embryonic lethal phenotypes [41], whereas mutations in any of the genes encoding for α3(IV)–α5(IV) cause tissue-specific defects that are related to different forms of the Alport syndrome [43, 44] and other diseases [45, 46]. Each chain of collagen IV contains a long triple-helical collagenous domain with frequent interruptions of the Gly-Xaa-Yaa repeats, flanked by a short N-terminal (7S) domain and a larger C-terminal globular (NC1) domain [41, 43].

Unlike most members of the collagen family, collagen IV is present only in the basement membranes of tissues [41]. Indeed, collagen IV is a major component of the Schwann cell basement membrane in peripheral nerves [3, 47, 48], suggesting its potential role in modulating the activity and function of Schwann cells or nerves. In fact, collagen IV can promote the attachment and spreading of Schwann cells through a mechanism that is mediated by α1β1 and α2β1 integrins [49], as well as enhance Schwann cell proliferation [50]. Moreover, collagen IV promotes peripheral axonal growth mediated by the binding of α1β1 integrin to its NC1 domain [51]. These findings provided an in vitro evidence supporting the contribution and underlying mechanisms for collagen IV in the regulation of Schwann cell and peripheral nerve functions. Furthermore, in vivo findings obtained from Trembler-J mice, an animal model for Charcot-Marie-Tooth disease, demonstrate that these mice display alterations in Schwann cell structure and ECM organization of peripheral nerves. Especially, these mice exhibit high levels of macrophage-derived matrix metalloproteinases (MMPs), thus inducing the decrease of collagen IV deposition in nerves [52]. Moreover, it has been demonstrated that the expression of PMP-22 in myelinating nerve fibers is associated with collagen IV [53]. The other evidence from sciatic nerve transected animals suggests that myelination and collagen IV

deposition can be detected in concurrence with regenerated fibers after receiving the treatment with electrospun tubes, indicating the correlation between collagen IV and myelination [54]. More importantly, collagen IV exhibits a beneficial effect in regeneration after sciatic nerve injury [55]. In addition, clinical findings show that the expression of collagen IV is enhanced in the peripheral nerves of patients, who were diagnosed as having myelin-related neuropathies, such as multiple sclerosis [56], Charcot-Marie-Tooth type 1 [57], and diabetic polyneuropathy [58]. The function of collagen IV in peripheral nerve is also supported by the evidence obtained from peripheral nerve sheath tumors. For example, it has been found that collagen IV is strongly deposited in the malignant peripheral nerve sheath tumor tissues [59–63], suggesting its potential role in peripheral nerve sheath tumor development. Collectively, these findings suggest that collagen IV may function as an important regulator involved in modulating myelination and may contribute to the onset of myelin-related peripheral neuropathies and peripheral nerve sheath tumors. However, further studies are still needed to clarify the role of collagen IV in myelination and peripheral nerve function using Schwann cell-specific deletion of collagen IV genes.

Collagen V

Collagen V is a minor component of collagen fibrils playing an important role in ECM organization [64, 65]. Collagen V usually co-polymerizes with collagen I and regulates the diameter of heterotypic collagen fibrils in lathyritic chicken [66] and bone [67]. The protein is composed of three polypeptide chains, α1(V), α2(V), and α3(V), encoded by distinct genes (*COL5A1*, *COL5A2*, and *COL5A3*, respectively) [68, 69]. Collagen V is widely deposited in multiple tissues as $[\alpha1(V)]_2\alpha2(V)$ heterotrimers [67, 70]. However, other forms, such as $[\alpha1(V)]_3$ homotrimers and α1(V)α2(V)α3(V) heterotrimers, have also been reported [67].

In addition to the three chains mentioned above, a collagen-like adhesive protein of 200 kDa (called p200) that binds with high affinity to the cell surface heparan sulfate proteoglycan syndecan-3 was isolated from the conditioned medium of Schwann cells. Purified p200 promotes the adhesion and spreading of Schwann cells, which is blocked by heparin, indicating that heparan sulfate proteoglycans function as receptors for p200 [71]. Among the tissues, p200 is only expressed in the ECM surrounding Schwann cell-axon units of developing sciatic nerves. The protein is first detected in mouse embryo between E15 and E18, and its deposition is only detectable during the first 2–3 weeks of postnatal development [31, 71], suggesting a unique role for p200 in peripheral nerve development. Subsequent studies identified p200 as a novel isoform of collagen V, named α4(V) collagen [72]. Purified α4(V) collagen isolated from Schwann cell conditioned medium promotes the migration of premyelinating Schwann cells and inhibits the outgrowth of axons from DRG. Further studies suggested that the effects of α4(V) collagen in promoting Schwann cell adhesion, spreading, and migration are exerted by the binding of its noncollagenous N-terminal domain to heparin [36], mediated by syndecan-3 [33]. In addition, the noncollagenous N-terminal domain of α4(V) collagen can be released constitutively by Schwann cells both in vitro and in vivo. In peripheral nerve tissues, this domain is found in the region of the outer Schwann cell membranes. After secretion, it binds to glypican-1 and perlecan, two proteins expressed on the cell surface of Schwann cells, and to ECM heparan sulfate proteoglycans, thus affecting Schwann cell behavior and function [34]. In mature myelinating Schwann cell-axon units, α4(V) collagen and its receptor syndecan-3 are highly concentrated at the nodes of Ranvier, which are structures strongly regulated by myelinating glia [73]. Altogether, these findings not only provide direct evidence supporting the role of α4(V) collagen in Schwann cell adhesion, spreading, and migration but also suggest the potential role of this protein in regulating peripheral nerve myelination. In agreement with this hypothesis, siRNA-mediated suppression of α4(V) collagen significantly inhibits Schwann cell myelination in vitro [35]. It should be pointed out that p200 was first considered as a novel collagen V isoform, named α4(V) collagen, due to the apparent differences between the full-length rat sequence and partial peptide sequence data from the human α3(V) chain. However, studies performing the cloning of the same gene from mouse and human demonstrated that p200 appears to be α3(V) collagen [3, 74]. Based on the findings mentioned above, further studies using genetic approaches are still needed to investigate in detail the in vivo effects of collagen V in myelination and peripheral nerve function.

Collagen VI

Collagen VI is a large ECM component, which has been shown to be abundantly expressed in several tissues [75, 76], including peripheral nerves [14, 77]. It is composed of three distinct polypeptide chains, α1(VI), α2(VI), and α3(VI), encoded by distinct genes (*COL6A1* to *COL6A3*, respectively) [75, 78]. Recent studies identified three novel collagen VI chains, called α4(VI), α5(VI), and α6(VI), which are coded by separate genes (*COL6A4* to *COL6A6*) and share a high degree of similarity with α3(VI) [79, 80]. Each collagen VI chain is characterized by a short triple helical domain, flanked by large N- and C-terminal globular ends composed of repeated motifs that are similar to the von Willebrand factor type A (vWF-A) module [75, 78, 79]. The α1(VI) and α2(VI) chains contain one N-terminal (N1) and two C-terminal (C1 and C2) vWF-A modules, whereas the α3(VI) chain contains ten N-terminal (N1 to N10) and two C-terminal (C1 and C2) vWF-A

modules, as well as three distinct domains (C3–C5) at the C-terminal end [75, 78]. The novel chains contain one N-terminal region composed of seven vWF-A modules and a C-terminal region made of two or three vWF-A modules, as well as one or two unique sequences [78–80]. It was demonstrated that α3(VI) undergoes a proteolytic processing, which leads to the release of the most C-terminal (C5) domain following collagen VI secretion [81]. The human *COL6A4* gene is disrupted into two pieces, due to a large chromosome inversion during primate evolution leading to the generation of a nonprocessed pseudogene that is not able to produce a functional α4(VI) chain in humans [80].

The synthesis and secretion of collagen VI requires the association of α1(VI), α2(VI), and α3(VI) in equimolar ratios. The novel chains may substitute for α3(VI) during the synthesis and assembly to form α1(VI) α2(VI)α4(VI), α1(VI)α2(VI)α5(VI), and α1(VI)α2(VI)α6(VI) isoforms [79, 80]. At difference from other collagens, collagen VI has a peculiar multistep pathway of intracellular assembly, which also involves extensive disulfide bond interactions. The association of distinct α-chains allows the formation of triple-helical "monomers" (three chains), followed by the assembly into "dimers" (six chains) and "tetramers" (12 chains) before secretion [75]. Once secreted, the tetramers associate by noncovalent interactions, giving rise to characteristic "beaded" microfilaments that are deposited in the ECM [75, 82]. The secreted collagen VI can regulate the properties of local microenvironment by interacting with a number of cell surface receptors and several other ECM components. Collagen VI microfilaments not only provide structural support for cells and connective tissues, by fine-tuning the local stiffness [83, 84] and the size of collagen I fibrils [85], but also regulate intracellular functions, such as apoptosis [76, 86, 87], proliferation [88], angiogenesis, and inflammation [89].

Given the important role of collagen VI in regulating cell functions, increasing evidence has demonstrated that this molecule is also critical for Schwann cell activities in peripheral nerves. Collagen VI expression is detectable in the mouse embryo at E10.5 in different locations, then rapidly increases in following days and remains at high levels during organogenesis, slowly decreasing after birth [90]. Collagen VI is abundant in both embryonic and adult peripheral nerves [14, 77], where it is produced by Schwann cells and endoneurial/perineurial cells, but not by neural crest cells or axons [14, 91]. Although collagen VI is broadly distributed in several tissues, expression of the genes coding for collagen VI chains is dynamically regulated and requires different *cis*-acting regulatory elements that confer proper levels of expression by different cell types. In agreement with this, an enhancer region located about 4.5 kb upstream the transcription start site of the *Col6a1* gene was shown to drive transcription in the peripheral nervous system [92]. Further studies suggest that *Col6a1* gene is highly expressed by mature Schwann cells which have undertaken myelination, but not by immature Schwann cells. Activation of *Col6a1* transcription is part of the differentiation program of Schwann cells from neural crest cells upon the initial stimulation with neuregulin. Furthermore, activation of this gene in sciatic nerves after birth is associated with the time of withdrawal of immature Schwann cells from the cell cycle, when they start to differentiate into myelinating Schwann cells [91]. Altogether, these findings highlight the contribution and underlying mechanisms of collagen VI expression in Schwann cell differentiation (Fig. 1).

In addition to involving in Schwann cell differentiation, collagen VI contributes to Schwann cell myelination. Recent findings demonstrated that addition of purified collagen VI to Schwann cells decreases the expression of myelin-associated glycoprotein, suggesting an inhibitory effect of collagen VI in Schwann cell myelination in vitro [14]. In vivo work showed that lack of collagen VI in *Col6a1*$^{-/-}$ mice, where targeted inactivation of the gene coding for α1(VI) chain prevents collagen VI assembly and secretion [86, 93], induces axon hypermyelination in the sciatic nerves of adult mice [14]. Myelination in the peripheral nerve is tightly regulated by specific signaling pathways. For instance, activation of FAK [94], AKT [95, 96], ERK [97, 98], and p38 [99, 100] and inhibition of vimentin [95], JNK, and c-Jun [101] signaling pathways are required for axon myelination in peripheral nerves. In agreement with the hypermyelination observed in *Col6a1*$^{-/-}$ nerves, collagen VI ablation is accompanied by the activation of FAK, AKT, ERK, and p38 signals and inhibition of vimentin, JNK, and c-Jun signals [14], suggesting that collagen VI affects peripheral nerve myelination by regulating both positive and negative myelination regulatory pathways.

It is well established that myelin thickness in the peripheral nerves should be properly maintained, and abnormal myelin thickness is closely related to the dysfunction of nerves in animals and humans [14, 17–21, 102]. For instance, axon hypermyelination can enhance the proportion of the surrounding wire volume and cause axonal damage, thus altering axonal functions [17, 103, 104]. In agreement with the hypermyelination of collagen VI deficient nerves, electrophysiological studies demonstrated that the NCVs are impaired in *Col6a1*$^{-/-}$ mice, with decreased Schwann cell internodal length. Moreover, behavioral studies demonstrated that *Col6a1*$^{-/-}$ mice exhibit an impairment of sensorimotor function, suggesting that deficiency of collagen VI induces functional deficits in peripheral nerves [14]. In addition to its direct role in Schwann cells and myelination, collagen VI interacts with other ECM molecules to modulate peripheral nerve structure and function. For example, von Willebrand A domain-related protein (WARP) is an ECM molecule that interacts with collagen VI in peripheral nerves. Ablation of WARP severely reduces collagen VI deposition in peripheral nerves, which not only leads to compromised peripheral nerve structure, such as the fusion of adjacent Schwann cells

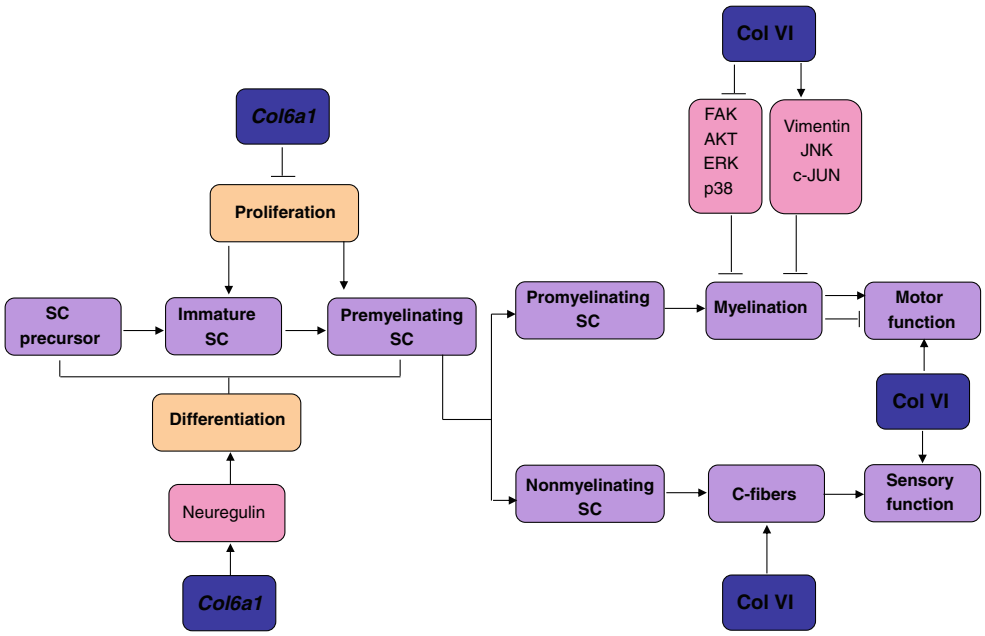

Fig. 1 The contribution of collagen VI in Schwann cell function, peripheral nerve myelination and function. Activation of *Col6a1* transcription is required for Schwann cell differentiation through modulation of axonal signal neuregulin and cell cycle. Lack of collagen VI in *Col6a1*$^{-/-}$ mice induces hypermyelination in peripheral nerve regulated by the activation of FAK, AKT, ERK, and p38 signals and inhibition of vimentin, JNK, and c-Jun pathways. The increased myelin in *Col6a1*$^{-/-}$ mice results in impaired motor functions. Furthermore, deficiency of collagen VI causes the disorganization of C fibers, thus inducing an impairment of sensory function. Thus, collagen VI is an important regulator for Schwann cell differentiation, myelination, and peripheral nerve function. *SC* Schwann cells, *Col VI* collagen VI

basement membranes, but also causes an impairment of sensorimotor function [77]. Altogether, these findings support the concept that collagen VI is not only involved in Schwann cell differentiation and myelination via distinct molecular mechanisms but also functions as an important factor for regulating peripheral nerve function (Fig. 1).

Collagen XV

Collagen XV is a large nonfibrillar collagen made of α1(XV) homotrimers and characterized by a highly interrupted triple-helical domain and large N- and C-terminal noncollagenous domains. This collagen has hybrid features of both collagens and proteoglycans, and it was found to correspond to a chondroitin sulfate proteoglycan located in specialized basement membranes [105, 106]. Collagen XV shows a high degree of similarity with collagen XVIII, and these two proteins form a distinct subgroup among the collagen family [40, 107–109]. In addition, both collagen XV and XVIII were shown to undergo proteolytic processing at their C-terminal end, giving rise to bioactive fragments of about 20 kDa, called endostatins, which display potent inhibitory activities on angiogenesis and tumor growth [110–113].

Collagen XV is widely distributed in the basement membrane zones of different tissues [105, 110]. Immunostaining studies demonstrated that collagen XV expression is higher during embryogenesis and it is abundant in several developing tissues, such as the heart, kidney, and lung. On the other side, a strong labeling for collagen XV is found in the endoneurium and perineurium of adult peripheral nerves. In addition, collagen XV is also deposited in the extrasynaptic and Schwann cell basement membranes of neuromuscular junction [114]. This expression profile suggests a potential role for collagen XV in the development and functional properties of peripheral nerves. Indeed, genetic studies in *Col15a1*$^{-/-}$ mice showed that lack of collagen XV causes polyaxonal myelination, loosely packed axons in C fibers, and less electron dense cytoplasm in Schwann cells, suggesting an impairment of peripheral nerve maturation and C fiber formation. Moreover, ablation of collagen XV leads to defective basement membrane assembly. Electrophysiological studies demonstrated that deficiency of collagen XV induces a decrease in NCVs, suggesting an impairment of sensory function in *Col15a1*$^{-/-}$ mice [115]. Laminin-411, another component of the basement membrane of peripheral nerves, was shown to be involved in axon segregation and myelination in peripheral nerves [116, 117]. Lack of laminin-411 in *Lama4*$^{-/-}$ mice causes an impairment of motor and tactile sensory functions, which is exacerbated by the simultaneous deficiency of collagen XV, although the sole ablation of collagen XV does not affect the motor function in mice [115]. Taken together, these findings point at collagen XV as an essential factor regulating peripheral nerve maturation and C fiber formation, as well as contributing to the modulation of another ECM molecule, laminin-411, that is involved in motor and sensory functions of peripheral nerves.

