THE ROLE OF SMALL LEUCINE-RICH PROTEOGLYCANS IN NON-SCARRING HUMAN ORAL MUCOSAL WOUND HEALING

by

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ABSTRACT

Small leucine-rich proteoglycans (SLRPs) decorin, biglycan, fibromodulin and lumican are extracellular matrix (ECM) molecules that regulate collagen fibrilogenesis, cell functions and activity of transforming growth factor-β (TGF-β). Thus, SLRPs may play critical roles in wound healing. In contrast to dermal wounds, gingival wounds regenerate with minimal scaring. However, the cellular and molecular mechanisms involved in this processes are not known. The aim of this study was to analyze the abundance of SLRPs, TGF-β and Endo180, the major collagen endocytosis receptor in fibroblasts, in normal unwounded gingiva and during wound healing. The association of Endo180 with decorin was also investigated during wound healing. We hypothesized that compared to normal unwounded tissue, gingiva shows distinct localization and altered accumulation of SLRPs, TGF-β and Endo180 during wound healing. To further analyze functions of SLRPs, we studied interaction of decorin with cultured gingival fibroblasts.

Double immunostaining was used to study the localization of SLRPs, Endo180 or TGF-β in tissue sections from normal human gingiva and up to 60 days after experimental wounding. The expression of Endo180 in cultured fibroblasts and keratinocytes was studied by immunoblotting and reverse transcriptase-polymerase chain reaction. To study interaction of cultured fibroblasts with decorin and decorin-induced signaling we used immunoblotting, function-blocking antibodies, pharmacological inhibitors, quantitative immunocytochemistry and RNA interference.

In normal gingiva and during wound healing, SLRPs localized to collagen in a site-specific manner. The immunoreactivity of SLRPs, TGF-β1, TGF-β3 and Endo180 was spatially and temporally regulated in myofibroblasts, pericytes, macrophages, endothelial and epithelial cells during wound healing. During wound healing, decorin colocalized with Endo180 in myofibroblasts. In cultured fibroblasts, decorin induced phosphorylation of distinct receptor tyrosine kinases leading to formation of reactive oxygen species (ROS) via the PI3K/mTOR signaling pathway. This was necessary for decorin endocytosis mainly via the clathrin-pathway.

SLRPs may play a role in gingival wound re-epithelialization, collagen fibrilogenesis, ECM remodeling and cell signaling. Specifically, increased abundance of fibromodulin, decorin and TGF-β3 relative to TGF-β1 may contribute to the reduced scaring during gingival wound healing. Decorin may interact with Endo180 to modulate its function and regulates cell signaling by inducing ROS formation.
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Chapters 2 to 5 of this thesis comprise published or submitted manuscripts for publication that have been supervised by Dr. Lari Häkkinen. Contributions of other collaborators are as follows:

Chapter 2: Drs. Hannu Larjava and Tara Habijanac participated in the collection of normal unwounded tissue samples and writing of the manuscript.

Chapter 3: Drs. Hannu Larjava and Tara Habijanac participated in the collection of the wound tissue samples. Dr. Ameneh Eslami participated in some of the immunohistochemical analysis. All co-authors also participated in the writing of the manuscript.

Chapter 4: Drs. Gouqiao Jiang performed the RT-PCR analysis, Dirk Wienke and Clare Isacke provided anti-Endo180 antibodies and participated in the writing of the manuscript.

Chapter 5: Drs. Gouqiao Jiang and Leeni Koivisto performed clathrin siRNA transfection and FACS analysis, respectively. Drs. Jyrki Heino and Johanna Jokinen provided the recombinant human decorin produced in E. coli. Dr. Paul Scott provided bovine decorin. All co-authors also participated in the writing of the manuscript.
CHAPTER 1: REVIEW OF THE LITERATURE

1.1 Overview of wound healing
The process of wound healing is comprised of overlapping phases of inflammation and repair in which platelets, fibroblasts, epithelial, endothelial and inflammatory cells interact with each other and extracellular matrix (ECM) molecules, growth factors and cytokines. Aberrations in this process may result in chronic non-healing wounds or deposition of excess collagen and scar formation. Wound healing consists of three partially overlapping phases: a) haemostasis and inflammation, b) re-epithelialization and granulation tissue formation, and c) maturation and tissue remodeling (Stadelmann et al., 1998a; 1998b). Figure 1.1 summarizes major wound healing events over time.

1.2 Early wound healing events and inflammatory phase
1.2.1 Haemostasis
Soon after wounding, glycoproteins expressed on the cell surface of platelets mediate their adhesion and aggregation to form hemostasis plaque (Midwood et al., 2004). Activation of prothrombin to the serine protease thrombin leads to converting fibrinogen into fibrin. Fibrin is then cross-linked by factor XIII to form blood clot. Fibrin and fibronectin cross-link together and form a network (Ehrlich and Krummel, 1996) that traps proteins and platelets, prevents further blood loss, facilitates cell migration and provides matrix scaffold for collagen deposition (Midwood et al., 2004). Platelets also release large amount of growth factors, cytokines and pro-inflammatory factors such as serotonin, bradykinin, prostaglandins, prostacyclins, thromboxane and histamine (Stadelmann et al., 1998). Thromboxanes and prostaglandins promote vasoconstriction to minimize blood loss, followed by vasodilation (Stadelmann et al., 1998).

1.2.2 Inflammatory response
Cytokines present in the blood clot attract inflammatory cells including polymorphonuclear leukocytes (PMNs) and macrophages into the wound. PMNs are involved in phagocytosis and clearance of debris, damaged cells and bacteria (Ehrlich and Krummel, 1996; Deodhar and Rana 1997). They also release large amount of inflammatory cytokines and growth factors (Martin et al., 1993). About two days after injury, macrophages replace PMNs and become predominant
cell type in the wound (Hubner et al., 1996) and continue to secrete major growth factors such as transforming growth factor-β1 (TGF-β1) and platelet drive growth factor (PDGF). In addition to phagocytosis and immune response, macrophages have been proposed to influence re-epithelialization, granulation tissue formation, matrix deposition and angiogenesis (Lin and Pollard, 2004). During inflammatory phase, fibroblasts and endothelial cells release antibacterial superoxide in order to protect the wound from infection. In addition, superoxidase induces cell signaling for further stimulation of growth factor release (Hopf et al., 1997). Taken together, the inflammatory response during wound healing provides pivotal conditions for resistance to wound infections and a bridge between earlier phases of wound healing and later stages when wound is repaired and stabilized.

1.2.3 Re-epithelialization

Immediately after injury, cytokines released from platelets activate keratinocytes. Migration of keratinocytes and hence re-epithelialization starts as early as two hours after wounding. Growth factors such as keratinocytes growth factor (KGF) and epidermal growth factor (EGF) induce proliferation and migration of keratinocytes (Putnins et al., 1999; Carrington and Boulton, 2005; Ceccarelli et al., 2007). The main sources of migrating keratinocytes during re-epithelialization process are basal keratinocytes from the wound edges, dermal appendages such as hair follicles, sweat glands and sebaceous glands and bone marrow derived keratinocyte stem cells (Badiavas et al., 2003; Badiavas and Falanga, 2003). Keratinocytes secrete proteases and plasminogen activator that activates plasmin, which dissolves the clot, debris, and parts of the ECM and promote cell migration (Ghersi et al., 2002; Etscheid et al., 2005). Migration of keratinocytes over the wound site is also enhanced by lack of contact inhibition and nitric oxide released from PMNs, keratinocytes and fibroblasts (Witte et al., 2002; Zhu et al., 2007). Epithelial cells continue migrating across the wound bed until cells from different sides meet in the middle, at which point contact between keratinocytes inhibits further migration. Subsequently, new layers of keratinocytes differentiate and give rise to a stratified epidermis. Additionally, wound contraction by myofibroblasts present in the granulation tissue accelerates wound closure by bringing wound edges closer together. Fast keratinocyte migration and re-epithelialization often leads to better wound healing outcomes and decreased scar formation (Li et al., 2006). In contrast, exposure to air and / or lack of moisture retards healing process (Gates and Holloway,
1992; Scanlon et al., 2005). Keratinocytes are also involved in angiogenesis, matrix production, chemoattraction and mitogenic activity by releasing vascular endothelial growth factor (VEGF), PDGF, and transforming growth factor-α (TGF-α) (Gillitzer and Goebeler, 2001).

1.3 Granulation tissue formation

The granulation tissue formation phase is characterized by fibroplasia in which the number of fibroblasts is increased in the wound. Soon after injury, local resident fibroblasts migrate into the wound site, undergo proliferation and constitute the granulation tissue. The number of fibroblasts that are involved in phagocytosis and deposition of new ECM in the wounded area peaks at one to two weeks post-wounding making them the dominant cells type in the granulation tissue (McDougall et al., 2006). Depending on their origin, fibroblasts in the wound granulation tissue show phenotypically and functionally distinct characteristics that determine how they respond to wound healing stimulation. Cells involved in the granulation tissue formation and wound healing are originated from different sources. For example, stem cells derived from muscle and adipose tissue, mesenchymal stem cell-like cells from surrounding healthy, unwounded tissue (Chunmeng et al., 2004), perivascular cells (Gould et al., 1977; Chen et al., 2006) and cells form the dermal sheath of the hair follicles (Jahoda and Reynolds, 2001; Gharzi et al., 2003; Richardson et al., 2005) have been suggested to contribute to granulation tissue formation. Granulation tissue is also populated by circulating blood-borne cells (Fathke et al., 2004) such as fibrocytes that can differentiate into myofibroblasts (Bucala et al., 1994; Abe et al., 2001; Jabs et al., 2005). Fibrocytes also secrete angiogenic factors that induce neovascularization during wound healing (Quan et al., 2004). Pericytes and bone marrow-derived endothelial progenitor cells (Garmy-Susini and Varner, 2005) that enter to the blood circulation in response to cytokine released from the injury also integrate into the granulation tissue at the sites of new blood vessel growth (Tepper et al., 2003). In addition, newly discovered relatively poorly characterized blood-derived Dot cells that are believed to promote scarless dermal wound healing also migrate to wound, differentiate and reside in the granulation tissue (Kong et al., 2008). Similarities in the structure and cell populations of both skin and oral mucosa suggest that in both tissues progenitor cells involved in wound healing may be recruited from similar origins with the exception that oral mucosa obviously lacks hair follicle-derived cells.
Granulation tissue also contains increased number of inflammatory cells such as macrophages and a provisional ECM that is mainly composed of fibronectin, type III collagen, glycosaminoglycans, proteoglycans and hyaluronan (Midwood et al., 2004). The provisional matrix provides hydrated matrix that facilitates migration of cells to the granulation tissue (Midwood et al., 2004). In addition, low oxygen environment stimulates neovascularization by inducing macrophages and platelets to secrete angiogenic factors such as fibronectin and increase growth factors that attract endothelial cells to the granulation tissue (Grazul-Bilska et al., 2003). Endothelial cells themselves secrete collagenases and plasminogen activator to degrade the clot ECM to facilitate their motility (Gross et al., 1982). Formation of new blood vessels continues increasingly in granulation tissue until after three to four weeks when their number decreases through apoptosis (Desmoulière et al., 1995).

Upon changes in ECM microenvironment caused by wounding, fibroblasts evolve into the proto-myofibroblasts that will subsequently differentiate to myofibroblasts characterized by the expression of α-smooth muscle actin (α-SMA) (Tomasek et al., 2002). Transition of fibroblasts to proto-myofibroblasts and to α-SMA expressing myofibroblasts is thought to be regulated by extra domain A (EDA) fibronectin, growth factors such as TGF-β and PDGF and by mechanical tension incurred from wounding and contraction (Tomasek et al., 2002). Myofibroblasts use α2β1 integrin to attach to collagen and pull collagen using actin-rich cytoskeleton that is linked to the cytoplasmic tail of α2β1 integrin (Eichler et al., 2006). Therefore, in addition to synthesis of ECM components, particularly type I collagen, myofibroblasts regulate wound contraction and ECM reorganization (Sappino et al., 1990a; 1990b; Grinnell, 1994; Ehrlich and Krummel, 1996). Around after two weeks, soon after the formation of new epithelium over the granulation tissue, the number of myofibroblasts starts to decrease through apoptosis (Desmouliere et al., 1995).

### 1.4 Maturation and tissue remodeling

During wound healing, ECM components undergo substantial changes that include transition from clot of fibrin and fibronectin to a mixture of hyaluronate, proteoglycans and collagen. Initially, collagen is deposited as a thin and randomly organized network that gradually is increased in thickness, rearranged, cross-linked and aligned (Clark, 1996). This leads to replacement of provisional matrix with collagen fiber bundles that more closely resembles to
normal unwounded tissue. During wound maturation and tissue remodeling phase, type III collagen, that is abundant during granulation tissue formation, is gradually degraded and type I collagen becomes dominant (Risteli et al., 1993). During several weeks to few months, as the remodeling phase progresses, the tensile strength of the wound increases with the strength reaching about 80% that of normal tissue (Habif, 1996). Depending on the size and location of the wound the maturation phase can last from months to years after the injury. However, balance in synthesis and degradation of collagen appears to be critical for a normal connective tissue remodeling and ECM reorganization. For example, in gingiva the degradation and remodeling of collagen-rich ECM are essential in maintaining normal oral mucosal connective tissue composition (McCulloch, 2004). In vitro studies suggest that the uptake and lysosomal degradation of collagen by fibroblasts comprises a major pathway in the turnover of collagen and connective tissue remodeling (Arora et al., 2000; McCulloch, 2004). The uptake of collagen by fibroblasts involves binding of collagen fibrils to the specific cell surface receptors. For example, phagocytosis of collagen by fibroblasts is mediated mostly by α2β1 integrin (Lee et al., 1996; Everts et al., 1996; Bhide et al., 2005). In addition, urokinase-type plasminogen activator receptor-associated protein (uPARAP)/Endo180 (CD280; henceforth referred to Endo180 in this thesis), an endocytic receptor expressed on the cell surface, also binds to and mediates endocytosis of collagen for lysosomal degradation (Engelholm et al., 2003; Mousavi et al., 2004; Wienke et al., 2003; East et al., 2003; Kjoller et al., 2004; Curino et al., 2005; Madsen et al., 2007) but its expression during wound healing is not known.

Matrix metalloproteinases (MMPs) released by fibroblasts cleave most of the ECM molecules and are also involved in the breakdown and remodeling during wound healing (Birkedal-Hansen et al., 1992; Steffensen et al., 2001). Collagenases such as MMP1, MMP2, MMP8, MMP9, MMP13 and MMP14 that degrade connective tissue collagen, gelatinases such as MMP2 and MMP9 that degrade basement membrane collagens and stromelysins such as MMP3, MMP10, and MMP11 that degrade ECM proteoglycans, laminin, fibronectin, and gelatin play an important role in ECM turnover and remodeling during wound healing (Porras-Reyes et al., 1991; Wysocki et al., 1993; Young and Grinnell, 1994; Takino et al., 1995; Agren, 1994; Bullen et al., 1995; Moses et al., 1996; Young et al., 1996; Imai et al., 1997). Thus, abnormalities in MMP activity and/or endocytosis or phagocytosis of ECM components may be associated with accumulation of excess collagen and scar formation (Lee et al., 2006).
1.5 Scar formation

In skin, abnormalities in wound healing process may lead to delayed healing or excess fibrosis and scar formation. Scars are fibrous tissues that replace normal tissue at the site of injury. When scaring occurs in skin it may result in significant cosmetic, functional and psychological impairments. Minor scars of skin are usually flat and pale with a trace of the original wound. Compared to other type of scars, they contain less collagen and connective tissue cells. In contrast, over-healing results in excessive collagen deposition and formation of keloids or hypertrophic scars (Kischer et al., 1982). They are characterized by excess amounts of thick unorganized collagen fibers that are randomly aligned as compared to normal basket-wave orientation in unwounded tissue. Hypertrophic scars are red, raised and itchy lumps on the skin and are limited in the boundaries of the original wound. Keloids that occur in about 10% of population are larger and grow beyond the original wound zone (Santucci et al., 2001).

Analysis of scar-inducing factors has been the center of attention in many studies. Excess activity of TGF-β1 released from platelets and inflammatory cells in the first phase of wound healing, failure to eliminate myofibroblasts from granulation tissue and reduced collagen breakdown at later time points have been considered as conditions that lead to formation of hypertrophic scars (Desmouliere et al., 1995; Ghahary et al., 1996). Although some observations did not detect changes in the population of myofibroblasts in hypertrophic scars, increased number of myofibroblasts has been observed in other studies (Sappino et al., 1990a; Ehrlich et al., 1994; Spyrou and Naylor, 2002). Furthermore, increased inflammatory response resulting in excess release of cytokines and fibrotic growth factors has been shown to promote keloid and hypertrophic scar formation (Hackam and Ford, 2002; Arabi et al., 2003).

1.6 Non-scaring fetal wound healing

Clinical observations and studies in human, rabbit, rat and mouse indicate that in contrast to adult skin, early gestation fetal skin exhibits a unique pattern of wound healing that leads to regeneration without scar formation. Several reasons for this difference have been proposed. For example, fetal wounds have substantially reduced inflammatory response in early stages of wound healing (Whitby and Ferguson, 1991; Cowin et al., 1998). Compared to fibrotic scar-borne wounds, lower numbers of PMNs, activated macrophages, T cells (Adolph et al., 1993) and B cells (Cowin et al., 1998) have been shown in scarless fetal wound healing (Julia et al.,
Fetal dermal fibroblasts are partially refractory to the effects of prostaglandine E2, a major inflammatory mediator associated with dermal wound healing (Sandulache et al., 2006). This effect may have significant correlation to the non-scarring wound healing in fetal tissue. Reactive oxygen species (ROS) produced during an active inflammatory response are potential fibrogenic byproducts of inflammation (Sbarra and Karnovsky, 1959; Finkel, 1998; Gamaley and Klyubin, 1999). Addition of hydrogen peroxidase, a member of ROS family to the early fetal wound upregulated fibrotic TGF-β1 level and induced scaring (Wilgus et al., 2005).

Increased activity of TGF-β1 is a hallmark of scar formation in adult skin (Shah et al., 1992; 1994; 1995; Wang et al., 2007) and coexpression of TGF-β1 and TGF-β2 aggravate fibrosis and scar formation (Shah et al., 1995). Interestingly, compared to adult murine, rat and human skin wound healing, fetal tissue exhibits lower levels and a more rapid clearance of both TGF-β1 and TGF-β2 during wound regeneration (Martin et al., 1993; Nath et al., 1994; Sullivan et al., 1995). Furthermore, addition of TGF-β1 decreased interstitial collagenase and induced fibrosis in fetal skin (Bullard et al., 1997). Consistent with in vivo observations, in vitro studies have shown that the expression of TGF-β1, TGF-β2, TGF-β1 and II receptors, Smad2, Smad3 and Smad4 is lower in fetal fibroblasts than postnatal fibroblasts (Colwell et al., 2007a). Increased number of myofibroblasts that produce collagen under the influence of TGF-β1 activity during wound healing has also been shown in scar tissue. Studies in sheep have indicated that myofibroblasts are absent in early scarless fetal wounds (Estes et al., 1994) but are present during healing at later stages when prominent scarring occurs (Whitby and Ferguson, 1991, McCluskey and Martin, 1995; Schor et al., 1996). Although the expression of TGF-β1 has been shown to be lower in fetal wounds, contradictory results exist suggesting that rapid midgestatational wound closure in a murine model is associated with increased TGF-β1 expression (Goldberg et al., 2007).

Interestingly, the balance between TGF-β1 and TGF-β3 may be important regulator of scar formation. TGF-β3 was shown to be higher in non-scarring fetal wounds and exogenously added TGF-β3 can also retard scar formation (Shah et al., 1995; Soo et al., 2000). Fetal fibroblasts also exhibited increased expression of TGF-β3 (Colwell et al., 2007b; Goldberg et al., 2007). It has been shown that expression of plasminogen activator inhibitor-1, a known TGF-β
downstream molecule and marker of fibrosis was increased in TGF-β3 gene knockout fetal mice (Li et al., 2006). PDGF is also considered a fibrogenic cytokine (Pinzani et al., 1996; Borkham-Kamphorst et al., 2004). Studies on comparing fetal and adult serum and scarless and fibrotic wounds showed that also PDGF level is lower in fetal serum and is produced for a shorter length of time in scarless fetal wounds (Olutoye et al., 1996; Price et al., 2003).

Accelerated re-epithelialization leads to faster wound closure and reduced scar formation (Li et al., 2006; Etscheid et al., 2005; Zhu et al., 2007). Notably, in contrast to adult skin wound, fetal wounds show faster re-epithelialization (Whitby et al., 1991; McCluskey et al., 1993). Several other reasons have been proposed to explain this difference. For example, epithelial cells at the edge of wounds in both chick and mouse embryos showed actin contractile force that promote early wound closure whereas adult tissue does not exhibit this feature (Martin and Nobes, 1992; McCluskey and Martin, 1995; Brock et al., 1996a; 1996b). Rapid upregulation of epidermal integrins by keratinocytes that facilitate cell adhesion and migration has also been considered to promote faster re-epithelialization in early fetal skin (Cass et al., 1998; Kirfel et al., 2003). Recent in vitro studies have also described unique characteristics of fetal keratinocytes that may differentially modulate the expression of key growth factors during wound healing in fetal and postnatal fibroblasts (Colwell et al., 2007a; 2007b). For example, keratinocytes modulate fetal TGF-β expression and its signaling mediators such as Smads and exhibit an overall antifibrotic influence on fetal fibroblasts in co-culture conditions.

Adult wounds show increased rate of angiogenesis while a marked decrease of neovascularization in fetal mouse wounds has been reported (Whitby and Ferguson, 1991). In a rat model of fetal wound healing, angiogenesis was not observed in day 16 of gestation (E16) wounds while gestation day 19 (E19) wounds showed increased neovascularization (Ihara et al., 1990). However, transplantation of fetal fibroblasts and fibroblast-like mesenchymal stem cells on the burn surface reduced cell infiltration and induced optimal angiogenesis and granulation tissue formation that provide conditions for accelerated healing of the burn wounds (Rasulov et al., 2006). Furthermore, treatment of chronic non-healing wounds with fetal murine mesenchymal stromal cells enhances wound healing possibly through mechanisms that involve upregulation of growth factor production, enhanced cell migration to the wounds bed, and accelerated neovascularization (Badillo et al., 2007).

Several differences have also been described between fetal and adult ECM components
such as proteoglycans, tenascin, collagen and matrix-degrading enzymes that influence wound regeneration or developing scars (Hallock et al., 1988; Merkel et al., 1988; Mast et al., 1991; Whitby et al., 1991; Adzick and Lorenz, 1994; Bullard et al., 1997). Hyaluronic acid that appears to be present at the highest levels in fetal wounds (Krummel et al., 1987; DePalma et al., 1989) can influence the structure, assembly and hydration of the ECM and was considered to support cell growth and migration, especially during development (Toole et al., 2000; 2002). Proteoglycans containing chondroitin sulfate glycosaminoglycan (GAG) chains (CS) exhibit anti-inflammatory property and stimulates the synthesis of proteoglycans and hyaluronic acid (McCarty et al., 2000). Therefore, significant amounts of CS containing proteoglycans produced in fetal but not adult wounds might be one of the reasons for absence of scar formation in the fetal wound (Whitby and Ferguson, 1991). Considering the fact that optimal deposition of collagen in terms of abundance and organization is essential for normal wound healing with minimal scaring, fetal skin wounds heal with deposition of fine reticular and basket-weave pattern identical to normal skin (Longaker et al., 1990; Whitby and Ferguson, 1991; Longaker et al., 1994). Interestingly, while type I collagen is most abundantly present in scar tissue, fetal ECM has a higher ratio of type III to type I collagen (Hallock et al., 1988; Merkel et al., 1988). Lysyl oxidase, an enzyme that cross-links collagen and elastin, have greater expression in E19 late-gestation wounds that heal with scar compared with E17 early-gestation scarless wounds suggesting a role for lysyl oxidase in scar formation in adult wounds (Colwel et al., 2006).

Increased expression of small leucine-rich proteoglycans (SLRPs) that have certain antifibrotic properties may play a pivotal role in scarless wound healing in fetal tissue. For example, expression of fibromodulin was found significantly increased in early fetal wounds compared to scar-forming fetal wounds at later gestation time (Soo et al., 2000). Conversely, decorin and biglycan expression was found to be down-regulated in scarless (E16) compared with scar (E18) wounds (Beanes et al., 2001) although decorin has been well characterized as an antifibrotic molecule that significantly reduces fibrosis and scar formation in adult tissue wound healing (Scott et al., 1995; Shah et al., 1995; Grisanti et al., 2005). Further studies are required to better understand the role of SLRPs in scar formation. MMPs and tissue inhibitors of MMPs (TIMPs) are key elements to maintain the appropriate balance between matrix production and degradation. Evidence suggest that increased expression of MMPs such as stromelysin-1 and gelatinase in mid-gestation human fetal skin contribute to scarless healing (Bullard et al., 1997).
1.7 Gingival vs. skin wound healing

Gingival tissue shares common characteristics with fetal skin in terms of faster re-epithelialization and wound healing with little or no scar formation. The cause of this difference is not well understood but components of saliva, leukocytes, growth factors and specific fibroblast subpopulations have been suggested as some of the factors present in oral mucosal wound healing (Noguchi et al., 1991; Varshney et al., 1997; Taichman et al., 1998; Stephens et al., 2001a; 2001b; Lepekhin et al., 2002). Saliva-treated cutaneous wounds have a reduced inflammatory reaction, faster epithelial coverage and show faster connective tissue regeneration (Varshney et al., 1997; Kagami et al., 2000; Ohshima et al., 2002a; 2002b). Furthermore, saliva contains growth factors such as EGF, VEGF and basic fibroblast growth factor (bFGF), that may promote wound healing (Skaleric et al., 1997; Taichman et al., 1998; Fujisawa et al., 2003).

Compared to skin, the number of macrophages, neutrophils and T-cells was found to be significantly lower in mouse oral wounds as compared to skin at very early stages of wound healing (Szpaderska et al., 2003). In addition, shortly after wounding, oral wounds contain fewer pro-inflammatory and fibrogenic factors such as TGF-β1 and interlukine-6, and a higher relative amount of anti-fibrogenic TGF-β3 (Bodner and Dayan, 1995; Häkkinen et al., 2000a; Szpaderska et al., 2003; Schrementi et al., 2008).

Interestingly, unique phenotype of oral mucosal fibroblasts compared to skin cells may also underlay fast wound regeneration in oral mucosa. Several in vitro studies have shown that oral fibroblasts produce increased levels of MMP2 and MMP3 than dermal fibroblasts (Stephens et al., 1996; 2001b; Sukotjo et al., 2003; Shannon et al., 2006) that may promote faster remodeling of the ECM. In addition, dermal and oral fibroblasts secrete different types and amounts of GAGs and proteoglycans. For example, human gingival fibroblasts produced larger dermatan sulfate proteoglycans due to larger dermatan sulfate chains than skin fibroblasts (Larjava et al., 1988). Differences in key cell functions such as migration (Lepekhin et al., 2002), adhesion properties to vitronectin, laminin, fibronectin, type I and IV collagen, expression of integrin receptors (Palaiologou et al., 2001) and response to TGF-β1 activity (Kahari et al., 1991; Lee and Eun, 1999) have also been reported between oral and dermal fibroblasts. For example, in skin and gingival fibroblasts TGF-β1 significantly increased the expression of biglycan that was correlated with increased type I procollagen gene expression. In contrast, the expression of decorin mRNA was markedly reduced by TGF-β1 in these cells. However, gingival cells were
more responsive to stimulation by TGF-β1 with respect to biglycan gene expression (Kahari et al., 1991). Compared to dermal fibroblasts, oral fibroblasts from gingiva exhibit a more fetal-like phenotype with higher contractile properties that enhance faster wound closure and tissue remodeling (Sloan, 1991; Irwin et al., 1994; Stephens et al., 2001a). However, this remains somewhat controversial as it was also reported that dermal fibroblasts in collagen gel possessed greater contraction potency than oral mucosal fibroblasts. However, oral mucosal fibroblasts showed an earlier collagen gel contraction that was independent of TGF-β (Lee and Eun, 1999). Table 1.1 summarizes some differences between molecular, cellular or physical characteristics of fetal and adult skin and gingival wound healing. In the current study gingival tissue was used as a model of non-scaring tissue to investigate spatial-temporal localization and expression of SLRPs.