Table 1 Expression and function of collagens in peripheral nerves and their related disorders

Collagen type	Location	Function	Related disorders	References
Collagens I and III	Epineurium, perineurium, and endoneurium	N.D.	Charcot-Marie-Tooth	[57]
Collagen II	Schwann cells	N.D.	N.D.	[118]
Collagen IV	Basement membrane of Schwann cells	Promoting Schwann cell adhesion, migration and proliferation, and axonal growth	Charcot-Marie-Tooth, multiple sclerosis, diabetic polyneuropathy, and peripheral nerve sheath tumor	[3, 47–52, 55–63]
Collagen V	Schwann cells	Promoting Schwann cell adhesion, migration and myelination, and inhibiting axonal growth	N.D.	[31, 34–36, 71, 72]
Collagen VI	Schwann cells, macrophages and endoneurial/perineurial cells	Involving in Schwann cell differentiation and myelination and modulation of motor and sensory function	Peripheral nerve function deficits	[14, 77, 91]
Collagen IX	Posterior sclerotome	Inhibiting sensory and motor neurite outgrowth, and neural crest cell migration	N.D.	[119, 120]
Collagen XIII	Dorsal root ganglia and nerves of the developing mouse fetus in mid-gestation	N.D.	N.D.	[121]
Collagen XV	Endoneurium, perineurium, and Schwann cell basement membrane of neuromuscular junction	Involving in nerve maturation, C fiber organization, and sensory function of peripheral nerves	Peripheral nerve function deficits	[114, 115]
Collagen XVI	Dorsal root ganglia	N.D.	N.D.	[122]
Collagen XXVIII	Nodes of Ranvier and nonmyelinating cells	N.D.	Charcot-Marie-Tooth	[123]

N.D. not determined

Conclusions and Perspectives

Collagens are a prominent component of the ECM. Various collagen types are abundantly deposited in the basement membranes of Schwann cells and in the ECM of peripheral nerves (Table 1), where they not only provide a structural support but also affect Schwann cell behavior and nerve function by triggering intracellular signals via binding to their cell surface receptors. Several members of the collagen family, such as collagens IV, V, VI, and XV, were shown to be key

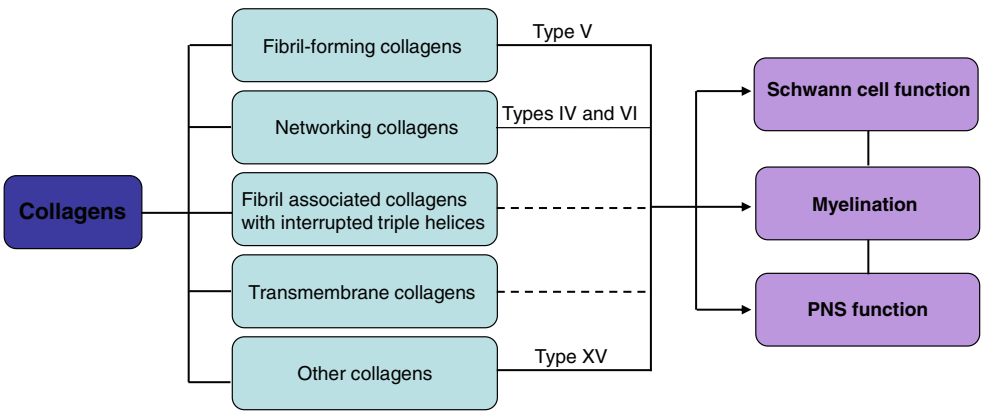

Fig. 2 Schematic diagram summarizing the role of collagens in Schwann cell function, peripheral nerve myelination and function. The diagram shows that there are at least four types of collagens, including collagens IV, V, VI, and XV, from three out of five collagen subgroups contributing to Schwann cell function, peripheral nerve myelination and function. It is still unknown whether other collagens are involved in these processes. *PNS* peripheral nervous system

regulators in the peripheral nerves, where they affect multiple Schwann cell activities (such as adhesion, spreading, proliferation, differentiation, and myelination) and/or peripheral nerve motor and sensory functions through distinct mechanisms (Fig. 2 and Table 1). Moreover, dysfunction or deficiency of collagens (such as collagens I, III, IV, VI, XV, and XXVIII) in nerves is related to peripheral neuropathies (Table 1). Collectively, these findings support the concept that collagens play a critical role in peripheral nerve myelination and function.

Although the increasing number of findings showing the contribution of collagens in peripheral nerve myelination and function, much work remains to be done regarding the role of different collagen molecules in these processes, as well as the underlying mechanisms. For example, our current knowledge regarding the role of the different members of the collagen family in peripheral nerve myelination and function is mainly restricted to few molecules, such as collagens IV, V, VI, and XV (Fig. 2). Further studies are needed to clarify whether and how other collagen types are involved. The contribution of collagens in regulating cell functions (e.g., cell growth, differentiation, and proliferation) and cell behaviors (e.g., adhesion and migration) is mediated by surface receptors, such as integrins, or by binding to an intermediary molecule that is then recognized by surface receptors [124]. Giving the fact that multiple classes of collagen receptors have been identified [124] and that one specific collagen type does not usually exhibit its cellular functions via a sole receptor, it would be of great interest to identify the receptors or intermediary binding molecules mediating the specific role of each collagen type in peripheral nerves. On the other hand, although some findings demonstrate that the expression profile of collagens, such as types IV, V, and VI, is changed in peripheral nerves of diabetic polyneuropathy and Charcot-Marie-Tooth type 1 patients [57, 58], further studies are needed to clarify the role and underlying molecular mechanisms of collagens in peripheral nerves in the context of peripheral neuropathies. In addition to providing a better understanding of the molecular mechanisms involved in mediating the effects of different collagens in peripheral nerve myelination and function, further findings in this field may provide novel evidence supporting collagens as key players in the onset and/or progression of peripheral neuropathies, thus providing novel targets for the therapy of peripheral neuropathies.

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Conflict of Interest The authors declare no conflict of interest.

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Review

Collagen VI in cancer and its biological mechanisms

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Collagen VI is a widely distributed extracellular matrix protein highly expressed in a variety of cancers that favors tumor growth and progression. A growing number of studies indicate that collagen VI directly affects malignant cells by acting on the Akt–GSK-3β–β-catenin–TCF/LEF axis, enhancing the production of protumorigenic factors and inducing epithelial–mesenchymal transition. Moreover, it affects the tumor microenvironment by increasing the recruitment of macrophages and endothelial cells, thus promoting tumor inflammation and angiogenesis. Furthermore, collagen VI promotes chemotherapy resistance and can be regarded as a potential biomarker for cancer diagnosis. Collectively, these findings strongly support a role for collagen VI as an important regulator in tumors and provide new targets for cancer therapies.

Collagen VI and its main properties

The extracellular matrix (ECM; see Glossary) in the tumor microenvironment is commonly deregulated and disorganized, thus contributing to cancer progression both directly, by promoting cancer cell growth and metastasis, and indirectly, by educating other microenvironment components [1]. Collagen VI is a major ECM protein composed of three major polypeptide chains – α1(VI), α2(VI), and α3(VI) – encoded by distinct genes (*COL6A1*, *COL6A2*, and *COL6A3*, respectively). The α1(VI) and α2(VI) chains have molecular weights of 140–150 kDa and extend for nearly 1000 amino acids, whereas the α3(VI) chain is three times larger (250–300 kDa) with several alternatively spliced variants ranging between 2500 and 3100 amino acids. Each chain is characterized by a short triple helical region flanked by large N- and C-terminal globular regions, which are composed of 200 amino acid motifs sharing similarity with the von Willebrand factor type A (vWF-A) module [2]. The α1(VI) and α2(VI) chains have one N-terminal (N1) and two C-terminal (C1 and C2) vWF-A modules, whereas the α3(VI) chain displays larger N- and C-terminal globular regions made of 12 vWF-A modules (N1–N10, C1, C2) and three distinct C-terminal domains (C3–C5) [2,3]. Interestingly, some studies indicate that α3(VI) undergoes proteolytic processing, leading to the release of the most C-terminal (C5) domain after collagen VI secretion [4] (Figure 1).

Recent studies led to the identification of three other collagen VI subunits – α4(VI), α5(VI), and α6(VI) – encoded by separate genes. These chains contain one N-terminal region made of seven vWF-A modules, a collagen triple helical region that is similar to that of α3(VI), and a C-terminal region containing two or three vWF-A modules as well as one or two unique sequences [5–8] (Figure 1).

Collagen VI is widely distributed in several tissues, including skeletal muscle, skin, lung, blood vessels, cornea, and intervertebral discs [2], as well as peripheral nerves [9], brain [10], myocardium [11], and adipose tissue [12]. Collagen VI contributes to the properties of the local ECM microenvironment by forming a discrete network of beaded microfilaments that interact with other ECM molecules and provide structural support for cells [13]. Furthermore, it triggers signaling pathways that regulate apoptosis [14], autophagy [15], proliferation, angiogenesis [16], fibrosis, and inflammation [16,17]. Given the role for collagen VI in regulating a number of cell and tissue processes, it is worth clarifying the molecular mechanism underlying the contribution of collagen VI to tumor progression. In this review, we summarize the current knowledge on the procarcinogenic function of collagen VI during tumor progression and discuss data indicating that collagen VI is a diagnostic biomarker for cancer and a modulator of chemotherapy

Glossary

Angiogenesis: the formation of new capillary blood vessels from pre-existing vessels.
Endotrophin (ETP): a soluble cleaved C5 fragment of the collagen α3(VI) chain that is secreted by various cell types, especially adipocytes.
Epithelial–mesenchymal transition (EMT): the process by which epithelial cells completely lose their epithelial traits, but acquire mesenchymal characteristics. EMT induces the loss of cell adhesion, decrease of E-cadherin expression, and increase of cell motility.
Extracellular matrix (ECM): the extracellular component in mammalian tissues that provides structural support to cells but also exerts several other functions.
Field cancerization: a concept proposed by Slaughter *et al.* in 1953 [86,87], which describes histologically abnormal epithelium surrounding oral squamous cell carcinoma within the aerodigestive region in relationship to the exposure to carcinogens. This concept now refers more broadly to describe multiple patches of premalignant disease, a higher-than-expected prevalence of multiple local and second primary tumors within the upper aerodigestive tract.
MMTV-PyMT mice: a transgenic mouse strain with mammary gland-specific PyMT expression controlled by the MMTV promoter, resulting in mammary tumors as well as in metastatic lesions of the lung and lymph nodes.
Tumor metastasis: the spread of primary tumor from one organ to another non-adjacent organ, or from one part to another part of the same organ.
Tumor microenvironment: the stromal components of tumor, which include ECM, blood vessels, lymphatic vasculature, bone marrow-derived cells, fibroblasts, and other cells.

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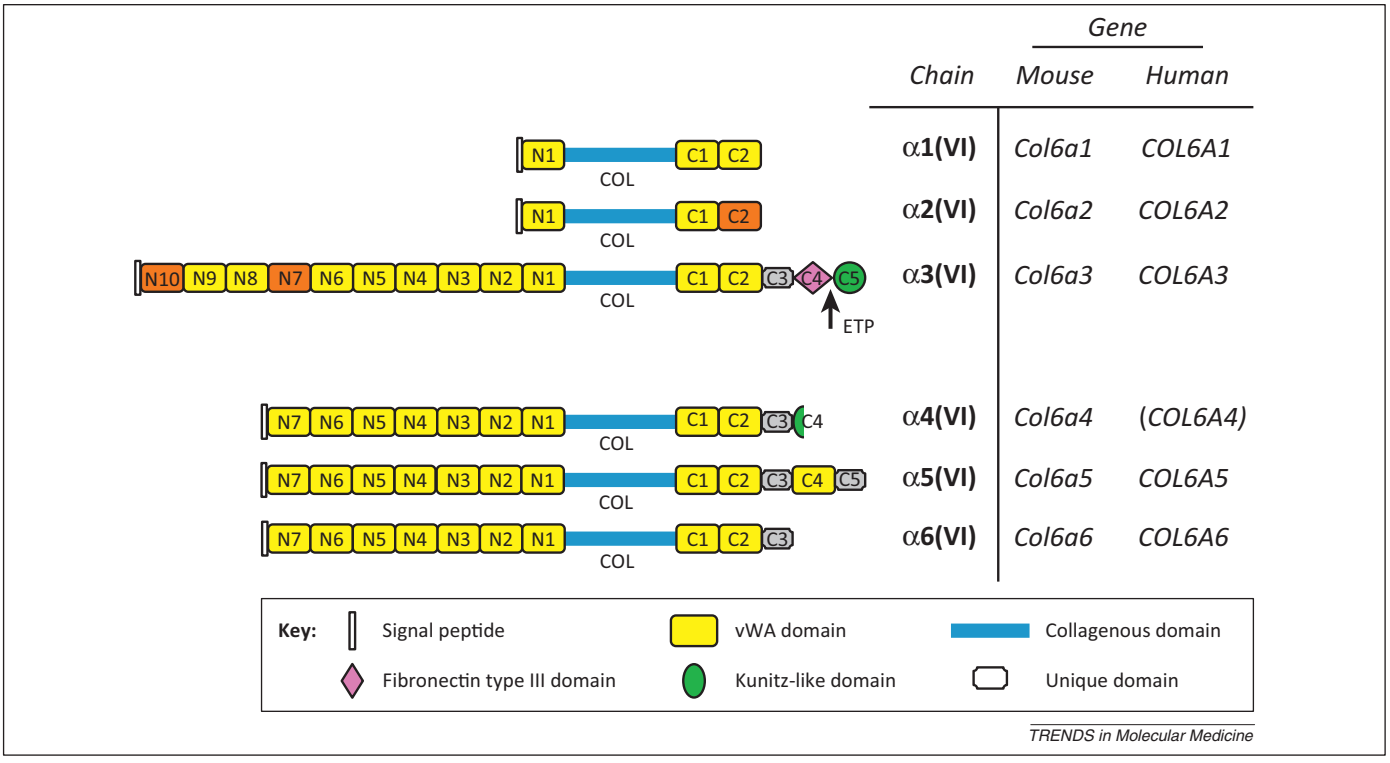

Figure 1. Schematic diagram of collagen VI domain structure. Usually, collagen VI contains α1(VI), α2(VI), and α3(VI) chains. Each chain is made of a short collagenous (COL) region flanked by a variable number of von Willebrand factor type A (vWF-A) modules. The α3(VI) chain contains three additional domains at the carboxyl-terminal end; the arrow marks the cleavage site that releases the most C-terminal domain, giving rise to the soluble endotrophin (ETP) peptide. The vWF-A modules depicted in orange were shown to undergo alternative splicing. The lower part of the diagram shows the domain structure of the three novel collagen VI chains described recently, which share a high degree of similarity with α3(VI). In humans, the *COL6A4* gene, coding for the α4(VI) chain, was broken into two pieces due to a large chromosome inversion and became a non-processed pseudogene [8].

resistance, two important aspects with extensive clinical applications.

The expression and function of collagen VI in tumors

Collagen VI is highly expressed in a variety of tumors. Table 1 lists the current knowledge of the expression and localization of collagen VI in human tumors. The expression of *COL6A1*, *COL6A2*, and *COL6A3* transcripts and the level of collagen VI protein in tumor tissues are much higher than those of the corresponding normal tissues [12,18–22]. Moreover, the cleaved C5 domain of collagen α3(VI), recently defined as endotrophin (ETP), displays markedly increased levels in biopsies from breast and colon cancers [16]. These clinical observations are well supported by experimental findings using different models. Collagen VI expression in tumor tissues of the mammary tumor virus-polyoma middle T antigen (MMTV-PyMT) transgenic mouse model is significantly increased during tumor progression [23]. A study on collagen α3(VI) in human biopsies and cell lines derived from pancreatic ductal adenocarcinoma (PDA), as well as in xenograft and transgenic mouse models for this aggressive cancer, showed a significant upregulation of *COL6A3*, with a specific pattern of alternative splicing [21]. Notably, tumor-specific α3(VI) isoforms were identified in several cancers by genome exon arrays, suggesting a dynamic process of tumor-specific alternative splicing for several exons of stromal *COL6A3*. Furthermore, the collagen VI ETP peptide is abundantly expressed in tumor tissues of MMTV-PyMT mice [16]. Taken together, these findings strongly support a role for collagen VI in tumorigenesis.

Several studies have demonstrated that collagen VI is an antiapoptotic factor for different cell types, including fibroblasts [24], muscle fibers [25], neurons [10,14], and endothelial cells [26]. In agreement with this, collagen VI exerts a potent effect in stimulating proliferation and preventing apoptosis of MCF-7 breast cancer cells *in vitro* (P. Bonaldo, unpublished). Notably, *in vivo* studies using the MMTV-PyMT mouse model have shown that ablation of collagen VI reduces proliferation and increases tumor cell apoptosis, whereas the ETP peptide exerts the opposite effect [16]. The antiapoptotic and prosurvival effects of collagen VI contributes to its tumor promoting effects in MMTV-PyMT transgenic and xenograft breast tumor models [12,16], and ETP reconstitution into collagen VI null ($Col6a1^{-/-}$) breast cancer cells rescues tumor growth, strongly suggesting that the promoting effect of collagen VI in breast tumorigenesis is exerted by ETP [16]. The *in vivo* role of collagen VI in tumor growth is further supported by a study investigating B16F10 melanoma allografts in the brains of wild type and $Col6a1^{-/-}$ mice, which showed that collagen VI ablation retards tumor growth by inducing deficits in the vascular basal lamina [26].

Collagen VI is also involved in tumor metastasis. It is highly expressed in human glioblastoma cells and has a potent ability to promote the adhesion and spreading of glioblastoma cells, thus enhancing the extension, penetration, and invasion of tumor cells in an autocrine manner

Table 1. Expression and localization of collagen VI in human tumors

Tumor types	Gene/protein	Location	Refs
Glioblastoma	Collagen VI	Adventitia of normal vessels and spindle cell proliferations of pathological vessels	[34]
Cutaneous neurofibromas	*COL6A1* and *COL6A2*	Endothelial cells	[33]
Melanoma	Collagen VI	Vascular area and tumor cells	[78]
Ovarian cancer	*COL6A3* and collagen VI	Tumor cells, extracellular and tumor-derived ovarian epithelial cells	[61,88]
Lung cancer	*COL6A3*	Tumor endothelium	[18]
Esophageal cancer	*COL6A3*	Tumor endothelium	[18]
Astrocytomas	*COL6A1*	N.D.	[22]
Juvenile angiofibromas	*COL6A1*, *COL6A2*, *COL6A3*, and collagen VI	Tumor endothelium	[19]
Pancreatic cancer	*COL6A3* and α3(VI)	Desmoplastic stroma	[21]
Breast cancer	Collagen VI	Tumor invasive front	[20]
	α3(VI) and its C-terminal domain	Near the vicinity of the adipocytes	[12]
	Cleaved C5 domain (ETP) of α3(VI)	N.D.	[16]
Colon cancer	*COL6A3*	Tumor endothelium	[18]
	Cleaved C5 domain (ETP) of α3(VI)	N.D.	[16]

N.D., not determined.

[27]. The motility of malignant human lung epithelial carcinoma cells is markedly upregulated by collagen VI treatment [28], suggesting that collagen VI promotes tumor metastasis, and quantitative secretome analysis shows that collagen α1(VI) is a metastasis-associated protein in lung cancer cells. *COL6A1* knockdown by RNA interference suppresses the metastatic ability of lung cancer cells, whereas overexpression of *COL6A1* has the opposite effect [29]. Furthermore, the ETP peptide enhances pulmonary metastasis of breast cancer by inducing transforming growth factor-beta (TGF-β)-dependent epithelial–mesenchymal transition (EMT) *in vivo* [16].

Although both experimental evidence and clinical data support the idea that collagen VI contributes to tumor growth and metastasis, it should be noted that exceptions to this pattern exist. For example, cells derived from several spontaneous mesenchymal tumors, such as fibrosarcomas, lack all three polypeptide chains required for the assembly and secretion of functional collagen VI, which may contribute to tumorigenicity and invasiveness of mesenchymal tumor cells. In the xenograft tumor model, fibrosarcoma cells are unable to synthesize and secrete collagen VI and the protein is not present in the tumor stroma [30]. Furthermore, in a transgenic mouse model of familial adenomatous polyposis treatment with sulindac, a nonsteroidal anti-inflammatory drug, reduces tumor mass by more than 80% and is accompanied by the upregulation of collagen VI mRNA and protein levels [31]. The apparently contradictory observations of opposite regulation of collagen VI are likely to be due to the different tumors and tumor microenvironments, and suggest that collagen VI may exert different functions in various tumors. Although further studies are needed to validate this hypothesis, these findings collectively support the concept of personalized therapy/medicine by targeting collagen VI in cancer therapy.

The role of collagen VI in tumor vascular networks

Angiogenesis, the new capillary blood vessels formed from pre-existing vasculature, is a typical tumor hallmark, exerting a pivotal role in cancer growth and metastasis [32]. In most solid tumors, blood vessels are significantly increased during cancer progression, and this neoangiogenic process is influenced by the tumor microenvironment, including ECM proteins [1]. As shown in Table 1, expression of collagen VI is mostly found in the tumor stroma, especially near or within blood vessels [18,19,33,34]. The evidence from NG2 null mice shows that tumor vasculature is aberrant, including impaired interaction of pericytes with endothelial cells, defective pericyte maturation, and vessel leakage, which may due to the loss of collagen VI anchorage [35]. These findings suggest a role for collagen VI in remodeling blood vessels in cancers, and direct evidence of collagen VI in remodeling tumor vessels has recently been provided by a study using B16F10 mouse melanoma cells grown in the brains of wild type and *Col6a1*$^{-/-}$ mice. It was shown that collagen VI deficiency affects the function of blood vessels by impairing pericyte maturation and inhibiting the sprouting and survival of endothelial cells, thus resulting in blood vessel leakage and inhibition of angiogenesis [26]. Several angiogenic modulators are derived from ECM molecules such as collagen IV and XVIII, including endostatin that functions as a potent angiogenic inhibitor [1], suggesting the value to identify collagen VI fragments potentially involved in tumor angiogenesis. Collagen VI ETP peptide was shown not only to function as a chemoattractant that recruits endothelial cells into the tumor microenvironment but also as a proangiogenic factor that significantly increases the migration and tubule formation of endothelial cells *in vitro*, and both functions can be abrogated by anti-ETP antibodies [16]. These findings strongly suggest that collagen VI, through ETP, plays a potent role in promoting angiogenesis by increasing the recruitment, migration, and tubule formation of endothelial cells. This hypothesis is further supported by the *in vivo* demonstration that ETP promotes tumor angiogenesis through upregulation of CD31, vascular endothelial growth factor receptor 2 (VEGFR2), and hypoxia-inducible factor 1 alpha (HIF1α), whereas anti-ETP antibodies have opposite effects [16]. Altogether, these findings highlight collagen VI as a key regulator involved in tumor vascular remodeling, and further studies on the underlying mechanisms will likely contribute to the design of more effective drugs targeting the tumor vasculature.

Collagen VI promotes tumor inflammation by recruiting macrophages

Inflammation plays a significant role in both tumor development and metastasis. The inflammatory tumor microenvironment is characterized by a large number of recruited inflammatory cells and activation of the inflammatory response [36]. Among the inflammatory cells, tumor-associated macrophages (TAMs) are the most notable cell type suppressing antitumor immunity and favoring tumor angiogenesis, growth, and metastasis [37–39]. Macrophages exhibit a spectrum of phenotypes, ranging from the classically activated (M1) phenotype to the alternatively activated (M2) phenotype which exhibit antitumor activity and favor tumor malignancy, respectively [38–41]. TAMs are mainly biased towards the M2 phenotype [38]. Although the underlying molecular mechanisms by which TAMs favor tumor development and metastasis are not completely understood, TAMs may represent significant new therapeutic targets.

Collagen VI is abundantly expressed by primary macrophages from humans [42] and mice, as well as by macrophage cell lines [43]. The synthesis and secretion of collagen VI by macrophages depends on their activation state, stage of differentiation, and cell density. M2 macrophages induced by TGF-β1, interleukin (IL)-4, IL-10, and IL-13 produce much more collagen α3(VI) than M1 macrophages induced by lipopolysaccharides (LPS) and interferon-gamma (INF-γ) [43], suggesting TAMs as one of the key providers for collagen VI in tumors. Notably, collagen VI significantly enhances the *in vitro* adhesion of macrophages, which suggests a potential role for collagen VI in macrophage recruitment [43]. These findings imply the presence of a feedback loop between collagen VI expression and macrophage recruitment/activation in tumors, which would favor tumor growth and progression. This concept is supported by the evidence that the collagen VI ETP peptide is able to promote tumor inflammation by increasing macrophage recruitment and upregulating the production of inflammatory factors, such as IL-6 and tumor necrosis factor-alpha (TNF-α), which is abrogated by anti-ETP antibodies [16]. Taken together, these findings not only indicate that collagen VI is a chemoattractant that promotes tumor progression by inducing inflammation by recruiting macrophages and increasing the production of inflammatory factors but also provide evidence that collagen VI contributes to tumor development and metastasis by modulating the immune system. Thus, a combination of targeted collagen VI inhibition with immunotherapy may represent an effective strategy for cancer therapies.

Collagen VI stimulates signal transduction

Several putative receptors for collagen VI have been proposed, such as β1-integrins and NG2/chondroitin sulfate proteoglycan (NG2/CSPG) [44–46]. These receptors, which are highly expressed in tumors and exert a potent role in modulating tumor progression [35,47], are able to mediate the effects of collagen VI on tumor growth and metastasis through several different mechanisms (Figure 2). Binding of collagen VI to NG2/CSPG in breast cancer cells triggers activation of the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor downstream of the

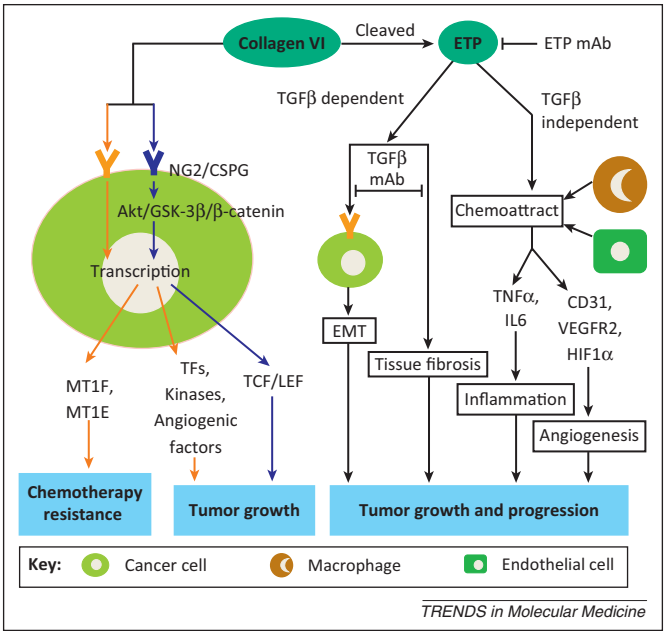

Figure 2. Schematic diagram of signaling pathways for collagen VI contributing to tumor progression and chemotherapy resistance. Collagen VI is highly expressed in tumors where it can act on tumor cells directly to promote tumorigenesis through several pathways, including upregulation of transcription factors (TFs), growth factors, protein kinases, and angiogenic factors, and activation of the Akt–GSK-3β–β-catenin–TCF/LEF pathway; or induce chemotherapy resistance by upregulation of metallothionein 1F/1E (MT1F/1E). The cleaved collagen VI ETP peptide, on the one hand, targets tumor cells to induce epithelial–mesenchymal transition (EMT) and fibrosis through the TGF-β-dependent pathway, and on the other hand, it acts on the tumor microenvironment to initiate tumor inflammation by recruiting macrophages and increasing TNF-α and IL-6 expression, or promote tumor angiogenesis by recruiting endothelial cells and upregulating CD31, VEGFR2, and HIF1α expression. Abbreviations: TCF/LEF, T cell factor/lymphoid enhancer factor; ETP, endotrophin; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; VEGFR2, vascular endothelial growth factor receptor 2; HIF1α, hypoxia-inducible factor 1 alpha.

Akt–GSK-3β–β-catenin pathway, thus enhancing tumor growth [12,13]. The Akt–GSK-3β–β-catenin–TCF/LEF axis is a prosurvival cascade with an important role in tumorigenesis and progression [48,49]. Cyclin D1 is a target of TCF/LEF, and it can be affected by GSK-3β and β-catenin activity [12]. Collagen VI treatment of MCF7 cells increases the expression and stability of cyclin D1 through NG2/CSPG [12], suggesting that this may be one mechanism underlying the role of collagen VI in tumor cell proliferation. Moreover, collagen VI enhances the expression of other transcription factors, such as ETR101, activating transcription factor (ATF)3, and ATF4, as well as CDC28 protein kinases 1, VEGF, calpain 4, IL-8, and angiopoietin-2 [12], which are known to promote tumor growth and angiogenesis.

EMT is a highly conserved and fundamental process that greatly contributes to the dissemination of single tumor cells from primary tumors, leading to metastasis [50]. EMT is considered as a biomarker for investigating whether or how field cancerization contributes to tumor development [51]. The collagen VI ETP peptide promotes tumor growth and metastasis at least in part mediated by enhanced TGF-β signaling, which contributes to EMT of tumor cells [16]. Thus, collagen VI facilitates tumor growth and metastasis by directly acting on tumor cells through upregulation of factors that stimulate tumor growth and metastasis, or by inducing EMT.

As discussed above, collagen VI is expressed in various tissues. Among them, adipose tissue is one of the most abundant sources of collagen VI [52]. The expression of collagen VI is upregulated in dysfunctional adipose tissues in obese and diabetic states, which are highly correlated with adipose tissue fibrosis, and ablation of collagen VI in these conditions inhibits the development of fibrosis [53,17], thus suggesting that collagen VI may be a link between obesity and fibrosis. Obesity is a high risk factor for breast cancer [54], and adipocytes are a major component of the mammary tumor stroma [55]. Further studies show that adipocyte-derived ETP promotes breast tumor growth and progression by upregulating tumor tissue fibrosis in a TGF-β-dependent manner [16]. These findings highlight collagen VI/ETP as an adipocyte-derived factor promoting breast tumor progression by enhancing tissue fibrosis.

Inflammation and angiogenesis are two major hallmarks of cancer [32]. The ETP peptide regulates to some extent both tumor inflammation and angiogenesis by recruiting macrophages and endothelial cells, as well as upregulating inflammatory and proangiogenic factors [16]. In addition, collagen VI can interact with other ECM components, such as collagen types I, II, IV, V, and XIV, fibronectin, and tenascin [46,56–58], in the tumor microenvironment. These ECM proteins are robustly expressed in tumors, and extensively affect tumor development and progression [1], suggesting that one potential mechanism for collagen VI in promoting tumor progression involves its binding and modulation of other ECM proteins. *In vivo* studies demonstrate that ablation of collagen VI is able to delay brain tumor progression due to deficits of vascular basal lamina at least in part by decreasing the deposition of collagen IV, collagen I, and laminin-11 in tumor vessels [26,35]. Mechanistic studies indicate that the decreased deposition of collagen VI elicited by ablation of collagen IV in brain tumor is mediated by NG2 [35]. Altogether, these findings throw light on the molecular mechanisms underlying the role of collagen VI in tumors and also provide potential targets for cancer treatment.

Role of collagen VI in tumor chemotherapy resistance

Resistance to chemotherapy is a common phenomenon in clinical treatment, and it is a major cause of death in cancer patients. However, the molecular mechanisms underlying this resistance are largely unknown. The ECM derived from tumor cells, stromal cells, or their interaction, was shown to inhibit the apoptosis induced by chemotherapeutic agents, thus promoting chemotherapy resistance in breast, lung, and pancreatic cancers by interactions with integrin receptors [59,60]. In the context of ovarian cancer, *COL6A3* is one of the most highly upregulated genes that are differentially expressed as a result of cisplatin resistance. Furthermore, culturing cisplatin-sensitive cells in the presence of collagen VI promotes *in vitro* resistance [61].

Metallothioneins are known to play a critical role in the promotion of cisplatin resistance [62], and collagen VI treatment induces a significant upregulation of metallothionein-1E and -1F in tumor cells [12], thus providing a mechanistic link for collagen VI involvement in cisplatin resistance (Figure 2). Among the differentially expressed genes in oxaliplatin-resistant versus oxaliplatin-sensitive ovarian carcinoma cell lines, *COL6A3* is the most highly upregulated gene with a 62-fold increase in oxaliplatin-resistant cells [63]. Taken together, these findings indicate that collagen VI contributes to chemotherapy resistance in an autocrine manner.

In addition to tumor cells, malignant solid tumors are also rich in myeloid cells, which favor tumor growth and metastasis [38,64,65]. Doxorubicin treatment in mouse tumor models induces higher myeloid cell infiltration in a C–C chemokine receptor type 2 (CCR-2)-dependent manner, which in turn induces chemotherapy resistance [66]. Among myeloid cells, TAMs are the most prominent cell type fostering tumor progression [39]. Interestingly, the response of tumor cells to chemotherapy is partly regulated by TAMs, as demonstrated by the fact that the combined treatment of chemotherapy with macrophage infiltration inhibition significantly decreases primary tumor growth and metastasis [67,68]. Further studies show that chemotherapy using taxol in the context of breast cancer significantly enhances macrophage infiltration, which protects tumor cells from chemotherapy-induced death, thus blunting the chemotherapeutic response by upregulating and activating cathepsin proteases [69]. Macrophage-induced chemotherapy resistance in myeloma is regulated by the interaction of P-selectin glycoprotein ligand-1 (PSGL-1)/selectins and ICAM-1/CD18 [70]. Moreover, TAM-derived milk-fat globule-epidermal growth factor 8 (MFG-E8) endows cancer stem cells with the ability to promote chemotherapy resistance [71]. MFG-E8 can directly target collagens, and *Mfge8*$^{-/-}$ macrophages exhibit defective collagen uptake [72]. One miRNA, miR-511 is robustly expressed by macrophages [73] and downregulated in human tumors [74,75], and strongly inhibits tumor cell proliferation [76]. As discussed above, macrophages abundantly express and secrete collagen VI [43], suggesting a potential role for miRNA-511 in the regulation of collagen VI expression. Indeed, overexpression of miRNA-511-3P, encoded by the *MRC1* gene and expressed in M2 macrophages, induces a significant downregulation of collagen VI expression in TAMs and evokes a genetic program that limits, rather than enhances, the protumoral function of TAMs, inhibiting tumor growth [77]. Thus, collagen VI may serve as an important link between TAMs and chemotherapy resistance. These findings not only shed new light on the molecular mechanisms underlying the ability of TAMs to blunt the response to chemotherapy but also provide novel targets for improving the efficacy of chemotherapy in patients. Thus, the combination of collagen VI inhibition and chemotherapy should be considered as a new potential promising strategy for cancer therapies.

Collagen VI as a potential serum biomarker for cancer diagnosis

The diagnostic implication of collagen VI for cancer patients is another interesting aspect that has been investigated in the context of melanoma and pancreatic cancer. Collagen VI levels in the sera of primary cutaneous melanoma patients (stage I/II) and distant melanoma metastatic patients (stage IV) is significantly increased compared

with healthy donors, although there is no difference between patients with stage I/II disease and those with stage IV [78]. Thus, collagen VI may be a potential serum biomarker for diagnosing melanoma and may also be applicable to pancreatic cancer patients. Indeed, collagen α1(VI) level in the sera of pancreatic cancer patients is dramatically increased compared with healthy controls [79]. Both melanoma and pancreatic cancer are aggressive malignant tumors and, unfortunately, patients who are diagnosed with these two types of cancer are nearly always at advanced stages, where the successes of radiation, chemotherapy, or combinatorial therapies are largely anecdotal. Therefore, the identification of serum biomarkers for such tumors is particularly important. The upregulation of collagen VI in the sera of melanoma and pancreatic cancer patients is a useful finding for the ultimate goal of developing serum diagnostic biomarkers in these aggressive tumors.

Concluding remarks and future perspectives

Collagen VI is a prominent promoter of tumor growth that is frequently overexpressed in human cancers. The actions of collagen VI inside malignant cells to promote tumorigenesis and progression are largely mediated by the Akt–GSK-3-β–β-catenin–TCF/LEF pathway, which activates transcription factors, growth factors, and kinases, leading to EMT through TGF-β signaling and chemotherapy resistance through upregulation of metallothioneins. Moreover, collagen VI acts on components of the tumor microenvironment, such as macrophages and endothelial cells, to promote inflammation and angiogenesis. Taken together, these collective findings point at collagen VI as an important regulator involved in tumor growth and progression.

Hanahan and Weinberg classified the pleiotropic changes of cancer into a number of hallmarks shared by most cancers and proposed that suppressing one or more hallmarks of cancer is promising for the development of new therapeutics and cancer therapies [32]. Collagen VI promotes tumorigenesis and progression by enhancing the proliferation, invasion, and metastasis of tumor cells, decreasing the apoptosis of tumor cells, as well as upregulating tumor angiogenesis and inflammation, therefore contributing to at least five hallmarks of cancer (Figure 3). Thus, blockade of collagen VI-regulated pathways or identification of negative mediators of collagen VI signals may have valuable implications in the setting of cancer therapies, with direct antitumor effects as well as indirect effects on the tumor microenvironment.

Although our knowledge of the role of collagen VI in tumorigenesis has increased in the past few years, several open questions remain regarding the molecular mechanisms underlying the effects of collagen VI in the context of tumors, as well as the value of this ECM molecule as a therapeutic target, biomarker, or prognostic indicator (Box 1). For example, the growth-stimulatory and prosurvival effects of collagen VI in breast cancer cells involve the NG2/CSPG receptor [12]. However, collagen VI receptors, such as integrins and NG2/CSPG, also serve as receptors for other ECM proteins [80,81]. Moreover, NG2 may interact with certain integrins, such as α4β1, to serve as a coreceptor facilitating signaling pathways involved in tumors

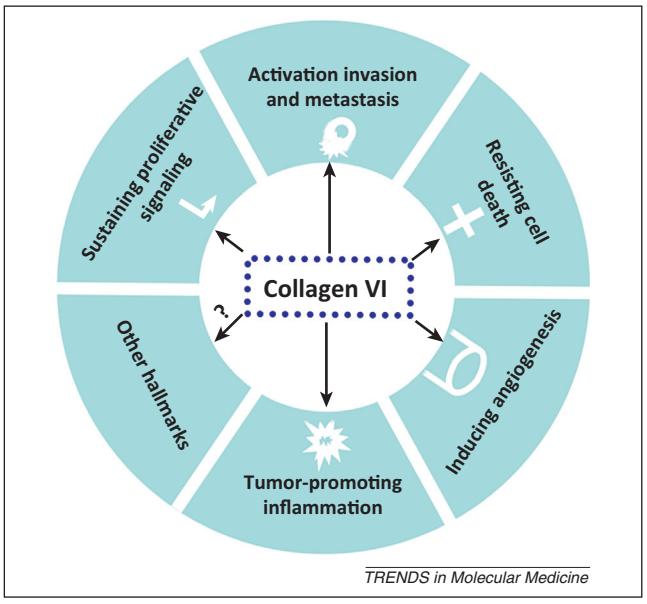

Figure 3. The contribution of collagen VI to cancer hallmarks. The diagram shows that collagen VI promotes tumorigenesis and progression by contributing to at least five hallmarks of cancer, including tumor-promoting inflammation, inducing angiogenesis, resisting cell death, activating invasion and metastasis, and sustaining proliferative signaling. It is still unknown whether collagen VI contributes to other hallmarks of cancer.