1.8 Small leucine-rich proteoglycans (SLRPs)

SLRPs are structurally related proteoglycans that are abundantly expressed in the ECM. SLRP family comprises of several members that have evolved from different genes (Figure 1.2a). SLRPs contain a core protein with up to 12 leucine-rich repeats (McEwan et al., 2006; Kalamajski et al., 2007) flanked by cysteine residues and GAG side chains that can be either chondroitin/dermatan sulfate, for example, in decorin and biglycan (Iozzo, 1998) or keratan sulfate in fibromodulin and lumican (Oldberg et al., 1989, Plaas et al., 1990) (Figure 1.2b). The GAG chains regulate several protein-protein, cell-cell and cell-matrix interactions while the leucine-rich repeats in the core protein are involved in collagen binding and modulate many biological processes, including cell migration, proliferation, adhesion, signaling, activity of growth factors and gene expression (Yamaguchi and Ruoslahti, 1988; Neame et al, 1989; Bidanset et al., 1992; Kobe and Deisenhofer, 1994; Svensson et al., 1995; Merle et al., 1999; Kinsella et al., 2000; Keene et al., 2000; Kresse and Schonherr, 2001).

Based on evolutionary protein conservation, the presence of cysteine-rich cluster in the N-terminal region, the number of the leucine-rich repeats and genomic organizations, SLRPs are categorized in three classes. Class I SLRPs include decorin (Krusius and Ruoslahti, 1986; Vetter et al., 1993; Danielson et al., 1993), biglycan (Fisher et al., 1989; Wegrowski et al., 1995) and asporin (Figure 1.2a).
Table 1.1: Cellular and molecular differences between fetal skin, adult skin and adult gingival tissue. See text for references.

<table>
<thead>
<tr>
<th>Cellular/Molecular/physical status</th>
<th>Fetal skin wound</th>
<th>Adult skin wound</th>
<th>Oral mucosal wound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound environment</td>
<td>Moist</td>
<td>Dry</td>
<td>Moist</td>
</tr>
<tr>
<td>Inflammatory cells / response</td>
<td>Less</td>
<td>More</td>
<td>Less</td>
</tr>
<tr>
<td>Reepithelialization</td>
<td>Faster</td>
<td>Slower</td>
<td>Faster (in vitro)</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>Decreased</td>
<td>Increased</td>
<td>?</td>
</tr>
<tr>
<td>Fibroblasts activities</td>
<td>Faster / higher</td>
<td>Slower / Lower</td>
<td>?</td>
</tr>
<tr>
<td>Myofibroblasts density</td>
<td>Lower</td>
<td>Higher</td>
<td>?</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>Higher</td>
<td>Lower</td>
<td>?</td>
</tr>
<tr>
<td>Glycosaminoglycan</td>
<td>Higher</td>
<td>Lower</td>
<td>?</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>Lower</td>
<td>Higher</td>
<td>Lower</td>
</tr>
<tr>
<td>Type III collagen</td>
<td>Higher</td>
<td>Lower</td>
<td>Higher</td>
</tr>
<tr>
<td>MMP-2, -3, -13</td>
<td>Higher</td>
<td>Lower</td>
<td>Higher</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Higher</td>
<td>Lower</td>
<td>Higher</td>
</tr>
<tr>
<td>Tenascin</td>
<td>Higher</td>
<td>Lower</td>
<td>Higher</td>
</tr>
<tr>
<td>PDGF</td>
<td>Lower</td>
<td>Higher</td>
<td>?</td>
</tr>
<tr>
<td>TGF-β1 level / activity</td>
<td>Lower</td>
<td>Higher</td>
<td>Lower</td>
</tr>
<tr>
<td>TGF-β3 level</td>
<td>Higher</td>
<td>Lower</td>
<td>Higher</td>
</tr>
<tr>
<td>Decorin</td>
<td>Lower</td>
<td>Higher</td>
<td>?</td>
</tr>
<tr>
<td>Biglycan</td>
<td>Lower</td>
<td>Higher</td>
<td>?</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>Abundant</td>
<td>Less</td>
<td>?</td>
</tr>
<tr>
<td>Lumican</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Fibroblast-mediated collagen contraction</td>
<td>Higher</td>
<td>Lower</td>
<td>Higher</td>
</tr>
<tr>
<td>Collagen deposition / orientation</td>
<td>Fast / fine / reticular basket-weaved</td>
<td>Slow / thick Paralleled</td>
<td>Fast ECM turnover</td>
</tr>
</tbody>
</table>

Wound healing outcomes

<table>
<thead>
<tr>
<th>Scar formation</th>
<th>Regeneration</th>
<th>Repair</th>
<th>Regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scar-less</td>
<td>Regeneration</td>
<td>Repair</td>
<td>Less scar</td>
</tr>
</tbody>
</table>
Decorin and biglycan show approximately 57% amino acid homology (Iozzo, 1998) and contain propeptide that is highly conserved across species (Neame et al., 1989). Decorin is mainly found in connective tissue ECM and is especially abundant in bone, skin, tendon and cartilage (Rosenberg et al. 1985; Fisher et al., 1989; 1991). Biglycan was originally isolated from bone and cartilage (Rosenberg et al, 1985; Fisher et al, 1989; 1991) but it is also expressed in the pericellular matrix of capillary endothelium and epithelium including kidney collecting tubules and skin (Bianco et al, 1990). Class II SLRPs consists of five members that include fibromodulin (Oldberg et al., 1989; Antonsson et al., 1993) and lumican (Grover et al., 1995; Hassell et al., 1998) with near to 48% protein sequence homology; keratocan (Tasheva et al., 1997) and PRELP (Grover et al., 1996) with about 55% protein identity; osteoadherin (Sommarin et al., 1998) with 37-42% protein identity to the other class II members. Fibromodulin is abundantly expressed in the ECM of articular cartilage (Heinegård et al., 1986) while lumican is abundantly present in cornea and normally expressed by stromal keratocytes. Lumican can also be found in lung (Dolhnikoff et al., 1998), kidney (Schaefer et al, 2000), muscles and articular cartilage (Funderburgh et al., 1991; Grover et al., 1995; Knudson and Knudson, 2001). Class III SLRPs consists of opticin (Hobby et al., 2000), epiphycan (Danielson et al., 1999) and mimecan (osteoglycin) (Tasheva et al., 2000) with about 40% protein sequence homology. Class III SLRPs have only six leucine-rich repeats in their core protein. Epiphycan contains either chondroitin sulfate or dermatan sulfate. Other members of the SLRPs family that have been characterized include ECM2, podocan, chondroadherin, and nyctalopin. In the present study we focused on analyzing the accumulation and function of decorin, biglycan, fibromodulin and lumican in scar-free human gingival wound healing. These SLRPs regulate cell proliferation, differentiation, migration and gene expression through binding to and interacting with multiple ECM molecules, cytokines, cell surface receptors and growth factors (Kresse et al., 1994; Schönherr et al., 1998; Markmann et al., 2000). Additionally, these SLRPs interact with collagen to regulate its structure and function. Therefore, these SLRPs may play a role in regulation of wound healing and scar formation.

Factors that regulate the expression of these SLRPs in different tissues needs to be elucidated but growth factors potentially play a pivotal role. For example, exogenous TGF-β down-regulated decorin expression in normal satellite muscle cells (Li et al., 2006) and fibroblasts isolated from skin and gingiva but increased biglycan expression in fibroblasts
(Kahari et al., 1991). However, gingival cells were more responsive to TGF-β1-induced biglycan gene expression. In fibroblasts isolated from wound granulation tissue and chronically inflamed tissues, TGF-β1 more potently increased the expression of biglycan and procollagen but had very little effect on fibromodulin and decorin expression as compared with normal fibroblasts (Häkkinen et al., 1996). EGF-mediated activation of ERK1/2 signaling pathway also down-regulated decorin gene expression in fibroblasts (Laine et al., 2000). Connective tissue growth factor (CTGF) has been shown to suppress the synthesis of biglycan but up-regulates decorin expression in vascular endothelial cells (Kaji et al., 2004). Interestingly, other factors, such as mechanical strain, increased biglycan but decreased decorin mRNA expression in vascular smooth muscle cells (Lee et al., 2001). On the other hand, decorin itself can regulate mechanical tensions in ECM. For example, overexpression of decorin by rat arterial smooth muscle cells showed enhanced contraction of type I collagen (Järveläinen, et al., 2004). However, contradictory results indicate that collagen matrices seeded with decorin-deficient embryonic fibroblasts have greater contraction, cell density and tensile strength (Ferdous et al., 2007).

1.9 SLRPs and gene knock-out studies
Table 1.2 summarizes phenotypical changes in single and double SLRP knockout mice. The first gene knockout studies of SLRPs in mice showed that deficiencies in decorin, biglycan, fibromodulin and lumican lead to defects in type I collagen fibrils. For example, decorin gene knockout mice showed similar abnormalities of collagen organization and structure in the skin connective tissue as compared to human Ehlers-Danlos syndrome, including irregular cross sectional diameter, hyperlaxivity, and reduced thickness and tensile strength and skin fragility (Dombi et al., 1993; Danielson et al., 1997; Mao and Bristow, 2001; Corsi et al., 2002). These mice show also abnormal fibril bundle morphology (Matheson et al., 2005) and unorganized collagen orientation and hyper-cellularity in periodontal ligament (Häkkinen et al., 2000b). Decorin-deficient mice also demonstrated alterations of mechanical properties in lung that resulted from defective collagen formation (Fust et al., 2005) and they had enhanced progression of diabetic nephropathy (Williams et al., 2007) and impaired angiogenesis during corneal wound healing (Schönherr et al., 2004). However, they showed accelerated revascularization in a skin wound model (Järveläinen et al., 2006).

Mice deficient in biglycan show broad pathological complications such as reduced bone
mass and osteoporosis-like phenotype (Xu et al., 1998), muscular dystrophy, osteoarthritis, ectopic tendon ossification, joint instability and mechanically compromised tendons (Ameye et al., 2002), increased fibril diameter in bone and skin dermis (Ameye et al., 2002; Corsi et al., 2002), osteoporosis and rapid reduction in the number of bone marrow stroma cells (Chen et al., 2002) and augmentation of osteoclast differentiation and activity resulted from defective osteoblasts that could potentially lead to LPS-induced osteolysis (Bi et al., 2006). Biglycan-deficient mice also showed spontaneous aortic dissection and rupture in mice aortas as a result of structural abnormalities of collagen fibrils and reduced tensile strength (Heegaard et al., 2007).

Phenotypical characteristics resulted from SLRPs double-deficient mice indicate functional overlap and critical role of SLRPs for collagen fibrilogenesis in normal and pathological conditions (Corsi et al., 2002). Although bone and skin is barely affected in decorin and biglycan deficient mice, respectively, the absence of both SLRPs was synergistic resulting in the severely reduced bone mass and pronounced skin fragility (Corsi et al., 2002). In addition, decorin/biglycan double-deficient mice exhibit more severe irregularity in collagen fiber cross-section displaying a wide variety of unorganized shapes and morphology (Corsi et al., 2002). Furthermore, fibroblasts in decorin/biglycan knockout mice were impaired in GAG synthesis as a result of defective xylosylprotein 4-β-galactosyltransferase I (Quentin et al., 1990; Corsi et al., 2002). Targeted disruption of both biglycan and decorin also induces phenotypical changes on enamel and dentin formation. For example, decreased mineralization of teeth in biglycan/decorin double knockout mice was observed although the effect was more prominent in the absence of decorin (Goldberg et al., 2005). Enamel formation was also dramatically decreased in decorin deficiency (Goldberg et al., 2005). In addition, biglycan/decorin double deficient mice exhibited impaired posterior frontal sutural fusion (Wadhwa et al., 2007).

Fibromodulin-null mice develop tendon ossification and osteoarthritis (Ameye et al., 2002) characterized with decreased and abnormally thin collagen bundle fibrils in tendon with irregular cross sections (Svensson et al., 1999; Ameye et al., 2002) and abnormal collagen fibril and fibril bundle morphology in the periodontal ligament (Matheson et al., 2005). In the adult mice and during postnatal development, lumican-deficiency resulted in opaque corneas with abnormally thick collagen fibrils, reduced corneal transparency and progressive corneal opacification (Chakravarti et al., 1998; 2006) that can be rescued by over expressing lumican in the tissue (Meij et al., 2007). Lumican-deficient mice also develop EDS-like skin laxity with substantial
reduction in skin tensile strength, loose dermis with abnormally collagen fibrils, increased skin lesions (Chakravarti et al., 1998) and abnormal collagen fibril morphology in the periodontal ligament (Matheson et al., 2005).

The collagen defects observed in fibromodulin/lumican double-deficient mice during tendon development is more severe than the single-deficient phenotypes in the earlier stages but comparable with the fibromodulin single-deficient mice at maturation (Ameye et al., 2002). This indicates that lumican and fibromodulin both regulate the initial steps of collagen fibrilogenesis, whereas fibromodulin, in addition, influences the fibril growth and maturation (Ezura et al., 2000). The eyes of lumican/fibromodulin-null mice show certain characteristics of high myopia including increased axial length, thin sclera, and retinal detachment (Chakravarti et al., 2003). Deletion of both lumican and fibromodulin gene in mice has also been shown to lead to marked abnormal collagen fibril and fibril bundle morphology in the periodontal ligament (Matheson et al., 2005). Biglycan/fibromodulin double-deficient mice develop severe premature osteoarthritis, ectopic tendon ossification and gait impairment (Ameye et al., 2002) and exhibit higher tendon plasticity and cartilage erosion than their wild-type counterparts (Ameye et al., 2002). Taken together, these data indicate that various pathological conditions including osteoporosis, osteoarthritis, muscular dystrophy, EDS, and corneal diseases resulted from SLRPs deficiency and point out the critical functional role of SLRPs in normal physiological condition, tissue development and homeostasis. Therefore, SLRPs may play a critical role during normal wound healing events and alteration in the expression of each of SLRPs may result in abnormal collagen fibrilogenesis and/or cell functions during this process. Wound healing response in the SLRP knockout mice will be discussed in detail below.

1.10 SLRPs and collagen fibrilogenesis

Collagen is the major insoluble fibrous protein in the ECM of bone, tendons and skin. Procollagen or molecular form of collagen is composed of triple helix of polypeptide chains (glysine-proline-hydroxyproline repeating triplet) containing globular C and N terminus. Once N- and C- ends are cleaved by procollagen peptidase the molecular collagen are cross-linked together and form fibrils. Collagen fibrils are packed together and form collagen fibers. Assembly of several collagen fibers together results in formation of collagen fiber bundles.
Table 1.2: Phenotypical characteristics of SLRPs gene deficiencies. See text for references.

<table>
<thead>
<tr>
<th>Gene deficiency</th>
<th>Phenotypical abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCN:</td>
<td>Irregular cross sectional diameter of collagen, hyperlaxivity, reduced thickness and tensile strength of skin, skin fragility, abnormal collagen fibrils and hypercellularity in the periodontal ligament, enhanced diabetic nephropathy, delayed excisional wound closure, impaired angiogenesis during corneal wound healing, accelerated revascularization during skin wound healing, defective mechanical property of lung due to impaired collagen formation, decreased enamel formation.</td>
</tr>
<tr>
<td>BGN:</td>
<td>Reduced bone mass, muscular dystrophy, osteoarthritis, osteoporosis, ectopic tendon ossification, joint instability, mechanically compromised tendons, increased fibril diameter in bone and skin dermis, rapid reduction in the number of bone marrow stroma cells, suppressed postnatal bone growth, spontaneous aortic dissection and rupture.</td>
</tr>
<tr>
<td>DCN / BGN:</td>
<td>More severe reduction in bone mass and skin fragility, more severe irregularity in collagen fiber cross-section, unorganized shapes and morphology of collagen, defective xylosylprotein 4-β-galactosyltransferase I, impaired glycosaminoglycan chains synthesis, decreased teeth mineralization.</td>
</tr>
<tr>
<td>FM:</td>
<td>Tendon ossification, osteoarthritis, decreased / abnormally thin collagen fiber bundles with irregular cross sections in tendon and periodontal ligament.</td>
</tr>
<tr>
<td>LUM:</td>
<td>Opaque corneas with abnormally thick collagen fibrils, reduced corneal transparency, progressive corneal opacification, EDS-like skin laxity with substantial reduction in skin tensile strength, loose dermis with abnormally collagen fibrils, abnormal collagen fibrils in the periodontal ligament.</td>
</tr>
<tr>
<td>FM / LUM:</td>
<td>More severe collagen defect during tendon development in the earlier stages, abnormal collagen fibril morphology in tendon and periodontal ligament, increased eye axial length, thin sclera, retinal detachment.</td>
</tr>
<tr>
<td>FM / BGN:</td>
<td>Severe premature osteoarthritis, ectopic tendon ossification, gait impairment, higher tendons plasticity, cartilage erosion.</td>
</tr>
</tbody>
</table>
The most studied characteristic of SLRPs is the property of decorin, biglycan, lumican and fibromodulin to bind to and regulate collagen fibrilogenesis. SLRPs regulate matrix assembly, formation, structural organization and orientation of collagen in normal and pathological conditions (Krusius and Ruoslahti, 1986; Grover et al., 1995; Ameye and Young 2002). For example, SLRPs can bind to type I collagen to delay fibrilogenesis and induce formation of thinner fibrils in vivo (Vogel et al., 1987; Hedbom et al., 1989; Font et al., 1998). While the GAG chain has been suggested to regulate intra-fibrillar spacing (Hahn and Birk, 1992) it appears that regulation of collagen fibrilogenesis is mediated by interaction of the collagen molecule with core protein of SLRPs. For example, biglycan can interacts with type VI collagen via its core protein and efficiently accelerates its organization and assembly into structured networks (Wiberg et al., 2002). Interactions between collagen and the core protein of decorin regulate the diameter of collagen fibrils as demonstrated in decorin knockout mice with irregularity in fibril thickness and morphology (Danielson et al., 1997). Previously, leucine-rich repeat 4-6 of the decorin core protein was considered as binding sites to type I collagen (Svensson et al., 1995; Keene et al., 2000; Bhide et al., 2005). Recent study has characterized Arg-207 and Asp-210 in the leucine-rich repeat 6 of decorin crucial for the binding to type I collagen (Kalamajski et al., 2007). However, at least two different binding sites for the interaction between decorin core protein and type I collagen that may not necessarily affect collagen fibrilogenesis have been described (Schönherr et al., 1995). In addition, decorin interacts with type I, II, and III collagens in vitro and decreases the diameter resulting in thinner fibers (Hocking et al., 1998; Douglas et al., 2006).

Findings in the structural and biophysical properties of decorin showed that it exists in solution as highly stable dimers and the assembly of decorin dimers occurs through the concave sides of the monomers (Scott et al., 2003; 2004). It has been proposed that dimerization of decorin through the concave surface of the leucine-rich domain mediates interaction of the molecule with collagen. This notion contradicts with widely accepted model that suggests that the inner concave face of the decorin leucine-rich repeat-domain binds to a single collagen triple helix (Ameye and Young, 2002; Weber et al., 1996) as the concave surfaces are mostly covered in the interface after dimerization. Instead, decorin dimers possess large contact areas that are associated with very high binding affinities (Scott et al., 2003). SLRPs might be able to exploit their dimeric nature to bridge different collagens (Nareyeck et al., 2004) and bind to more than
one different type of collagen.

Conflicting data exists as to whether biglycan interacts with fibrillar collagens. For example, while some reports suggest that biglycan does not bind to fibrillar collagens (Svensson et al., 1995) others have indicated that biglycan interacts with collagen VI and efficiently regulates collagen VI assembly and organization into structural networks (Hocking et al., 1996; Wiberg et al., 2001). A distinct interaction of the core protein of biglycan with collagen VI at the border between the N-terminal non-triple helical and triple helical domains has been characterized (Wiberg et al., 2001). Collagen type II bound significantly more SLRPs in fibrils than collagen I and III, with more biglycan than decorin bound by all three collagen types (Douglas et al., 2007). Biglycan has also been reported to form dimers in solution (Liu et al., 1994).

Fibromodulin interact with both types I and II collagens (Hedbom and Heinegård. 1989; Viola et al., 2007). Binding of fibromodulin to type I collagen occurs in Glu-353 and Lys-355 residues in the leucine-rich repeats of the molecule (Kalamajski and Oldberg, 2007). It appears that binding of fibromodulin to disulphide and the C-terminal region of type I collagen is critical for the control of fibrilogenesis (Font et al., 1998). Lumican regulates type I collagen assembly into fibrils in various connective tissues such as a highly organized collagenous matrix in the cornea (Chakravarti et al., 2000). Regulation of collagen fibril thickness and spacing by lumican in the cornea is critical for corneal transparency and further emphasizes the key role of SLRPs in collagen fibrilogenesis (Chakravarti et al., 1998).

SLRPs have been shown to bind to collagen in the gap region of the D-period (Scott, 1988). Binding competition experiments indicated that biglycan and decorin bind to the same site in type I collagen. However, decorin shows a higher affinity to bind to collagen (Schönherr et al., 1995). Although lumican exhibits a lower affinity to bind to type I collagen, fibromodulin and lumican compete for the binding to collagen on the same binding site but distinct from the binding site of decorin and biglycan (Hedbom and Heinegård, 1993; Svensson et al., 2000). Therefore, fibromodulin and lumican do not affect binding of decorin to collagen. Interestingly, interaction of SLRPs with collagen protects the collagen fibrils from cleavage by collagenases. Studies show that binding of recombinant decorin, fibromodulin or lumican to collagen type I and II masked cleavage site of collagen and decreased MMP-1 and MMP-13 mediated degradation of both types of collagen (Geng et al., 2006).
1.11 Interaction of SLRPs with extracellular matrix molecules

Several studies have shown that in addition to their function in ECM, SLRPs can regulate cellular behavior, such as cell migration and proliferation. These are important cellular functions during embryonic development, tissue repair and tumor growth (Iozzo, 1999; Saika et al., 2000; Kresse and Schonherr, 2001). SLRPs exert their function by interacting through their core protein or GAG chains with other ECM components, cell surface receptors or soluble signaling molecules. Table 1.3 highlights the ECM and cell-associated molecules that interact with the core protein of SLRPs.

Decorin can modulate cell adhesion. Interaction of decorin core protein with fibronectin resulted in inhibition of human skin fibroblasts adhesion to fibronectin (Winnemöller et al., 1991). Decorin is also able to strongly bind to and inhibit C1q complement activity in human endothelial cells (Krumdieck et al., 1992) and U937 cells (Groeneveld et al., 2005), suppress C1q-induced MCP-1 and IL-8 production by these cells and act as inhibitors of activation of complement cascade, cellular interactions and C1q-mediated proinflammatory cytokine production (Groeneveld et al., 2005). Decorin is a metalloprotein that binds to Zn2+. The Zn2+-binding sites are localized to the N-terminal domain of the decorin core protein (Yang et al., 1999). In the ECM, zinc is required for the activity of MMPs which are responsible for the degradation of structural ECM components (Woessner, 1991). Thus, by binding to zinc decorin may modulate ECM remodeling. In addition, decorin binds to several cell surface receptors and regulates cell signaling and functions that will be described below.

Biglycan is also expressed and/or interacts with a variety of cell types including kidney collecting tube epithelial and glomerular endothelial cells (Schaefer et al., 2000), blood vessel endothelial cells (Shimizu-Hirota et al., 2004), myofibroblasts of the testis (Ungefronen et al., 1995), chondrocytes (Demoor-Fossard et al., 1998) and skin and gingival fibroblasts (Kahari et al., 1991). The localization of biglycan in pericellular matrix of embryonic mesenchymal cells or on the surface of CHO cells and rat embryo fibroblasts as well as its ability to bind fibronectin suggest that biglycan is also involved in cell adhesion (Fleischmajer et al., 1991; Bidanset et al., 1992). Like decorin, biglycan strongly binds to the complement component C1q (Krumdieck et al., 1992) and down-regulates proinflammatory effects mediated by C1q (Groeneveld et al., 2005). Similar to decorin, biglycan is also a Zn2+-binding protein (Liu et al., 1994; Yang et al., 1999).
Table 1.3: Molecular interactions of SLRPs mediated by their core protein.

<table>
<thead>
<tr>
<th>SLRPs</th>
<th>Decorin</th>
<th>Biglycan</th>
<th>Fibromodulin</th>
<th>Lumican</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM and / or cell associated molecules</td>
<td>Collagen types I, II, VI, and XII Glycoprotein-1, Fibronectin, Matrilin-1 Plasmin Plasminogen Apolipoprotein Tropoelastin Collectin Fibrinogen MAGP1 Thrombospondin-1 Von Willebrand factor Tenascin-X Endosomal protein Filamin-A</td>
<td>Collagen types I, VI Glycoprotein-1 MAGP1 Amelogenin, Matrilin-1 α-Sarcoglycan γ-Sarcoglycan Apolipoprotein Tropoelastin Collectin</td>
<td>Collagen types I, II, III</td>
<td>Bacterial LPS</td>
</tr>
<tr>
<td>Proteases</td>
<td>MMP-2, MMP-3, MMP-7, MMP-13,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth factors /cytokines</td>
<td>TGF-β1,2,3</td>
<td>TGF-β1,2,3</td>
<td>TGF-β1,2,3</td>
<td>Chemokine (CXCL1) Fas-ligand</td>
</tr>
<tr>
<td>Cell surface Receptors</td>
<td>EGFR IGF1R LRP-1 26, 51, 110 kDa proteins SR-A, α2β1 integrin</td>
<td></td>
<td></td>
<td>Integrin β1 subunit</td>
</tr>
<tr>
<td>Inflammatory factors</td>
<td>C1q Mannose-binding lectin</td>
<td>C1q Mannose-binding lectin</td>
<td>C1q Factor H</td>
<td></td>
</tr>
<tr>
<td>Elements / ions</td>
<td>Zn++</td>
<td>Zn++</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fibromodulin has been detected at the protein level in the cytoplasm of the B-cell chronic lymphocytic leukemia and lymphomatic mantle cell (Mikaelsson et al., 2005) and was strongly expressed in the alveolar bone and periodontal ligament fibroblasts (Qian et al., 2003). Fibromodulin can also directly bind to C1q of the complement and activate it by the classical pathway. Thus, fibromodulin may influence inflammatory response such as antigen phagocytosis and cells destruction (Sjöberg et al., 2005). The lumican core protein was shown to bind to cell surface receptors of mouse peritoneal macrophages and regulate rapid cell adhesion and migration (Funderburgh et al., 1997). In melanoma cells lumican core protein interacts with β1 integrin and enhances cell adhesion resulting in decreased migration (D’Onofrio et al., 2007; 2008).

1.12 Interaction of SLRPs with cells
In addition to binding to ECM molecules and growth factors and other soluble mediators, SLRPs also interact with cell surface receptors. For example, several cell surface receptors expressed by different cells appear to bind to decorin. However, it is unclear whether these interactions are cell type specific or can occur in a single cell simultaneously. For example, decorin can directly bind to α2β1 integrin in platelets (Guidetti et al., 2002), scavenger receptor A (SR-A) in murine macrophages (Santigo-garcia et al., 2002), insulin-like growth factor-1 receptor (IGF1R) in kidney fibroblasts and endothelial cells (Schonherr et al., 2005; Paye, 2006), EGFR in human skin fibroblasts (Feugaing et al., 2007) and squamous carcinoma cells (Moscatello et al., 1998), low-density lipoprotein receptor-related protein-1 (LRP-1) in Chinese hamster ovary (CHO) cells (Brandan et al., 2006) and myoblasts (Cabello-Verrugio, 2007) and to more poorly characterized 26-kDa and 51-kDa cell membrane proteins in chondrocytes and endothelial cells (Gotte et al., 1995; Hausser et al., 1996) and a 110-kDa protein in human skin fibroblasts (Hausser and Kresse, 1991; Feugaing et al., 2007) (Figure 1.3). The binding of decorin to α2β1 integrin and SR-A mediates cell adhesion whereas LRP-1, 26-kDa, 51-kDa and 110-kDa molecules are involved in decorin internalization. The 26-kDa, 51-kDa or 110-kDa proteins also mediate binding and internalization of biglycan but it is not clear whether these molecules are cell surface receptors or cytoplasmic endosomal proteins. Interaction of decorin with receptor tyrosine kinases LRP-1, IGF1R and EGFR regulates also cell signaling (Moscatello et al., 1998; Schonherr et al., 2005; Feugaing et al., 2007; Gaultier et al., 2006; Brandan et al., 2006; Schaefer
et al., 2007). For example, decorin can induce expression of the endogenous cyclin-dependent kinase inhibitor p21<sup>WAF1</sup> (De Luca et al., 1996; Nash et al., 1999) and a cell cycle arrest in colon cancer cells (Ständler et al., 1999), and suppress tumor cell growth in vivo (Santra et al., 1997; Schönherr et al., 2001). Growth-suppressive properties of the soluble decorin and its core protein also were shown in murine tumor cells, normal human endothelial cells (Schönherr et al., 2001), periodontal fibroblasts (Häkkinen et al., 2000b) and macrophages in vitro (Xaus et al., 2001). Markedly, increased expression of decorin in normal human diploid fibroblasts at quiescence (Coppock et al., 1993) and its absence in transformed lymphocytes and ovarian cancer cells (Ioizzo et al., 1999; Nash et al., 2002) also points out a key role for decorin in the control of cell proliferation. Lumican also suppresses cell proliferation and promotes Fas-Fas ligand mediated apoptosis in cornea (Vij et al., 2004; 2005). Lumican regulates toll-like receptor 4 (TLR4) signaling pathway and the bacterial lipopolysaccharide-induced innate immune response mediated by its core protein (Wu et al., 2007).

SLRPs may also indirectly interact with cell surface receptors via other molecules such as type I collagen. Type I collagen binds to the surface of fibroblasts via α2β1 integrin and mediates cell adhesion and participates in collagen phagocytosis (Goldberg, 1982; Goldberg and Burgeson, 1982). In the sprouting endothelial cells, decorin modulates cell adhesion to type I collagen by interacting with α2β1 integrin via its GAG chain at a site distinct from the collagen I binding domain. In addition, activation of IGF1R signaling by decorin appeared to be necessary for α2β1 integrin-dependent adhesion and migration of endothelial cells on collagen. This was mediated by decorin-induced cytoskeletal and focal adhesion reorganization through activation of small GTPase Rac (Feidler et al., 2008).

In addition, collagen can bind to and activate the discoidin domain receptors (DDR1 and DDR2). These receptors are collagen-activated tyrosine kinase receptors (Mohan et al., 2001) that have been found to modulate cell proliferation and MMPs expression in response to collagen stimulation (Shrivastava et al., 1997; Vogel et al., 1997). Thus, activation of DDRs triggers downstream signaling that is known to regulate the ECM turnover. Collagen can also bind to fibroblasts by a 47-KDa-heat shock/stress protein (Nagata et al., 1986), DPP IV that is a membrane-associated collagen-binding glycoprotein (Bauvois, 1988) and by Endo180 that mediates collagen turnover by endocytosis (East et al., 2003; Wienke et al., 2003; Thomas et al., 2005). It is not known if SLRPs regulate interaction of collagen with these molecules.
Interestingly, decorin can potently inhibit collagen phagocytosis by fibroblasts by an undefined mechanism (Bhide et al., 2005). One of the candidate molecules mediating this function is Endo180.

1.13 uPARAP/Endo180 (CD280)

Endo180, a member of macrophage mannose receptor family (Engelholm et al., 2001a) is a multi-domain transmembrane glycoprotein (Behrendt et al., 2000), and is involved in cell adhesion, migration, uptake and lysosomal degradation of collagen by fibroblasts (Martinez-Pomares et al., 1999; Sheikh et al., 2000; Engelholm et al., 2001a; 2003). The domain structure of Endo180 consists of an NH2-terminal, a cysteine-rich domain, a fibronectin type II-like domain (FNII) that is associated with collagen binding (Ancian et al., 1995a; 1995b), eight C-type lectin-like domains (CTLDs 1-8) that are involved in carbohydrate binding, a transmembrane domain, and a short COOH-terminal cytoplasmic tail (Bateman, 1999; Engelholm et al., 2001a) (Figure 1.4). Endo180 is continuously recycled between clathrin-coated pits at the plasma membrane and intracellular endosomes. Increased expression of Endo180 has been found in osteoblasts and osteocytes at sites of endochondral and intramembranous ossification during development (Engelholm et al., 2001b) in chondrocytes of embryo and neonate at areas of active cartilage deposition (Wu et al., 1996), in tumor endothelium and in breast cancer cells (St Croix et al., 2000; Schnack-Nielsen et al., 2002). In addition, Endo180 has been shown to localize in macrophages (peripheral monocytes), dermal and umbilical endothelial cells and specific subsets of fibroblasts isolated from skin (Sheikh et al., 2000).

Internalization and lysosomal degradation of collagen by fibroblasts has been considered as a major pathway in connective tissue turnover and remodeling (Kielty et al., 1993; Segal et al., 2001). Endo180 binds to the C-terminal region of type I collagen (Thomas et al., 2005) and to type IV and V collagens (Wienke et al., 2003) and takes part in collagen uptake and lysosomal degradation (Wienke et al., 2003; Sulek et al., 2007). Endo180 regulates uptake and degradation of collagen by fibroblast by a mechanism that involves extracellular cleavage of collagen by MMP-14 and possibly other MMPs followed by Endo180-mediated endocytosis (Madsen et al., 2007). In addition, Endo180 regulates cell migration (Engelholm et al., 2003a; Sturge et al., 2003), cell-matrix adhesion (Thomas et al., 2005) and signaling (Sturge et al., 2006) and it may play an important role in wound healing.

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1.14 SLRPs and low-density lipoprotein receptor-related protein -1 (LRP-1)
LRP-1, a 600-kDa cell surface receptor is a member of the LDL receptor family. The molecular structure of LRP-1 consists of an 85-kDa short C-terminal and 515-kDa large N-terminal subunits. The large subunit of the LRP-1 molecule contains four ligand-binding domains that constitute most of the extracellular portion (Krieger and Herz 1994; Willnow, 1999). LRP-1 regulates receptor-mediated endocytosis and degradation (Warshawsky et al., 1996) and cell signaling by binding to and interacting with various ECM molecules such as fibronectin (Salicioni et al., 2002; Gonias et al., 2004), matrix metalloproteinases (Yang et al., 2001), thrombospondin (Godyna et al., 1995; Mikhailenko et al., 1995; Orr et al., 2003), plasminogen activators (Herz et al., 1992; Bu et al., 1992) and growth factors, including CTGF (Segarini et al., 2001; Yang et al., 2004), PDGF (Boucher et al., 2002; Loukinova et al., 2002) and TGF-β (Huang et al., 2004; Tseng et al., 2004). Endo180 mRNA was significantly increased in LRP-1-deficient MEF-2 cells by a mechanism involving Jun amino-terminal kinase (Gaultier et al., 2006). Thus, LRP-1 also regulates expression of Endo180.

Biochemical analysis of LRP-1 molecule indicates that phosphorylation of the LRP-1 tyrosine residue mediates signal transduction while serine-phosphorylated LRP-1 regulates endocytosis (van der Geer, 2002). LRP-1 gene disrupted mice did not survive longer than 9 days after gestation suggesting a pivotal role of LRP-1 in embryonic development (Herz et al., 1992). It has been shown that decorin binds to and is internalized by LRP-1 in CHO cells and myoblasts (Brandan et al., 2006). Of note, interaction of decorin with LRP-1 is required for Smad pathway activation and regulation of TGF-β signaling in myoblasts (Cabello-Verrugio and Brandan, 2007). Interestingly, decorin binds to several of the ECM molecules that also bind and interact with LRP-1, including fibronectin, MMPs, thrombospondin, plasminogen activators, PDGF and TGF-β. It is not known whether other SLRPs interact with LRP-1.

1.15 SLRPs and epidermal growth factor receptor (EGFR)
EGFR is a member of the ErbB family of receptor tyrosine kinases. Dimerization of EGFR induces intracellular protein-tyrosine kinase activity leading to autophosphorylation of tyrosine residues in the C-terminal domain of EGFR. Autophosphorylation of EGFR triggers downstream activation and signaling of the MAPK, Akt and JNK pathways leading to DNA synthesis and regulation of cell migration, adhesion, and proliferation (Mendelsohn and Baselga, 2000; Chen...
and Wang, 2001; Kagiyama et al., 2003). The carboxy terminal tyrosine residues on EGFR, Tyr1068 and Tyr1173, are the major sites of receptor phosphorylation (Batzer et al., 1994). Decorin binds to EGFR via its leucine-rich repeat 6 in the core protein and induces dimerization and down-regulation of EGFR from the cell surface in human squamous carcinoma cells (Santra et al., 2002). In squamous carcinoma cells and tumor stroma of colon cancer cells expressing high levels of EGFR, interaction of decorin with EGFR triggers signaling cascades that lead to phosphorylation of MAPK, calcium influx, induction of the cyclin-dependent kinase inhibitor p21 leading to growth suppression (Montgomery et al., 1995; Santra et al., 2002). Thus, decorin suppresses cancer cell growth and metabolism by blocking EGFR function in vivo (Reed et al., 2005). More specifically, it induces endocytosis of the EGFR via caveolar pathway that leads to EGFR degradation and attenuation of its signaling. Decorin also suppresses cell growth and survival by a mechanism that involves caspase-3 activation in human carcinoma cells (Seidler et al., 2006).

However, the effect of decorin may be cell-type specific. For example, in endothelial cells, decorin enhances EGFR-mediated phosphorylation of protein kinase B (Akt) and p21 level by a MAPK-independent pathway (Schönherr et al., 2001). Furthermore, although decorin reduced growth of human gingival fibroblasts, this effect was not related to EGFR signaling (Häkkinen et al., 2000b). In human and mouse skin fibroblasts, EGFR mediates uptake and internalization of decorin probably by modulating receptor phosphorylation and signaling events (Feugaing et al., 2007). Thus, there appears to be a complex feedback mechanism at least in certain cells that regulates EGFR signaling and decorin endocytosis. In contrast to decorin, biglycan does not induce phosphorylation of the EGFR (Moscatello et al., 1998). It is not known whether other SLRPs interact with EGFR.

1.16 SLRPs and insulin-like growth factor-1 receptor (IGF1R)
The IGF1R is a receptor tyrosine kinase that consists of a heterotetramer linked by disulfide bridges. IGF1 and IGF2 bind to two extracellular domains of IGF1R with high affinity. This interaction leads to autophosphorylation of the receptor and activates several signaling molecules including PI3K, phosphoinositol-3, 4,5-trisphosphate, phosphorylation of Akt, protein kinases C and A (Cui and Almazan, 2007). IGF1R signaling leads to the inhibition of pro-apoptotic factors, the activation of survival factors and cell differentiation or proliferation (Vincent et al.,
IGF1R gene knock-out mice exhibit growth suppression and lethality (Liu et al., 1993). Decorin has been shown to bind and interact with IGF1R in endothelial cells leading to strong activation of the receptor by phosphorylation of Tyr1131 (Schönherr, et al., 2005). The physiological relevance of the decorin/IGF1R interaction was studied in inflammatory angiogenesis in the cornea and unilateral ureteral obstruction, a model of renal fibrosis. In both conditions the expression of IGF1R was increased in decorin knocked out-mice indicating that decorin may play an important role in the regulation of IGF1R expression (Schönherr, et al., 2005). In endothelial cells, decorin binds to IGF1R leading to phosphorylation and activation and subsequent down-regulation of the receptor (Schönherr et al., 2005). Recently, the involvement of decorin in the regulation of IGF1R/ mTOR/ p70 S6 kinase signaling pathway in the translational regulation of fibrillin-1 expression in renal fibroblasts has been studied (Schaefer et al., 2007). In these cells, decorin binds to and induces phosphorylation of IGF1R and subsequently enhanced synthesis of fibrillin-1 (Schaefer et al., 2007).

1.17 SLRPs and transforming growth factor-β (TGF-β)

TGF-β exists in three known isoforms in human, TGF-β1, TGF-β2 and TGF-β3. TGF-βs play crucial roles in tissue regeneration, embryonic development and regulate multiple cellular functions, including cell growth, differentiation, proliferation and apoptosis. In addition TGF-β1 functions as a key regulator of inflammation and immunity (Shull et al., 1992; Gorelik and Flavell, 2000). All these function are also critical processes in wound healing. Several in vitro and in vivo studies have shown that SLRPs can interact with TGF-β and regulate its activity. The low level or activity and production of growth factors/cytokines by fetal cells, especially TGF-β1, appears to be a major factor in the absence of excess collagen deposition and scar formation in fetal wound healing (Shah et al., 1995). It is not known whether SLRPs play role in modulating TGF-β activity during fetal wound healing. However, overexpression of TGF-β1 resulted in marked lung fibrosis, which was significantly reduced by concomitant overexpression of decorin using adenoviral decorin gene transfer (Kolb et al., 2001a). In addition, a single administration of adenovirus containing decorin gene in mouse lungs effectively blocked the fibrogenic response to bleomycin by inhibition of TGF-β1 (Kolb et al., 2001b). Overexpression of decorin using recombinant adenovirus containing decorin cDNA has also been shown to block excess level of exogenous TGF-β1 and its signaling during lung growth and differentiation.
Thus, SLRPs may suppress TGF-β activity also during scarless wound healing. Decorin, biglycan and fibromodulin bind to TGF-β1 through their core proteins and inhibit TGF-β1 fibrogenic activity by preventing the interaction of TGF-β with its cell surface receptors (Yamaguchi et al., 1990; Hildebrand et al., 1994; Giri et al., 1997; Soo et al., 2000; Kolb et al., 2001a; 2001b). For example, increased expression of decorin down-regulates binding of TGF-β to its transducing receptors leading to attenuation of TGF-β signaling in myoblasts during skeletal muscle differentiation (Droguett et al., 2006). Removal of the CS/DS chains of decorin or biglycan increased binding affinity and interaction with TGF-β suggesting that the GAG chains may hinder the interaction of the core proteins with TGF-β (Hildebrand et al., 1994). Two binding sites on SLRP core proteins has been suggested for TGF-βs, Kd values ranging from 1 to 20 nM for a high-affinity binding site and 50 to 200 nM for the lower-affinity binding site (Hildebrand et al., 1994). Binding competition experiments showed that fibromodulin had a higher affinity to TGF-β1 than biglycan and decorin. However, decorin and biglycan had a higher affinity to TGF-β3 than fibromodulin. Fibromodulin is the only SLRP that appears to bind to latent TGF-β to some degree (Hildebrand et al., 1994). All three SLRPs also bind to TGF-β2 and TGF-β3 but it is unclear whether they modulate the activity of these TGF-β isoforms.

In addition to scavenging TGF-β1, SLRPs also modulate TGF-β signaling. For example, decorin suppresses TGF-β1 signaling through a mechanism that involves serine-240 phosphorylation of Smad2 molecule leading to down-regulation of TGF-β-induced plasminogen activator inhibitor-1 expression in mesangial cells (Abdel-Wahab et al., 2002). Conversely, decorin can also promote TGF-β signaling as decorin-deficient myoblasts have a diminished TGF-β response that can be restored by decorin re-expression. In myoblasts, decorin interacts with LRP-1 and modulates activation of the Smad pathway through PI3K activity (Cabello-Verrugio and Brandan, 2007). In addition, interaction of decorin core protein with TGF-β1 increased binding of TGF-β1 to its type I, II and betaglycan receptors in osteoblast-like cells leading to enhanced inhibitory effect of TGF-β1 on cell proliferation (Takeuchi et al., 1994). Thus, decorin, and maybe other SLRPs, may either upregulate or downregulate TGF-β activity and signaling depending on the cellular context.

Expression of each of the three SLRPs can also be affected by increased level/activity of
TGF-β1 to a different extent. Compared to decorin and biglycan, fibromodulin expression was most rapidly increased in response to TGF-β1 in cartilage explants (Burton-Wurster et al., 2003). However, earlier studies indicated that TGF-β1 upregulated biglycan but down-regulated decorin mRNA levels in human skin fibroblasts in culture (Kahari et al., 1991). In granulation tissue fibroblasts, TGF-β1 also increased biglycan mRNA expression but had very little effect on decorin or fibromodulin expression (Häkkinen et al., 1996).

1.18 SLRPs and endocytosis

Endocytosis of ECM molecules is critical for tissue turnover and regulates the abundance of molecules in the ECM. In addition, it regulates cell-surface receptor expression and receptor signaling (Nicholas et al., 1999). Two major endocytosis mechanisms, receptor-mediated endocytosis via clathrin-coated pits and caveolar raft-mediated endocytosis operate in various cell types (Helenius and Marsh, 1982). Clathrin-mediated endocytosis is the specific uptake of large extracellular molecules such as proteins, membrane localized receptors and ion-channels antigens, growth factors, pathogens and recycling receptors (Takei and Haucke, 2001). Caveolae are flask shaped invaginations that consist of the protein caveolin-1 with a bilayer enriched in cholesterol detergent-resistant membrane domains or glycolipid rafts (Harder and Simons, 1997). Fibroblasts and other cells of mesenchymal origin are known to efficiently internalize decorin and biglycan by receptor-mediated endocytosis (Hausser et al., 1989; 1992). This may be an important mechanism that regulates the abundance of SLRPs in the ECM and may also regulate cell signaling induced by SLRPs. Decorin directly binds to the EGFR resulting in dimerization, endocytosis and degradation of the receptor (Zho et al., 2005). Curiously, EGFR signaling is involved in decorin internalization but the exact mechanism is unclear (Feugaing et al., 2007). Interaction of decorin with LRP-1 also regulates endocytosis of decorin (Brandan et al., 2006). In addition, the poorly characterized 26-kDa and 51-kDa proteins that are present at the plasma membrane and in endosomes appear to mediate endocytosis of decorin and biglycan in bovine aortic endothelial cells (Hausser et al., 1989; 1991; 1998). In addition to decorin (Brandan et al., 2006), the classical clathrin-mediated endocytosis pathway was suggested as a major route for the internalization of biglycan (Götte et al., 2004). In vivo studies also indicated that endothelial cells are involved in the endocytosis and clearance of decorin from blood plasma (Götte et al., 1995). Taken together, SLRPs interact with several different receptors expressed in various cell
types. These interactions regulate cell signaling, gene expression, growth and modulate endocytosis (turnover) of SLRPs. It appears, though, that these interactions may be cell type specific and very little is known about interactions of SLRPs with fibroblasts that are pivotal cells for wound healing. It is also not known whether lumican or fibromodulin are endocytosed by cells.

1.19 SLRPs and wound healing

SLRPs regulate key cell functions involved in wound healing such as collagen assembly, cell adhesion, growth, gene expression and activity of growth factors. However, very little is known about the expression of SLRPs in wound healing. Earlier studies demonstrated the localization of SLRPs, namely biglycan and decorin, in human gingival wound repair and chronically inflamed human periodontium (Oksala et al., 1995; 1997). In earlier phases of wound healing decorin was localized at the wound edge on collagen fibers where keratinocytes initiate migration leading to re-epithelialization. Decorin and biglycan were localized in the primitive granulation tissue and areas under the wound epithelium containing newly synthesized collagen fibers (Oksala et al., 1995). At later time points of oral mucosal wound healing, decorin localized in the subepithelial granulation tissue followed by increased staining intensity in the deeper granulation tissue (Oksala et al., 1995). During skin wound healing in a pig model, while mRNA levels for biglycan and fibromodulin were not significantly changed at earlier time points, mRNA levels of decorin and fibromodulin showed marked increase 14 days after injury with a second peak by days 56–70 post-wounding (Wang et al., 2000; Gallant et al., 2004). Decorin appears to be involved in neovascularization because endothelial cells express decorin during angiogenesis in vitro and in vivo during wound healing (Järveläinen et al., 1992; Nelimarkka et al., 2001). However, its function in this process is unclear because decorin gene-deficient mice showed accelerated cutaneous wound healing (Järveläinen et al., 2006) whereas other studies have indicated that targeted deletion of decorin gene results in impaired angiogenesis in cornea (Schönherr et al., 2004). Interestingly, fibromodulin may be able to partially compensate lack of decorin in endothelial cells during angiogenesis (Schönherr et al., 2004).

In an experimental wounding in mouse cornea, wound epithelium showed marked expression of lumican in the early phases of wound healing suggesting lumican may be associated with keratinocytes adhesion or migration (Saika et al., 2000). In contrast, another
study of corneal wound healing found that lumican expression was initially low but was then upregulated at later stages of re-epithelialization suggesting a role of lumican in epithelial cell differentiation (Carlson et al., 2003). Interestingly, compared to wild-type mice, the healing process of an experimental wounding in corneal epithelial cells was significantly delayed in lumican-null mice (Saika et al., 2000) showing that lumican plays a role in re-epithelialization.

In normal and pathological conditions or during wound healing, ECM macromolecules, including SLRPs are proteolytically degraded. For example, MMP-13 can degrade decorin biglycan, fibromodulin and lumican. Although degradation of decorin and lumican was limited, cleavage of biglycan and fibromodulin was extensive (Monfort et al., 2006). Cleavage of fibromodulin by MMP-13 can result in specific fragments that are also detected in interleukin-1-stimulated bovine articular cartilage in vitro and may influence joint disease progression (Heathfield et al., 2004). Decorin and its degradation products have also been detected in early wound fluid (Penc et al., 1998). The functional consequence of this is not clear but cleavage of decorin by MMP-2, MMP-3 and MMP-7 can result in the release of TGF-β leading to potentially increased TGF-β signaling (Imai et al., 1997). Furthermore, release of ECM-associated SLRPs may allow the soluble molecules to interact with cells in the surrounding tissue. For instance, it was observed that a low molecular weight fraction of connective tissue proteoglycan inhibited collagen synthesis by normal and scleroderma fibroblasts (Maquart et al., 1985).

1.20 SLRPs and scar formation

Abnormal wound healing often results in overproduction and accumulation of collagen and formation of scars or hypertrophic scars as a response to prolonged/increased activity of TGF-β1 (Shah et al., 1994; Lin et al., 1995). There is indirect evidence that reduced abundance of decorin may contribute to hypertrophic scar formation. For instance, in hypertrophic scars, TGF-β1 activity was increased while level of decorin was reduced (Scott et al., 1995; Zhang et al., 2004; 2007). In addition, fibroblasts isolated from hypertrophic scars produced reduced amount of decorin (Scott et al., 1998). Expression of decorin mRNA in burn wounds was also downregulated during the first 12 months after injury, significantly increased between 12 and 36 months when resolution of hypertrophic scarring generally is considered to start and returned to level comparable with normal skin after 36 months (Sayani et al., 2000). Decorin was also found markedly reduced for the first 3 months in red Duroc pig wound healing that resulted in
hypertrophic scar formation (Zhu et al., 2003; 2004). Administration of recombinant human decorin inhibits cell proliferation and downregulates TGF-β1 production in hypertrophic scar fibroblasts (Zhang et al., 2007). Therefore, decorin may be considered as a candidate for therapeutic approach to reduce hypertrophic scar formation.

Decreased levels of fibromodulin have also been implicated in scar formation in fetal and adult rat wound healing (Soo et al., 2000). Fibromodulin-deficient adult mice exhibit increased scar formation (Patel et al., 2005). In both fetal and adult rat wound repair, fibromodulin showed an inverse relationship with TGF-β1 activity during scar formation (Soo et al., 2000). Studies comparing adult and fetal skin wound healing revealed that fibromodulin abundance in fetal wounds improved collagen architecture and prevented scar formation (Soo et al., 2000). Recent studies using adenoviral-mediated overexpression of fibromodulin showed reduced scarring and improved outcomes of wound healing in full-thickness incisional wounds in rabbit (Stoff et al., 2007). Interestingly, adenoviral induced overexpression of fibromodulin downregulated expression of TGF-β1 and TGF-β2 but increased antifibrotic TGF-β3 (Stoff et al., 2007). The role of biglycan or lumican in scar formation has not been studied previously.

Taken together, SLRPs play a pivotal role in the regulation of collagen fibrilogenesis, cell functions, signalling and activity of TGF-β that are key processes in wound healing. Thus, SLRPs may play pivotal role in the regulation of wound healing. However, systematic information about the function, localization and changes in the expression of SLRPs over time during wound healing in human is lacking. For example, to date, there is little systematic information describing the spatiotemporal localization of SLRPs in gingival wound healing as a non-scaring wound healing model. Studying differences in the cellular and molecular mechanisms between gingiva and skin wound healing that results in scar formation may provide information to better control scarring and/or tissue fibrosis.

1.21 Objectives, rationales and hypothesis

To date, there is little systematic information describing the spatiotemporal localization of SLRPs in gingival wound healing as a non-scaring wound healing model. In this study, we will investigate which cell types in human gingiva associate with decorin, biglycan, fibromodulin and lumican. The information will be compared with the expression and localization of SLRPs in gingival tissue over time during wound healing. In addition to SLRPs, accumulation of TGF-βs
in gingival wound healing will be studied and correlated with the abundance of SLRPs. We hypothesize that compared to normal, unwounded tissue, gingiva shows distinct localization and altered accumulation of SLRPs and TGF-β during wound healing.

Our unpublished preliminary data also indicate that decorin interacts with the collagen receptor Endo180 in vitro. Therefore, the localization and expression of Endo180 will be studied during gingival wound healing and compared to localization of decorin. **Our hypothesis is that decorin and Endo180 colocalize to distinct wound cells providing evidence that they may interact during wound healing.**

Decorin appears to be a powerful signaling molecule and appears to interact with several cell surface receptors. However, these interactions appear cell type specific. It is not known whether decorin interacts with multiple receptors simultaneously in a single cell type and what is the role of these receptors in decorin-induced signaling. Therefore, we will identify decorin receptors in human gingival fibroblasts and study their function in more detail. **Our hypothesis is that in human gingival fibroblasts, decorin interacts with several receptors at the same time and those interactions induce distinct cellular signaling pathways important in wound healing.**

### 1.22 Significance

Burn wounds or traumatic injuries in skin develop hypertrophic scars that can have devastating consequences ranging from tissue disfigurement to organ dysfunction. In contrast, gingival wounds heal with little or no scar formation. Therefore, better understanding of cellular and molecular mechanisms involved in oral mucosal wound healing may provide valuable information that can be used to prevent dermal scars. Studying the role of SLRPs may provide information for improvement of wound healing and clinical therapeutic approaches to minimize scar formation after injury.
Figure 1.1: Wound healing events over time.