[82]. Thus, it is essential to identify the specific receptors involved in mediating collagen VI effects in tumors of different types and stages, and also extensively investigate the underlying molecular mechanisms. Although current knowledge indicates that collagen VI contributes to at least five hallmarks of cancer (Figure 3), further studies are needed to assess whether collagen VI is involved in others. For example, integrins and NG2/CSPG are widely expressed in immune cells, such as B lymphocytes [83], macrophages [84,85], and neutrophils [85]; however, it is largely unknown whether binding of collagen VI to these receptors can mediate effects that modulate the immune response to tumors. The detailed knowledge of biomarkers and prognostic indicators will be highly valuable for clinicians in diagnosing tumors and prescribing cancer therapy. As discussed above, the serum levels of collagen VI in melanoma and pancreatic cancer patients are higher than

Box 1. Outstanding questions

- Among the different receptors for collagen VI, which one mediates signals in tumors?
- Are α4(VI), α5(VI), and α6(VI) expressed in tumors and involved in tumorigenesis and progression?
- How ETP peptide is cleaved from α3(VI) in tumors?
- In addition to ETP, can other domains of collagen VI promote tumorigenesis and progression?
- In addition to macrophages and endothelial cells, can collagen VI act on other bone marrow-derived cells?
- Can collagen VI serve as a critical link between malignant cells and the tumor microenvironment?
- Does collagen VI contribute to all hallmarks of cancer?
- Does collagen VI inhibition in tumors have adverse effects?
- Can collagen VI serve as a biomarker and prognostic indicator in a wide range of cancer types?
- Can we achieve successful clinical outcomes in cancer patients by blocking or inhibiting collagen VI?

those in healthy donors and the expression of collagen VI in certain tumors is associated with tumor grades [22], highlighting the potential value of collagen VI as a biomarker and prognostic indicator in tumors. Given the fact that these findings are currently few in number and for only some types of cancers, further studies are needed.

Altogether, our current understanding of the mechanisms involved in the different effects mediated by collagen VI is only partial; it is unmistakable that this ECM molecule plays a key role on tumor growth and drug resistance. Recent studies have provided valuable information on collagen VI as a key regulator in tumors, and it can be anticipated that in the future additional studies will grant us a detailed understanding of the underlying molecular mechanisms. In addition to increasing our knowledge of cancer biology, these studies will likely provide novel therapeutic targets for cancer treatment.

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Autophagy-mediated regulation of macrophages and its applications for cancer

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Keywords: autophagy, tumor-associated macrophages, cancer, macrophage production, macrophage polarization

Abbreviations: ATG, autophagy-related; Atg8–PE, Atg8–phosphatidylethanolamine; CCL2, chemokine (C–C motif) ligand 2; CSF1, colony stimulating factor 1 (macrophage); CSF2/GM-CSF, colony-stimulating factor 2 (granulocyte-macrophage); CuIIa, cucurbitacin IIa; HSCs, hematopoietic stem cells; IL6, interleukin 6 (interferon, beta 2); LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MTOR, mechanistic target of rapamycin; NFKB, nuclear factor of kappa light polypeptide gene enhancer in B-cells; RB1CC1, RB1-inducible coiled-coil 1; ROS, reactive oxygen species; rVP1, recombinant capsid viral protein 1; TAMs, tumor-associated macrophages; TLR, toll-like receptor; ULK, unc-51 like autophagy activating kinase

Autophagy is a highly conserved homeostatic pathway that plays an important role in tumor development and progression by acting on cancer cells in a cell-autonomous mechanism. However, the solid tumor is not an island, but rather an ensemble performance that includes nonmalignant stromal cells, such as macrophages. A growing body of evidence indicates that autophagy is a key component of the innate immune response. In this review, we discuss the role of autophagy in the control of macrophage production at different stages (including hematopoietic stem cell maintenance, monocyte/macrophage migration, and monocyte differentiation into macrophages) and polarization and discuss how modulating autophagy in tumor-associated macrophages (TAMs) may represent a promising strategy for limiting cancer growth and progression.

Introduction

Autophagy is an evolutionarily conserved homeostatic pathway that is widely occurring in eukaryotic cells.[1] Induction of autophagy can generate lots of amino acids and other building blocks, which are required for cellular homeostasis. Moreover, autophagy is necessary for quality control for both organelles and proteins.[2-4] Autophagy is induced upon stimulation by various extrinsic and intrinsic cellular stress conditions, such as reactive oxygen species (ROS), endoplasmic reticulum stress, bacterial infection, and hypoxia, in order to clear damaged organelles, protein aggregates, and intracellular pathogens. Thus, autophagy is crucial for the maintenance of cellular homeostasis.[2-4] Based on different functions and mechanisms, 3 major forms of autophagy have been described.[5,6] Microautophagy allows for the degradation of portions of the cytoplasm, which are directly enwrapped by the lysosomal membrane. Macroautophagy (hereafter referred to as "autophagy") is responsible for the degradation of bulk cytoplasm, long-lived proteins, and entire organelles, through the formation of a double-membrane compartment, called an autophagosome, which is subsequently targeted to lysosomal digestion. In contrast to these 2 types of autophagy, which mediate both selective and nonselective degradation, chaperone-mediated autophagy only degrades individual, unfolded soluble proteins in a selective manner.[7]

The autophagic process (**Fig. 1**) requires a set of evolutionarily conserved proteins, most of which are known as autophagy-related (ATG) proteins, functioning at different steps.[8] A kinase complex containing ULK1/ATG1, ATG13, RB1CC1/FIP200, and C12orf44/ATG101 is critical for autophagy induction.[9] A different set of complexes, which contain BECN1/Beclin 1, PIK3C3/VPS34, PIK3R4/VPS15, and either ATG14 and AMBRA1 (autophagy/Beclin 1 regulator 1), or UVRAG (UV radiation resistance associated) and SH3GLB1/Bif-1 (SH3-domain GRB2-like endophilin B1) is required for the nucleation and expansion of the phagophore, the initial sequestering compartment.[6,10] Autophagosome formation then requires 2 ubiquitin-like conjugation systems, ATG12–ATG5 and LC3/Atg8–phosphatidylethanolamine (LC3-II/Atg8–PE). In yeast, these proteins are involved in elongation and maturation of the phagophore. In mammals, there are 2 subfamilies of Atg8 proteins; the LC3 subfamily acts at the elongation stage, whereas the GABARAP proteins function later in autophagosome maturation.[11] Autophagy regulation is partly based on the phosphorylation and dephosphorylation of ATG proteins. The major upstream actor in these intracellular pathways is the MTOR (mechanistic target of rapamycin) kinase, which inhibits autophagy by regulating mRNA and protein levels of critical components, and also by direct phosphorylation of the autophagy machinery. For example, MTOR can phosphorylate ATG13, thus inhibiting the activity of ULK1 and autophagy. Given its critical role as a sensor for nutrients, MTOR is able

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to regulate translation by controlling the activity of some specific molecules involved in protein synthesis, such as EIF4EBP1/4E-BP1 (eukaryotic translation initiation factor 4E binding protein 1) and RPS6KB/p70S6K (ribosomal protein S6 kinase, 70 kDa, polypeptide 1). MTOR plays a major role in regulating switches between anabolic and catabolic metabolism, in order to stabilize cell viability in energy stress conditions.[12] MTOR is also regulated by some extracellular growth factors, such as IGF1 (insulin-like growth factor 1 [somatomedin C]), through class III phosphatidylinositol 3-kinase, AKT/PKB (v-akt murine thymoma viral oncogene homolog 1), and AMPK (AMP-activated protein kinase) pathways.[5]

A large amount of experimental data demonstrates that autophagy functions as a key mechanism for the regulation of biological activities in both physiological conditions (e.g., cell/tissue homeostasis and development) and pathological conditions (e.g., cancer and neurodegenerative diseases), and autophagy dysfunction is associated with various diseases.[3,4,13] The role of autophagy in cancer is extremely complex. On the one hand, autophagy can act as a tumor suppressor by eliminating oncogenic protein substrates, unfolded proteins, and damaged organelles, and by preventing oxidative stress and genomic instability. On the other hand, autophagy can function as a tumor promoter in established cancers, by providing substrates that allow tumor cells to overcome nutrient limitation and hypoxia.[6,14] Most studies investigating the role of autophagy in tumors have sampled cancer cells.[6,14,15] However, the solid tumor also includes nonmalignant resident stromal cells, such as cancer-associated fibroblasts, endothelial cells, and bone marrow-derived cells, all of which extensively affect tumor growth and progression.[16,17] Recent studies have begun to unveil the significance of autophagy in the tumor microenvironment, a condition defined as the "autophagic tumor stroma."[18] In established tumors, the autophagic tumor stroma is able to provide essential nutrients to cancer cells, remodel other components of the tumor microenvironment, and increase DNA damage and genetic instability of cancer cells, as well as decrease cancer cell apoptosis, thus representing an important regulator for tumor growth and progression.[18] Moreover, it was recently demonstrated that autophagy and inflammation work synergistically in the tumor microenvironment to facilitate tumor growth and metastasis.[19] These findings highlight the role of autophagy in inflammatory cells in affecting tumor progression, thus pointing at this process as a target for cancer therapies.

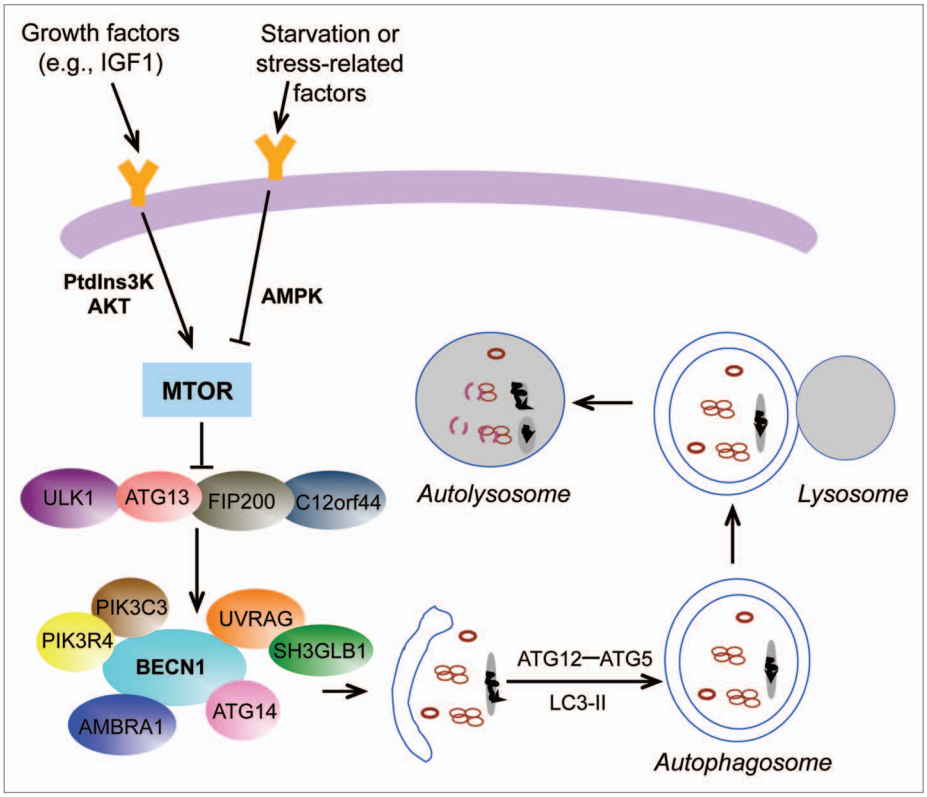

Figure 1. Schematic diagram summarizing the regulation of autophagy in mammalian cells. Following stimulation by growth factors, such as IGF1, MTOR is activated, whereas this pathway is inhibited upon stress conditions, such as starvation. MTOR inhibition is required to activate the ULK complex, since MTOR is able to induce the phosphorylation of ATG13, leading to reduction of ULK1 activity. By sensing the activation of the ULK complex, the BECN1 complex is activated leading to the nucleation of a phagophore. By means of 2 ubiquitin-like conjugation systems, generating ATG12–ATG5 and LC3-II, the membrane is elongated to form a double-membraned vesicle, the autophagosome. Finally, the autophagosome fuses with the lysosome, forming an autolysosome, where the cargo is digested by lysosomal enzymes and the degraded material released into the cytoplasm for recycling by the cell.

Among the inflammatory cells, macrophages are the most prominent cell type in the tumor microenvironment. Both experimental and clinical findings over the past decade demonstrated that tumor-associated macrophages (TAMs) favor malignant progression by suppressing antitumor immunity, by stimulating angiogenesis, and by enhancing tumor cell proliferation, migration, and invasion.[16,20,21] Autophagy is an important component of innate immunity by macrophages regulated by both toll-like receptors (TLRs) and intracellular pathogens. For example, lipopolysaccharide (LPS) can induce the formation of autophagosomes in macrophages, which is regulated through a TICAM1/TRIF (toll-like receptor adaptor molecule 1)-dependent TLR4 signaling pathway.[22] *Listeria monocytogenes* is an intracellular pathogen that can activate the autophagy pathway in macrophages via a MAPK/ERK (mitogen-activated protein kinase)-dependent TLR2 and Nucleotide Oligomerization domain 2 (NOD2) signaling pathways.[23] Increasing evidence demonstrates that autophagy can modulate the activity of

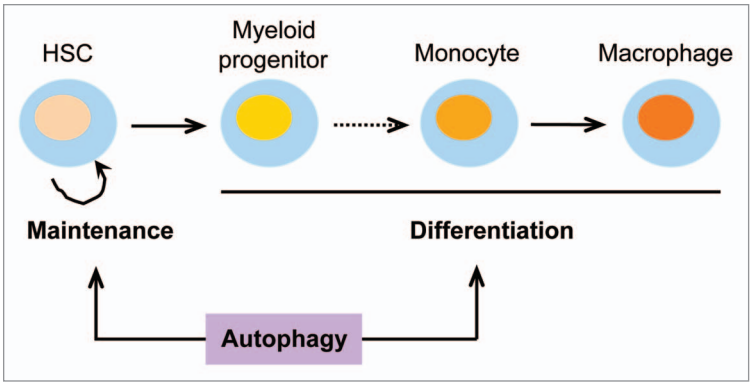

Figure 2. Schematic diagram of the roles of autophagy in macrophage production. Autophagy is involved in the maintenance and differentiation of HSCs, as well as in the differentiation of monocytes into macrophages. It is not yet known whether autophagy also plays a role in mediating the differentiation of HSCs into monocytes (dotted line).

macrophages and their response to different stimuli,[22,24,25] thus highlighting the connections among autophagy, macrophages, and cancer, and suggesting the potential to enhance an antitumor response by modulating autophagy in macrophages. Given the major roles of macrophages and autophagy in tumor progression and their correlation in biological activities, it is valuable to clarify the contribution and underlying molecular mechanisms of autophagy-mediated regulation of macrophages, and their implications for cancer. In the next sections we will summarize the significance of autophagy in regulating macrophage production and polarization, and discuss the value of autophagy modulation with regard to the protumoral functions of TAMs.

Role of Autophagy in Macrophage Production

TAMs are a type of cells that have a short half-life and cannot proliferate in tumor tissues.[26] In order to maintain high TAM levels, these cells require continuous replenishment throughout tumor growth and progression. TAMs are derived from bone marrow progenitor cells, a process that involves different steps, including the maintenance of hematopoietic stem cells (HSCs), the production of monocytes, the recruitment of monocytes into tumors, the differentiation of monocytes into macrophages, and the polarization of macrophages into TAMs.[20,27] In this section, we discuss experimental evidence demonstrating that autophagy is necessary for macrophage production (**Fig. 2**), even in the absence of cancer.

Role of autophagy in hematopoietic stem cell maintenance

HSCs reside in the bone marrow niche and are generally in a quiescent state.[28] However, HSCs undergo distinct programs in response to stimulation, including self-renewal and differentiation. The balance of quiescence, self-renewal, and differentiation is tightly regulated in HSCs in order to maintain their physiological functions, and when this balance is not properly executed it may induce hematopoietic malignancies.[29] Bone marrow that hosts HSCs is usually a hypoxic environment, where a low-oxygen niche limits ROS production, thus providing long-term protection of HSCs from ROS stress.[30] ROS production and metabolic rate are increased when HSCs transit from a quiescent state to a proliferation/differentiation state, a process that is mediated by the MTOR pathway.[30,31] Activation of AKT, an upstream regulator of MTOR, decreases autophagy in HSCs and promotes myeloid proliferation,[32] whereas deletion of *Rptor/Raptor* (regulatory-associated protein of MTOR, complex 1), encoding a component of MTORC1 (MTOR complex 1), enhances autophagy and decreases this myeloid cell population (**Table 1**).[33] Moreover, HSC self-renewal can be restored by treatment with antioxidants or rapamycin.[31,39] RB1CC1 is an important regulator of autophagy, and conditional ablation of *Rb1cc1* in HSCs causes perinatal lethality and severe anemia, with a marked increase of HSC proliferation, ROS levels, and mitochondrial mass (**Table 1**).[34] These findings provide indirect evidence that autophagy is potentially involved in the maintenance of HSCs.

Notably, recent studies have provided direct evidence for the concept that autophagy functions as an important determinant for HSC fate. For example, a study demonstrated that autophagy is highly activated in HSCs in humans and that this process is required for the self-renewal and differentiation of HSCs.[40] Inhibition of autophagy by 3-methyladenine or by *Atg5* siRNA-mediated knockdown results in a complete blockade of the differentiation and self-renewal of HSCs.[40] Inhibition of autophagy in HSCs by conditional ablation of *Atg7* impairs the production of lymphoid and myeloid progenitors, thus suggesting that *Atg7* is essential for HSC maintenance in a cell-autonomous fashion (**Table 1**).[35,36] The effect of autophagy in HSC maintenance is also displayed under metabolic stress conditions, when autophagy is robustly induced by a pathway regulated by FOXO3A to protect HSCs against apoptosis. Moreover, HSCs from aged mice have the ability to exhibit an intact FOXO3A-induced proautophagic gene program, and this ongoing autophagy is

Table 1. Summary of studies related to the roles of autophagy in macrophage polarization using mouse models deficient in autophagic components

Mice	Key findings	Refs.
Rptor knockout	Autophagy is enhanced, myeloid cell population is decreased	33
Rb1cc1 knockout in HSCs	Increase of HSC proliferation, ROS levels, and mitochondrial mass	34
Atg7 knockout in HSCs	Impaired production of lymphoid and myeloid progenitors	35, 36
Atg7 knockout	CSF1-induced differentiation of monocytes into macrophages is significantly hampered	37, 38
Atg5 knockout	M2-polarized macrophages are forced to produce a high level of M1-like cytokines	61, 62

required for protecting HSCs against apoptosis and mitigating metabolic stress.[41] Altogether, these findings highlight autophagy as a key mechanism for the maintenance and for the proper function of HSCs. However, it is also very important to consider that targeting of autophagy in HSCs may lead to several side effects including loss of HSC function, anemia, myeloproliferation and, ultimately, development of leukemia.[29,35,36] Thus, manipulation of autophagy in HSCs should be treated with extreme caution.