- **Inflammation**
  - Bleeding
  - Complement activation
  - Coagulation
  - Platelets,
  - Macrophages,
  - Polymorphonuclear leukocytes,
  - Phagocytosis,
  - Cytokine release

- **Granulation tissue formation**
  - Fibroplasia
  - Angiogenesis
  - ECM deposition
  - Wound contraction

- **Maturation / tissue Remodeling**
  - Apoptosis
  - Decreased cellularity
  - Decreased number of blood vessels
  - ECM turnover
  - Tensile strength increase

Re-epithelialization

Modified from Clark, The Molecular and Cellular Biology of Wound Repair, 1996
Figure 1.2: Family members and structure of small leucine-rich proteoglycans.

a) Family members and classification of SLRPs

<table>
<thead>
<tr>
<th>Class</th>
<th>SLRPs</th>
</tr>
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<tbody>
<tr>
<td>Class I:</td>
<td>Decorin, Biglycan, Asporin.</td>
</tr>
<tr>
<td>Class II:</td>
<td>Fibromodulin, Lumican, Keratocan, PRELP, Osteoadherin.</td>
</tr>
<tr>
<td>Class III:</td>
<td>Opticin, Epiphycan, Mimecan (Osteoglycin).</td>
</tr>
<tr>
<td>Others:</td>
<td>ECM2, Podocan, Chondroadherin, Nyctalopin.</td>
</tr>
</tbody>
</table>

b) Schematic illustration of SLRPs molecular

DS: Dermatan sulafate, CS: Chondroitin sulfate, KS: Keratan sulfate, TS: Tyrosine sulfate
Modified from: Iozzo and Murdoch, 1996
Figure 1.3: Schematic diagram of cell surface receptors that bind decorin.
Figure 1.4: Schematic illustration of Endo180 molecular structure.

Modified from: Wienke et al., 2003


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CHAPTER 2: Colocalization of the collagen-binding proteoglycans decorin, biglycan, fibromodulin and lumican with different cells in human gingiva

A version of this chapter has been published:

2.1 ABSTRACT

**Background and objective:** Decorin, biglycan, fibromodulin and lumican are structurally related molecules that belong to the family of small leucine-rich proteoglycans (SLRPs). These SLRPs are secreted extracellular matrix molecules that interact with type I collagen and regulate collagen fibrillogenesis. They may also modulate cell functions that are important in maintenance of connective tissue structure. The aim of this study was to localize decorin, biglycan, fibromodulin and lumican in human gingiva.

**Methods:** Localization of decorin and its proform (prodecorin), biglycan, fibromodulin and lumican and mature and proform of type I collagen was studied by immunohistochemical staining of frozen tissue sections from healthy human attached gingiva. Double immunostaining with anti-SLRP or anti-type I procollagen antibodies and specific markers for different connective tissue cells was used to study association of these molecules with cells.

**Results:** The mature and proforms of decorin and collagen and biglycan, fibromodulin and lumican showed distinct localization in the extracellular matrix where they associated with type I collagen fiber bundles. Prodecorin also localized to the epithelial basement membrane zone. Fibroblasts, myofibroblasts, endothelial cells and pericytes showed immunoreactivity for procollagen, prodecorin, biglycan and fibromodulin while lumican associated with fibroblasts and myofibroblasts only. Biglycan and fibromodulin were also associated with macrophages. Basal epithelial cells of the gingival epithelium showed immunoreactivity for biglycan, fibromodulin and lumican.

**Conclusions:** Decorin, biglycan, fibromodulin and lumican associate with type I collagen and may collaborate to regulate collagen fibrillogenesis in human gingiva. Each of the SLRPs showed a distinct association with different connective tissue cells suggesting that the cells produce these molecules and/or that the cells interact with them. Localization of biglycan, fibromodulin and lumican at the epithelial cells suggests novel functions for these SLRPs in human gingival epithelium.
2.2 INTRODUCTION

The family of small leucine-rich proteoglycans (SLRP) consists of at least 13 structurally related molecules that have evolved from different genes (Ameye and Young, 2002). They are widely expressed in the extracellular matrix (ECM) of various hard and soft connective tissues where they are involved in different specialized functions. For example, SLRPs are important in the maintenance of the mineralized matrix of bones and teeth, the transparency of the cornea, the tensile strength of the skin and tendons, and the viscoelasticity of blood vessels. All members of the SLRP family consist of a protein core and one or more glycosaminoglycan side chains that can be chondroitin, dermatan, or keratan sulphate. The core protein of SLRPs has a characteristic central domain with multiple leucine-rich repeats. This domain is responsible for most of the functional activities of these molecules although some of the functions are also mediated by the glycosaminoglycan chains (Iozzo, 1998).

The most studied characteristic of SLRPs is the property of class I SLRPs decorin and biglycan and class II SLRPs lumican and fibromodulin to regulate collagen fibrillogenesis. Gene knockout studies showed that targeted deletion of one or more of these SLRP genes caused abnormal collagen fibril morphology (Ameye and Young, 2002; Reed and Iozzo, 2002). For example, altered collagen fibrils were found in skin and periodontal ligament in the absence of decorin (Danielson et al., 1997). Fibromodulin-null mice had also structurally abnormal collagen fibrils and tissue organization in tendons (Svensson et al., 1999), and lumican-null mice had opaque corneas with abnormally thick collagen fibrils (Chakravarti et al., 2000). In decorin- and lumican-null mice the abnormal collagen fibril morphology resulted in functional defects evidenced by reduced tensile strength and fragility of skin (Danielson et al., 1997; Chakravarti et al., 1998). Targeted disruption of biglycan also caused abnormal type I collagen fibrils in mice (Ameye et al., 2002; Corsi et al., 2002). Thus, decorin, biglycan, fibromodulin and lumican appear to regulate collagen fibrillogenesis *in vivo*. The regulation of collagen fibrillogenesis by decorin, fibromodulin and lumican has been attributed to their property to bind type I collagen. However, it is not clear whether biglycan interacts directly or indirectly with type I collagen. Binding of the core protein of decorin to type I collagen regulates the kinetics of collagen fibrillogenesis and the diameter of and the distance between the fibrils (Vogel et al., 1984). Binding of fibromodulin to collagen also retards the formation of collagen fibrils (Hedbom et al., 1989).
Besides influencing the assembly of the ECM, SLRPs regulate cell functions including cell adhesion, proliferation, differentiation and apoptosis. These effects are cell-type specific and are mediated by binding of the SLRPs to certain growth factors, by SLRP-induced intracellular signaling cascades or by regulation of cell-ECM interactions (Ameye and Young, 2002). For example, decorin, biglycan and fibromodulin regulated activity of transforming growth factor beta (TGF-β) by binding to it and decorin also modulated TGF-β-induced signaling cascades (Yamaguchi et al., 1990; Hildebrand et al., 1994; Zhao et al., 1999; Abdel-Wahab et al., 2002). Overexpression of decorin regulated expression of several matrix metalloproteinases and cytokines in gingival fibroblasts (Al Haj Zen et al., 2003) and decorin also reduced growth of periodontal fibroblasts in vitro and in vivo by a novel mechanism (Häkkinen et al., 2000). Also, biglycan and lumican regulate cell growth. In pancreatic cancer cells, biglycan inhibited cell growth by a mechanism that may have involved SMAD4 signaling pathway (Weber et al., 2001; Chen et al., 2002). Overexpression of lumican suppressed anchorage-independent growth of melanoma cells in vitro and in vivo (Vuillermoz et al., 2004). Decorin, biglycan and lumican regulate also cell adhesion and migration (Winnemoller et al., 1991; 1992; Saika et al., 2002; Schaefer et al., 2003; Tufvesson et al., 2003).

The gingival connective tissue consists of a dense network of type I collagen fibril bundles that provide firmness to the gingiva and attach the gingiva to the tooth and alveolar bone (Narayanan and page, 1985). Type I collagen is also one of the major cell adhesion molecules that regulates functions of the connective tissue cells (White et al., 2004). Gingiva has a fast turnover rate of collagen allowing it to adapt to changing functional demands (Sodek and Ferrier, 1988; Sodek, 1977). Given the multiple functions of SLRPs in the regulation of collagen fibrillogenesis and cell functions they may play an important role in gingival homeostasis. Previous studies have shown that decorin and biglycan are expressed in human gingiva (Häkkinen et al., 1993). Human gingival fibroblasts also express fibromodulin in culture (Häkkinen et al., 1996) but localization of fibromodulin or lumican in human gingiva is not known. The purpose of this study was to analyze the localization and which cell types in human gingiva express collagen binding proteoglycans decorin, biglycan, fibromodulin and lumican. The findings showed that decorin, biglycan, fibromodulin and lumican associated with type I collagen fiber bundles suggesting that they collaborate to regulate collagen fibrillogenesis in gingiva. As these SLRPs cover the collagen fibers, they may also modulate cell interactions with
type I collagen. The SLRPs also showed association with distinct connective tissue and epithelial cells suggesting that various gingival cells participate to produce or metabolize these molecules and/or that these molecules interact with cells to regulate their function.

2.3 MATERIALS AND METHODS

2.3.1 Tissue samples

Tissue samples from palatal attached gingiva were obtained from seven healthy individuals between 23-43 years of age with the approval from the Office of Research Services, Behavioral Research Ethics Board, Biosafety and Animal Care, University of British Columbia. After collection, tissues were immediately embedded in optimal cutting temperature (OCT) compound (Tissue-Tek®), snap frozen in liquid nitrogen, and stored at -86°C until sectioning. After cutting, cryostat sections (6 μm) were transferred to 3-aminopropyltriethoxysilane-coated slides, air dried, and kept in -86 °C until use. The absence of inflammation was confirmed by hematoxylin and eosin staining of representative sections from each sample (not shown).

2.3.2 Antibodies

List of antibodies used for immunostaining, concentrations and their providers are listed in Table 2.1 (a). Schematic illustration of gingival tissue constituents stained with those antibodies is shown in Table 2.1 (b).

2.3.3 Immunohistochemical staining

Tissue samples were shortly thawed at room temperature before fixation using -20 °C acetone for 5 minutes. Samples were then rehydrated with phosphate buffered saline (PBS) for 5 min. For immunostaining using ABC avidin/peroxidase system (Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, CA, USA), sections were incubated in 0.3% hydrogen peroxide in methanol for 30 minutes in order to quench endogenous peroxidase activity. After washing with PBS, sections were incubated with appropriate normal blocking serum (Vectastain ABC Kit) for 60 minutes at room temperature and then incubated with a primary antibody against the core protein of the SLRP, collagen, procollagen or various cell specific markers (Table 2.1) in PBS containing bovine serum albumin (BSA; 1mg/ml) and Triton X-100 (0.01%) at 4 °C overnight.
After washing with PBS, sections were incubated with biotinylated anti-rabbit or anti-mouse antibodies for 60 minutes, rinsed again and then reacted with ABC avidin / peroxidase reagent (Vectastain Elite Kit, Vector Laboratories Inc.). Once optimal color intensity was obtained, reaction was halted by immersing the tissue sections in distilled water. The sections were then air-dried and mounted using VectaMount™ (Vector Laboratories Inc.). The slides were examined using Axiolab E light microscope (Carl Zeiss, Germany).

For double immunofluorescence staining, sections were blocked with PBS containing BSA (10 mg/ml) and Triton X-100 (0.01 %) for 30 minutes at room temperature followed by overnight incubation with the primary antibody (Table 2.1) as above. The sections were then washed and incubated with second primary antibody (Table 2.1) for one hour at room temperature. After washing the samples were incubated with appropriate Alexa-conjugated secondary antibodies (Alexa 488 and Alexa 596; Molecular Probes Inc., Eugene, OR, USA) against both primary antibodies for 1 hour at room temperature. The control samples were incubated with corresponding concentrations (2-5 μg/ml) of non-immune rabbit or mouse IgG instead of primary antibodies and then exposed to secondary antibodies as above (Fig. 2.1, E and F). The slides were mounted using Immuno-mount solution (Thermo Shadon, Pittsburgh, PA, USA), examined by a Zeiss Laser Confocal Scanning Microscope 10 (LSM 10) (Carl Zeiss, Germany), and images were captured using Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada). The relative staining intensity was recorded for each molecule at different tissue locations by using two to three representative sections stained with both immunoperoxide and immunofluorescence methods from three subjects. Relative immunostaining intensity was scored -, +, ++, or +++ by two examiners. (-) represented no immunoreactivity and the most intense reaction was given (+++).

2.4 RESULTS

2.4.1 Localization of various connective tissue cells in human gingiva

In order to localize different connective tissue cells in healthy human attached gingiva we immunostained tissue sections with antibodies that recognize all fibroblasts (Ab-1), myofibroblasts and perivascular cells (Ab-1 and α-SMA), endothelial cells (CD31) and macrophages (CD68) (Table 2.1). Ab-1 positive fibroblasts were numerous throughout the connective tissue (Fig. 2.1A, arrowheads). Additionally, blood vessels showed positive
immunoreaction for the Ab-1 antigen (Fig. 2.1A, arrows). α-SMA positive myofibroblasts were also evenly scattered throughout the connective tissue (Fig. 2.1B, arrowheads). The density of myofibroblasts appeared less than that of fibroblasts expressing the Ab-1 marker. α-SMA positive cells also localized at many of the blood vessels (Fig. 2.1B, arrows). Because both the anti-Ab-1 and anti-α-SMA antibodies were produced in mouse we were not able to perform double immunolocalization experiments using these two antibodies in the same sections. However, by comparing parallel serial sections it was possible to differentiate the Ab-1 positive and α-SMA negative (Ab-1+/α-SMA-) cells from Ab-1 and α-SMA positive (Ab-1+/α-SMA+) cells (Table 2.3). Macrophages were most abundant at the subepithelial connective tissue although some cells were noted also in the deeper areas (Fig. 2.1C, arrowheads). CD31 positive blood vessels localized in the papillary and deep connective tissue (Fig. 2.1D).

2.4.2 The proform and mature form of type I collagen show distinct localization in human gingiva

The proform of type I (procollagen) was present throughout the entire connective tissue where it localized in the ECM (Fig. 2.2 and Table 2.2) and associated with cells (Fig. 2.3 and Table 2.3). Compared to deep connective tissue, more intense staining was localized in the ECM of papillary connective tissue (Figs 2.2A-B and Table 2.2) where a band-like staining was localized most abundantly at and below the epithelial basement membrane zone (Figs 2.2A-B and Table 2.2). This expression pattern was different from the fully processed (mature) form of type I collagen that showed relatively even distribution throughout the connective tissue and was organized into thick collagen fiber bundles (Fig. 2.2C and Table 2.2). Type I procollagen colocalized with Ab-1+/α-SMA- fibroblasts and all α-SMA+ myofibroblasts and perivascular cells (Figs 2.3A-C and Table 2.3). It also colocalized with capillary endothelial cells (Figs 2.3D-F and Table 2.3). However, macrophages did not show any colocalization (Figs 2.3G-I and Table 2.3). The association of procollagen and SLRPs with distinct cells and epithelium were consistent among all the subjects used for the study.
2.4.3 Distinct localization of the proform and mature form of decorin in human gingiva

The proform of decorin (prodecorin) localized in the connective tissue ECM (Fig. 2.2 and Table 2.2) and associated with certain cells (Figs 2.2 and 2.4 and Table 2.3). In the ECM it showed the most intense staining in the deep connective tissue (Fig. 2.2D and Table 2.2) where it localized on the collagen fiber bundles (Fig. 2.2E). Also, a strong, distinct line of prodecorin staining was noted at the epithelial basement membrane zone while epithelium did not show any specific staining (Figs 2.2D and F and Table 2.2). Double immunostaining showed that prodecorin colocalized with Ab-1+/α-SMA- fibroblasts and some of the α-SMA+ myofibroblasts and perivascular cells (Figs 2.4A-F and Table 2.3). It also associated with endothelial cells (Figs 2.4G-I and Table 2.3) but no colocalization with macrophages was noted (Figs 2.4J-L and Table 2.3). Immunostaining of fully processed decorin using an antibody that recognizes the mature protein showed that decorin localized on collagen fiber bundles throughout the connective tissue (Figs 2.2G-I). In contrast to prodecorin, papillary connective tissue and areas close to epithelium stained most strongly for mature decorin (Fig. 2.2I and Table 2.2). No decorin immunoreactivity localized in the epithelium (Figs 2.2G and I and Table 2.2) and no colocalization with Ab-1, α-SMA, CD31 or CD68 positive cells was noted (Table 2.3).

2.4.4 Biglycan associates with collagen, connective tissue and epithelial cells in human gingiva

Biglycan showed immunoreactivity throughout the connective tissue but the most abundant staining localized to the deep connective tissue (Fig. 2.2J and Table 2.2) and colocalized with collagen fiber bundles (Figs 2.2J-K). Additionally, basal epithelial cells showed cell membrane-associated biglycan staining (Figs 2.2L and Table 2.2). In these cells, most abundant staining localized against the basement membrane zone. Double immunostaining revealed that biglycan also associated with Ab-1+/α-SMA- fibroblasts and α-SMA+ perivascular cells (Figs 2.5A-C and Table 3) and myofibroblasts (Figs 2.5D-F and Table 2.3). In addition, a distinct colocalization of biglycan and endothelial cells (Figs 2.5G-I and Table 2.3) and macrophages was observed (Figs 2.5J-L and Table 2.3).
2.4.5 Fibromodulin associates with collagen, connective tissue and epithelial cells in human gingiva

Fibromodulin was most abundantly localized in the deep connective tissue where it localized on collagen fiber bundles (Figs 2.2M-N and Table 2.2). Basal epithelial cells also showed strong immunoreactivity of fibromodulin around the cell membranes (Figs 2.2M and O and Table 2.2). Double immunostaining showed that fibromodulin colocalized with Ab-1+/α-SMA- fibroblasts and αSMA+ perivascular cells (Figs 2.6A-C and Table 2.3) and myofibroblasts (Figs 2.6D-F and Table 2.3). It also colocalized with endothelial cells (Figs 2.6G-I and Table 2.3) and macrophages (Figs 2.6J-L and Table 2.3).

2.4.6 Lumican associates with collagen, fibroblasts, myofibroblasts and epithelial cells in human gingiva

Lumican localized abundantly throughout the connective tissue. The expression of lumican appeared to be less intense in the papillary than in the deeper connective tissue (Fig. 2.2P and Table 2.2). In the connective tissue, lumican was localized on collagen fiber bundles but not with blood vessels (Fig. 2.2Q). In the rete ridge areas of the epithelium, lumican localized on the cell membranes of basal cells (Fig. 2.2R and Table 2.2). This staining appeared most intense in the cell membrane facing the basement membrane. At the connective tissue papilla area, lumican staining was noted around the basal cells (Fig. 2.2R). Lumican also associated with Ab-1+/α-SMA- fibroblasts and α-SMA+ myofibroblasts (Figs 2.7D-F and Table 2.3) but no colocalization with perivascular α-SMA+ cells (Figs 2.7A-C and Table 2.3), endothelial cells (Figs 2.7G-I and Table 2.3) or macrophages was noted (Figs 2.7J-L and Table 2.3).

2.5 DISCUSSION

Previous studies have shown the distribution and expression of SLRPs in dental and periodontal tissues of different animal species. For example, matrix deposition of decorin and biglycan was investigated during odontogenesis in the rat incisor. Administration of growth hormone to the dwarf rats markedly elevated the expression of both proteoglycans (Zhang et al., 1995). In addition, a recent study on the adult rat periodontium has localized fibromodulin in the gingival epithelium and gingival and periodontal fibroblasts as well as their surrounding ECM. Strong expression of fibromodulin was noted in the palatal gingival tissues and at the interfaces of the
periodontal ligament with alveolar bone and cementum (Qian et al., 2004). In bovine cementum, lumican and fibromodulin were found to be located almost exclusively in nonmineralized portions such as precementum and the pericementocyte area (Cheng et al., 1996; Matias et al., 2003). Although SLRPs have been studied in periodontal tissues of animal models, there is lack of evidence about the localization of these molecules and their interactions with collagen fibers in human gingiva. Previously, we demonstrated that decorin and biglycan are expressed in human gingiva and periodontal ligament (Hakkinen et al., 1993). In the present study, we showed that fibromodulin and lumican are also expressed in human gingiva. In the ECM, decorin showed the most abundant staining in the papillary connective tissue while biglycan, fibromodulin and lumican where more abundant in the deep connective tissue suggesting that they may serve for different functions in the areas where they accumulate.

These SLRPs associated with type I collagen fiber bundles in the ECM. Type I collagen is a cell adhesion molecule for connective tissue cells and interaction of cells with collagen regulates several key signaling pathways involved in cell proliferation, survival, gene expression and collagen turnover (Lee et al., 1996; Ivaska and Heino, 2000; East and Isacke, 2002). It is not known, however, whether SLRPs modulate these interactions in gingival cells. SLRPs are also important regulators of collagen fibrillogenesis in vitro (Vogel et al., 1984; Hedbom et al., 1989; Rada et al., 1993). Consequently, single gene knockout of each of the SLRPs lead to altered collagen fibrillogenesis in vivo (Ameye and Young, 2002; Reed and Iozzo, 2002; Danielson et al., 1997, Svensson et al., 1999; Chakravarti et al., 2000). Thus, our findings that the four SLRPs colocalized to collagen fiber bundles suggest that these SLRPs collaborate to regulate collagen fibrillogenesis in human gingiva. There is evidence that these four SLRPs may have distinct but also synergistic functions (Ameye and Young, 2002). Therefore, collagen fibrillogenesis is likely tissue and tissue site specific and depends on multiple variables including level of expression of individual SLRPs and collaboration between the SLRPs. In order to find out whether the SLRPs could interact with gingival cells we studied whether immunoreactivity for the SLRPs associated with different cells in gingiva.

To identify different connective tissue cells we used antibodies that recognize specific molecules expressed by fibroblasts (Ab-1), myofibroblasts and pericytes (α-SMA), macrophages (CD68) and endothelial cells (CD31). The anti-fibroblast antigen (Ab-1) antibody recognizes CD90/Thy-1 that is expressed by fibroblasts, neurons, certain CD34+ blood stem cells and
activated endothelial cells (Saalbach et al., 1996; 1998; 1999; 2000). α-SMA is a cytoskeletal protein that is expressed by myofibroblasts and perivascular cells (pericytes) (Gabbiani, 2003). Differentiation of myofibroblasts and expression of α-SMA is induced during organogenesis, wound healing and in certain pathological conditions including fibrosis where these cells are involved in secretion and organization of ECM molecules (Powell et al., 1999). Pericytes exist in all organs where they associate abluminally with all vascular capillaries and postcapillary venules and regulate local blood flow (Hirschi and D'Amore, 1996). In healthy human gingiva, α-SMA positive cells were located both within the ECM and in association with blood vessels. Therefore, the former cell population represents myofibroblasts while the latter population represents pericytes. By comparing parallel serial sections it was possible to differentiate the Ab-1+/α-SMA- fibroblasts from Ab-1+/α-SMA+ myofibroblasts and show that the fibroblasts were more abundant than myofibroblasts. Interestingly, myofibroblasts do not usually reside in e.g. normal skin connective tissue (data not shown). Therefore, a unique property of human gingiva appears to be the presence of myofibroblasts in the healthy tissue. Our findings also support previous findings showing that a proportion of fibroblasts isolated from healthy human gingiva express α-SMA in culture (Hakkinen et al., 1996; Arora and McCulloch, 1994; Giannopoulou and Cimasoni, 1996).

By using double immunostaining we showed that distinct SLRPs associated with various connective tissue cells. Cell-association of SLRP immunoreactivity can have several explanations. First, it suggests that the cells participate to secrete these molecules to the ECM. Previous findings have shown that human periodontal fibroblasts and myofibroblasts from wound or chronically inflamed granulation tissue produce at least decorin, biglycan and fibromodulin in culture (Hakkinen et al., 1996; Larjave et al., 1992). Second, cells may bind SLRPs to remove them from the ECM by endocytosis and/or phagocytosis. This would facilitate ECM turnover taking place in gingiva. Comparable turnover mechanisms have been described for collagens, decorin and biglycan in fibroblasts (Hausser et al., 1992; Wienke et al., 2003; Everts et al., 1996; Engelholm et al., 2003). Third, SLRPs may interact with the cells to modulate their functions. This is supported by findings showing that decorin regulated growth and gene expression of human periodontal fibroblasts (Hakkinen et al., 2000; Al Haj Zen et al., 2003).

Fibroblasts are considered to be the major cell type responsible for the maintenance and turnover of the connective tissue ECM in tissue homeostasis. Our findings suggest that in human
gingiva myofibroblasts, macrophages, pericytes and endothelial cells also participate in this process. The double immunostainings showed that procollagen, prodecorin, biglycan, fibromodulin and lumican associated with both fibroblasts and myofibroblasts. In contrast to myofibroblasts, pericytes did not show colocalization with lumican although they showed immunoreactivity for the other ECM molecules. Macrophages showed colocalization with biglycan and fibromodulin while endothelial cells of gingival blood vessels associated with procollagen, prodecorin, biglycan and fibromodulin. Previous studies of other tissues have shown that pericytes synthesize decorin (Ungefroren et al., 1995) whereas decorin is absent from resting capillaries (Bosse et al., 1993). However, decorin appears to be involved in neovascularization because endothelial cells express decorin during angiogenesis in vitro and in vivo (Jarvelainen et al., 1992; Nelimarkka et al., 2001). Corneal endothelial cells and cultured endothelial cells undergoing angiogenesis in vitro also synthesize type I collagen (Jarvelainen et al., 1992; Kay et al., 1996). Based on these findings localization of prodecorin and procollagen to gingival blood vessels suggests that these vessels are undergoing constant remodeling. This is further supported by our finding that gingival blood vessels showed immunoreactivity for the anti-Ab-1 antibody that recognized CD90/Thy-1 that is expressed by activated endothelial cells (Saalbach et al., 2000). The finding that biglycan and fibromodulin also associated with endothelial cells is novel and their function remains to be shown.

Our findings showing different tissue distribution and cell association of prodecorin and procollagen in gingiva suggest that there are site-specific variations in the processing of procollagen and prodecorin molecules. Gingiva, like other connective tissues, is composed of phenotypically and functionally different fibroblast populations (Boedin et al., 1984; Hakkinen and Larjava, 1992). Differences in e.g. synthesis of collagen, glycosaminoglycans and decorin have been noted in gingival fibroblast clones and subpopulations (Larjave et al., 1992; Hassel et al., 1983; Fries et al., 1994). Thus, the spatial differences in the abundance, localization and processing of the SLRPs and procollagen may result from presence of phenotypically different fibroblast subpopulations in the tissue. Furthermore, our findings suggest that fibroblasts, myofibroblasts, pericytes, macrophages and endothelial cells produce distinct SLRPs which can lead to variable expression of these molecules at different tissue sites. Epithelial cells were recently shown to regulate collagen and decorin expression by fibroblasts (Shephard et al., 2004) which may also account for the increased localization of decorin, prodecorin and procollagen in...
the subepithelial connective tissue. The abundant immunoreactivity of prodecorin underneath the epithelium found in the present study is the first demonstration that prodecorin associates with the basement membrane zone.