Autophagy in the regulation of monocyte/macrophage recruitment

In most solid tumors, TAM density is significantly higher than in the surrounding normal tissues. Generally, TAMs first originate from monocytes that are recruited into tumors by chemoattractants, including chemokines and cytokines released from both tumor cells and stromal cells. Among these chemoattractants, CCL2 (chemokine [C–C motif] ligand 2) is the one exerting a prominent activity in recruiting monocytes into tumors.[16] Conversely, CCL2 is able to protect monocytes against apoptosis in the tumor microenvironment, by upregulating anti-apoptotic proteins and inhibiting CASP8/caspase-8 cleavage, and it also induces hyperactivation of autophagy in these cells,[42] thus suggesting a role of autophagy in CCL2-induced monocyte recruitment. Cucurbitacin IIa (CuIIa), a member of the cucurbitacin family, exerts a wide spectrum of pharmacological activities including anticancer and anti-inflammatory activities, which can inhibit macrophage proliferation and migration as well as enhance LPS-induced autophagy in macrophages, suggesting that enhancement of autophagy may contribute to the anti-inflammatory activity of CuIIa in vitro.[43] However, further studies are needed to investigate the potential role of autophagy in regulating the anticancer activity of CuIIa by inhibiting in vivo macrophage recruitment. Although these data suggest a possible role of autophagy in monocyte recruitment, the connections of autophagy with regulation of monocyte recruitment by CCL2 and antiinflammatory activity of CuIIa are still uncertain and represent an intriguing subject for future investigation.

Recombinant capsid viral protein 1 (rVP1) suppresses growth and metastasis of tumor cells by inducing apoptosis and by modulating CCL2 production.[44] Furthermore, rVP1 also acts on host immune cells and promotes macrophage migration by inducing autophagy in these cells.[45] Mechanistic studies suggested that the phosphorylation levels of MAPK3/ERK1 and MAPK1/ERK2 and the activity of MMP9 (matrix metalloproteinase 9 [gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase]) are increased upon rVP1 treatment, leading to autophagy upregulation and macrophage migration by a mechanism dependent on WIPI1 (WD repeat domain, phosphoinositide interacting 1), WIPI2, ATG5, and ATG7, but not on BECN1.[45] In the larval wound model, autophagy is required for the recruitment of blood cells into wound sites and for the spreading of macrophages.[46] Taken together, these findings provide evidence demonstrating that autophagy is an important mechanism for mediating macrophage migration. However, further studies are needed to investigate how autophagy contributes to monocyte/macrophage recruitment in the tumor microenvironment.

Role of autophagy on monocyte differentiation into macrophages

The half-life of monocytes in blood is very short, with approximately 3 days in humans and 1 day in mice, where they are programmed to undergo apoptosis in the absence of stimulation.[47] However, when stimulated by inflammatory factors, they activate survival pathways, migrate into distinct tissues, and then differentiate into macrophages, dendritic cells, or osteoclasts.[48] CSF1 (colony stimulating factor 1 [macrophage]) is the main factor that can induce monocyte differentiation into macrophages and activate survival pathways.[49] Several lines of data imply the involvement of autophagy in monocyte differentiation into macrophages. When monocytes are stimulated to differentiate into macrophages, autophagy is induced via increased expression and phosphorylation of ULK1.[37,38] Studies involving the inhibition of autophagy by pharmacological agents, siRNA approaches or *Atg7* knockout mice show that the CSF1-driven differentiation of monocytes into macrophages is significantly hampered (**Table 1**).[37,38] CSF2/GM-CSF (colony-stimulating factor 2 [granulocyte-macrophage]) is another important factor that can drive the differentiation of monocytes into macrophages, and it was demonstrated that autophagy is induced during monocyte differentiation into macrophages triggered by CSF2 in vitro and by thioglycolate in vivo.[47] Interestingly, CSF2 is able to promote monocyte survival and differentiation into macrophages by MAPK/JNK and by inducing the dissociation of BECN1 from BCL2 (B-cell CLL/lymphoma 2), thus stimulating autophagy, whereas blockade of autophagy has an inhibitory effect on CSF2-induced monocyte differentiation into macrophages.[47]

CSF1-induced monocyte differentiation into macrophages is a process that requires the activation of CASP3/caspase-3 and CASP8 by modulating the AKT signaling pathway,[50] suggesting a role for properly regulated apoptosis in monocyte differentiation into macrophages. Even if CASP8 may cleave specific substrates required for monocyte differentiation, explaining the requirement for its limited activation during differentiation, CASP8 is considered as the upstream enzyme in the proteolytic caspase cascade whose activation is required for monocyte differentiation into macrophages.[51] Several studies have demonstrated that there is a crosstalk between apoptotic and autophagic pathways. For example, BCL2 can bind to BECN1 to inhibit BECN1-mediated autophagy,[52] whereas BECN1 can be cleaved by caspases, and its C-terminal fragment has the ability to amplify the apoptotic response.[53,54] Moreover, death stimuli can trigger calpain-mediated cleavage of ATG5 to promote mitochondrial-mediated apoptosis.[55] Altogether, these findings point at autophagy as an essential mechanism for monocyte differentiation, and they suggest that inhibition of autophagy may be a promising strategy for impairing macrophage production in tumors.

Autophagy-Mediated Control of Macrophage Polarization

Macrophages are heterogeneous and can display divergent phenotypes and functions dependent on distinct tissue microenvironments.[16,56] For instance, macrophages can be divided into

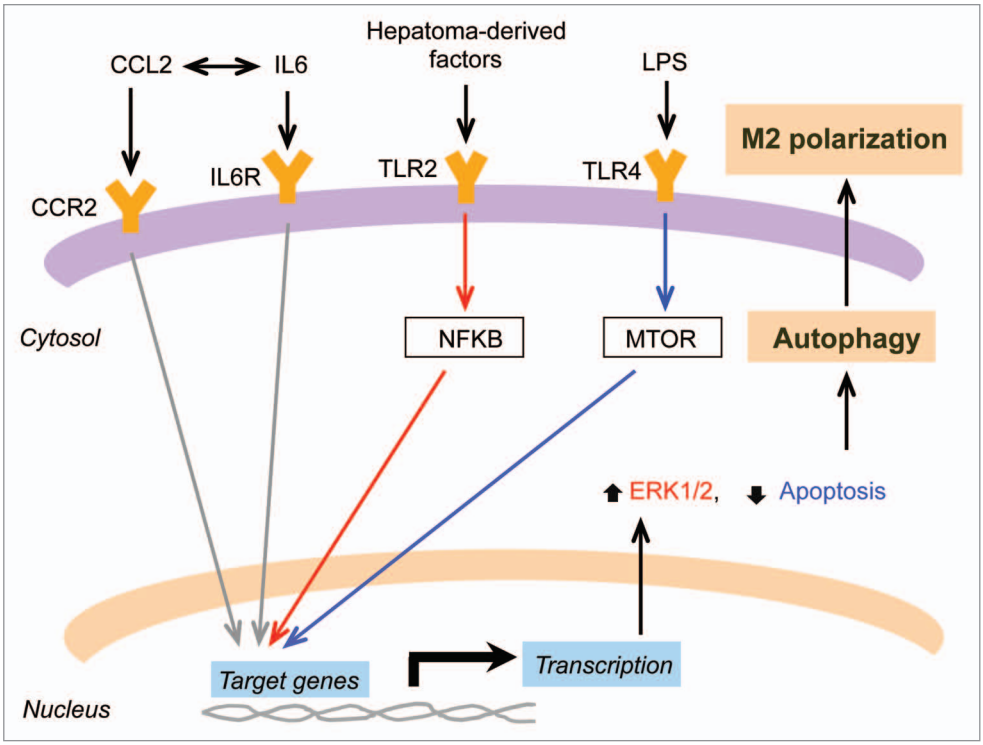

Figure 3. Schematic diagram of signaling pathways for tumor-derived factors contributing to macrophage polarization via induction of autophagy. Expression of IL6 and CCL2 in the tumor microenvironment is regulated in a reciprocal manner. Induction of autophagy triggered by binding of IL6 and CCL2 to IL6R (interleukin 6 receptor) and CCR2 (chemokine [C–C motif] receptor 2), respectively, is essential for macrophage polarizaton to the M2 phenotype. Following binding to TLR2, the hepatoma-derived factors are able to stimulate macrophage polarization to the M2 phenotype by controlling NFKB homeostasis through selective autophagy. Moreover, M2 macrophages can be induced by autophagy triggered by LPS or bacteria, which is modulated by the MTOR pathway via activation of TLR4.

classically activated (M1 phenotype) and alternatively activated (M2 phenotype), according to the T helper cell type (Th)1/Th2 dichotomy.[16,48] M1 macrophages stimulate a Th1 response against intracellular microorganisms and tumor cells by activating an immune response, whereas M2 macrophages are immunosuppressive cells, which promote angiogenesis as well as tissue repair and remodeling.[16,56,57]

We have recently reviewed the findings suggesting that macrophage polarization is triggered by polarization-related factors in the tumor microenvironment.[16] By sensing the stimulation, several intracellular signaling pathways, such as NFKB (nuclear factor of kappa light polypeptide gene enhancer in B-cells) and MTOR, are thought to modulate this process.[16] NFKB is a transcriptional factor that can be regarded as a pivotal link between inflammation and cancer,[58,59] and it also plays a central role in the regulation of macrophage polarization. It has been shown that both M1 and M2 macrophage polarization in the tumor microenvironment require the NFKB pathway,[60] and isolated TAMs from various tumors exhibit low NFKB activity.[60,61] However, the molecular mechanisms by which NFKB is essential for M2 macrophage polarization and is downregulated in TAMs remain to be investigated experimentally. Interestingly, recent studies have demonstrated that hepatoma-derived TLR2-related ligands are able to polarize macrophages toward the M2 phenotype by controlling RELA/NFKB p65 (v-rel avian reticuloendotheliosis viral oncogene homolog A) through selective autophagy.[62,63] Hepatoma-derived TLR2 signals lead to the ubiquitination of RELA, thus forming aggresome-like structures in macrophages, which can be degraded by SQSTM1/p62-mediated autophagy.[62,63] Inhibition of autophagy through pharmacological and genetic approaches can rescue NFKB activity and force M2-polarized macrophages to produce a high level of M1-like cytokines (**Table 1**).[62,63] Furthermore, mechanistic studies demonstrated that TLR2 signals can promote the sustained phosphorylation of MAPK1 and MAPK3, thus stimulating autophagy-dependent NFKB regulation.[62,63] These studies highlight that the role of NFKB in macrophage polarization is regulated by SQSTM1/p62-mediated selective autophagy. However, it has been shown that the role of NFKB in regulating TAM polarization and function is complex, which is exhibited in context-and gene-dependent manners.[60] The specific role of NFKB with respect to the synthesis of tumor-promoting genes and M2 macrophage polarization remains to be fully investigated.

MTOR is an evolutionarily conserved protein kinase regulating autophagy,[64,65] which is also critical in the regulation of monocyte polarization into TAMs. In LPS-stimulated monocytes, inhibition of the MTOR pathway by rapamycin leads to polarization toward the M1 phenotype, whereas activation of this pathway by knockdown of the MTOR repressor TSC2 (tuberous sclerosis 2) exerts the opposite effect.[66] CCL2 and IL6 (interleukin 6 [interferon, β 2]) are 2 abundant cytokines in the tumor microenvironment, and their expression in myeloid cells is induced in a reciprocal manner. CCL2 and IL6 have a potent effect in inducing autophagy and inhibiting apoptosis in macrophages, as well as in stimulating macrophage polarization toward the M2 phenotype. Inhibition of CASP8 is able to promote autophagy in macrophages and increase M2 macrophage polarization. Inhibition of autophagy under these circumstances attenuates M2 macrophage polarization, which directly indicates that autophagy plays a key role in macrophage polarization.[67] Sorafenib is an antiangiogenic agent that has been approved for cancer treatment. However, some studies also demonstrated that antiangiogenic drugs may, in some conditions, accelerate

cancer progression.[68-70] Moreover, TAMs can be recruited when sorafenib is administered, thus promoting the progression of hepatocellular carcinoma.[71] Interestingly, recent studies showed that sorafenib exerts a potent effect on macrophages by inducing autophagy and suppressing the expression of CD80, a marker of the M1 phenotype, suggesting the possible correlation between autophagy and macrophage polarization and highlighting the protumorigenic effect of sorafenib through modulation of macrophage polarization by autophagy.[72] Altogether, these findings support a key role of autophagy in the regulation of macrophage polarization in the tumor microenvironment (**Fig. 3**).

The Significance of Macrophage Autophagy for Cancer

Our current understanding of the contribution of autophagy in controlling macrophage production, polarization, and function in cancer remains limited. Nonetheless, as we discussed above, it is now well established that autophagy plays a crucial role in macrophage production by regulating HSC maintenance, monocyte/macrophage recruitment, and monocyte differentiation into macrophages. Data obtained from patient biopsies indicate that TAM density is correlated with poor prognosis in most human cancers.[16] Tumor angiogenesis and progression are also affected by macrophage density in animal cancer models. For instance, inhibition or enhancement of macrophage density in tumors by genetic and pharmacological approaches, respectively, inhibits or promotes tumor angiogenesis, growth, and progression.[16,21] These findings highlight the significance of autophagy-mediated macrophage production in promoting cancer progression. The induction of autophagy in macrophages is triggered by TLR ligands,[22,63,73] suggesting the potential role of TLR signaling in modulating macrophage function by autophagy. Indeed, TLR2 deficiency induces a significant reduction of autophagy and macrophage infiltration in liver tissues, and promotes hepatocarcinogenesis, suggesting a potential role of TLR2 in tumorigenesis by modulation of autophagy in macrophages.[74] Future studies should aim at using genetic approaches that specifically inhibit or facilitate autophagy in macrophages or their precursors, thus helping to establish in detail the roles of autophagy in regulating macrophage production, tumor growth, and progression in vivo.

As discussed above, macrophages exhibit a spectrum of phenotypes including M1 and M2 phenotypes, which exert anticancer activity and favor tumor progression, respectively.[16,21,75,76] TAMs are mainly polarized toward the M2 phenotype, that promotes tumor angiogenesis, growth, and metastasis.[16,21] However, it should be noted that some exceptions to this pattern exist. For instance, TAMs are biased toward the M1 phenotype in nonprogressing, regressing, and early-stage tumors,[16,21,75,77] suggesting that the phenotype of TAMs can be polarized by the local tumor microenvironment. Interestingly, recent studies demonstrated that HRG (histidine-rich glycoprotein) inhibits tumor growth and metastasis by inducing TAM polarization toward the M1 phenotype through downregulation of PGF (placental growth factor).[77] ADM/adrenomedullin(22–52), an antagonist of ADM receptors, suppresses tumor growth by skewing TAMs polarization to the M1 phenotype through downregulation of ADM in an autocrine-dependent manner.[75] These findings suggest that the identification of potential targets that polarize TAMs toward the M1 phenotype should be a promising anticancer strategy. By sensing the stimulation from the tumor microenvironment, macrophages are polarized to specific phenotypes through different signaling pathways, including the induction of autophagy.[16] Several studies have shown that treatments targeting autophagy can modify the activation states of macrophages.[62,63,66,67,72] These findings should encourage studies to develop genetic and pharmacological approaches to skew TAM polarization to the M1 phenotype by targeting autophagy. For example, even if TLR2 deficiency causes a reduction of macrophage infiltration, this ablation also induces a significant suppression of autophagy and a reduction in the expression of TNF/TNFα (tumor necrosis factor), IFNG (interferon, gamma) and CXCL2 (chemokine [C–X–C motif] ligand 2) in liver tissues, indicating an increase of M2 macrophage polarization, which in turn promotes hepatocarcinogenesis.[74] Notably, recent findings highlight that activation of the MTOR-TSC2 pathway, a key regulator of autophagy, is critical for macrophage polarization toward the M2 phenotype to promote tumor angiogenesis and growth in mouse hepatocellular carcinoma models, whereas inhibition of this pathway exerts the opposite effects.[66] Thus, the polarization of macrophages regulated by autophagy may represent a promising and effective strategy for liver cancer therapies. In this respect, it is important to consider that the role of autophagy in cancer cells depends on different factors, such as tumor type, stage, and genetic context. Therefore, further studies are needed to assess whether and how these factors affect the function of autophagy modulation in macrophages.

Concluding Remarks and Future Perspectives

A number of studies indicate that autophagy extensively regulates the response of macrophages to microenvironmental stimuli, and may modulate the function of TAMs in tumors. They also provide clear evidence that autophagy functions as a key determinant for macrophage production, by modulating HSC maintenance, monocyte differentiation into macrophages, and monocyte/macrophage recruitment, as well as for macrophage polarization. Macrophage production and polarization are 2 key events for the contribution of macrophages in promoting tumor growth and progression. Therefore, modulation of autophagy in macrophages by controlling these parameters represents a promising and effective strategy for anticancer therapies.

Although our knowledge of the role of autophagy in controlling macrophages has increased in the last few years, several open questions remain to be addressed regarding the molecular mechanisms underlying the effects of autophagy in macrophage production and activation, and the effects of macrophage autophagy in the context of tumors, as well as the value of macrophage autophagy as a target for anticancer therapies. Although autophagy is required for HSC maintenance and differentiation as discussed above, it remains to be defined whether autophagy is also required for HSC differentiation into monocytes, an

important stage for macrophage production. The role of autophagy in the maturation of other types of hematopoetic cells has been clearly established. For instance, independent findings indicate that autophagy is essential for the maturation of red blood cells by the clearance of mitochondria, and functional studies demonstrated that the impairment of mitochondrial autophagy by elimination of BNIP3L/Nix, ULK1, or ATG7 causes serious defects in the maturation and function of red blood cells.[78-80] Although a number of experiments indicate that autophagy is essential for macrophage production and activation, further studies are needed to validate this concept in the context of tumors in vivo by specifically targeting autophagy in TAMs. In addition to increasing the understanding of the mechanisms regulating macrophage autophagy during cancer progression, prospective findings in this field may provide novel therapeutic targets for cancer therapy.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER ONE

Role of Macrophage Polarization in Tumor Angiogenesis and Vessel Normalization: Implications for New Anticancer Therapies

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Abstract

Angiogenesis, the formation of new capillary blood vessels from preexisting vasculature, is one of the hallmarks of cancer that is pivotal for tumor growth and metastasis. Tumor vessels are known to be abnormal, with typically aberrant, leaky and disordered vessels. Thus, the combination of angiogenesis inhibition and vessel normalization is a potential strategy for anticancer therapy. The solid tumor is composed of not only cancer cells, but also the nonmalignant resident stromal cells, such as bone-marrow-derived cells (BMDCs) and cancer-associated fibroblasts (CAFs). Tumor-associated macrophages (TAMs) are the most abundant cell components of BMDCs, which play a significant role in promoting tumor progression. Accumulating evidences from both patient biopsies and experimental animal models have shown that TAMs function in tumor angiogenesis and vessel abnormalization in a density- and phenotype-dependent manner.