Our findings also showed for the first time that SLRPs were localized in human gingival epithelium. Previous studies have localized lumican to the basal epithelial cells of injured mouse corneal epithelium and biglycan to developing human skin epithelium (Bianco et al., 1990; Saika et al., 2000). In the present study biglycan, fibromodulin, and lumican showed distinct and partially different localization in basement membrane zone and basal epithelium suggesting that these molecules may serve for different functions in these locations. The specific function of these molecules in the gingival epithelium remains to be shown.

2.6 Acknowledgements:
We thank Dr. Larry Fisher (NIH, NIDR, Bethesda, MD, USA) for anti-prodecorin and anti-biglycan antibodies, Dr. Peter Roughley (Genetics Unit, Shriners Hospital for Crippled Children, and Department of McGill University, Montreal, Quebec, Canada) for the anti-lumican antibody and Dr. Anna Plaas (Shriners Hospital for Crippled Children, Tampa, FL, USA) for the anti-fibromodulin antibody. We also thank Mr. Cristian Sperantia for expert technical assistance. The study was supported by Canadian Institutes of Health Research (CIHR).
Table 2.1: a) List of antibodies used for immunostaining of gingival tissue constituents.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Reference / Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal anti-decorin</td>
<td>1:100</td>
<td>Krusius and Ruoslahti, 1986</td>
</tr>
<tr>
<td>Polyclonal anti-fibromodulin</td>
<td>1:2000</td>
<td>Plaas and Wong Palms, 1993</td>
</tr>
<tr>
<td>Polyclonal anti-collagen type I</td>
<td>1:300</td>
<td>Biodesign international, Kennebunk, ME, USA</td>
</tr>
<tr>
<td>Polyclonal anti-procollagen type I</td>
<td>1:10000</td>
<td>Chemicon International, Temecula, CA, USA</td>
</tr>
<tr>
<td>Polyclonal anti-biglycan (LF 106)</td>
<td>1:1000</td>
<td>Fisher et al, 1989</td>
</tr>
<tr>
<td>Monoclonal anti-CD90, Fibroblast (Ab-1)</td>
<td>1:10</td>
<td>Oncogene Research Products, San Diego, CA, USA</td>
</tr>
<tr>
<td>Monoclonal anti-α-Smooth Muscle Actin</td>
<td>1:100</td>
<td>Sigma, Saint Louis, MO, USA</td>
</tr>
<tr>
<td>Monoclonal anti-CD31, Endothelial cells</td>
<td>1:200</td>
<td>BD Biosciences, Mississauga, ON, Canada</td>
</tr>
<tr>
<td>Monoclonal anti-CD68, Macrophages</td>
<td>1:100</td>
<td>Dako Cytomation Inc. Mississauga, ON, Canada</td>
</tr>
</tbody>
</table>

b) Schematic illustration of gingival tissue constituents.
Table 2.2: Relative immunostaining intensity SLRPs and collagen in human gingiva.

<table>
<thead>
<tr>
<th></th>
<th>Basal Epithelial Cells</th>
<th>Basement Membrane Zone</th>
<th>Papillary Connective Tissue</th>
<th>Deep Connective Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procollagen</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Collagen</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Prodecorin</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Decorin</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Biglycan</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Lumican</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

- : no immunoreactivity; + : weakest; ++ : intermediate; +++ : strongest immunostaining intensity. The relative intensity compares staining of each molecule between different tissue sites, not between different molecules.

Table 2.3: Association of SLRPs with different connective tissue cells in human gingiva.

<table>
<thead>
<tr>
<th></th>
<th>Fibroblasts (Ab-1+/α-SMA-)</th>
<th>Myofibroblasts and pericytes (α-SMA+)</th>
<th>Endothelial cells (CD31+)</th>
<th>Macrophages (CD68+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procollagen</td>
<td>+</td>
<td>*</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Decorin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Prodecorin</td>
<td>+</td>
<td>*</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Biglycan</td>
<td>+</td>
<td>*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>+</td>
<td>*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lumican</td>
<td>+</td>
<td>*</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- : No colocalization; + : Colocalization
* Myofibroblasts and perivascular cells
** Myofibroblasts only
Figure 2.1: Immunolocalization of connective tissue cells in gingiva.

A: gingival connective tissue contained abundantly Ab-1 positive fibroblasts throughout the connective tissue (arrowheads). Also blood vessels showed immunoreactivity for the Ab-1 antibody (arrows). B: α-SMA positive myofibroblasts were scattered throughout the connective tissue (arrowheads). α-SMA positive perivascular cells were also noted in many of the blood vessels (arrows). C: Macrophages were most abundant at the subepithelial connective tissue although some cells were noted also in the deeper areas (arrowheads). D: CD31 positive cells localized at blood vessels in the papillary and deep connective tissue. E and F: control samples reacted with rabbit or mouse non-immune IgG instead of primary antibodies. *Non-specific background staining; E: epithelium; CT: connective tissue. Magnification bar = 50 μm.
Figure 2.2: Immunolocalization of SLRPs and type I collagen in gingiva.
Figure 2.2: Immunolocalization of SLRPs and type I collagen in gingiva.
A and B: Type I pro-collagen showed most intense staining in the papillary connective tissue (arrowheads in A). A band-like staining was localized most abundantly at and below the basement membrane zone of epithelium (arrows in B). C: Type I collagen showed even distribution throughout the connective tissue. D and F: A distinct line of prodecorin staining was localized at the basement membrane zone (arrows). E: Prodecorin also localized on the collagen bundle fibers (arrows) and around certain blood vessels (arrowhead). G and H: Decorin was widely present on collagen fiber bundles throughout the connective tissue (arrows). I: Papillary connective tissue and areas close to epithelium stained strongly for decorin. J-L: Biglycan showed most abundant staining in the deep connective tissue (J) and on collagen bundle fibers (arrows in K). Biglycan was also present around blood vessels (arrowheads in J). Biglycan showed cell membrane-associated staining in basal epithelial cells (arrows in L). M and N: Fibromodulin localized most abundantly in the deep connective tissue (M) where it localized on collagen fiber bundles (arrows in N). O: Basal epithelial cells showed distinct immunoreactivity of fibromodulin around the cell membranes (arrows). P and Q: Lumican localized also most abundantly in the deep connective tissue (P) on collagen fiber bundles (arrows in Q). R: In the rete ridge areas, lumican localized on the cell membranes of basal epithelial cells. On these cells most abundant staining localized against the basement membrane (arrowheads). At the connective tissue papilla area, lumican was localized around the basal cells (arrows). E: Epithelium; DCT: Deep connective tissue; PCT: Papillary connective tissue; *: Non-specific background staining. Magnification bar = 50 μm.
Figure 2.3: Type I procollagen associates with endothelial and α-SMA positive cells.

A-C: Type I procollagen colocalized with α-SMA positive fibroblasts located in the extracellular matrix (arrowheads) and around blood vessels (arrows). D-F: Procollagen also colocalized with capillary endothelial cells (arrows). Inserts show higher magnification of cross sections of representative blood vessels. G-I: Macrophages did not show any colocalization with type I procollagen. A, D, and G: Type I procollagen staining. B: α-SMA staining. E: CD31 (endothelial cell) staining. H: CD68 (macrophage) staining. C, F, and I: Corresponding merged images with yellow color indicating colocalization. Magnification bar = 50 μm.
Figure 2.4: Prodecorin associates with α-SMA positive cells.

Figure 2.5: Biglycan associates with α-SMA positive cells, endothelial cells and macrophages.

Figure 2.6: Fibromodulin associates with α-SMA positive cells, endothelial cells and macrophages.

Figure 2.7: Lumican associates with myofibroblasts.

A-C: Lumican did not show colocalization with α-SMA expressing perivascular cells. D-F: However, α-SMA positive myofibroblasts in the connective tissue (arrowheads) showed colocalization with lumican. G-L: No colocalization of lumican with endothelial cells (G-I) or macrophages (J-L) was noted. A, D, G, and J: Lumican staining. B and E: α-SMA staining. H: CD31 (endothelial cells) staining. K: CD68 (macrophage) staining. C, F, I and L: Corresponding merged images with yellow color indicating colocalization. Magnification bar = 50 μm.
2.7 REFERENCES


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Krusius T, Ruoslahti E. Primary structure of an ECM proteoglycan core protein deduced from cloned cDNA. *Proc Natl Acad Sci U S A.* 1986;83:7683-7.


CHAPTER 3: Localization of small leucine-rich proteoglycans and transforming growth factor-β in human oral mucosal wound healing

A version of this chapter has been submitted for publication:

3.1 ABSTRACT

Wound healing in oral mucosa is fast and results in little scar formation as compared to skin. The biological mechanisms underlying this property are poorly understood but may provide valuable information about the factors that promote wound regeneration. Small leucine-rich proteoglycans (SLRPs) decorin, biglycan, fibromodulin and lumican are extracellular matrix (ECM) molecules that regulate collagen fibrillogenesis, inhibit transforming growth factor-β (TGF-β) activity and reduce scarring. In the present study, we analyzed accumulation of SLRPs and TGF-β during non-scarring human oral mucosal wound healing. Biopsies were collected from healthy volunteers from unwounded tissue and from standardized experimental wounds 3-60 days post-wounding. Localization of SLRPs, TGF-β1 and TGF-β3 was analyzed by immunohistochemical staining and quantitated by image analysis. Double immunostaining was used to study localization of SLRPs or active TGF-β to distinct cells. Decorin, biglycan, fibromodulin and TGF-β isoforms showed significantly increased accumulation in the wound ECM and distinct wound cells while the abundance of lumican in the ECM was strongly reduced during wound healing. Localization and abundance of fibromodulin, lumican and TGF-β isoforms was also spatio-temporally regulated in the wound epithelium. The findings suggest that SLRPs regulate wound re-epithelialization and connective tissue regeneration during oral mucosal wound healing.

3.2 INTRODUCTION

Wound healing is a well-coordinated, complex process that restores tissue integrity after injury. It involves spatially and temporally tightly regulated interactions of various cells, extracellular matrix (ECM) components and soluble factors. Although wound healing restores tissue integrity it does not always result in complete regeneration of tissue structure and function but rather results in formation of a scar tissue. For example, 67% of patients suffering from cutaneous burn wounds develop hypertrophic scars that can have devastating consequences ranging from esthetic concerns to tissue disfigurement or organ dysfunction (Bombaro et al., 2003). Better understanding of the biological mechanisms involved in wound healing is needed to prevent scar formation. Interestingly, surgical or traumatic wounds in the oral mucosa share common characteristics with fetal skin healing and heal by regeneration with very little or no scar formation (Häkkinen et al., 2000; Szpaderska et al., 2003; Ferguson and O'Kane, 2004;
Schrementi et al., 2008). The reason for the absence of scar formation in oral mucosa is not
known but may depend on factors present in oral cavity (saliva and specific microflora),
inhertently different composition of the ECM and/or phenotypically unique cells (Häkkinen et al.,
2000; Szpaderska et al., 2003).

The hallmark of scar formation in skin is increased accumulation of abnormally organized
collagen-rich ECM as a response to increased activity of transforming growth factor-β1 (TGF-
β1; Cutroneo et al., 2003). The low level of growth factor and cytokine activity in fetal skin or
gingival cells, particularly TGF-β1, may be a major factor for the absence of scar formation
(Ferguson and O’kane, 2004). In addition, abundance of the anti-fibrogenic TGF-β3 relative to
TGF-β1 may play a role (Ferguson and O’kane, 2004; Schrementi et al., 2008). Most of the
TGF-β is secreted as a latent molecule and the factors that promote or inhibit the activation
process are important for its biological function (Sheppard, 2006). The small leucine-rich
proteoglycans (SLRPs) are structurally and functionally related ECM molecules. SLRPs decorin,
biglycan, fibromodulin and lumican are abundantly expressed in connective tissues, including
human gingiva, and interact with type I collagen to coordinately regulate fibrillogenesis and
collagen turnover (Ameye and Young, 2002; Bhide et al., 2005; Matheson et al., 2005; Geng et
al., 2006). In addition, SLRPs may serve as signaling molecules and appear to regulate ECM
deposition. For example, recombinant decorin reduces collagen and TGF-β1 synthesis in
cultured hypertrophic scar fibroblasts (Zhang et al., 2007). This effect may at least partially be
mediated by the ability of decorin, like biglycan and fibromodulin, to bind to active TGF-β
resulting to inhibition of its biological activity (Hildebrand et al., 1994). This function of SLRPs
may also be significant in vivo as in experimental lung or peritoneal fibrosis or
glomerulonephritis, adenoviral-mediated decorin overexpression resulted in decreased TGF-β
activity and reduced fibrosis (Kolb et al., 2001) and a single injection of decorin prevented
subconjunctival scarring in an animal model (Grisanti et al., 2005). Furthermore, adenoviral
overexpression of fibromodulin reduced scar formation in a rabbit skin wound healing model
and suppressed expression of profibrotic TGF-β1 and TGF-β2 and increased expression of
antifibrotic TGF-β3 in cultured fibroblasts (Stoff et al., 2007). Thus, SLRPs may collaborate to
regulate key wound healing events, including collagen deposition, turn-over and activity of
TGF-β. However, systematic information about spatio-temporal accumulation of SLRPs in
wound healing is lacking as data about individual SLRPs come from different animal models
and short-term or cross-sectional studies. In rat skin wounds, decorin, biglycan and fibromodulin had spatio-temporally distinct expression during transition from fetal to adult wound healing (Soo et al., 2000). Expression of decorin also appeared temporally increased during skin wound healing in a pig model while fibromodulin and biglycan levels remained relatively constant (Wang et al., 2000). In human gingival wounds, decorin and biglycan showed differential localization in the primitive granulation tissue ECM at the early stages of wound healing (Oksala et al., 1995) and in human skin wounds, decorin was localized to the newly-formed blood vessels (Nelimarkka et al., 2001). Lumican expression has only been studied in corneal wound healing where it was down-regulated during wound healing (Carlson et al., 2003). In the present study, we compared the spatio-temporal accumulation of SLRPs decorin, biglycan, fibromodulin and lumican with TGF-β1 and anti-fibrogenic TGF-β3 during human oral mucosal wound regeneration.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Tissue samples

Preparation of the experimental wounds and collection of the wound sample at different time points has been described previously (Honardoust et al., 2006). Briefly, full thickness wounds (10 mm long, 2 mm wide and 2 mm deep) were made in the human adult palatal masticatory mucosa at least 3 mm away from the gingival margins of the teeth and distal to the canine in 16 healthy volunteers. The subjects were all nonsmokers, healthy males and females (22-35 years of age) with no systemic disease, periodontal or gingival disease or medication. A punch biopsy (4 mm diameter) was collected from unwounded tissue (day 0 sample) and from the wounds 3, 7, 14, 28 or 60 days after wounding, embedded in Optimal Cutting Temperature Compound (Sakura Finetek Inc., Torrance, CA, USA) and immediately frozen in liquid nitrogen. Tissue sections (6 μm) were cut using a 2800 Frigocut Cryostat Microtome (Leica, Nussloch, Germany), placed on 3-aminopropyltriethoxysilane-coated slides and stored at -86°C until use. Human experimentation was approved by the Research Ethics Board of the University of British Columbia and complies with the ethical rules for human experimentation that are stated in the 1975 Declaration of Helsinki.
3.3.2 Antibodies

To analyze the localization and abundance of target molecules the following antibodies were used: polyclonal anti-prodecorin (LF 110; recognizes mostly cell-associated decorin; Fisher et al., 1995), monoclonal anti-decorin (MAB143; recognizes mature, ECM-associated decorin; R&D Systems Inc., Minneapolis, MN, USA), polyclonal anti-biglycan (LF 106; recognizes cell-associated biglycan; Fisher et al., 1989), monoclonal anti-biglycan (M01; recognizes extracellular matrix-associated biglycan; Abnova Co., Taipei, Taiwan), polyclonal anti-fibromodulin (Plaas et al., 1993) polyclonal anti-lumican (Grover et al., 1995) polyclonal anti-TGF-β1 (Promega, Madison, WI, USA), polyclonal anti-TGF-β3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal anti-active TGF-β1 (Hahm et al., 2007; sc-146, Santa Cruz Biotechnology). To identify different connective tissue cells, monoclonal antibodies that recognize specific molecules expressed by myofibroblasts and pericytes (α-smooth muscle actin, α-SMA; Sigma, Saint Louis, MO, USA), endothelial cells (CD31; BD Biosciences, Mississauga, ON, Canada), and macrophages (CD68; Dako Cytomation Inc., Mississauga, ON, Canada) were used. Appropriate Alexa-conjugated anti-rabbit-594 or anti-mouse-488 (Molecular Probes Inc., Eugene, OR, USA) was used as the secondary antibody.

3.3.3 Immunohistochemical staining

The immunofluorescence staining protocol has been described previously (Honardoust et al., 2006). Briefly, tissue samples were thawed, air dried, fixed with ice-cold acetone for 5 minutes, rehydrated with phosphate buffered saline (PBS) and blocked with PBS containing bovine serum albumin (BSA; 10 mg/ml) and Triton X-100 (0.01 %) for 30 minutes at room temperature. The primary antibody diluted in PBS containing BSA (1 mg/ml) and Triton X-100 (0.01%) was incubated on tissue sections overnight at 4°C. The tissue sections were then washed and incubated with the appropriate secondary antibody for one hour at room temperature followed by mounting using Immuno-mount solution (Thermo Shadon, Pittsburgh, PA, USA). For double immunofluorescence staining, the tissue sections were incubated with the first primary antibody as above. Sections were then washed and incubated with the second primary antibody for one hour at room temperature followed by incubation with appropriate secondary antibodies against both primary antibodies for one hour at room temperature. Samples were examined by using Nikon.
E600 D-Eclipse Laser Confocal Microscope equipped with Nikon D-Eclipse C1 digital camera and images captured using EZ C1 2.3 software (Nikon Instech. Co., Kawasaki, Japan). Control immunostainings performed by using appropriate non-immune serum instead of the primary antibody or by omitting the primary antibody incubation step gave negative staining results (data not shown).

3.3.4 Picrosirius red staining
Tissue sections were fixed with 4% paraformaldehyde in a 0.1 M phosphate buffer containing 7.5% sucrose for 30-60 minutes at room temperature and rinsed in distilled water and 95% alcohol. Tissue section were then incubated in alkaline alcohol (95% alcohol with ammonium hydroxide, pH > 8.0) for 10 minutes at 60°C and then quickly rinsed in distilled water. The slides were immersed in picrosirius red (100 ml of saturated aqueous picric acid containing 0.1 g of Garr’s Sirius Red F3B; BDH Chemicals, Toronto, ON, Canada) for 60 minutes at room temperature. Tissue section were finally washed with 1% acetic acid for several times, mounted using 50% glycerol in PBS and visualized with Zeiss Axioplan 2 Imaging Microscope using differential interference contrast filter (Carl Zeiss, Jena, Germany) and images were captured using QICAM digital camera (Q-Imaging, Surrey, BC, Canada) and Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada).

3.3.5 Quantitative image analysis
To assess immunostaining intensity in the connective tissue, five representative sections from three to four subjects at each time point were immunostained as above. Standardized images were captured with equal setting for offset (background darkness) and exposure time (overall brightness). Using Image J software (http://rsb.info.nih.gov/ij/), ten 40x40 μm areas were randomly selected from subepithelial connective tissue (area from basement membrane zone to the depth of 100 μm) or from deep granulation/wound connective tissue (area 100–400 μm deep from basement membrane zone) and staining intensity of each area was measured on a min-max range gray scale. The scale valued from 0-255 where 0 and 255 represent pure black and pure white, respectively. Statistical analysis was performed on values obtained from the mean from different subjects by one-way ANOVA and Dunnett’s Multiple Comparison Test. Values of p < 0.05 were considered to be statistically significant.
The relative number of different connective tissue cells per unit area in the wound granulation tissue as compared to the unwounded tissue was scored by two independent, calibrated examiners and the results were averaged. The relative number of cells in the wound tissue was given a numerical score where 1.0 represented equal cell number to unwounded tissue and values 0.25, 0.5 and 0.75 represented 75, 50 or 25% lower cell number as compared to unwounded tissue. Values 1-3 indicated fold increase in cell number in the wound tissue as compared to unwounded tissue. For analysis of the number of different connective tissue or wound cells showing colocalization with SLRPs or active TGF-β1, the double immunostained tissue sections were examined visually and scores given by the examiners on the same tissue sections were averaged. Different connective tissue cells were identified with antibodies against the cell type specific molecules (see above). The following scale was used to score the number of different connective tissue cells showing positive immunoreactivity (colocalization) for SLRPs or active TGF-β1 per unit area of tissue relative to unwounded tissue: -: no cells showing immunoreactivity; +: same number of cells/unit area showing immunoreactivity as in unwounded tissue; ++++: the highest number of cells/unit area showing immunoreactivity. The relative immunostaining intensity of SLRPs, TGF-β1, active TGF-β1 and TGF-β3 in wound epithelium was scored as follows: -: not present; +: the lowest relative staining intensity; ++++: the highest relative staining intensity.

3.4 RESULTS

All wounds healed without complications and after 60 days the original wound areas were clinically indistinguishable from the normal mucosa confirming the scar-free healing (not shown). After 3 days, the wounds were filled with a fibrin clot and the wound epithelium had started to migrate across the wound space. After 7 days, the wound epithelium had completely covered the wound. In unwounded tissue the number of macrophages was relatively low compared to myofibroblasts (Table 3.2b). However, during wound healing the number of macrophages started to increase and peaked at day 7 (Figure 3.1A) when the formation of a granulation tissue had started. After 14 days, the inflammation was significantly reduced, the number of new blood vessels, pericytes and myofibroblasts peaked and a notable wound contraction was underway. After 60 days, the cellularity of the wound connective tissue was decreased to the level of unwounded tissue except of a small area in the deep wound connective
tissue that still showed higher cellularity (Figure 3.1A). In order to assess collagen deposition and organization we used picrosirius red staining (Figure 3.1B). Primitive, randomly organized collagen fiber bundles were detected in the wounds at day 7 after which their thickness and abundance increased. At day 14 and in the deep wound connective tissue at day 28, the collagen fiber bundles were aligned perpendicularly against the wound edges indicating wound contractions. At day 60, the collagen organization and structure in the subepithelial wound connective tissue was normalized but the deep wound connective tissue showed small areas where collagen was still organized to parallel bundles that appeared thinner than in unwounded tissue (Figure 3.1B). These areas also showed somewhat increased cell density as compared to other parts of the connective tissue (not shown).

Accumulation of SLRPs was spatio-temporally distinctly regulated during wound healing. Prodecorin (Figure 3.2) and probiglycan (Figure 3.3) abundance was increased as compared to unwounded tissue in cells invading to the wound already at day 3 and remained increased during the most active ECM deposition until day 28. Appearance of prodecorin and probiglycan producing cells was followed by increasing accumulation of corresponding mature molecules in the ECM at day 14 (Figures 3.2 and 3.3). In contrast to fibromodulin that showed increasing localization especially into the wound cells over time, lumican abundance decreased in the ECM during wound healing (Figure 3.4). In the same deep wound connective tissue locations at day 60 where collagen organization was not quite normalized (Figure 3.1Bi), relative abundance of decorin was elevated (Figure 3.2L) while that of lumican (Figure 3.4L) was reduced as compared to unwounded tissue. Staining intensity of fibromodulin was also strongly increased at this area but the staining associated mostly with connective tissue cells (Figure 3.4F).

The number of different cell types showing immunoreactivity for different SLRPs changed dynamically during wound healing (Table 3.1 and Figure 3.5). For example, the number of myofibroblasts that were positive for prodecorin increased strongly during healing and peaked at day 14. This coincided with active wound contraction by myofibroblasts. Some cells that did not associate with SLRPs in the unwounded tissue started to show positive immunoreactivity at certain stages of wound healing. For example, macrophages became positive for prodecorin and lumican (Table 3.1). The number of vascular endothelial cells and pericytes that were positive for prodecorin, probiglycan and fibromodulin increased strongly during wound healing (Table
In addition, some of the endothelial cells and pericytes started to show cell-associated staining of lumican at the later stages of wound healing (Table 3.1 and figure 3.5).

Immunostaining intensity of TGF-β1 and TGF-β3 increased strongly during wound healing (Figure 3.6). Relative abundance of TGF-β1 was significantly increased at 7, 14 and 28 days post-wounding (about 2.7-fold increased compared to unwounded tissue). Most abundant relative staining intensity of TGF-β3 occurred also at this time interval but it was more strongly increased relative to unwounded tissue (about 4-fold maximum increase) as compared to TGF-β1. Most abundant accumulation of active TGF-β1 occurred at the early wound healing at day 7, especially in macrophages (Table 3.1 and Figure 3.6). The number of myofibroblasts showing positive staining for active TGF-β1 peaked at day 14 (Table 3.1 and Figure 3.5). In addition, active TGF-β1 was abundantly present in endothelial cells and pericytes especially at the later stages of wound healing (Table 3.1).

Keratinocytes in the unwounded oral epithelium showed positive staining for probiglycan, fibromodulin and lumican (Table 3.2a and Figure 3.3 and 3.4). Immunostaining intensity of lumican increased in the migrating epithelium already 3 days after wounding, while staining intensity of fibromodulin was reduced in the migrating epithelium but was then gradually strongly increased when the migrating epithelial fronts had joined and were undergoing maturation (Table 3.2a and Figure 3.4). While we did not detect active TGF-β1 in the epithelium in unwounded tissue or at any stages of wound healing, TGF-β1 and TGF-β3 abundance was strongly increased in the wound epithelium especially from day 7 showing peak staining intensity at day 28 (Table 3.2a and Figure 3.6). At day 60, however, the staining intensity of TGF-β1 and TGF-β3 in the wound epithelium was very weak similar to unwounded tissue (Table 3.2a and Figure 3.6).

### 3.5 DISCUSSION

The cellular and molecular mechanisms underlying scar-free healing in oral mucosa are not well understood but may provide important information about the biological processes that regulate tissue regeneration. In the present study, we show for the first time the localization and accumulation of SLRPs, decorin, biglycan, fibromodulin and lumican, in relation to TGF-β1 and TGF-β3 in scar-free human oral mucosal wound healing over time. The major finding in the present study was that decorin, fibromodulin and TGF-β3 showed extended accumulation in the
wound connective tissue over time while abundance of lumican was significantly reduced as compared with unwounded tissue. Previous studies have linked increased abundance of fibromodulin to scar-free fetal wound healing and reduced abundance of decorin to hypertrophic scar formation in skin (Scott et al., 1998; Sayani et al., 2000). Furthermore, likely through their ability to bind and inactivate TGF-β, decorin and fibromodulin both inhibit TGF-β1-dependent fibrosis or scar formation in vivo (Grisanti et al., 2005; Stoff et al., 2007) suggesting that the prolonged accumulation of these SLRPs may reduce scar formation in gingiva. Increased abundance of anti-fibrotic TGF-β3 relative to TGF-β1 can also protect wound healing from scar formation (Ferguson and O’Kane, 2004). In the present study, active TGF-β1 was present in the wounds mostly during the early wound healing stage when accumulation of SLRPs was relatively low. After increasing accumulation of decorin and biglycan to the ECM from day 7 to day 28, abundance of active TGF-β1 decreased while that of total TGF-β1 and TGF-β3 increased. Thus, SLRPs may reduce activity of TGF-β while promoting its retention in the ECM during later stages of wound healing. To confirm this, functional studies addressing the role of SLRPs in regulating TGF-β activity during wound healing and scar formation are obviously needed. There is no previous data about the expression or localization of lumican in scar formation and it remains to be shown what is the function of lumican in this process.

All four SLRPs showed distinct spatio-temporal localization and staining intensity in the ECM during wound healing. Thus, their abundance appears differently regulated during wound healing and they may serve for different functions. In addition, each of the SLRPs showed distinct association with different wound connective tissue and/or epithelial cells. Association of immunoreactivity for SLRPs with distinct cells may have different explanations. It may mean that the cells are induced to produce these SLRPs differently during wound healing. Alternatively, SLRPs may localize to the pericellular matrix or to the cells surface allowing the cells to interact with the SLRPs. It is becoming more clear that certain cells can interact with SLRPs by using distinct cell surface receptors and this interaction regulates cell signaling, growth, apoptosis and gene expression (Hocking et al., 1998; Kresse et al., 2001). In addition, certain cells, including fibroblasts, are able to endocytose decorin and biglycan (Gotte et al., 2004), which may be an important mechanism to regulate the abundance of these molecules in the ECM during wound healing.
Previous studies have identified certain properties of SLRPs that regulate cell functions that are relevant for wound healing. In the present study, the number of myofibroblasts that showed positive immunostaining for prodecorin increased strongly at day 14 which coincided with active wound contraction by myofibroblasts. Thus, decorin may regulate wound contraction as suggested by finding showing that it promotes cell-mediated collagen contraction in vitro (Jarvelainen et al., 2004). Similar to a recent study of mouse corneal wound healing (Schonherr et al., 2004), prodecorin, probiglycan and fibromodulin were also localized abundantly to newly forming blood vessels in gingival wounds. In addition, some of the blood vessels and pericytes became positive for lumican. Therefore, it is possible that SLRPs regulate neovascularization during wound healing. Based on previous studies, role of decorin in neovascularization is somewhat unclear because decorin-deficiency appears to either accelerate or delay revascularization depending on the experimental model (Schonherr et al., 2004; Jarvelainen et al., 2006). Biglycan expression is also increased in endothelial cells during wound healing in vitro and may promote cell migration (Kinsella et al., 1997) while lumican appears to inhibit angiogenesis (Albig et al., 2007).

Interestingly, some cells that did not associate with SLRPs in the unwounded tissue started to show positive immunoreactivity during wound healing. For example, macrophages became positive for prodecorin and lumican. This suggests that macrophages either started to express these molecules as a response to wound healing, interacted with them or endocytosed them in the process of ECM turnover. An alternative explanation would be that phenotypically different macrophages that express SLRPs populate the wound at different stages of healing. This is supported by previous studies that have identified different macrophage phenotypes in human gingiva (Pernu et al., 2001).

Wound epithelium showed also distinct accumulation of SLRPs and TGF-β during wound healing. The abundance of fibromodulin was reduced in the migrating epithelium but was then gradually strongly increased when the migrating epithelial fronts had joined and were undergoing maturation. While we did not detect active TGF-β1 in the epithelium, TGF-β1 and TGF-β3 abundance was strongly increased in the wound epithelium in parallel with fibromodulin. The function of fibromodulin in epithelium is not clear and it is not known if it regulates TGF-β retention or activation in this location. In corneal wounds, lumican promotes re-
epithelialization (Yeh et al., 2005) and it may serve for similar function in gingival wounds as its abundance was strongly increased during re-epithelialization.

Although the gingival wounds appeared clinically completely regenerated with no clinical signs of scar formation at 60 days, the deep wound connective tissue showed small areas where collagen organization and cellularity was not quite normalized. Wound remodeling can proceed for several months or even years after the initial injury (Compton et al., 1989) and, therefore, wounds collected at later time points than 60 days after wounding would need to be studied to find out whether the tissue structure and composition will regenerate completely over time.

Taken together, the findings in the present study showed that accumulation of SLRPs and TGF-β is distinctively spatio-temporally regulated during gingival wound healing and partially resembles scar-free healing in fetal wounds.

3.6 Acknowledgments
We thank Dr. Tara Habijanac, Mr. Cristian Sperantia and Mr. Andre Wong for expert technical assistance. The study was supported by the Canadian Institutes of Health Research.
Table 3.1: Relative number of connective tissue cells showing immunoreactivity for SLRPs or active TGF-β1 during wound healing.

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0-60: days post-wounding; MFB: α-SMA expressing myofibroblasts; PC: α-SMA expressing pericytes associated with blood vessels; EC: CD31 expressing endothelial cells.; MQ: CD68 expressing macrophages. -: No colocalization; +: Same number of cells / unit area showing colocalization as in unwounded tissue; ++++: The highest number of cells / unit area showing colocalization relative to unwounded tissue; /+++: In 60 d wounds, a small area in the deep wound connective tissue showed higher number of cells (++) that colocalized with the target molecules as compared to unwounded tissue while most part of the tissue showed similar relative number.
Table 3.2: a) Relative staining intensity of SLRPs and TGF-βs in wound epithelium.

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<th>Days Post-Wounding</th>
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<td>Active TGF-β1</td>
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<td>TGF-β3</td>
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</table>

-: No immunoreactivity; +: The lowest relative staining intensity; ++++: The highest relative staining intensity.

b) Average number of different cell types per unit area in normal, unwounded gingival connective tissue.

Cells were counted from 5 to 10 randomly selected fields from parallel sections from three subjects. Results show mean +/- SD.
Figure 3.1: Abundance of connective tissue cells and collagen organization during wound healing.

A: Relative number of different connective tissue cells in the wound as compared to the unwounded tissue. Myofibroblasts were identified as cells with typical fibroblast morphology and showing positive immunoreaction for α-SMA. Pericytes were identified as cells that were associated with blood vessels and showed immunoreactivity for α-SMA. Endothelial cells and blood vessels were identified based on positive immunoreaction for CD34 and macrophages for CD68. Results show mean +/- s.d. from five parallel sections from three to four subjects (*: p< 0.05; **: p< 0.01). Cells were counted from 5 to 10 randomly selected fields from parallel sections of each subject. B: Collagen organization at different time points after wounding. A-E: Representative images from picrosirius red stained sections from unwounded tissue (day 0) and at 7, 14, 28 and 60 days after wounding. a-i: higher magnification images from A-E, respectively. Magnification bar = 50 μm.
Figure 3.2: Localization and relative abundance of prodecorin and decorin during wound healing.

A-F: Cell-associated staining of prodecorin in normal unwounded tissue (0) or in 3-, 7-, 14-, 28- and 60-day wounds. G-L: Localization of mature decorin to ECM in normal and wounded tissue. CT: connective tissue; E: epithelium; SEGT: subepithelial granulation tissue; DGT: deep granulation tissue; SEWCT: subepithelial wound connective tissue; DWCT: deep wound connective tissue. Magnification bar = 100 μm. M and N: Relative staining intensity of prodecorin (M) and decorin (N) in subepithelial (SE) or deep (Deep) unwounded connective tissue or wound granulation/connective tissue. Results show mean +/- s.d. from five parallel sections from three to four subjects (*: p< 0.05; **: p< 0.01).
Figure 3.3: Localization and relative abundance of probiglycan and biglycan during wound healing.

A-F: Cell-associated localization of probiglycan in normal unwounded tissue (0) and in 3-, 7-, 14-, 28- and 60-day wounds. Arrows indicate epithelium. G-L: Localization of mature biglycan to ECM in unwounded tissue and in wounds. Arrowheads (I) point abundant biglycan staining at the basement membrane zone. CT: connective tissue; E: epithelium; SEGT: subepithelial granulation tissue; DGT: deep granulation tissue; SEWCT: subepithelial wound connective tissue; DWCT: deep wound connective tissue. Magnification bar =100 μm. M and N: Relative staining intensity of probiglycan (M) and biglycan (N) in subepithelial (SE) or deep (Deep) unwounded connective tissue or wound granulation/connective tissue. Results show mean +/- s.d. from five parallel sections from three to four subjects (*: p< 0.05; **: p< 0.01).
Figure 3.4: Localization and relative abundance of fibromodulin and lumican during wound healing.

A-F: Localization of fibromodulin in normal unwounded tissue (0) and in 3-, 7-, 14-, 28- and 60-day old wounds. Arrows indicate epithelium. G-L: Localization of lumican in unwounded tissue and wounds. Arrows indicate epithelium. Arrow (H) points distinct lumican immunoreactivity at the tip of migrating wound epithelium. CT: connective tissue; E: epithelium; SEGT: subepithelial granulation tissue; DGT: deep granulation tissue; SEWCT: subepithelial wound connective tissue; DWCT: deep wound connective tissue. Magnification bar = 100 µm. M and N: Relative staining intensity of fibromodulin (M) and lumican (N) in subepithelial (SE) or deep (Deep) unwounded connective tissue or wound granulation/connective tissue. Results show mean +/- s.d. from five parallel sections from three to four subjects (*: p< 0.05; **: p< 0.01).
Figure 3.5: SLRPs and active TGF-β1 localize to distinct granulation tissue cells in the 14-day-old wounds.

Representative images from mid-granulation tissue from 14-day old wounds. A: Prodecorin colocalized with some α-SMA expressing myofibroblasts (arrowheads) while some myofibroblasts did not associate with it (circles). Blood vessel-associated α-SMA expressing cells (pericytes) showed immunoreactivity for prodecorin (arrows). F: Prodecorin colocalized with endothelial cells in the blood vessels (arrows). K: Some macrophages (arrowheads) showed positive staining for prodecorin. B: Probiglycan colocalized with α-SMA expressing myofibroblasts (arrowheads). Pericytes showed also immunoreactivity for probiglycan (arrows). G: All blood vessels and endothelial cells (CD31 positive cells) strongly stained for probiglycan (arrows). L: Probiglycan colocalized with macrophages (CD68 positive cells; arrowheads). C: Fibromodulin colocalized with myofibroblasts (arrowheads) and pericytes. H: Fibromodulin associated with all blood vessels (arrows). M: Macrophages did not show immunoreactivity to fibromodulin (arrowheads). D: Myofibroblasts (arrowheads) and pericytes colocalized with lumican. I: Lumican associated with blood vessels (arrows). N: Macrophages did not show immunoreactivity to lumican (arrowheads). E: Active TGF-β1 colocalized with myofibroblasts (arrowheads). Some α-SMA expressing cells associated with blood vessels (pericytes) showed immunoreactivity to active TGF-β1 (arrows) but some did not (circle). J: Active TGF-β1 colocalized with some endothelial cells (arrow) while some endothelial cells were negative (arrowheads). O: Macrophages showed immunoreactivity for active TGF-β1 (arrowheads). Magnification bar = 50 μm.
Figure 3.6: Localization and relative abundance of TGF-β1, active TGF-β1 and TGF-β3 during wound healing.

Representative images of tissue sections immunostained with polyclonal antibodies against TGF-β1, active TGF-β1 and TGF-β3. A-F: Localization of TGF-β1 in normal unwounded tissue (0) and in 3-, 7-, 14-, 28- and 60 days after wounding. B: TGF-β1 is absent in migrating keratinocytes facing the blood clot (arrows). C-E: Arrows indicate wound epithelium showing strong immunoreactivity for TGF-β1. F: Arrows indicate wound epithelium with weak immunostaining for TGF-β1. G-L: Localization of active TGF-β1 in unwounded and wound tissue. Active TGF-β1 is absent in normal and wound epithelium (arrows). M-R: TGF-β3 immunoreactivity in unwounded and wound tissue. N: TGF-β3 shows distinct immunoreactivity at migrating keratinocytes facing the blood clot (arrows). O-Q: Arrows indicate strong TGF-β3 expression in wound epithelium. R: Arrows indicate weak immunoreactivity of TGF-β3 at the wound epithelium. CT: connective tissue; E: epithelium; SEGT: subepithelial granulation tissue; DGT: deep granulation tissue; SEWCT: subepithelial wound connective tissue; DWCT: deep wound connective tissue. Magnification bar = 100 μm. S, T, and U: Relative staining intensity of TGF-β1 (S), active TGF-β1 (T) and TGF-β3 (U) in subepithelial (SE) or deep (Deep) unwounded connective tissue or wound granulation/connective tissue. Results show mean +/- s.d. from five parallel sections from three to four subjects (*: p < 0.05; **: p < 0.01).
3.7 REFERENCES


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CHAPTER 4: Expression of Endo180 is spatially and temporally regulated during wound healing

A version of this chapter has been published:

4.1 ABSTRACT
Interactions of cells with the extracellular matrix are important for normal wound healing and may be altered in aberrant wound healing leading to scar formation. Remarkably, wound healing in the adult human gingiva does not usually result in scar formation and serves as a model for wound regeneration. Endo180 (CD280) is a cell surface receptor that has novel functions to bind and internalize collagens and regulate cell migration that are key processes in wound healing. The aim of this study was to examine the expression of Endo180 during human gingival wound regeneration by immunohistochemical methods. In the unwounded normal gingiva, Endo180 was expressed in the basal epithelial cells, fibroblasts, myofibroblasts, pericytes, macrophages and endothelial cells. In the early wounds, Endo180 was expressed in the migrating keratinocytes facing the blood clot or granulation tissue. At the later phases of wound healing, expression of Endo180 was spatio-temporally regulated in the differentiating wound epithelium, adjacent normal epithelium and in subsets of wound myofibroblasts, pericytes, macrophages and endothelial cells until the expression was returned to the pre-wounded level 60 days after wounding. Growth factors involved in the epithelial and connective tissue wound healing upregulated the expression of Endo180 in cultured human keratinocytes and fibroblasts. The findings suggest that Endo180 plays a role in wound re-epithelialization and connective tissue remodeling. Endo180 colocalized with collagen-binding small leucine-rich proteoglycan decorin in wound fibroblasts in vivo. Thus, decorin may interact with Endo180 or modulate Endo180-collagen interaction during wound healing.

4.2 INTRODUCTION
Wound healing of skin or mucosa involves tightly regulated processes that restore the epithelial barrier and the connective tissue structure and function. These processes are regulated by cell-extracellular matrix (ECM) interactions that control cell migration and proliferation, ECM deposition and degradation and gene expression. These events are orchestrated by the release and activation of growth factors and cytokines at the wound site. Wound healing in human skin does not usually occur by regeneration but results in formation of collagen-rich scars, hyperthrophic scars or keloids (Trojanowska et al., 1998; Ferguson and O’Kane, 2004). Interestingly, wound healing in the adult human gingiva does not usually result in any type of scar formation. The reason for this is not known but may depend on the unique phenotype of gingival cells, special
composition of the gingival ECM or factors present in saliva (Schor et al., 1996; Häkkinen et al.,
2000; Colwell et al., 2003; Szpaderska et al., 2003; Ferguson and O’Kane, 2004). Thus, human
gingiva provides a model to study the processes that may be important in wound regeneration.

During early wound healing, signals that regulate cell migration and proliferation are

During early wound healing, signals that regulate cell migration and proliferation are
crucial for wound reepithelialization and granulation tissue formation. At the later stages, the
mechanisms that control ECM deposition and degradation (remodeling) regulate connective
tissue regeneration. Migration of keratinocytes and connective tissue cells is regulated by
interactions of cells with the ECM. Initially, the cells use the proteins present in the clot and
connective tissue for cell adhesion. These ECM molecules are also subjected to a well-controlled
localized proteolysis involving the plasminogen activation cascade and secretion and/or
activation of matrix metalloproteinases (MMPs). This focalized proteolysis is crucial for cell
migration (Romer et al., 1996; Steffensen et al., 2001). At the later stages of healing, ECM
remodeling is mediated in part by extracellular proteolysis and by intracellular degradation. The
former pathway involves tightly regulated secretion and activation of proteolytic enzymes,
including the MMPs, by the tissue cells. The intracellular degradation pathway requires
internalization of ECM molecules by various pathways of endocytosis and/or phagocytosis
followed by proteolysis by lysosomal enzymes (Everts et al., 1996; East and Isacke, 2002; Seto
et al., 2002; Everts et al., 2003).

Recently, a novel cell surface receptor Endo180 (also called urokinase plasminogen
activator receptor associated protein, uPARAP) or CD280 was described (Isacke et al., 1990;
East and Isacke, 2002; Behrendt, 2004). Endo180 is a type I transmembrane protein and belongs
to the macrophage mannose receptor family. It is composed of an N-terminal cysteine-rich
domain followed by a fibronectin type II domain, eight C-type lectin-like domains, a single pass
transmembrane domain and a short cytoplasmic domain (East and Isacke, 2002; Behrendt, 2004).
Experiments using Endo180-deficient cells or cells expressing mutated Endo180 protein domains
have revealed three main functional roles for Endo180. First Endo180 is a novel collagen
internalization receptor mediating uptake of collagens for lysosomal degradation (Wienke et al.,
2003; Kjoller et al., 2004; Curino et al., 2005). Second, expression of Endo180 regulates cell
migration (Engelholm et al., 2003; Sturge et al., 2003). Finally, Endo180 forms a trimolecular
complex with urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) (Behrendt
et al., 2000). Thus, Endo180 may be involved in cell functions crucial for the early (cell
migration) and late (remodeling) wound healing stages. However, nothing is known about the expression of Endo180 during wound healing. We hypothesize that Endo180 expression may exhibit spatiotemporal changes in distinct gingival wound cells during wound healing. Thus, we analyzed the expression of Endo180 during human gingival wound regeneration and in cultured fibroblasts and keratinocytes. The findings showed that Endo180 expression was spatiotemporally regulated in distinct epithelial and connective tissue cells during wound healing. In cultured human gingival fibroblasts and keratinocytes, the expression of Endo180 was increased by growth factors involved in epithelial and connective tissue wound healing. These findings suggest that Endo180 is involved in early cell migration and connective tissue remodeling phases of wound healing. In vivo, collagen is decorated with small leucine-rich proteoglycan decorin therefore, decorin may regulate interaction of Endo180 with collagen. Our unpublished in vitro findings also suggest that decorin interacts with Endo180. Therefore, we also hypothesize that Endo180 and decorin colocalize in fibroblasts during wound healing. Our findings showed colocalization of decorin with Endo180 suggesting that this collagen-binding small leucine-rich proteoglycan interacts with Endo180 and/or modulates interaction of Endo180 and collagen during wound healing.

4.3 MATERIALS AND METHODS

4.3.1 Tissue samples

In order to create experimental wounds, two full thickness wounds (~10 mm long, 2 mm wide and 2 mm deep) were made in the palatal gingiva at least 3 mm away from the gingival margins of the teeth and distal to the canine in four healthy volunteers. The wounds were made in the palatal mucosa with at least 10 mm of space between each wound. The tissue obtained from the initial wound (normal unwounded tissue) was rinsed in physiologic saline and embedded in Tissue-Tek Optimal Cutting Temperature compound (O.C.T.; Sakura Finetek, Inc., Torrance, CA, USA) and immediately frozen in liquid nitrogen. Wounds were then allowed to heal for a period of 1, 3, 7, 14, 28 or 60 days. A punch biopsy (4 mm diameter) was taken from each wound at the indicated time-points and immediately frozen in liquid nitrogen as above. The samples were then stored at -86°C until sectioning. Tissue sections (6 μm) were cut using a 2800 Frigocut Cryostat Microtome (Leica, Nussloch, Germany). After cutting, sections were transferred to 3-aminopropyltriethoxysilane-coated slides, air-dried and kept in -86°C until use. The
experimental protocol for this study was approved by the Clinical Research Ethics Board of the University of British Columbia and included obtaining an informed consent from all of the subjects.

4.3.2 Immunohistochemical staining

To localize Endo180 expressing cells, anti-human Endo180 monoclonal antibodies A5/158 (1:2 dilution of hybridoma supernatant; Sheikh et al., 2000) and E1/183 (1:5 dilution of hybridoma supernatant; Isacke et al., 1990) and a cross-species-reactive anti-Endo180 polyclonal antibody (1:100 dilution; Isacke et al., 1990) were used. To identify myofibroblasts and pericytes an antibody that recognizes α-smooth muscle actin (α-SMA) was used (1:100 dilution; Sigma, St. Louis, MO, USA). Macrophages were identified using an anti-CD68 antibody (1:100 dilution; DakoCytomation Inc., Mississauga, ON, Canada) and endothelial cells using an anti-CD31 antibody (1:200; BD Biosciences, Mississauga, ON, Canada). Additionally, a monoclonal antibody (Ab-1) that recognizes CD90/Thy-1 (1:500 dilution; Oncogene Research Products, San Diego, CA, USA) was used to localize all fibroblasts (Saalbach et al., 1998). To localize decorin polyclonal anti-prodecorin (LF 110; recognizes mostly cell-associated decorin; Fisher et al., 1995) was used. For immunofluorescence staining, the tissue samples were shortly thawed at room temperature before fixation using -20°C acetone for 5 min. Samples were then rehydrated with phosphate-buffered saline (PBS) for 5 min and blocked with PBS containing bovine serum albumin (BSA; 10 mg/ml) and Triton X-100 (0.01%) at room temperature for 30 min followed by overnight incubation with the primary antibody (anti-Endo180 antibody) in PBS containing BSA (1mg/ml) and Triton X-100 (0.01%) at 4°C. For double immunostainings, the sections were then washed and incubated with the second primary antibody at room temperature for 1 h as above. After washing with PBS, the samples were incubated with appropriate Alexa anti-rabbit 596 and anti-mouse 488 secondary antibodies (1:100 dilution; Molecular Probes, Inc., Eugene, OR, USA) at room temperature for 1 h. Negative control stainings were performed by omitting the primary antibody incubation step. The slides were mounted using Immuno-mount solution (Thermo Shadon, Pittsburgh, PA, USA) and examined by a Zeiss Laser Confocal Scanning Microscope 10 (Carl Zeiss, Jena, Germany). Finally, images were captured using Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada). In order to block Fc-receptors expressed by macrophages (Mellman et al., 1988), a set of samples were blocked with different concentrations (2-50 μg/ml) of purified human IgG (Sigma) before incubation with the primary
antibody. The same staining results were obtained as in sections blocked with BSA indicating that there was not non-specific binding of true antibodies to Fc-receptors (data not shown).

For semiquantitative analysis, five representative sections from two to four individuals at each time point were stained with the immunofluorescence method and scored by two independent examiners. The relative number of different connective tissue cells in the wound granulation tissue as compared to the unwounded tissue was scored as follows: -: no cells present; +: the lowest relative number of cells; ++++: the highest relative number of cells. The relative proportion (%) of cells expressing Endol80 within each cell population during wound healing was assessed using the following scale: -: no cells expressing Endol80; 100 %: all cells expressing Endol80. The relative immunostaining intensity of Endol80 in epithelial and connective tissue cells was compared within each cell population using the following scale: N/A: no cells present; +: the lowest relative staining intensity; +++++: the highest relative staining intensity.

4.3.3 Reverse transcription - polymerase chain reaction (RT-PCR)

The human gingival fibroblast culture (HGF-DC27) was established from a healthy male subject as described previously (Hakkinen and Larjava, 1992). The human HaCaT keratinocyte line was a generous gift from Dr. Norbert Fusenig (German Cancer Center, Heidelberg, Germany; Boukamp et al., 1988). To analyze Endol80 mRNA expression after growth factor treatment, HGF-DC27 (1x10^4/cm^2) and HaCaT cells (1x10^4/cm^2) were seeded on 35 mm cell culture dishes (Corning Incorporated, Corning, NY, USA) in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco BRL Life Technologies, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco Life Technologies) and cultured for 48 h. HaCaT cells were then incubated with DMEM supplemented with 1% FBS, 10% FBS or 1% FBS containing 10 ng/ml final concentration of transforming growth factor-β1 (TGF-β1; Chemicon, Temecula, CA, USA), epidermal growth factor (EGF; Invitrogen, Carlsbad, CA, USA), heparin binding EGF-like growth factor (HB-EGF; Oncogene Research Products) or keratinocyte growth factor (KGF; Upstate Cell Signaling, Lake Placid, NY, USA) for 96 h with medium change after 48 h. HGF-DC27 were treated with DMEM supplemented with 1% FBS, 10% FBS or with 1% FBS and 10 ng/ml TGF-β1, EGF or platelet derived growth factor (PDGF; Sigma) as above. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Inc., Mississauga, ON, Canada). RNA concentrations were determined using
an RNA/DNA spectrophotometer (GeneQuant, LKB Biochrom, Ltd., Cambridge, UK). Total RNA (1 μg) was reverse-transcribed using Oligo (dT) primer with the SuperScript First-Strand Synthesis System (Invitrogen Life Technologies). Reverse-transcribed DNA (4 μl) was used for each PCR reaction in a reaction mixture (50 μl) containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 10 mM dNTPs, 10 μM each of oligonucleotide primers and 1μl of 2 units/μl DNA polymerase. Different reverse-transcribed DNA dilutions (1:4, 1:8, 1:16 and 1:32) were subjected to PCR to ensure that the reactions were occurring in the linear range of amplification. PCR was performed as follows: initial denaturation step at 94 °C for 90 s followed by 35 cycles of denaturation at 94°C, annealing at 62°C, and extension at 72°C, each for 40 s. A final extension step was carried out at 72°C for 5 min. RT-PCR of β-actin was performed as a control reaction for normalization of total RNA. The primer sequences for Endo180 were the following: 5'-GTCTTCCTCATCTTCAGCCA-3' and 5'-GTTTCCCTGGATGGTGTAGA-3' corresponding to 170-190 bp and 566-586 bp of Endo180 cDNA, respectively. The primers for β-actin were 5'-GAGACCTTCAACACCCAGCC-3 and 5'-GGCCATCTCTTGCTCGAAGTC-3 (Redlich et al., 2001). RT-PCR products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide (0.5 μg/ml) for visualization under a UV light source, photographed using a Nikon E995 digital camera (Nikon Corporation, Tokyo, Japan) and quantified by using NIH Image software (http://rsb.info.nih.gov/nih-image). The experiments were performed three times with similar results.

4.3.4 Western blotting

HaCaT cells (1x10⁴/cm²) and HGF-DC27 (1x10⁴/cm²) were seeded in 35 mm cell culture dishes in DMEM containing 10% FBS and cultured for 48 h before treatment with growth factors or 1% or 10% FBS as above. Cells were then washed with ice-cold PBS, lysed in tris-buffered saline (TBS, pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 mM ethylene glycol-β-aminoethyl-NNN’N’-tetraacetic acid and 100 mM octylglucoside on ice for 10 min, collected using a rubber policeman, vortexed and centrifuged at 12,000 RPM at 4°C for 5 min. Supernatants were collected, and the total protein concentration was determined using a protein-assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of proteins from cell lysates were separated in SDS-polyacrylamide gels (4%/7.5%) in non-reducing conditions.
After electrophoresis, the proteins were transferred onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Quebec, PQ, Canada) at 4°C overnight. The nonspecific protein-binding sites were blocked by incubating the membranes with TBS containing 0.1% Tween-20 (TBS-T) and 5% non-fat dry milk at room temperature for 1 h. The membranes were then incubated with the anti-Endo180 (E1/183) monoclonal antibody (1:10 dilution of the hybridoma supernatant) in 3% non-fat dry milk in TBS-T at 4°C overnight. After washing, the membranes were incubated with an anti-mouse peroxidase-conjugated secondary antibody (1:2000 dilution; Amersham Biosciences) in TBS-T containing non-fat dry milk at room temperature for 1 h. After washing, the blots were reacted with enhanced chemiluminescence detection reagent (Amersham Biosciences) and exposed to Kodak Biomax X-ray film (Eastman Kodak Company, Rochester, NY, USA). The band intensities were quantified using NIH image software. In order to normalize the band intensities, the same membranes were immunoblotted with a polyclonal antibody against β-actin (1:5000 dilution; Abcam, Cambridge, MA, USA) followed by incubation with a peroxidase-conjugated anti-rabbit secondary antibody (1:2000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The experiments were performed two times for HGF-DC27 fibroblasts and three times for HaCaT cells with similar results.