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This chapter will discuss the evidence for the factors and signaling pathways that are involved in macrophage recruitment and polarization in the tumor microenvironment, and it summarizes the role and underlying molecular mechanisms of macrophage polarization in tumor angiogenesis and vessel normalization. In addition, an overview of the potential of targeting TAM polarization for anticancer therapy will be provided.

1. INTRODUCTION

Angiogenesis, the formation of new capillary blood vessels from preexisting vasculature, is one of the hallmarks of cancer (Hanahan and Weinberg, 2000). The tumor cell population gained the most attention so far due to its capability of secreting proangiogenic factors that are critical in initiating tumor angiogenesis. However, tumor is not an island but rather an ensemble performance of nonmalignant resident stromal cells (also called tumor microenvironment), which includes cancer-associated fibroblasts (CAFs), endothelial cells, bone-marrow-derived cells (BMDCs), and extracellular matrix (ECM) (Ahn and Brown, 2008; Chan et al., 2009; Joyce, 2005). Among the tumor microenvironment, BMDCs are the major stromal cell population, which represents about 15–20% of total cells in solid tumors (Du et al., 2008). Several findings demonstrate that BMDCs are directly proportional to angiogenesis within the development of tumor (Balkwill and Coussens, 2004; Schmid et al., 2011; Yang et al., 2004, 2008), thus suggesting their important role in regulating tumor angiogenesis. BMDCs constitute extremely heterogeneous populations, which consist of CD45+ vascular modulatory cells, endothelial progenitor cells (EPCs), and pericyte progenitor cells (PPCs) (De et al., 2005; Du et al., 2008; Grunewald et al., 2006; Kopp et al., 2006; Lyden et al., 2001). CD45+ vascular modulatory cells make up the largest group of BMDCs. Such cells consist of several subtypes, including tumor-associated macrophages (TAMs) and immature monocytes including Tie2+ monocytes (TEMs), VEGFR1+ hemangiocytes, and CD11b+ myeloid cells (Du et al., 2008). In the tumor microenvironment, EPCs can incorporate into the vasculature and mature into endothelial cells, while PPCs can envelop blood vessels and differentiate into pericytes and vascular smooth muscle cells. Of the multiple stromal cell types in solid tumors, TAMs are most significant for fostering tumor angiogenesis and progression (Condeelis and Pollard, 2006). It was shown that the level of infiltrating macrophages is positively correlated with tumor angiogenesis and poor prognosis in cancer patients (Lewis and Pollard, 2006). Macrophage depletion in mouse tumor models results in decrease of vascular density. Conversely, overexpression of the colony-stimulating factor-1

(CSF-1) induces the enhancement of macrophage recruitment, causing an increase of tumor angiogenesis (Lewis and Pollard, 2006; Lin et al., 2001).

Angiogenesis is well known to promote tumor growth and metastasis. However, unlike the healthy vasculature, tumor vessels are highly chaotic, poorly organized and dysfunctional (vessel abnormalization), due to the excessive production of proangiogenic factors (De et al., 2011; Jain, 2005). These abnormalities of tumor vessels result in a hypoxic tumor microenvironment and represent physiological barriers for the delivery of cancer therapeutic agents (Fokas et al., 2012). The restoration of the balance between pro- and antiangiogenic factors production in tumor microenvironment may play a pivotal role in modulating the normalization of the structure of tumor blood vessels (Jain, 2005). Some important molecules, such as vascular endothelial growth factor (VEGF) (Jain, 2005), placental growth factor (PlGF) (Fischer et al., 2007; Fokas et al., 2012; Hedlund et al., 2009; Van et al., 2010), platelet-derived growth factors (PDGFs), angiopoietins, HIF-prolyl hydroxylases (PHD) (De et al., 2011), Rgs5 (Hamzah et al., 2008), CD160 receptor (Chabot et al., 2011), nitric oxide (NO) and EGF receptor (Kashiwagi et al., 2008), derived from either tumor cells or stromal cells, were reported to participate in regulating vessel normalization. In addition, intracellular signaling pathways, such as PI3K/mTOR pathway (Fokas et al., 2012; Qayum et al., 2012), are also involved in regulating vessel normalization. Understanding molecular mechanisms of vessel normalization may ultimately lead to more effective therapeutic strategies against cancer.

Interestingly, VEGF ablation in inflammatory cells promotes vessel normalization and vessel maturation (Stockmann et al., 2008), suggesting that inflammatory cells, such as macrophages, play a significant role in these processes. Depending on the activation states induced by the microenvironment, macrophages can be designated as either classically activated (M1 phenotype) or alternatively activated (M2 phenotype) (Sica et al., 2008). The different phenotypes of macrophages may exhibit opposing effects in blood vessels modeling and tumor progression. M2, rather than M1, macrophages were defined as the proangiogenic phenotype due to their ability to secrete factors that promote angiogenesis (Lamagna et al., 2006). Moreover, M2 macrophages induce vessel abnormalization, while M1 macrophages lead to vessel normalization (Rolny et al., 2011). These evidences suggest the significant role of TAM polarization from the M2 phenotype to the M1 phenotype in the regulation of tumor angiogenesis and vessel normalization. Thus, tumor vascular network, including angiogenesis and vessel normalization, is affected by the dynamic changes in macrophages phenotypes.

The current review will summarize a comprehensive overview about the basic biology of macrophages, monocytes recruitment and the polarization of macrophages in the tumor microenvironment, as well as the function and underlying mechanisms of TAM polarization in the regulation of tumor angiogenesis and vessel normalization. Furthermore, the potential of targeting TAMs in the tumor microenvironment for anticancer therapy by tumor angiogenesis inhibition and vessel normalization will also be discussed.

2. MACROPHAGES AND THEIR PHENOTYPES

Macrophages are a major population in the mononuclear phagocytic lineage deriving from bone marrow progenitor cells (Doulatov et al., 2010). In contrast to the other mononuclear phagocytic lineages, such as dendritic cells (DCs), macrophages have a longer lifespan (from hours to possibly years) and display proteolytic and catabolic activities, such as phagocytosis, which allow them to be highly effective in the ingestion of pathogens, in the clearance of dead cells and debris, and in the remodeling of tissues (Galli et al., 2011). Macrophages can be subcategorized according to their anatomical locations, and tissue-specific resident macrophages include osteoclasts in bone, alveolar macrophages in lung, histiocytes in interstitial connective tissues, microglia in brain, Kupffer cells in liver and splenic macrophages in spleen. However, they may have similar functional abilities following the appropriate stimuli (Galli et al., 2011). To distinguish them from other cell types, macrophages can be characterized by their specific markers. In mice, macrophages express CD11b, F4/80, and CSF-1R, but not Gr1. In human, they display specific expression of CD68, CD163, CD312, CD115 and CD16 (Qian and Pollard, 2010). On the other hand, macrophages themselves are also heterogeneous, and they can be divided into M1 and M2 phenotypes according to the Th1/Th2 dichotomy (Gordon and Taylor, 2005). M1 phenotype macrophages induce type I helper T cell (Th1) response. This population of macrophages is activated by bacterial moieties, such as lipopolysaccharide (LPS), and Th1 cytokine interferon-gamma (IFN-γ). In contrast, M2 phenotype macrophages are involved in type II helper T cell (Th2) response, and are activated by cytokines secreted from Th2 cell, such as interleukin 4 (IL-4) and IL-13. These two phenotypes of macrophages can be characterized by their different patterns of gene and protein expression (Table 1.1). For example, M1 macrophages display a high expression of major histocompatibility complex (MHC) class II, IL-12 and tumor necrosis factor-alpha (TNF-α), and also generate reactive

Table 1.1 Genes and molecules differentially expressed in M1 and M2 phenotype macrophages

Highly expressed genes and molecules in M1 macrophages	Membrane receptors	*IL2RA*, *IL15RA* (Martinez et al., 2006); TLR2, TLR4, CD16, CD32, CD64, CD80, CD86, IL-1R type I, CCR7 (Mantovani et al., 2002)
	Cytokines and growth factors	*IL15*, *ECGF1* (Martinez et al., 2006); TNF-α, IL-1, IL-6, IL-12 and IFN-γ (Mantovani et al., 2002)
	Chemokines	*CCL15*, *CCL19*, *CCL20* (Martinez et al., 2006); CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, CCL5 (Mantovani et al., 2002)
	Effector molecules	iNOS, ROI (Mantovani et al., 2002)
Highly expressed genes and molecules in M2 macrophages	Membrane receptors	*CD209*, *CD36*, *MS4A4A*, *MS4A6A TLR5*, *CLECSF13*, *TGFBR2*, *CXCR4* (Martinez et al., 2006); Scavenger receptor A/B, CD163, CD206, CD14, CD23, IL-1R type II, IL7R, CCR2, CXCR1 and CXCR2 (Mantovani et al., 2002)
	Cytokines and growth factors	*IGF1* (Martinez et al., 2006); IL-1ra, IL-10, IL-4 and IL-13 (Mantovani et al., 2002); MSF (Solinas et al., 2010)
	Chemokines	*CCL13*, *CCL23* (Martinez et al., 2006); CCL17, CCL22, CCL24, CCL16 and CCL18 (Mantovani et al., 2002)
	Effector molecules	Arginase I (Mantovani et al., 2002)

CLECSF13, C-type lectin superfamily member 13; *ECGF1*, endothelial cell growth factor 1; *IGF1*, insulin-like growth factor 1; *IL2RA*, interleukin 2 receptor α chain; *MS4A4A*, membrane-spanning 4-domains, subfamily A, member 4A; *TGFBR2*, transforming growth factor-beta (TGF-β) receptor type 2. CCR7, chemokine (C–C motif) receptor; CXCL8, chemokine CXC motif ligand 8; CXCR1, C-X-C chemokine receptor 1; IL-1R type I, interleukin 1 receptor type I; ROI, reactive oxygen intermediates; TLR2, Toll-like receptor 2.

oxygen species (ROS) and NO (Qian and Pollard, 2010). Conversely, M2 macrophages show high expression of mannose receptor (CD206), galactose receptor, arginase I, IL-10, IL-1 decoy receptor, and IL-1RA, and low expression of IL-12 (Biswas and Mantovani, 2010; Gordon and Taylor, 2005; Mantovani et al., 2002; Mantovani, 2008). Recent study demonstrated that M2 macrophages specifically express and secrete migration-stimulating factor (MSF), a truncated isoform of fibronectin that is considered as a new marker for M2 macrophages (Solinas et al., 2010). Given their different gene expression profiles, the functions of M1 and M2 macrophages are also different. M1 macrophages are able to kill microorganisms and tumor cells by activating immune responses. By contrast, M2 macrophages are generally considered as immunosuppressive cells, which promote angiogenesis, and tissue remodeling and repair (Mantovani et al., 2002). TAMs are always biased toward the M2 phenotype (Mantovani and Sica, 2010). Monocytes recruitment into tumors, the polarization of macrophages in tumors and its functions and underlying mechanisms in promoting tumor malignancy and in remodeling vascular network including angiogenesis and vessel normalization, are discussed below.

3. RECRUITMENT OF MONOCYTES INTO TUMORS

It has been shown that there are higher numbers of macrophages in tumor tissues than surrounding normal tissues (Murdoch et al., 2008). These cells initially originate from monocytes that extravasate across the tumor vessels from blood and differentiate into TAMs following their recruitment into tumors. The chemoattractants including chemokines and cytokines secreted from both malignant and stromal cells that contribute to the recruitment of monocytes (Murdoch et al., 2008, 2004). Among these chemoattractants, chemokine (C–C motif) ligand 2 (CCL2, also known as monocyte chemoattractant protein-1, MCP-1) is the main one (Bottazzi et al., 1983, 1992; Loberg et al., 2007; Murdoch et al., 2008), which is widely expressed in multiple types of tumors (Bingle et al., 2002; Loberg et al., 2006; Murdoch et al., 2008). Both the experimental studies using CCL2 overexpression and knockdown approaches, and the investigation on human tumor biopsies support the concept that CCL2 plays a pivotal role in monocytes recruitment into tumors (Murdoch et al., 2004). Recent studies showed that CCL2 is responsible for the recruitment of inflammatory monocytes (which express CCR2, the receptor for CCL2), which in turn promotes tumor metastasis. Inhibition of CCL2–CCR2 signaling or

depletion of tumor cell-derived CCL2 blocks the recruitment of monocytes, inhibits tumor metastasis and prolongs the survival of tumor-bearing mice (Qian et al., 2011). Other chemokines, such as CCL3 (macrophage inflammatory protein-1α, MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (MCP-3), CXCL8 (IL-8), and CXCL12 (stromal cell-derived factor 1, SDF-1) (Murdoch et al., 2004, 2008; Scotton et al., 2001), S100A8 and S100A9 (Hiratsuka et al., 2006, 2008), are also involved in monocytes recruitment into tumors or/and premetastastic sites. Furthermore, these chemokines directly stimulate monocytes to express some related molecules/chemoattractants that contribute to not only monocyte recruitment but also tumor progression. For example, CCL5 promotes the expression of CCL2, CCL3, CCL4, CXCL8 and CCR1 in human monocytes, thus induces a positive amplification loop for chemokines production in tumors that accelerate monotytes recruitment (Locati et al., 2002; Murdoch et al., 2004).

In addition to chemokines, cytokines are also implicated in the recruitment of monocytes into tumors. VEGF and macrophage colony stimulating factor (M-CSF) are two prominent cytokines that are commonly produced by tumors. They promote monocytes recruitment by acting tyrosine kinase receptors (Mantovani et al., 2004). It was shown that CSF-1 and its receptor, CSF-1R, are overexpressed in a wide variety of human tumors, and they are positively correlated with macrophage infiltration in tumors (Murdoch et al., 2004). Studies in mouse models suggested that overexpression of CSF-1 in tumors promotes TAM infiltration (Dorsch et al., 1993), while CSF-1 knockout decreases this infiltration (Lin et al., 2001). In addition to its proangiogenic effect, VEGF also functions as a chemotactic factor that is responsible for monocytes recruitment. It was shown that VEGF promotes monocytes migration through its receptors, VEGF-R1 (Barleon et al., 1996; Sawano et al., 2001) and VEGF-R2 (Dineen et al., 2008). VEGF-R1 depletion in macrophages induces the decrease of macrophage migration in response to VEGF stimulation (Hiratsuka et al., 1998). In vivo studies demonstrated that the elevated VEGF expression is positively correlated by the number of macrophage infiltration in breast cancer (Leek et al., 2000). Furthermore, recent studies suggested that VEGF expression in skin cancers is also correlated with macrophage infiltration (Linde et al., 2012). These findings highlight the important role of M-CSF and VEGF in monocytes recruitment into tumors.

More recent studies demonstrated that some other factors, such as tissue factor (TF), transcription factor and chitinase, are also involved in monocytes recruitment. TF expression in tumor is usually correlated with metastasis in

experimental settings and poor prognosis in human tumors (Gil-Bernabe et al., 2012; Laubli and Borsig, 2010; Palumbo and Degen, 2007). TF-induced clot formation indirectly enhances tumor cell survival and metastasis by recruiting macrophages. Genetic or pharmacologic inhibition of coagulation, by induction of TF pathway inhibitor expression, abrogates macrophage recruitment and tumor cell survival (Gil-Bernabe et al., 2012). These data suggested that TF is a significant player in macrophage recruitment into tumors. The expression of forkhead Box m1 (Foxm1) transcription factor is increased in many human tumors (Kalin et al., 2011; Myatt and Lam, 2007), and its expression is associated with poor prognosis in cancer patients (Xia et al., 2012a, 2012b). Balli et al. (2011) generated a mouse model with macrophage-specific Foxm1 deletion, and found that this deletion decreases macrophage recruitment into tumors by downregulating some chemokines, such as IL-6, CCL3 and MIP-2, and chemokine receptors CXCR1 and CXCR4, suggesting Foxm1 transcription factor is required for macrophage migration. Chitinase 3-like 1 (CHI3L1) is a member of chitinase family, which is expressed in several types of human cancers (Eurich et al., 2009; Johansen et al., 2006). CHI3L1 expression in tumors is positively associated with macrophage infiltration and poor prognosis. Overexpression of CHI3L1 in macrophages enhances their abilities of migration in vitro, while CHI3L1 antibody has the opposite effect. Mechanistic studies demonstrated that CHI3L1-induced macrophage recruitment is mediated by the upregulation of IL-8 and CCL2, and activation of mitogen-activated protein kinase (MAPK) signaling pathway (Kawada et al., 2011). These studies provide further insights into the mechanisms of in monocytes recruitment into tumors.