4.3.5 Statistical Analysis
The differences between various treatment groups and control were analyzed using one way ANOVA followed by Dunnett’s Multiple Comparison Test for comparisons of multiple groups. A value of $P < 0.05$ was considered statistically significant.

4.4 RESULTS
4.4.1 Wound healing
Clinical observations of the 1- to 60-day wounds showed a continuous progression of healing (changes in the color, outline and size of the wound, and degree of epithelial covering), and all wounds healed without complications. The outline of the wound margin could still be delineated for certain individuals after 28 days of healing but after 60 days the original wound area was indistinguishable from the normal mucosa and no clinical scar formation occurred (not shown). All wound samples were processed for histological analysis with hematoxylin and eosin staining. Fluorescence from eosin-stained collagen fibrils, when viewed under a fluorescence microscope
using the rhodamine filter, was used to analyze collagen organization during healing. In normal non-wounded tissue, epithelium formed the typical long rete ridges that extended relatively deep into the connective tissue (Fig. 4.1A). In the normal connective tissue, thick collagen fibril bundles were arranged to the typical basket-wave organization (Fig. 4.1B). The 1-day wound margins were clearly defined with a fibrin clot and a multitude of inflammatory cells established within the wound. The epithelium had started to proliferate and migrate down the wound margins (Fig. 4.1C). 3 days after wounding, the wound space was still filled with a fibrin clot containing numerous inflammatory cells. The epithelium had migrated for some distance through the blood clot but did not completely cover the wound area (Fig. 4.1E). Some endothelial cells and the first macrophages had started to migrate into the blood clot from the connective tissue at the wound edge. No fibroblasts were noted within the wound at this point (Table 4.1). By day 7, the epithelium had migrated to completely cover the wound bed and an increase in epithelial thickness was noted (Fig. 4.1G). Granulation tissue with unorganized extracellular matrix was formed beneath the wound epithelium (Fig. 4.1G and H). The granulation tissue contained many endothelial cells and macrophages and the first fibroblasts also appeared in the wound. Some of the fibroblasts expressed α-SMA suggesting that myofibroblast differentiation had started. Also α-SMA positive perivascular cells (pericytes) were noted in the granulation tissue (Table 4.1). 14 days after wounding, the wound epithelium had become still thicker and more organized as compared with the 7-day wounds (Fig. 4.1I). The granulation tissue had developed to form a primitive wound connective tissue where wound fibroblasts had started to align perpendicularly against the wound margins along with some similarly oriented collagen fibers indicating wound contraction (Fig. 4.1I and J). The wound connective tissue contained abundantly α-SMA positive myofibroblasts, perivascular cells, endothelial cells and macrophages (Table 4.1). From 28 (Fig. 4.1K) to 60 (Fig. 4.1M) days, the thickness and organization of the wound epithelium became gradually more normal, and by day 60 the epithelial rete ridges were reformed at the wound site (Fig. 4.1M). After 28 days, the number of myofibroblasts in the wound connective tissue was slightly reduced as compared to day 14 (Table 4.1) and myofibroblasts were all organized perpendicularly against the wound edges. Also the vascularization was slightly reduced and the number of macrophages was normalized to resemble the normal tissue (Table 4.1). As compared to the 14-day wounds, the size of the wound connective tissue was reduced and collagen was clearly oriented perpendicularly against the wound edges at 28 days (Fig. 4.1L). In the 60-day
wounds, the number of connective tissue cells was normalized to resemble normal tissue (Table 4.1) but many fibroblasts were still oriented perpendicularly against the wound edges, unlike the more random orientation in the unwounded areas (Fig. 4.1M). While most of the wound collagen showed a random basket-wave organization typical to the normal unwounded tissue, some collagen fibers showed still perpendicular arrangement against the wound margins (Fig. 4.1N).

### 4.4.2 Expression of Endo180 in the wound epithelium

In the unwounded normal tissue, Endo180 as detected with the monoclonal antibody A5/158 (Sheikh et al., 2000) showed moderate relative immunostaining intensity in the basal epithelial cells. In the epithelium facing the connective tissue papilla, Endo180 localized around the cell membrane of the basal cells, while in the rete pegs it showed most abundant staining against the basement membrane (Fig. 4.2A-C). Similar staining pattern was observed with the second monoclonal anti-Endo180 antibody (E1/183, Isacke et al., 1990) and with the polyclonal (Isacke et al., 1990) anti-Endo180 antibody (data not shown). The relative immunostaining intensity of Endo180 was strongly upregulated 1 day after wounding around the cell membrane of migrating epithelial cells facing the blood clot (Table 4.2 and Fig. 4.2D and E). In the unwounded area close to the wound margins, Endo180 showed variable staining and occasionally increased expression was noted around the cell membranes in the keratinocytes at the tips of the rete ridges (Fig. 4.2F). From day 3 to day 7, the staining intensity of Endo180 in the wound keratinocytes remained upregulated (Table 4.2) and Endo180 was now localized most abundantly in the basal cell membrane facing the granulation tissue (Fig. 4.2G, H, J and K). Staining intensity in the unwounded area close to wound increased especially in the basal cell membrane facing the basement membrane (Fig. 4.2I). After 7 days, strong immunoreactivity was noted also in two to three immediately suprabasal cell layers in many locations of the unwounded area close to the wound (Fig. 4.2L). During the maturation of the wound epithelium from day 14 to day 28, the relative expression of Endo180 was gradually increased around the basal and immediately suprabasal wound keratinocytes (Table 4.2 and Fig. 4.2M, N, P and Q). Also the basal and two to three suprabasal cell layers in the unwounded area close to wound showed strong immunoreactivity (Fig 4.2. O and R). At 28 days, the most abundant staining in the basal cells of unwounded area was localized in the cell membrane facing the basement membrane zone while in the immediately suprabasal layers the immunoreactivity was strong around the cells (Table 4.2)
and Fig. 4.2R). Still after 60 days when the new rete pegs were formed in the wound epithelium, the relative immunostaining intensity of Endo180 remained elevated around the basal keratinocytes as compared to the normal tissue (Table 4.2 and Fig. 4.2S and T). At this time point, the expression of Endo180 was normalized in the epithelium close to the wound (Table 4.2 and Fig. 4.2U).

4.4.3 Expression of Endo180 in the connective and granulation tissue cells

In order to find out cell-type specific expression of Endo180 in the connective tissue cells we used double immunostaining with the anti-Endo180 antibody and cell-type specific markers. Expression of α-SMA was used as a marker for myofibroblasts, while macrophages and endothelial cells were identified by the immunoreactivity for CD68 and CD31, respectively. Although both myofibroblasts and perivascular cells (pericytes) expressed α-SMA, we were able to distinguish these two cell types by association of pericytes to blood vessels. The antibody against Thy-1 (CD90) was used to identify all fibroblasts.

In the normal unwounded human gingiva, Endo180 colocalized with the perivascular cells (pericytes; Fig. 4.3A-C), all α-SMA positive fibroblasts (myofibroblasts; Fig. 4.3D-F), Thy-1 positive cells (representing all fibroblasts) (Fig. 4.3G-I) and with the endothelial cells (Fig. 4.3J-L) and macrophages (Fig. 4.3M-O). Endo180 was also expressed by the Thy-1 positive cells that showed the typical fibroblasts morphology that were negative for α-SMA immunoreactivity, representing fibroblasts (not shown). During wound healing, expression of Endo180 was spatio-temporally regulated within each cell population (Tables 4.3 and 4.4). After 7 days, only a subpopulation of myofibroblasts expressed Endo180 (Table 4.3) and the staining in these cells was less intense as compared with the unwounded tissue (Table 4.4). However, from the day 14 to day 60 the proportion of myofibroblasts that expressed Endo180 was gradually returned to the pre-wounded level (Table 4.3). After 14 and 28 days, the number of myofibroblasts in the wound peaked (Table 4.1) and the relative staining intensity of Endo180 was strongly increased in these cells as compared with the unwounded tissue (Table 4.4 and Figs 4.4 and 4.5). The first α-SMA expressing pericytes were observed in the granulation tissue after 3 days but these cells did not express Endo180 (Table 4.3). From day 7 to day 60 the number of pericytes gradually increased (Table 4.1). At the same time increasing proportion of pericytes started to express Endo180 (Table 4.3 and Figs 4.4 and 4.5). The relative staining intensity of
Endol80 in pericytes was initially low and gradually increased from day 7 until it was returned to the pre-wounded level at day 60 (Table 4.4).

The first endothelial cells started to emerge in the granulation tissue at day 3 and the density of blood vessels exceeded that of unwounded tissue up to day 60 when their number was returned to the pre-wounded level (Table 4.1). The number of blood vessels peaked at 7 and 14 days (Table 4.1). Only about half of the wound blood vessels and endothelial cells expressed Endol80 at days 3-14 after which the proportion of Endol80 positive blood vessels started to increase. At day 60, all endothelial cells in the blood vessels showed immunoreactivity for Endol80 (Table 4.3). The relative staining intensity of Endol80 in endothelial cells in the granulation tissue was initially lower than in unwounded tissue until the expression peaked at 14 days (Table 4.4).

The number of macrophages was increased in the granulation tissue at day 3, 7 and 14 as compared with unwounded tissue (Table 4.1). While all macrophages in the unwounded tissue expressed Endol80, only about 25% of these cells in the granulation tissue showed positive staining (Table 4.3). Only after 60 days all macrophages at the wound connective tissue expressed Endol80 (Table 4.3). Until day 60, the relative staining intensity of Endol80 was reduced in the wound macrophages as compared with the unwounded tissue (Table 4.4).

4.4.4 Regulation of Endol80 expression in keratinocytes and fibroblasts by growth factors

As the findings showed that expression of Endol80 was spatially and temporally regulated during wound healing we wanted to find out whether growth factors involved in wound healing can regulate the expression of Endol80 in human keratinocytes and fibroblasts. To this end, we treated human keratinocytes and fibroblasts with growth factors and analyzed the expression of Endol80 protein and mRNA by immunoblotting and semi-quantitative RT-PCR, respectively. Immunoblotting results from fibroblasts showed that TGF-β1, EGF and PDGF increased the expression of Endol80 by about 2-, 2.5- and 2-fold, respectively (Fig. 4.6A). Compared to 1% FBS, fibroblasts treated with 10% FBS also showed about a 1.5-fold increase in the expression of Endol80 (Fig. 4.6A). Similarly, the Endol80 mRNA expression was increased by TGF-β1, EGF, and PDGF by about 3-, 3- and 2.5-fold, respectively. Fibroblasts treated with 10% FBS showed also a 2-fold increase in the Endol80 mRNA (Fig. 4.6B). The strongest relative
immunostaining intensity of Endo180 was observed in myofibroblasts that expressed α-SMA at 14-day wounds (Table 4.4). To determine whether the upregulation of Endo180 expression may be because of changes in the fibroblast phenotype to myofibroblasts we treated fibroblasts with the growth factors and assessed the phenotype of fibroblasts by α-SMA immunostaining. Only TGF-β1 induced α-SMA expression but other growth factors did not (data not shown).

The immunoblotting results showed that in HaCaT keratinocytes expression of Endo180 was up-regulated by 10% FBS, TGF-β1, EGF, HB-EGF and KGF treatment by 3-, 2.5-, 3-, 4- and 4.5-fold, respectively (Fig. 4.6C). Consistent with the immunoblotting data, RT-PCR results showed that the Endo180 mRNA was upregulated by TGF-β1 and EGF by about 3-fold, while HB-EGF and KGF upregulated it by about 3.5-fold (Fig. 4.6D). Treatment with 10% FBS also induced about a 3.5-fold increase in the Endo180 mRNA expression (Fig. 4.6D).

4.4.5 Decorin and Endo180 colocalized in distinct cells during wound healing

Our preliminary observations indicate that decorin binds to Endo180 in vitro (data not shown). During wound healing, interaction of decorin with Endo180 may modulate Endo180 / collagen binding and regulate cellular uptake of collagen and tissue remodeling. In addition, by binding to Endo180 at the cell surface, decorin may partly alter cell signaling leading to changes in Endo180-mediated cell functions, such as migration and / or differentiation. Interestingly, from 3-60 days post-wounding, decorin and Endo180 colocalized in cells in granulation tissue that showed typical fibroblasts morphology (Fig. 4.7A-O). In the 3-day wound, colocalization of decorin and Endo180 was observed in some of the fibroblastic cells that appeared to be migrating from connective tissue toward granulation tissue (Fig. 4.7A-C). The number of cells that showed positive immunoreactivity for both decorin and Endo180 at the same time was gradually increased from day 7 to 28 in GT until after 60 days when the number of cells stained for both molecules decreased (Fig. 4.7D-O).

4.5 DISCUSSION

Remarkably, when compared to skin, wound healing in adult human gingiva shows faster re-epithelialization and heals by regeneration with no or very little scar formation. The reason for this is not known but may depend on the inherently unique phenotype of the gingival cells, composition of the gingival ECM or factors present in saliva that promote healing (Schor et al.,
1996; Häkkinen et al., 2000; Colwell et al., 2003; Szpaderska et al., 2003). Thus, gingival wound healing can be used as a model to study wound regeneration in the adult human tissues. Endo180 is a cell membrane receptor that is involved in the regulation of cell migration and mediates uptake of collagen by endocytosis (East and Isacke, 2002; Behrendt, 2004), both important processes in wound healing. In the present study, we examined the expression of Endo180 in the normal human gingiva and during gingival wound regeneration. Interestingly, Endo180 was consistently expressed in the basal cell layer of the normal gingival epithelium. A previous immunohistochemical study did not find Endo180 expression in the human epidermis (Sheikh et al., 2000). Thus, the gingival epithelium may have a unique property to express Endo180. Our unpublished findings have shown that certain locations of the human epidermis contain isolated cell clusters of keratinocytes that show immunoreactivity for Endo180 suggesting that Endo180 expression is not necessarily restricted entirely to the human gingival keratinocytes. The human gingival epithelium is different from the epidermis, for example, in pattern and repertoire of cytokeratin expression (Barrett et al., 1998). Cell turnover rate in the gingival epithelium is also higher than in the skin (Rowat and Squier, 1986). It is possible that the consistent expression of Endo180 in the gingival epithelium reflects these unique phenotypic or functional characteristics in vivo.

During wound healing, expression of Endo180 was spatially and temporally regulated in the epithelium. Expression of Endo180 was upregulated at 1 and 3 days after wounding at the migrating wound keratinocytes facing the blood clot or granulation tissue. In certain cells, Endo180 associates with uPAR and uPA to form a trimolecular complex (Behrendt et al., 2000). Binding of uPA to uPAR initiates focalized plasminogen activation that is a key process in the proteolytic degradation of the fibrin clot and keratinocyte migration in wound healing (Romer et al., 1996). The uPAR-associated Endo180 promotes cell migration (Engelholm et al., 2003) and regulates uPA/uPAR-mediated cell chemotaxis in vitro (Sturge et al., 2003). Thus, Endo180 may regulate the uPA/uPAR-mediated clot degradation and migration of the epithelial cells in the early stages of gingival wound healing. Similar to our findings, previous studies have shown strong expression of uPAR and uPA in the migrating keratinocytes at the edge of incisional skin wounds (Morioka et al., 1987; Solberg et al., 2001), supporting the idea that Endo180 may associate with uPAR in the migrating keratinocytes.
Keratinocytes stop migrating when the reepithelialization is completed. In the human gingival wounds, this process occurred within 7 days after wounding. The expression of Endo180 was gradually increased in the basal and immediately suprabasal wound keratinocytes from day 7 to day 28 and remained elevated up to 60 days after wounding. This associated with a gradual increase in the thickness and maturation of the wound epithelium. The parallel increase of Endo180 expression with the increasing maturation of the wound epithelium suggests that Endo180 may also be involved in the regulation of proliferation and differentiation of the wound epithelium.

The expression of Endo180 was gradually increased in the areas of unwounded epithelium close to the wound from 1 to 28 days after wounding. Cells in the unwounded epithelium close to the wound become activated upon wounding, undergo cell proliferation, show increased uPA expression and are the main source of migrating keratinocytes for reepithelialization in the early time points of wound healing (Jaakkola et al, 1998). Thus, the activation of keratinocytes appears to involve upregulation of Endo180 expression. In order to find out whether some of the growth factors involved in wound healing regulate Endo180 expression in keratinocytes, we treated cultured keratinocytes with different concentrations of serum or with TGF-β1, EGF, HB-EGF or KGF. Treatment of the cells with 10% FBS or any of these growth factors increased both the Endo180 mRNA and protein levels as compared with the cells treated with 1% FBS. Our unpublished observations have shown that there was no difference in the expression of Endo180 in the cultured keratinocytes located at the edge of an experimental scratch wound compared to cells away from the wound edge, indicating that wounding itself does not induce Endo180 expression. Thus, growth factors present in the wound are likely to induce upregulation of Endo180 expression in keratinocytes after wounding in vivo.

In the normal unwounded gingival connective tissue, Endo180 was expressed uniformly by the different connective tissue cells (fibroblasts, myofibroblasts, pericytes, macrophages, and endothelial cells). Previous studies have shown Endo180 expression in the human dermal and intralobular breast fibroblasts, in the ductal breast carcinoma myofibroblasts, in the dermal and breast carcinoma macrophages and in subsets of endothelial cells in the placenta (Isacke et al., 1990; Sheikh et al., 2000; Schnack Nielsen et al., 2002; Wienke et al., 2003). In culture, the highest expression of Endo180 has been demonstrated in the mesenchymal cells, including fibroblasts, MG-63 human osteosarcoma cells, macrophages, mouse osteogenic cells and
endothelial cells, while the hematopoetic cell lines have shown no or very little expression (Isacke et al., 1990; Sheikh et al., 2000; Engelholm et al., 2001). Our findings show for the first time that Endo180 is expressed by multiple cell types involved in tissue maintenance in the same connective tissue.

During the gingival connective tissue healing, expression of Endo180 changed over time. From 3 to 28 days after wounding only a subpopulation of myofibroblasts, pericytes, macrophages and endothelial cells expressed Endo180. However, in the 60-day-old wounds, as in the normal tissue, all of these connective tissue cells expressed Endo180. During time, also the relative staining intensity of Endo180 changed within each of these cell populations. In the 7-day-old wounds, α-SMA-positive myofibroblasts appeared for the first time and these cells showed relatively weak immunoreactivity for Endo180 as compared with the cells in the unwounded tissue. However, after 14 and 28 days, there was a strong upregulation of Endo180 staining intensity in myofibroblasts. This coincided with the wound contraction and alignment of collagen fibrils in the wound ECM. In addition to the synthesis of ECM components, particularly type I collagen, myofibroblasts attach to collagen by integrin-type collagen receptors and contract the collagen network. Endo180 functions as a collagen receptor and binds to type I, IV and V collagens (Wienke et al., 2003; Engelholm et al., 2003). Thus, Endo180 may function as a co-receptor with integrins in myofibroblasts and regulate wound contraction. Given the importance of Endo180 in mediating the endocytosis of collagen (Wienke et al., 2003; East et al., 2003, Engelholm et al., 2003), Endo180 may also facilitate removal of excess collagen from the ECM which is important to normalize the structure of the collagen-rich wound connective tissue and prevent scar formation. This role for Endo180 is supported by studies showing Endo180 expression in cells involved in ECM remodeling in other tissue locations, including the human dermal, kidney and intralobular breast fibroblasts, human dermal macrophages, subset of placental endothelial cell and tumor-associated myofibroblasts and macrophages in breast carcinoma. Endo180 is also expressed at the sites of active bone formation and cartilage deposition in young mice (Isacke et al., 1990; Wu et al., 1996; Sheikh et al., 2000; Engelholm et al., 2001; Schnack Nielsen et al., 2002; Wienke et al., 2003; Howard et al., 2004). More direct evidence of the role of Endo180 in collagen endocytosis is based on studies using genetic ablation of Endo180 expression or function in mice and cell lines showing reduced collagen uptake and remodeling (East et al., 2003; Engelholm et al, 2003; Curino et al., 2005).
present study, the expression of Endo180 was upregulated in the human gingival fibroblasts by treatment with 10% FBS or with TGF-β1, EGF or PDGF, suggesting that these growth factors play a key role in the regulation of Endo180 expression also in vivo. Increased Endo180 expression did not depend on myofibroblast phenotype (expression of α-SMA) because only TGF-β1 induced α-SMA expression in the cultured cells (not shown). Our preliminary observations indicate that decorin binds to Endo180 in vitro. Endo180 colocalized with collagen-binding small leucine-rich proteoglycan decorin in wound fibroblasts in vivo. During wound healing, interaction of decorin with Endo180 may modulate Endo180 / collagen binding and regulate cellular uptake of collagen and tissue remodeling. The findings suggest that Endo180 plays a role connective tissue remodeling. In addition, by binding to Endo180 at the cell surface, decorin may partly alter cell signaling leading to changes in Endo180-mediated cell functions, such as migration and/or differentiation.

Although the number of macrophages was increased in the wounds at day 7 and 14 as compared with the unwounded tissue, only a subpopulation of these cells showed expression for Endo180. The expression of Endo180 in macrophages gradually increased until day 60 when all macrophages at the wound connective tissue, as in the normal unwounded tissue, showed immunoreactivity for Endo180. Tissues contain different macrophage populations that exhibit subtype-specific phenotypes and perform diverse cellular functions. For example, two functionally distinct subpopulation of macrophages showed opposite but complementary functions in the injury and recovery phases of wound healing in liver (Duffield et al., 2005). Thus, it is possible that the heterogeneity in Endo180 expression reflects the functional differences among the different macrophage populations during wound healing. Macrophages have been shown to internalize collagens (Deporter 1979; Lucattelli et al., 2003), and they also express uPAR that promotes cell migration (Gu et al., 2005). However, the function Endo180 in macrophages is still unclear.

Angiogenesis is a critical step in wound healing. In order to form new blood vessels, endothelial cells must proliferate and migrate to the wound bed. The upregulation of Endo180 expression in the tumor endothelium (St. Croix et al., 2000) suggests a role for Endo180 in tumor angiogenesis. In the present study, the first endothelial cells were noted in the granulation tissue in the 3-day-old wounds. At this time point, only half of the endothelial cells showed weak immunoreactivity for Endo180. In the later time points, up to 28 days after wounding, a larger
proportion of endothelial cells started to express Endo180. Notably, the small and/or new forming blood vessels showed little or no expression, whereas the large blood vessels strongly expressed Endo180. As in the unwounded tissue, all endothelial cells in the 60-day-old wound connective tissue showed immunoreactivity for Endo180. A previous study also found that in the placenta, the larger blood vessels expressed Endo180, while the smaller ones were negative (Sheikh et al., 2000). Thus, Endo180 may be more involved in the later development or maintenance of the blood vessels rather than early migration of endothelial cells during wound healing.

In addition to myofibroblasts, perivascular cells (pericytes) express α-SMA (Gabbiani, 2003). Pericytes exist in all organs where they associate abluminally with all vascular capillaries and postcapillary venules and regulate local blood flow (Hirschi and D’Amore, 1996). In the healthy human gingiva and in the wounds, α-SMA positive cells were located both within the ECM and in association with blood vessels. Therefore, the former α-SMA positive cell population represents myofibroblasts while the latter population represents pericytes. In the normal gingiva, all pericytes showed immunoreactivity for Endo180. The first pericytes appeared in the wound granulation 3 days after wounding but their number was dramatically increased only in the 14-day-old wounds. In contrast, the new blood vessels started to appear abundantly in the wounds already at day 7. Thus, most developing blood vessels, especially at the 7-day-old wounds, did not have pericytes present. Only about 25% of pericytes in the 7-day-old wounds expressed Endo180 and only after 60 days all of the wound pericytes, like pericytes in the normal gingiva, showed immunoreactivity for Endo180. Also the relative staining intensity of Endo180 in pericytes increased over time. Thus, Endo180 is expressed most abundantly also in pericytes at the later stages of blood vessel formation after wounding.

Taken together, the findings in this study showed for the first time the expression of Endo180 in human keratinocytes during gingival wound healing and in cell culture.Expression of Endo180 was spatio-temporally regulated in epithelium and in distinct connective tissue cell subpopulations during the early and late wound regeneration stages. These findings suggest a role for Endo180 in the cell migration and ECM remodeling during wound healing. Colocalization of decorin with Endo180 in distinct cells during wound healing suggests a role of SLRPs in the regulation of Endo180-mediated cell function and collagen turnover.
4.6 ACKNOWLEDGMENTS

We thank Mr. Cristian Sperantia and Dr. Tara Habijanac for expert technical assistance. The study was supported Canadian Institutes of Health Research (LH) and Breakthrough Breast Cancer and The Welcome Trust (CMI).

Table 4.1: The relative number of different connective tissue cells in the wound as compared to the unwounded tissue.

<table>
<thead>
<tr>
<th></th>
<th>Unwounded tissue</th>
<th>Wound (days after wounding)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Myofibroblasts(^1)</td>
<td>++*</td>
<td>-</td>
</tr>
<tr>
<td>Pericytes(^2)</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Endothelial cells(^3)</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Macrophages(^4)</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

*Results compare the relative number of cells in the wound at each indicated time point after wounding to the relative number of the same cell type in the unwounded tissue.

\(^1\) \(\alpha\)-SMA expressing cells that were not associated with blood vessels

\(^2\) \(\alpha\)-SMA expressing cells that were associated with blood vessels

\(^3\) Cells that showed immunoreactivity for anti-CD31 antibody

\(^4\) Cells that showed immunoreactivity for anti-CD68 antibody

-: no cells present; +: the lowest relative number of cells; ++++: the highest relative number of cells
Table 4.2: The relative staining intensity of Endo180 in the epithelial cells during wound healing.

<table>
<thead>
<tr>
<th>Unwounded tissue</th>
<th>Wounded tissue (days after wounding)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Epithelium of the unwounded tissue</td>
<td>+</td>
</tr>
<tr>
<td>Wound epithelium</td>
<td>N/A</td>
</tr>
<tr>
<td>Epithelium close to the wound</td>
<td>N/A</td>
</tr>
</tbody>
</table>

+: the lowest relative staining intensity; +++: the highest relative staining intensity; N/A: not applicable.
Table 4.3: The relative proportion (%) of cells expressing Endo180 within each connective tissue cell population during wound healing.*

<table>
<thead>
<tr>
<th>Percent of cells expressing Endo180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwounded tissue</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Myofibroblasts(^1)</td>
</tr>
<tr>
<td>Pericytes(^2)</td>
</tr>
<tr>
<td>Endothelial cells(^3)</td>
</tr>
<tr>
<td>Macrophages(^4)</td>
</tr>
</tbody>
</table>

*In total 100 to 500 of each cell type were counted from three parallel samples at each time point.

\(^1\) α-SMA expressing cells that were not associated with blood vessels
\(^2\) α-SMA expressing cells that were associated with blood vessels
\(^3\) Cells that showed immunoreactivity for anti-CD31 antibody
\(^4\) Cells that showed immunoreactivity for anti-CD68 antibody
Table 4.4: The relative immunostaining intensity of Endo180 in the connective tissue cells during wound healing.

<table>
<thead>
<tr>
<th>Unwounded tissue</th>
<th>Wound (days after wounding)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Myofibroblasts¹</td>
<td>+++</td>
</tr>
<tr>
<td>Pericytes²</td>
<td>+++</td>
</tr>
<tr>
<td>Endothelial cells³</td>
<td>+++</td>
</tr>
<tr>
<td>Macrophages⁴</td>
<td>+++</td>
</tr>
</tbody>
</table>

*The relative staining intensity between the unwounded and wounded tissues was compared within each cell population.