4. MACROPHAGES AND MACROPHAGE POLARIZATION IN THE TUMORIGENESIS

4.1. Macrophages and Cancer

Studies from patient biopsies strongly suggest that macrophages contribute to tumorigenesis. Table 1.2 lists the current knowledge on the correlation between TAM levels and patient survival in a wide range of human tumors. The clinical data suggest that TAM density is correlated with poor prognosis in most tumors. These clinical observations are well supported by experimental studies using macrophages depletion or overexpression approaches. For example, genetic ablation of the M-CSF in different murine tumor models, such as the polyoma middle T (PyMT) oncoprotein breast cancer model, the colon cancer spontaneous model (Oguma et al., 2008)

Table 1.2 The correlation between TAMs density and prognosis or angiogenesis in different forms of human cancer

Tumor type	Prognosis	Angiogenesis	References
Stomach	Good	No	(Ohno et al., 2003)
Colorectal	Good	No	(Funada et al., 2003)
Melanoma	No correlation	No	(Piras et al., 2005)
Ovary	Poor	Increase	(Orre and Rogers, 1999)
Cervix	Poor	Increase	(Salvesen and Akslen, 1999)
Lung (NSCLC)	Poor	Increase	(Koukourakis et al., 1998)
Glioma	Poor	Increase	(Nishie et al., 1999)
Breast	Poor	Increase	(Leek et al., 1999)
Prostate	Poor	Increase	(Lissbrant et al., 2000)
Endometrial	Poor	Increase	(Ohno et al., 2004)
Bladder	Poor	Increase	(Hanada et al., 2000)
Kidney	Poor	Increase	(Hamada et al., 2002)
Squamous cell carcinoma	Poor	Increase	(Koide et al., 2004)
Malignant uveal melanoma	Poor	Increase	(Makitie et al., 2001)
Follicular lymphoma	Poor	No	(Farinha et al., 2005)
Hepatocellular	Poor	Increase	(Zhu et al., 2008)
Thyroid	Poor	No	(Ryder et al., 2008)
Cholangiocarcinoma	Poor	No	(Subimerb et al., 2010)
Sarcoma	Poor	No	(Fujiwara et al., 2011)
Lymphoma	Poor	No	(Harris et al., 2012)
Pancreatic	Poor	Increase	(Kurahara et al., 2012)

"No" indicates without detection. NSCLC, non-small-cell lung carcinoma.

and the osteosarcoma xenotransplant model (Kubota et al., 2009), significantly reduces the macrophage density in these tumors, which in turn inhibits tumor growth and progression (Lin et al., 2001). In contrast, overexpression of CSF-1 in wild-type tumors increases macrophage recruitment and accelerates tumor growth and metastasis. Furthermore, studies using small interfering RNAs or antibodies to inhibit CSF-1 expression in MCF-7 xenografts confirmed these findings, showing that TAMs depletion is associated with the reduction of tumor growth and angiogenesis (Abraham et al., 2010; Aharinejad et al., 2004; Paulus et al., 2006). In addition, specific depletion of macrophages with clodronate-encapsulated liposomes also demonstrates a significant growth reduction of several forms of tumor (Qian and Pollard, 2010). It should be noted, however, that although the experimental and clinical data largely support the hypothesis that macrophages promote tumor growth and progression, there are exceptions. As shown in Table 1.2, a few tumors, such as stomach and colorectal tumor, have the correlation of high TAM density with good prognosis. In animal models, similar observations were found for liver macrophages, which have the function to kill the circulating tumor cells, and depletion of such cells induces the enhancement of metastasis (van der Bij et al., 2005b) and tumor differentiation (Oosterling et al., 2005). Taken together, these data suggest that TAMs may also exhibit antitumorigenic properties (Bingle et al., 2002; van der Bij et al., 2005a) although they are primarily considered as protumorigenic (Condeelis and Pollard, 2006; Pollard, 2004). The different functions of TAMs may depend on their activation states induced by the different tumor microenvironments (Watkins et al., 2007). Following their recruitment into the tumor site, monocytes can be "educated" by the tumor microenvironment and differentiate into different phenotypes, including M1 and M2 macrophages (Sica et al., 2008). Such differences of the TAM phenotype in different tumors may explain their distinct functions in modulating tumorigenesis and progression.

4.2. Macrophage Polarization in the Tumor Microenvironment

In regressing, nonprogressing or early stage of tumors, TAMs mainly resemble the M1 phenotype, while in malignant and advanced tumors, TAMs are biased toward the M2 phenotype (Biswas and Mantovani, 2010; Chen et al., 2011; Lamagna et al., 2006; Qian and Pollard, 2010; Rolny et al., 2011). These findings further suggest that the phenotype of TAMs can be modified by microenvironmental triggers in the tumor. A recent study showed that TAM phenotype is switched from M1 to M2 during the tumor growth

(Chen et al., 2011), which not only supports the concept that macrophage phenotypes can be changed according to different tumor microenvironments but also provides a new insight into the dynamic nature of TAM phenotypes during the tumor growth. Given the opposing effects of M1 and M2 macrophages for tumor progression, there is a great deal of interest in elucidating the factors that regulate macrophage polarization in the tumor microenvironment, as well as their underlying molecular mechanisms.

4.2.1. Macrophage Polarization-Related Factors in the Tumor Microenvironment

Macrophage polarization-related factors have been recently reviewed, and these include M2 phenotype polarization factors, such as IL-4, IL-10, IL-13, IL-21, IL-33, CCL2 and transforming growth factor-β (TGF-β), and M1 phenotype polarization factors, such as IFN-γ and LPS (Biswas and Mantovani, 2010; Galli et al., 2011). Macrophage polarization in the tumor microenvironment is regulated by a number of factors including growth factors, chemokines, interleukins and other molecules (Table 1.3). We herein put an emphasis on summarizing recent research progress in this topic.

VEGF plays an important role in tumor angiogenesis and progression. A recent study demonstrated that VEGF-A-induced skin carcinogenesis depends on the alternative activation of macrophages, suggesting a role for VEGF-A in M2 macrophage polarization. However, this effect is indirect since VEGF-A itself does not induce M2 macrophage polarization in vitro (Linde et al., 2012). Similar to VEGF, adrenomedullin (ADM) is another angiogenic peptide, and it is widely expressed in a variety of tumors (Zudaire et al., 2003). In melanoma, TAMs are the major source of ADM, and TAM-derived ADM upregulates CD206 expression and arginase I production, and downregulates inducible NO synthase (iNOS) production, thus shewing macrophages toward the M2 phenotype in an autocrine manner (Chen et al., 2011).

As mentioned above, CCL2 is the main chemoattractant that is responsible for the recruitment monocytes into the tumor sites (Murdoch et al., 2008). IL-6, one of the most abundant cytokines in the tumor microenvironment, is typically expressed by cancer cells (Siegall et al., 1990). Interestingly, expression of CCL2 and IL-6 in myeloid cells is induced in a reciprocal manner. CCL2 expression in macrophages shows a twofold increase upon IL-6 treatment, and IL-6 expression exhibits fivefold upregulation upon CCL2 stimulation. Therefore, CCL2 and IL-6 induce an amplification loop that affects tumor microenvironment, including TAMs and cancer cells.

Table 1.3 The factors involved in regulating TAM polarization

TAM polarization	Factor types	Major factors and References
M2 polarization	Growth factors	VEGF (De, 2012; Linde et al., 2012); ADM (Chen et al., 2011); PlGF (Rolny et al., 2011); TGF-β, GM-CSF (Biswas and Mantovani, 2010; Galli et al., 2011)
	Chemokines	CCL2 (Roca et al., 2009)
	Interleukins	IL-4, IL-10, IL-13, IL-21, IL-33 (Biswas and Mantovani, 2010; Galli et al., 2011); IL-6 (Roca et al., 2009)
	Others	DcR3 (Tai et al., 2012); ANG-2 (Coffelt et al., 2010)
M1 polarization	HRG (Rolny et al., 2011); AMA (Chen et al., 2011); Celecoxib (COX-2 inhibitor) (Nakanishi et al., 2011); MENK (Chen et al., 2012a)	

GM-CSF, granulocyte/macrophage colony stimulating factor.

Moreover, each member of this loop can induce macrophage polarization to M2 phenotype by upregulation of CD206+ and CD14+/CD206+ cell populations, via the inhibition of caspase-8 cleavage and enhanced autophagy (Roca et al., 2009).

Decoy receptor 3 (DcR3) is a soluble protein belonging to the TNF receptor family and that is overexpressed in cancer cells. DcR3 expression in tumors is correlated with poor prognosis in a variety of tumors. DcR3 modulates TAMs polarization to M2 phenotype by downregulating MHC class II expression through an epigenetic mechanism (Tai et al., 2012). Angiopoietin 2 (ANG-2) is an angiogenic peptide released by endothelial cells, which plays an essential role in modulating angiogenesis and vascular homeostasis (Augustin et al., 2009). Besides its angiogenic function, ANG-2 increases the expression of M2-like genes, such as IL-10, CD206 and CCL17, in macrophages in vitro. Furthermore, in vivo studies showed that ANG-2 overexpression in a mouse tumor model induces higher vascular density and M2 TAM infiltration (Coffelt et al., 2010). Altogether, these findings shed light on the critical factors skewing macrophages toward M2 phenotype in the tumor microenvironment, which in turn promote angiogenesis, tumor growth and metastasis, and also provide some potential targets for anticancer therapies.

The potential molecules involved in TAMs polarization from M2 to M1 phenotype are also documented. Histidine-rich glycoprotein (HRG) is a heparin-binding protein, and its expression is downregulated in tumors (Rolny

et al., 2011). Both pharmacological and genetic approaches indicate that HRG inhibits tumor angiogenesis, growth and metastasis by promoting immune responses (Rolny et al., 2011; Tugues et al., 2012), especially by skewing TAM polarization away from the M2 phenotype to a tumor-inhibiting M1 phenotype via downregulation of PlGF (Rolny et al., 2011). Adrenomedullin22-52 (AMA), an antagonist of ADM receptors, inhibits tumor growth by inducing M2 macrophages polarization to M1 phenotype through downregulation of ADM expression in an autocrine manner (Chen et al., 2011). Cyclooxygenase-2 (COX-2) is constitutively expressed in various tumors, and TAMs are considered as the major source of COX-2 (Inaba et al., 2003). It has been shown that celecoxib, an inhibitor of COX-2, induces TAM polarization from M2 to M1 phenotype in intestinal tumors (Nakanishi et al., 2011). Met-enkephalin (MENK) is an endogenous neuropeptide, which has the ability to inhibit tumor growth and metastasis (Kuniyasu et al., 2010). The expression of MENK in tumors, such as colorectal cancer, is associated with tumor-infiltrating immune cells (Ohmori et al., 2009). It was recently demonstrated that MENK induces TAM polarization to M1 phenotype, as indicated by the downregulation of CD206 and arginase I (Chen et al., 2012a). Taken together, these evidences provide novel insights into potential targets for efficient tumor therapeutic strategies through TAM polarization from M2 to M1 phenotype.

4.2.2. *Molecular Mechanisms of Macrophage Polarization in the Tumor Microenvironment*

Nuclear factor-kappaB (NF-κB) signaling is a key regulator that links inflammation and cancer (Ben-Neriah and Karin, 2011; Grivennikov et al., 2010). The potential role of NF-κB signaling in macrophages polarization was validated in both in vitro and in vivo experiments. It was shown that macrophages polarization to M2 phenotype requires NF-κB activation. When NF-κB signaling is specifically inhibited by blocking IκB kinase (IKK)β, the major activator of NF-κB, TAMs are switched to M1 phenotype, which in turn inhibits tumor growth (Hagemann et al., 2008). On the other hand, overexpression of p50 NF-κB overexpression in TAMs inhibits M1 inflammatory responses and antitumor resistance (Saccani et al., 2006). It was recently demonstrated that Wnt5a induces a negative feedback loop of NF-κB activation in macrophages and has the ability to skew macrophage polarization to M2 phenotype in breast cancer patients (Bergenfelz et al., 2012). Notch signaling is another important mediator in the determination of M1 versus M2 polarization of TAMs. Notch signaling activation induces macrophage polarization to M1 phenotype, whereas blockade

of this signaling causes macrophage polarization to M2 phenotype (Wang et al., 2010). These studies support the important function for NF-κB and Notch signaling pathways in macrophage polarization. Furthermore, several transcription factors, such as signal transducer and activator of transcription 6 (Stat6), peroxisome proliferator-activated receptor-gamma (PPARγ), and c-Myc, also play important roles in this process (Bouhlel et al., 2007). Stat6 is a facilitator of PPARγ-regulated gene expression in macrophages (Szanto et al., 2010), which is responsible for epigenetic changes mediated by the histone demethylase Jumonji domain containing 3 (Jmjd3), which in turn leads to M2 polarization (Ishii et al., 2009). c-Myc is required for the polarization of macrophages into M2 phenotype by inducing a significant set of M2-related genes through Stat6 and PPARγ pathways (Pello et al., 2012).

Autophagy is a key cellular process widely occurring in eukaryotic cells (Reggiori and Klionsky, 2002). In macrophages, autophagy is an important regulator of innate immunity (Xu et al., 2007). Moreover, the induction of autophagy is pivotal for the survival and differentiation of monocytes (Zhang et al., 2012b). CCL2 and IL-6 treatment induces macrophages skewing to M2 phenotype, a process that is also accompanied by induction of autophagy. Inhibition of caspase-8 can mimic similar functions in macrophages. However, combination treatment with autophagy inhibitors or lysosomal protease inhibitors, and caspase-8 inhibitor results in a significant decrease in CD206+ and CD14+/CD206+ cell populations when compared with caspase-8 inhibitor treatment alone, in agreement with the concept that autophagy plays a significant role in M2 macrophage polarization (Roca et al., 2009). The mTOR kinase is a major regulator of autophagy (Jung et al., 2010), and mTOR positively regulates immune cell activation (Weichhart and Saemann, 2008). The TSC2–mTOR signaling pathway is critical for macrophage polarization, as demonstrated by the fact that when this pathway is inhibited by rapamycin or activated by TSC2 knockdown, LPS-stimulated monocytes are induced to differentiate into M1 or M2 macrophages, respectively (Chen et al., 2012b).

These findings highlight that the characterization of the molecular mechanisms involved in macrophage polarization within the tumor microenvironment is essential for improving our understanding of cancer biology. In summary, upon stimulation by different factors in the tumor microenvironment, macrophage gene transcription is regulated through specific signaling pathways and transcription factors, thus leading to macrophage polarization to M1 or M2 phenotypes to exert different functions in tumor angiogenesis, growth and progression (Fig. 1.1).

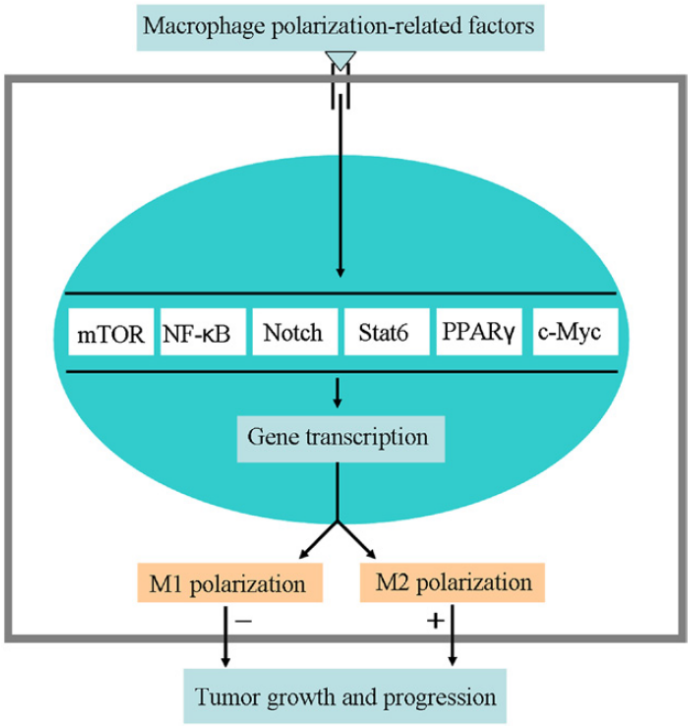

Figure 1.1 ***Schematic representation of signaling pathways in macrophage polarization.*** Macrophage polarization-related factors in the tumor microenvironment exert their effects through macrophage surface receptors to trigger intracellular signaling pathways, such as mTOR, NF-κB, Notch, Stat6, PPARγ and c-Myc, which in turn induce gene transcription. By sensing the differently transcribed gene products, macrophages are polarized to classically activated (M1) or alternatively activated (M2) phenotype, which, respectively, inhibits and promotes tumor angiogenesis and progression. For color version of this figure, the reader is referred to the online version of this book.

5. ROLE OF TAMs AND THEIR POLARIZATION IN TUMOR ANGIOGENESIS

5.1. Role and Mechanisms of TAMs

In most tumors, blood vessels are dramatically increased during the transition from benign to malignant states, a process regarded as an angiogenic switch and that is influenced by the tumor microenvironment (Qian and Pollard, 2010). The potential role of macrophages in modulating tumor angiogenesis was first proposed by Sunderkotter et al. in 1991 (Sunderkotter et al., 1991). After that, a variety of studies have shown that TAMs are often found in the surrounding of blood vessels of solid tumors (Leek et al., 1996; Negus et al., 1997; Ohno et al., 2004). As shown in Table 1.2, studies in human tumors demonstrate a positive correlation between blood vessel density and the

number of TAMs in vessel areas (Leek and Harris, 2002; Onita et al., 2002). The proangiogenic function of TAMs was also thoroughly investigated in animal cancer models. Accumulating evidences show that TAM depletion results in the decrease of tumor angiogenesis (Lin et al., 2001, 2006), while TAM enhancement exhibits the opposing effect (Zhang et al., 2010). For example, it has been shown that genetic depletion of macrophages in PyMT mammary tumor model delays the angiogenic switch, whereas restoration of macrophage infiltration rescues the vessel phenotype (Lin et al., 2006).

In addition to the functional studies mentioned above, much interest has been given to the mechanistic insights on the proangiogenic function of TAMs. Hypoxia is a common feature of solid tumors, and macrophages are often attracted to the hypoxic areas of tumor site due to the secretion of hypoxia-induced chemoattractants by tumor cells. Such chemoattractants include VEGF, endothelin, endothelial monocyte activating polypeptide II (EMAP II) (Murdoch et al., 2004) and CCL2 (Murdoch et al., 2008). Once TAMs are attracted to the hypoxic areas, this microenvironment promotes the metabolic adaptation of TAMs to hypoxia by upregulating hypoxia-inducible factors (HIF)-1, HIF-2 and VEGF (Burke et al., 2002; Lewis and Pollard, 2006; Murdoch and Lewis, 2005). VEGF-A functions as a potent mitogen for endothelial cells by binding to VEGFR1 and VEGFR2 (Squadrito and De, 2011). Genetic studies showed that TAM-derived VEGF-A is essential for angiogenesis in the PyMT mammary tumors (Lin et al., 2007). Restoring VEGF-A expression in macrophage-deficient PyMT tumor model induces the increase of tumor angiogenesis (Lin et al., 2007). These data indicate that VEGF is a key regulator of the proangiogenic activity of TAMs. Further studies suggested that hypoxia also upregulates the expression and secretion of ADM by macrophages (Cejudo-Martin et al., 2002), which are often regulated by HIF and VEGF (Fang et al., 2009; Oladipupo et al., 2011). A recent study showed that TAM-induced endothelial cell migration and tubule formation are inhibited by treatment with an ADM neutralizing antibody (Chen et al., 2011). These findings demonstrate that ADM can function as a novel pivotal factor of TAMs in facilitating tumor angiogenesis. TAMs also have the ability to release a number of other proangiogenic factors, including growth factors (such as PlGF, basic fibroblast growth factor (bFGF), M-CSF, PDGF, heparin-binding epidermal growth factor (HB-EGF), macrophage-inhibitory factor (MIF), platelet activating factor (PAF) and TGF-β), and cytokines (such as IL-1, IL-8, TNF-α and MCP-1) (Dirkx et al., 2006; Fischer et al., 2007). Recent studies have increased our understanding about TAM-derived

factors involved in angiogenesis. In solid tumors, the hypoxic condition often induces apoptosis of tumor cells (Weinmann et al., 2004). The apoptotic tumor cells can upregulate prostaglandin E2 (PGE2) production from macrophages to promote angiogenesis (Brecht et al., 2011). Semaphoring 4D (Sema4D) is a proangiogenic molecule that acts through its receptor, plexin B1 (Conrotto et al., 2005). In the tumor microenvironment, TAMs are the major source of Sema4D, which is critical for tumor angiogenesis and vessel maturation, as demonstrated by the impaired angiogenesis and vessel maturation in Sema4D knockout mice (Sierra et al., 2008). In addition to producing proangiogenic factors in the hypoxic condition, TAMs also promote angiogenesis by downregulating the expression of angiogenesis inhibitors, such as vasohibin-2 (Shen et al., 2012).

Apart from the secretion of proangiogenic factors, TAMs also express a number of angiogenesis-modulating enzymes, such as COX-2, iNOS, and matrix metalloproteinases MMP-2, MMP-7, MMP-9, and MMP12 (Klimp et al., 2001; Lewis and Pollard, 2006). TAM-derived MMP-9 was shown to be crucial for angiogenesis development in estrogen-treated K14-HPV16 transgenic mice, a model of human cervical carcinogenesis (Giraudo et al., 2004). Cathepsin proteases were also implicated in human tumor progression (Mohamed and Sloane, 2006). In the tumor microenvironment, TAMs are the primary source of the high levels of cathepsin protease activity in pancreatic cancer and mammary tumor. Removal of TAM-derived cathepsin B or S in these tumors impairs tumor angiogenesis, suggesting their critical roles in mediating TAMs effects on angiogenesis (Gocheva et al., 2010).

The ability of TAMs to secrete angiogenic factors is regulated by specific signaling pathways or transcription factors. Flt-1 is a tyrosine kinase receptor that binds to VEGF-A/B and PlGF and promotes angiogenesis (Olofsson et al., 1998; Sawano et al., 1996). Flt-1 is expressed by both endothelial cells and TAMs, and Flt-1 deficiency in TAMs impairs tumor angiogenesis, thus suggesting an important role of Flt-1 in mediating TAM-induced angiogenesis (Kerber et al., 2008). Activation of the Stat3 transcription factor mediates the function of TAMs in angiogenesis by upregulating several proangiogenic factors, such as VEGF and bFGF (Kujawski et al., 2008). In macrophage and tumor cell coculture systems, the expression of proangiogenic factors VEGF-A and VEGF-C in macrophages is dramatically upregulated. This effect is significantly reduced when the NF-κB signaling pathway is inhibited (Wu et al., 2012). It was recently shown that TSC2–mTOR signaling pathway also plays an important regulatory function in this process. Tubule formation is reduced or increased when endothelial

cells are cocultured with macrophages treated with either rapamycin or TSC2 siRNA, respectively (Chen et al., 2012b).

In summary, when TAMs are attracted to the hypoxic areas of tumor site, they produce a large body of proangiogenic factors in addition to angiogenesis-modulating enzymes, under the regulation of specific signaling pathways (i.e. NF-κB and mTOR) and transcription factors (i.e. Stat3), which together or partly contribute to tumor angiogenesis (Fig. 1.2). On the other hand, TAMs may also promote tumor angiogenesis by affecting other components, such as cancer cells, in the tumor microenvironment. For example, the interaction of mouse breast cancer cells and TAMs leads to the upregulation of Fra-1, a member of the FOS transcription factor family, which in turn induces activation of the IL-6/JAK/Stat3 signaling pathway in TAMs. This leads to increased release of the proangiogenic factors MMP9,VEGF and TGF-β by cancer cells, thus promoting tumor angiogenesis (Luo et al., 2010).

5.2. Role of Macrophage Polarization

Although most studies reported that TAMs usually favor tumor angiogenesis, this effect is largely dependent on TAM phenotypes. Increasing evidence demonstrate that TAMs normally exhibit the M2 (proangiogenic) phenotype, which promotes endothelial cell proliferation and tumor angiogenesis (Lewis and Pollard, 2006). However, in some cases, such as in the

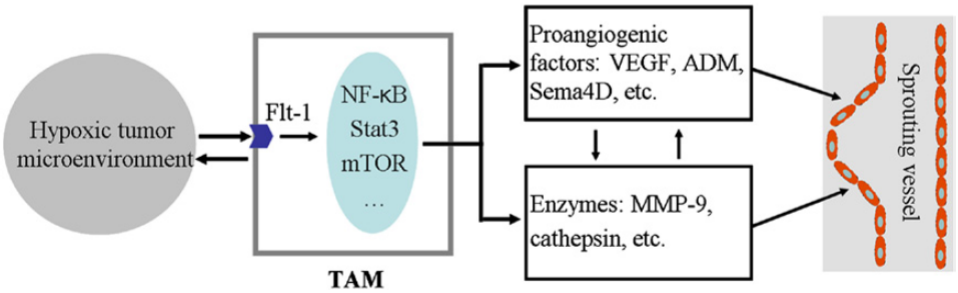

Figure 1.2 ***Proangiogenic function of macrophages and its molecular nature in tumors.*** Following the attraction into hypoxic areas in tumors, signals from tumor microenvironment induce macrophages activation by their cell surface receptors. During the process of macrophage activation, specific signaling pathways (including NF-κB and mTOR) and transcription factors (such as Stat3) are activated, leading to the secretion of proangiogenic factors (such as VEGF, PlGF, Sema4D, bFGF, IL-1, ADM and IL-8), and the production of angiogenesis-modulated enzymes (such as cathepsin proteases, COX-2 and matrix metalloproteinases MMP-2, MMP-7, MMP-9, and MMP-12). These proangiogenic factors and enzymes contribute to tumor angiogenesis. For color version of this figure, the reader is referred to the online version of this book.

early stage of tumors as well as in regressing and nonprogressing tumors, TAMs mainly resemble the M1 (antiangiogenic) phenotype, which causes angiogenesis inhibition and antitumor immune response, due to secretion of IFN-γ, IL-12 (Lamagna et al., 2006; Tsung et al., 2002) and/or TNF-α (Blankenstein et al., 1991; Lamagna et al., 2006). In vitro studies showed that the conditioned medium from resting macrophages inhibits endothelial cells proliferation, while the conditioned medium from activated macrophages displays the opposite effect (Pakala et al., 2002). Resting macrophages inhibit the proliferation of endothelial cells by releasing a variety of endothelial cell growth inhibitors, such as oncostatin M (OSM) and leukemia inhibitory factor (LIF) (Takashima and Klagsbrun, 1996). Furthermore, recent findings showed that TAM polarization from the M2 to the M1 phenotype by HRG or AMA inhibits tumor angiogenesis in vivo (Chen et al., 2011; Rolny et al., 2011). These evidences support the concept that the different macrophage activation states contribute to their opposite effects in regulating tumor angiogenesis. It is conceivable to target TAMs in order to limit tumor angiogenesis by modifying their activation status toward the antiangiogenic phenotype. Thus, the combination of the inhibition of TAM recruitment and their polarization from the M2 to the M1 phenotype should be considered as a new potential strategy in antiangiogenic studies and therapies.

6. ROLE OF TAMs AND THEIR POLARIZATION IN VESSEL NORMALIZATION

In the hypoxic microenvironment, TAMs produce an excess of angiogenic molecules, which not only promote tumor angiogenesis but also induce tumor vessel abnormalization. By impairing oxygen delivery, abnormal vessels trigger a vicious cycle of nonproductive angiogenesis, which creates a hostile microenvironment from where cancer cells escape through leaky vessels, thus rendering tumors less responsive to chemoradiation (De et al., 2011; Jain, 2005). Indeed, antiangiogenic vessel normalization strategies not only improve the chemotherapy drug delivery but also convert the malignant invasive and metastatic tumors into more benign and less aggressive cancers (De et al., 2011; Rolny et al., 2011). Thus, these strategies are gaining more attention, and are emerging as a new concept in cancer therapies (Jain, 2001).

Past work on treatment strategies aimed at vessel normalization mostly focused on the abnormalities of vascular endothelial cells and pericytes. However, the other components in the tumor microenvironment, such as

inflammatory cells (Stockmann et al., 2008), also play an important role in this process. It was recently reported that CCR2-dependent myeloid cells infiltration in tumors induces low response to chemotherapeutic agents, while CCR2 depletion reverses this phenomenon (Nakasone et al., 2012). Moreover, macrophages also have the ability to increase tumor resistance to chemotherapy, which is demonstrated by the fact that the survival of tumor-bearing mice is enhanced by the blockade of macrophage recruitment, in combination with chemotherapeutic agents (Denardo et al., 2011). Macrophage-induced chemotherapy resistance is, at least in part, mediated by the formation of abnormal and hypoperfused blood vessels, which limit the delivery of chemotherapeutic agents. Thus, it is conceivable that TAM depletion increases the efficacy of chemotherapy, which is due to the normalization of blood vessels, which enhances the blood flow to tumor and then increases the delivery of chemotherapeutic agents (De and Lewis, 2011). These findings reveal that myeloid cells, especially macrophages, function as an important player in blood vessel abnormalization, and limit the delivery of chemotherapeutic agents into tumors.

Recent studies have demonstrated that M2 macrophages depletion by clodronate treatment normalizes tumor blood vessels, while M1 macrophage depletion is not involved in this process, thus revealing an important role of TAMs and their polarization away from M2 to M1 phenotype in tumor vessels normalization (Huang et al., 2011; Rolny et al., 2011). This is the first indication that links TAM polarization to vessel normalization, thus implying that "re-education" of TAMs is a promising cancer therapeutic strategy by inducing tumor vessel normalization. The underlying molecular mechanisms of TAMs and their polarization in vessel normalization are discussed below and are summarized in Fig. 1.3.

PlGF, a VEGF family member, is not necessary for physiological angiogenesis but selectively promotes angiogenesis in diseased states (Carmeliet et al., 2001; Fischer et al., 2008). PlGF is highly expressed in tumors and exerts pleiotropic functions in promoting tumor angiogenesis and growth (Loges et al., 2009). Genetic or pharmacological blockage of PlGF induces vessel normalization in mouse spontaneous tumor models, without affecting the tumor blood vessel density (Van et al., 2010). Mechanistic studies in mice suggest that PlGF effects in tumor vessel disorganization are, in part, mediated by changes of TAM polarization. Macrophage loss of PlGF induces vessel maturation and reduces vessel leakage and remodeling, which in turn increase tumor oxygenation and response to chemotherapy (Rolny et al., 2011). PlGF blockage normalizes tumor vessels by shifting

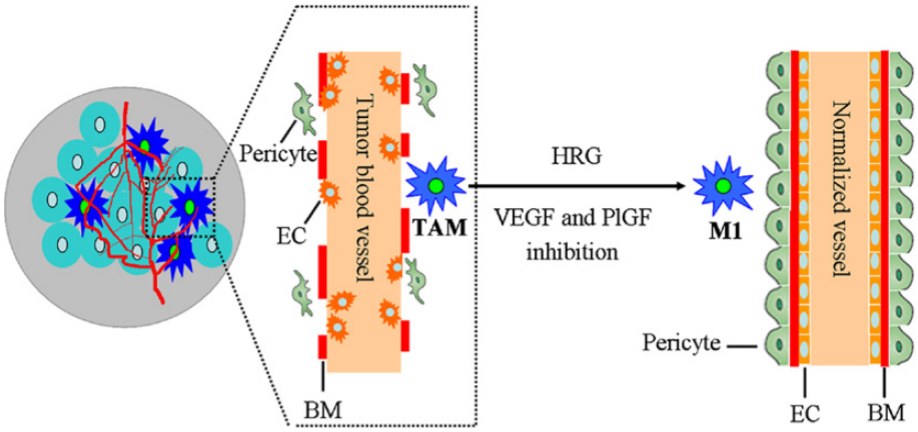

Figure 1.3 ***Role and underlying mechanisms of TAMs and their polarization away from M2 to M1 phenotype in vessel normalization.*** In established solid tumors, blood vessels always exhibit structural and functional abnormalities, which may be induced by the excess of proangiogenic factors derived from TAMs skewing to the M2 phenotype. Tumor vessel normalization is emerging as a new therapeutic strategy for cancer. Myeloid cells, such as macrophages, are involved in tumor vessel disorganization. Genetic depletion of VEGF in myeloid cells or PlGF in macrophages induces tumor vessel normalization. Furthermore, HRG as well as the blockade of PlGF or VEGF signals in TAMs contribute to TAM skewing toward the M1 phenotype and tumor vessel normalization. BM, basement membrane; EC, endothelial cell. For color version of this figure, the reader is referred to the online version of this book.

TAM phenotype from M2 to M1, without affecting TAM density (Rolny et al., 2011).

VEGF is expressed in TAMs (usually M2 phenotype) of both human and mouse mammary tumors (Qian and Pollard, 2010). Given the importance of VEGF for angiogenesis in health and disease, it has been become the prime target for antiangiogenic therapies in cancer (Ellis and Hicklin, 2008). VEGF inhibition not only inhibits tumor angiogenesis and tumor progression but also induces vessel normalization (Jain, 2005). The blockade of VEGF expression results in increased pericyte coverage and vessel maturation, and decreased tumor vessel permeability (Abramovitch et al., 1999; Tong et al., 2004; Weisshardt et al., 2012). Interestingly, myeloid cell-derived VEGF is also very important in this process, as demonstrated by the fact that VEGF deletion in these inflammatory cells promotes vessel normalization and maturation (Stockmann et al., 2008). Recent studies demonstrated that VEGF-A is a potent chemoattractant for macrophages and that the majority of macrophages recruited to VEGF-A-expressing tumors exhibit M2 phenotype, thus implying an important role of VEGF-A in macrophage recruitment and polarization (De, 2012; Linde et al., 2012). These data strongly support

a mechanism where TAMs regulates vessel normalization by the distinct expression and production of VEGF in M1 or M2 macrophages. Further studies suggest that VEGF inhibition induces tumor vessel normalization in part by the remodeling of the vascular basement membrane and the surrounding ECM (Vosseler et al., 2005; Winkler et al., 2004). It is well established that ECM influences tumor growth and progression and regulates vascular morphogenesis in tumors (Bauer et al., 2009). Integrins is a family of heterodimeric receptors that link the surface of cells to different ECM components, thus mediating the transduction of cell-ECM signals. Some integrins, such as α5β3, α5β5, α5β1 and α6β5, are upregulated in tumors (Joyce, 2005). β3-intergrin is a promoter of endothelial cell survival during tumor angiogenesis (Brooks et al., 1994), and it is expressed in macrophages (Friggeri et al., 2010; Zhang et al., 2012a). Additionally, β3-intergrin seems to play a significant role in macrophage polarization, as demonstrated by findings that β3-intergrin knockout mice display impaired muscle regeneration and increased fibrosis due to the infiltration of macrophages that are polarized to M2 phenotype (Zhang et al., 2012a). Furthermore, β3-intergrin-binding peptide has the ability to induce vessel normalization in spinal cord injury mouse mode (Han et al., 2010). However, more studies are needed to clarify if TAMs produce β3-intergrin, and the significance of TAM-derived β3-intergrin for macrophage polarization and vessel normalization in tumors.

7. CONCLUDING REMARKS

Given the significant role of angiogenesis in solid tumor growth and metastasis, antiangiogenic strategies offered a promising perspective for cancer therapies. Bevacizumab, a monoclonal antibody targeting VEGF, is the first antiangiogenic agent used in clinic. When administered as a single agent in patients with solid tumors, bevacizumab produces modest responses (Cobleigh et al., 2003; Yang et al., 2003), but this treatment does not have the benefits of long-term survival (Mayer, 2004). Interestingly, when administered in combination with chemotherapeutic agents, bevacizumab can produce an unprecedented increase in cancer patients survival compared with chemotherapy alone (Hurwitz et al., 2004). These findings highlight the relevance of anti-VEGF activity in tumor angiogenesis inhibition and vessel normalization, and also pave the way for the development of new strategies in antiangiogenic therapies. Tumor progression is modulated by the synergistic effects coming from both tumor cells and stromal cells. Tumor angiogenesis regulation by TAMs, the most abundant stromal

cells, has largely been investigated and was shown that macrophages depletion decreases tumor angiogenesis and progression (Lin et al., 2001, 2006), thus pointing at TAMs as a potential target for cancer therapies. Moreover, increasing evidence suggest that macrophage polarization away from the M2 phenotype to the M1 phenotype can be regarded as another important strategy for cancer therapies (Sica et al., 2008). Recent studies indicate that HRG functions in vivo to repress M2 macrophage skewing, which results in both tumor angiogenesis inhibition and vessel normalization, while PlGF displays the opposing effects (Rolny et al., 2011). A number of findings support the concept that TAMs are educated by tumor cells and tumor microenvironment, and "re-education" of TAMs is now emerging as a novel strategy for cancer therapies via tumor angiogenesis inhibition and vessel normalization (Fig. 1.4). However, much work remains

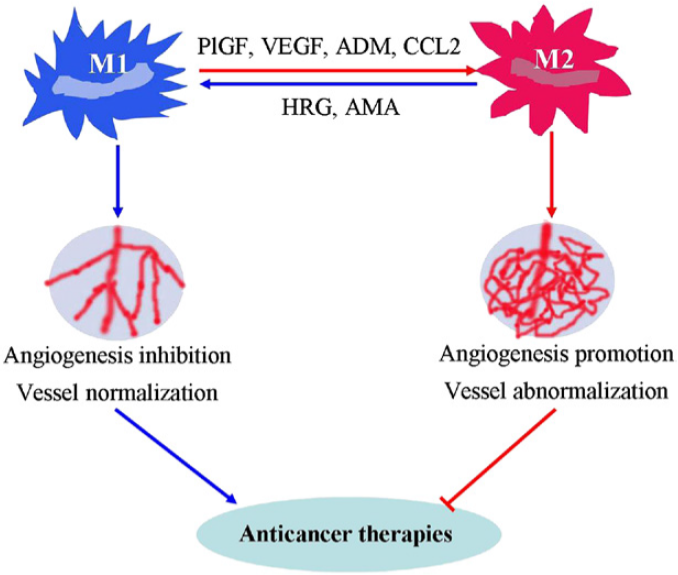

Figure 1.4 ***Cancer therapeutic strategy by targeting TAM polarization to inhibit tumor angiogenesis and normalize tumor vessels.*** TAMs exhibit the proangiogenic M2 phenotype, which not only promotes tumor angiogenesis but also leads to the abnormal tumor vessels. Conversely, M1 macrophages are considered as antiangiogenic and have the opposing effect in the modulation of vascular network. The phenotype of macrophages can be shifted based on the tumor microenvironment. For example, HRG and AMA, an antagonist of ADM receptors, or MENK can polarize TAMs away from the M2 to the M1 phenotype, which in turn inhibits tumor angiogenesis and/or normalizes tumor vessels. By contrast, proangiogenic factors such as PlGF, VEGF, ADM and CCL2 have the opposing effects. New cancer therapeutic strategies are being developed not only for inhibiting tumor angiogenesis but also for normalizing tumor vessels by the "re-education" of TAM polarization from an M2 phenotype to an M1 phenotype. For color version of this figure, the reader is referred to the online version of this book.

to be done in order to get a clear understanding of the communication between TAMs and tumor cells or other types of stromal cells, and how such communication processes contribute to TAM "re-education." In the meanwhile, more molecules that induce angiogenesis inhibition and vessel normalization by skewing TAMs toward M1 phenotype, as well as the mechanistic basis of these processes, need to be identified. Identifying the key elements in these anticancer therapeutic strategies will be the main challenge in the future.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare that they have no financial interests.

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