¹ α-SMA expressing cells that were not associated with blood vessels
² α-SMA expressing cells that were associated with blood vessels
³ Cells that showed immunoreactivity for anti-CD31 antibody
⁴ Cells that showed immunoreactivity for anti-CD68 antibody

N/A: no cells present; +: the lowest relative staining intensity; ++++: the highest relative staining intensity.
Figure 4.1: Histological analysis of representative gingival biopsies at different time points of wound healing.

A, C, E, G, I, K, and M: Hematoxylin and Eosin (H & E) staining; B, D, F, H, J, L, and N: H & E stained samples viewed under fluorescent light through rhodamine channel to visualize fluorescence from eosin-stained collagen fibers. C-F: Arrowheads indicate the migrating front of the wound epithelium. Arrows indicate the connective tissue-granulation tissue border. J: Arrows indicate collagen fibers that are aligned perpendicularly against the wound margins. E: epithelium; CT: connective tissue; FC: fibrin clot; GT: granulation tissue; WCT: wound connective tissue. Magnification bar = 50 μm.
Figure 4.2: Expression of Endo180 during wound healing.

Representative images of tissue sections immunostained with the monoclonal antibody against Endo180 (mAb A5/158) in the normal unwounded gingiva and at different time points after wounding. A: Insert shows the connective tissue cells expressing Endo180. D and E: Arrowheads indicate the migratory front of wound epithelial cells. G: Insert shows connective tissue cells at the wound margin displaying strong Endo180 expression. M: Insert shows wound connective tissue cells that show strong immunostaining for Endo180. E: epithelium; CT: connective tissue; FC: fibrin clot; GT: granulation tissue; WCT: wound connective tissue. Magnification bar = 50 μm.
Figure 4.3: Expression of Endo180 by connective tissue cells in the normal tissue.

(A-C) In the unwounded normal gingiva, Endo180 colocalized with all α-SMA-expressing vascular-associated cells (pericytes; arrows). (D-I) All α-SMA positive fibroblasts (myofibroblasts; arrowheads in D-F) and Thy-1 positive fibroblasts (arrowheads in G-I) also expressed Endo180. (J-O) Endo180 also associated with endothelial cells (arrows in J-L) and macrophages (arrowheads in M-O). (A and D) α-SMA staining; (G) Thy-1 staining; (J) CD31 (endothelial cells) staining; (M) CD68 (macrophage) staining; (B, E, H, K, and N) Endo180 staining; (C, F, I, L, and O) Corresponding merged images with yellow color indicating colocalization. Magnification bar = 50 μm.
Figure 4.4: Endo180 localizes to a subset of myofibroblasts and blood vessels in the 14-day-old wounds.

(A-C) The number of myofibroblasts that expressed Endo180 increased in the wound connective tissue, and the relative staining intensity of Endo180 was strongly increased in these cells as compared with unwounded tissue (arrowheads). Only a proportion of pericytes (α-SMA expressing blood vessels) expressed Endo180 in the wound connective tissue (circles and arrows). (D-F) Endo180 did not colocalize with the new, forming blood vessels (CD31 expressing endothelial cells; arrows) or (G-I) macrophages (CD68 expressing cells; circles). (A) α-SMA staining; (D) CD31 (endothelial cells) staining. (G) CD68 (macrophage) staining; (B, E, and H) Endo180 staining; (C, F, and I) Corresponding merged images with yellow color indicating colocalization. Magnification bar = 50 μm.
Figure 4.5: Endo180 localizes to the wound connective tissue cells in the 28-day-old wounds.

(A-C) The number of myofibroblasts peaked, the relative staining intensity of Endo180 was strongly increased and Endo180 colocalized with more than 75% of myofibroblasts in the granulation tissue (arrowheads). (D-F) A proportion of CD31-expressing endothelial cells showed immunoreactivity for Endo180 (arrows), while some still did not express Endo180 (arrowheads). (G-I) Also many of the macrophages showed immunoreactivity for Endo180 (arrows). (A) α-SMA staining; (D) CD31 (endothelial cells) staining; (G) CD68 (macrophage) staining; (B, E, and H) Endo180 staining; (C, F, and I) corresponding merged images with yellow color indicating colocalization. Magnification bar = 50μm.
**Figure 4.6:** Growth factors upregulate the expression of Endo180 in fibroblasts and keratinocytes *in vitro*. Immunoblotting of Endo180 (A) and semi-quantitative RT-PCR of Endo180 mRNA expression (B) in human gingival fibroblasts treated with 1% or 10% FBS or with 1% FBS and TGF-β1, EGF or PDGF (10 ng/ml) for 96 h. Immunoblotting of Endo180 in HaCaT keratinocytes (C) and semi-quantitative RT-PCR of Endo180 mRNA expression in HaCaT keratinocytes (D) that were treated with 1% or 10% FBS or with 1% FBS and TGF-β1, EGF, HB-EGF or KGF (10 ng/ml) for 96 h. Immunoblotting and RT-PCR images shown are representative examples of two to three separate experiments. For immunoblotting, equal amounts of proteins were loaded into gels and the band intensities were normalized against the level of β-actin in the same samples. PCR results were quantitated using values obtained from 1:4 dilution of the RT template and normalized against β-actin mRNA levels in the same set of samples. Quantitations show mean +/- s.d. of two to three separate experiments. *; P < 0.05, **; P < 0.01 (one way ANOVA and Dunnett’s Multiple Comparison Test).
Figure 4.7: Decorin and Endo180 colocalized in distinct cells during wound healing.

(A-C) Endo180 and decorin colocalized in some migrating fibroblasts (arrows). (D-L) The number of fibroblasts expressing both Endo180 and decorin gradually increased from 7-day to 28-day (Arrows). (M-O) After 60 days the number of cells that expressed both Endo180 and decorin decreased (arrows).
4.7 REFERENCES


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Chapter 5: Decorin-induced formation of reactive oxygen species is critical for decorin endocytosis by human gingival fibroblasts

A version of this chapter is being prepared for publication:

5.1 ABSTRACT
Decorin is a small leucine-rich proteoglycan normally present abundantly in extracellular matrix where it regulates collagen fibrillogenesis and activity of growth factors. Interaction of decorin with cells may regulate its uptake and its abundance in the ECM during wound healing. The aim of this study was to identify receptors/mechanisms involved in the interaction of decorin with fibroblasts from non-scaring gingival tissue. We hypothesize that decorin may interact with multiple cell surface receptors in gingival fibroblasts simultaneously that regulate decorin endocytosis and cell signaling. In the present study, we show that in primary fibroblasts from human oral mucosal gingiva, decorin core protein associates with epidermal growth factor receptor (EGFR), insulin-like growth factor-1 receptor (IGF1R) and low density lipoprotein receptor-related protein-1 (LRP-1) on the cell surface and induces rapid clustering and tyrosine phosphorylation of these receptors leading to activation of mTOR pathway and PI3K, NADPH oxidase and mitochondrial electron transport-dependent formation of reactive oxygen species (ROS). Signaling through this pathway was critical for decorin endocytosis via the clathrin-mediated pathway and its lysosomal degradation. In endosomes, decorin colocalized mostly with LRP-1 and to a lesser degree with EGFR but not with IGF1R. Thus, decorin signaling through EGFR, IGF1R and LRP-1 and subsequent mTOR activation and induction of ROS formation regulate decorin endocytosis and provides a mechanism by which fibroblasts regulate decorin abundance in the extracellular matrix. ROS are also powerful regulators of gene expression, cell growth and survival, and decorin-mediated ROS formation may also modulate other fibroblast functions.

5.2 INTRODUCTION
Decorin is a multifunctional extracellular matrix protein that belongs to the family of small leucine-rich proteoglycans. It is composed of a protein core encompassing characteristic leucine-rich repeat moieties and a single chondroitin or dermatan sulphate glycosaminoglycan chain (McEwan et al., 2006). Based on the characterization of the crystal structure and x-ray scattering data, arch-shaped decorin molecules are present as dimers in physiological conditions (Scott et al., 2003; Scott et al., 2004). Decorin interacts with collagens and many other extracellular matrix proteins, growth factors, cytokines and cell surface receptors. It plays a role in various physiological and pathological conditions by regulating collagen fibrillogenesis and activity of
growth factors. In addition, it interacts with cells and regulates cell growth, adhesion, migration and gene expression (Kresse and Schonherr, 2001; Tufvesson and Westergren-Thorsson, 2003; Lamoureux et al., 2007).

Interestingly, interactions of cells with decorin appear to be mediated by several distinct receptors and lead to different functional outcomes depending on the cell type. For instance, α2β1 integrins and class A scavenger receptors (SR-A) serve as cell adhesion receptors for decorin in platelets and murine macrophages, respectively (Guidetti et al., 2002; Santigo-Garcia et al., 2003) while decorin inhibits adhesion of fibroblasts and osteoblasts to certain extracellular matrix proteins (Winnemoller et al., 1992; Huttenlocher et al., 1996; Merle et al., 1997). In kidney fibroblasts and endothelial cells, decorin binds to insulin-like growth factor-1 receptor (IGF1R) and induces its phosphorylation and the activation of downstream PKB/Akt pathway. In renal fibroblasts, this pathway results in activation of mTOR and p70S6K and induction of fibrillin-1 expression (Schonherr et al., 2001; Schonherr et al., 2005; Schaefer et al., 2007). In epithelial carcinoma and sarcoma cells, decorin binds and activates epidermal growth factor receptor (EGFR) leading to activation of downstream signaling involving the MAPK pathway and growth suppression (Moscatello et al., 1998; Iozzo et al., 1999; Santra et al., 2002). On the other hand, in Chinese hamster ovary cells and myoblasts, decorin specifically binds to low density lipoprotein receptor-related protein-1 (LRP-1; Brandan et al., 2006), and in myoblasts, this interaction regulates transforming growth factor-β (TGF-β)-signaling by a mechanism involving PI3K (Cabello-Verrugio and Brandan, 2007).

In addition to specific signaling functions, decorin-cell interactions are important in the regulation of extracellular abundance of decorin. This has biological significance as extracellular matrix-associated decorin regulates for instance collagen fibrillogenesis and the activity of TGF-β1, a key growth factor involved in wound healing and fibrosis (Lamoureux et al., 2007). Cell lines from various tissue origins, including skin fibroblasts, endothelial cells, epithelial cells and tumor cells, have been shown to endocytose decorin and target it to lysosomal degradation (Seidler et al., 2006; Feugaing et al., 2007). However, the mechanism of decorin endocytosis may vary depending on cell type. For example, relatively poorly characterized 26-kDa and 51-kDa cell membrane-associated proteins in mesangial cells, skin fibroblasts, chondrocytes and endothelial cells appear to interact with decorin and be involved in its endocytosis and lysosomal degradation (Hausser and Kresse, 1991; Hausser et al., 1992; Hausser et al., 1993; Götte et al.,
On the other hand, in Chinese hamster ovary cells SR-A and LRP-1 and in myoblasts LRP-1 mediate decorin uptake (Santiago-Garcia et al., 2003; Brandan et al., 2006). Thus, decorin interacts with multiple cell surface receptors that may be involved in decorin-induced cell signaling events and/or decorin endocytosis in a cell type specific manner. However, it is unclear whether these multiple interactions occur in a single cell type at the same time and what is the collective outcome of these interactions.

During embryonic development, oral mucosal gingival fibroblasts derive from the cranial neural crest while many of the other mesenchymal cell lines, including skin fibroblasts, originate from the mesoderm (Palmer and Lubbock, 1995; Schmidt-Ullrich and Paus, 2005). Not surprisingly, fibroblasts isolated from gingiva show distinct cellular phenotype and functions in vitro when compared for instance to skin fibroblasts. These differences may also have significance in vivo. For example, the turnover rate of the extracellular matrix in gingiva is high and wound healing in gingiva proceeds faster and results to less scar formation than in skin (Schor et al., 1996; Häkkinen et al., 2000b; Ferguson and O’Kane, 2004; Lallier, 2004; Shannon et al., 2006). Decorin is abundantly present in gingiva where it regulates collagen fibrillogenesis (Häkkinen et al., 1993; Häkkinen et al., 2000a; Alimohamad et al., 2005; Matheson et al., 2005). In normal gingiva and at certain stages of gingival wound healing, decorin localizes to the pericellular matrix of fibroblasts likely allowing the cells to interact with it (Oksala et al., 1995; Honardoust et al., 2008). In fact, decorin regulates growth of gingival fibroblasts in vitro and in vivo, but unlike in tumor cells, decorin-mediated growth suppression does not appear to involve EGFR-mediated signaling (Häkkinen et al., 2000a). In the present study, we investigated the interaction of decorin with human gingival fibroblasts in more detail. The findings showed that decorin associated via its core protein with EGFR, IGF1R and LRP-1 and induced phosphorylation and downstream signaling from these receptors, leading to rapid increase in intracellular reactive oxygen species (ROS) formation that was necessary for decorin endocytosis by the clathrin-mediated pathway and for its lysosomal degradation. This provides a mechanism by which fibroblasts regulate decorin abundance in the extracellular matrix. ROS are powerful regulators of other signaling events, gene expression, cell growth and survival (Hancock et al., 2001), and decorin-mediated ROS formation may also modulate fibroblast functions important in tissue homeostasis, wound healing and pathological conditions.
5.3 EXPERIMENTAL PROCEDURES

5.3.1 Reagents

Rabbit anti-IGFIR, anti-p-LRP-1 (Tyr457) against the extracellular domain of the receptor and anti-Rab7, mouse anti-cavolin-1 and anti-ERK1/2, goat anti-clathrin and anti-p-EGFR (Tyr1173) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Function blocking mouse anti-IGFIR (αIR3) and anti-LRP-1, rabbit anti-LAMP1, anti-Rab5 and anti-β-actin antibodies were obtained from Abcam, Inc. (Cambridge, MA, USA). Recombinant human receptor-associated protein (RAP) was from Calbiochem (San Diego, CA, USA), rabbit anti-Rab11 antibody from BD Transduction Laboratories (San Jose, CA, USA), rabbit anti-integrin αv subunit (L230) and mouse anti-α2β1 (BHA2.1) integrin antibodies from Chemicon, Inc. (Temecula, CA, USA). Mouse anti-decorin (DS-1) antibody against bovine decorin developed by Dr. A. Robin Poole (Rosenberg et al., 1985) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained in Iowa, Department of Sciences (Iowa City, IA, USA). Rabbit anti-decorin (LF-94) antibody against bovine decorin was from Dr. Larry Fisher (Matrix Biochemistry Unit, CSDB, DIR, National Institute of Health/NIDCR, Bethesda, MD, USA; Fisher et al., 1995), rabbit anti-decorin antibody from Dr. Tom Krusius (The Finnish Red Cross Blood Service, Helsinki, Finland; Krusius and Ruoslahti, 1986), mouse anti-human decorin antibody (Mab143) from R&D Systems Inc. (Burlington, ON, Canada), rabbit anti-p-ERK1/2, anti-p-p70S6k (Ser371), anti-p-mTOR (Ser2448) and anti-m-TOR antibodies from Cell Signaling Technology, Inc. (Boston, MA, USA) and mouse anti-p-IGFIR (Tyr1131) antibody from Upstate Cell Signaling Solution Co. (Lake Placid, NY, USA). Recombinant human platelet-derived growth factor-BB (rhPDGF), bovine decorin isolated from articular cartilage, tyrphostin AG1478 and AG1024, E64d cathepsin inhibitor, fucoidan, polyinosinic acid, monensin, N-acetyl-L-cysteine (NAC), diphenyleneiodonium chloride (DPI), filipin III, rapamycin, ebselen, allopurinol, rotenone, L-NAME hydrochloride, apocynin, everolimus, FITC-conjugated cholera toxin B (CTB), lactose analog isopropyl β-galactoside (IPTG), reduced glutathione and 2-mercaptoethanol and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, Missouri, USA). Alexa-488 and Alexa-594 conjugated secondary antibodies, rhodamine-conjugated transferrin, 2′7′-dichlorodihydrofluoresceine diacetate (DCF), trypsin/EDTA (0.25%/1 mM), antibiotic-antimycotic mixture for cell culture (100x concentrate; 10,000 U/ml penicillin, 10,000 μg/ml streptomycin.
and 25 μg/ml amphotericin B), fetal bovine serum (FBS), 4'-6-diamidino-2-phenylindole (DAPI), clathrin siRNA (UAAUCCAAUUCGAAGACCAAU), LRP-1 siRNA (AAGACUUGCGAGCCCCAAGCAG; Wu et al., 2004), scrambled control siRNA (ACUUCGACACAUAGCGACUGdTdT), recombinant human EGF (rhEGF), Lipofectamine 2000 and Opti-MEM I medium were purchased from Invitrogen (Eugene, OR, USA). LY294002, SB415286 and U0126 were from Biomol International, Inc. (Plymouth Meeting, PA, USA). Fully glycosylated recombinant human decorin produced in human 293 kidney cells was from EMP Genentech (Denzlingen, Germany) and mouse anti-EGFR (Ab-3) antibody and recombinant human decorin produced in S/21 cells from Oncogene Research Products (San Diego, CA, USA). Recombinant human insulin-like growth factor (rhIGF-I), competent *E. coli* BL21 cells, Pfu-polymerase and BamHI and EcoRI enzymes were from Promega (Madison, WI, USA). NucleoSpin RNA II kit was from Macherey-Nagel, Inc. (Bethlehem, PA, USA), T4 DNA ligase from Boehringer Mannheim (Mannheim, Germany), QIAquick PCR Purification Kit and QIAprep Spin Miniprep from Qiagen (Mississauga, ON, Canada), Luria Bertaini (LB) plates from Biokar Diagnostics (Pantin Cedex, France), ampicillin from Roche Molecular Biochemicals (Basel, Switzerland) and Slide-A-Lyzer (molecular weight cut-off 10,000) from Pierce (Rockford, IL, USA). iScript Select cDNA Synthesis Kit, 2x iQ SYBR Green I Supermix and chromatography columns were obtained from Bio-Rad Laboratories (Mississauga, ON, Canada). Glutathione Sepharose 4B, pGEX-KT expression vector, Hybond-ECL nitrocellulose membrane and ECL™ chemiluminescence detection reagent were purchased from GE Healthcare (Little Chalfont, UK). Hydrogen peroxidase (30%) and cell culture glass coverslips were from Fisher Scientific (Fair Lawn, New Jersey, USA), Kodak X-ray film from Biomax (Rochester, NY, USA), and Dulbecco’s Modified Eagle’s Medium (DMEM) from Gibco BRL Life Technologies (Rockville, MD, USA). PCR primers for human decorin were from CyberGene AB (Huddinge, Sweden) and for β-actin, ALG9 and clathrin heavy chain from Integrated DNA Technologies, Inc. (San Diego, CA, USA). Cell culture consumables were obtained from Falcon (Franklin Lakes, NJ, USA) and Corning (Acton, MA, USA).

5.3.2 Generation of human GST-decorin fusion protein

The core protein of decorin contains 12 repeats of leucine-rich motifs (LRRs; McEwan et al., 2006). In the present study, decorin core protein (LRR1-12) was produced as a glutathione-S-
transferase (GST) fusion protein. DNA encoding this fragment was generated by PCR using human decorin cDNA as a template (ATCC 3934022). The decorin fragment was amplified by PCR (Hybaid PCRSprint; Thermo Fisher Scientific, Waltham, MA, USA) using the following primers: (f) 5’-GGC GGA TCC GAC ACA ACT CTG CTA GAC CTG CAA-3’; (r) 5’-CAG GAA TTC TCC AGG TGG GCA GAA GTC ACT-3’. The forward primer contained a BamHI restriction site at the 5’-end and EcoRI restriction site was introduced into the 5’-end of the reverse primer. The initial denaturation step was at 95°C for 5 min. Pfu-polymerase was then added (80°C, 5 min), followed by 25 cycles of 1 min at 95°C, 1 min at 50°C and 2.5 min at 72°C. PCR product and pGEX-KT expression vector were then digested with BamHI and EcoRI enzymes. The digestion products were purified using QIAquick PCR Purification Kit, the insert and vector ligated with T4 DNA ligase at 4°C over night and transformed into competent E. coli BL21 cells. To select for transformants, cells were grown on LB plates containing ampicillin (100 µg/ml). Selected colonies were amplified in 2xYT-medium containing ampicillin (50 µg/ml) followed by processing for small-scale plasmid DNA preparations using QIAprep Spin Miniprep kit. The clones were identified by restriction enzyme digestion and gel analysis. The DNA sequences were verified by sequencing. For the production of the recombinant decorin GST-fusion protein, a single colony of E. coli cells containing recombinant pGEX plasmid was grown in 2xYT-medium containing ampicillin (100 µg/ml) until the optical density at 600 nm reached 0.2 – 0.8. The protein expression of pGEX is under the control of the tac-promoter, which was induced using the lactose analog IPTG (0.4 mM) at 25°C for 5 h. Different IPTG concentrations, induction temperatures and induction times were tested in order to minimize the formation of inclusion bodies. Next, cells were harvested and lysed by mild sonication, and detergent (Triton X-100) was added to a final concentration of 1%. After incubation at 4°C for 30 min, dissolved proteins were cleared of cellular debris by centrifugation (17,000 rpm, rotor SS-34, Sorvall RC5C; Thermo Fisher Scientific). Glutathione Sepharose 4B was then added to the cleared lysate, and the GST-fusion proteins bound to the glutathione-sepharose were separated from the supernatant by centrifugation (1,700 rpm, rotor BS4402/A, Heraeus Megafuge 1.0R; Thermo Fisher Scientific) and transferred to chromatography columns. Unbound proteins were removed with phosphate-buffered saline (PBS; pH 7.4). Finally, the fusion protein was eluted with reduced glutathione (30 mM) and dialyzed against PBS in a Slide-A-Lyzer. The fusion protein was characterized by using SDS-PAGE (not shown).
5.3.3 Cell culture

Human gingival fibroblast cultures were established from four healthy human subjects as described previously (Häkkinen et al., 1992). All key experiments were performed using three to four different cell strains between passages 5-12. Fibroblasts were cultured in DMEM containing 10% FBS and antibiotic-antimycotic mixture until they reached 95% confluency. Cells were then harvested using trypsin/EDTA solution, washed with culture medium and seeded for different experiments.

5.3.4 Decorin treatment of cultured cells

Fibroblasts were seeded on 12 mm glass coverslips and cultured in their normal growth medium for 48 h. Cells were incubated at 4°C for 5 min in DMEM before treatment with equimolar amount of bovine skin or cartilage decorin, chondroitinase ABC-treated decorin (Kuc and Scott, 1997), recombinant human decorin proteoglycan produced in human 293 kidney cells, Sj/21 cells or the GST-decorin (LRR1-12) fusion protein at 4°C for 20 min. Unbound decorin was carefully removed by two washes with cold PBS. A set of samples was then fixed with cold 4% formaldehyde containing 5% sucrose in PBS for 20 min (time 0 samples). The other cultures were transferred to 37°C to induce decorin internalization. After various periods of time, these samples were fixed and processed for immunostaining. In a set of experiments, cells were treated with rhodamine-conjugated transferrin (50 μg/ml; a specific marker for clathrin-mediated endocytosis; Hansen et al., 1993) or FITC-conjugated CTB (2 μg/ml; a marker of caveolae-mediated endocytosis; Orlandi and Fishman, 1998) with decorin and/or inhibitors (see below).

5.3.5 Immunostaining and quantitation of fluorescence intensity

Immunostaining was used to analyze decorin cell surface binding and endocytosis and to colocalize decorin with cell surface receptors and intracellular markers of endocytosis pathways. For immunostaining of cell surface molecules, fixed cells were left non-permeablized, while for detecting internalized decorin and other intracellular molecules, they were permeablized with 0.5% Triton X-100 at room temperature for 4 min. Samples were then incubated with BSA (10 mg/ml) and glycine (1 mg/ml) in PBS containing 1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$ (PBS+) at room temperature for 30 min to block nonspecific binding sites, followed by an incubation with the primary antibody against human (for samples treated with recombinant human decorin) or
bovine decorin (for samples treated with bovine decorin) or against GST (for human recombinant decorin GST-fusion protein) in PBS containing 1 mg/ml BSA at room temperature for 60 min. For double immunofluorescence staining, cells were then washed twice with PBS and incubated with the second primary antibody as above. In all experiments, the first primary antibody was made in a different animal species than the second primary antibody. After washing, the samples were incubated with appropriate Alexa 488 and/or Alexa 594 conjugated secondary antibodies as above. In order to stain nucleae, the samples were incubated with DAPI (10 μM) at room temperature for 5 min. The coverslips were mounted with 50% glycerol in PBS. Control immunostainings were performed omitting one of the primary antibodies at the time and showed no nonspecific staining (data not shown). The samples were examined by a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Jena, Germany) or by Nikon E600 D-Eclipse Laser Confocal Microscope (Nikon Instech Co., Kawasaki, Japan). Images were captured using Northern Eclipse (Empix Imaging, Mississauga, ON, Canada) or EZ C1 2.3 software (Nikon Instech Co.) for Zeiss Axioplan 2 and Nikon E600 D-Eclipse microscopes, respectively. To quantitate fluorescence intensity, standardized images from five representative microscopic fields per sample were captured by single rapid scans at the same time using a x20 objective with standardized digital camera setting for offset (background darkness) and exposure time (overall brightness) for all samples. Using ImageJ software (http://rsb.info.nih.gov/ij/), relative fluorescence intensity from about 50 cells per field (determined by counting nucleae stained with DAPI) was measured using a scale where 0 and 255 represent the lowest and the highest staining intensity, respectively. Statistical analysis was performed on values obtained from the means of three to four parallel experiments by one-way ANOVA and Dunnett’s Multiple Comparison Test. Values of p<0.05 were considered to be statistically significant.

5.3.6 Fluorescence activated cell-sorting (FACS) analysis of decorin endocytosis
Fibroblasts were grown in their normal growth medium and released by trypsin/EDTA incubation as above. The cells were counted, and equal aliquots were suspended in 100 μl of cold DMEM in microcentrifuge tubes and kept at 4°C for 10 min. Cells were then treated with 50 μg/ml of bovine decorin at 4°C to allow binding of decorin to the cell surface. After 20 min, cells were collected by centrifugation and washed three times with cold DMEM to remove unbound decorin. A set of cells was placed on ice (time 0 samples) while parallel sets were
transferred to 37°C to induce decorin endocytosis. Decorin endocytosis was terminated after 5 or 30 min by placing the cells on ice. The cells were then processed for immunostaining of bovine decorin using the anti-bovine decorin antibody (DS-1) as above except that the cells were kept suspended in the staining solutions on ice during the whole procedure. Control samples were prepared by omitting the primary antibody incubation step. Fluorescence emission intensities from cell surface-associated decorin were measured in about 5x10^4 cells in each sample by using the FACSCalibur System (BD Biosciences, Mississauga, ON, Canada) flow cytometer. Three parallel samples were used in each experiment and the experiment was repeated three times. Statistical analysis was performed as above.

5.3.7 Analysis of decorin-induced ROS formation

Fibroblasts were cultured on coverslips as described above and then treated with 10 μM DCF diluted in PBS+ at 37°C for 60 min. DCF is an indicator of ROS formation that is nonfluorescent. When the acetate groups of the molecule are removed by intracellular esterases DCF becomes oxidized and fluorescent (Robinson et al., 1988). After DCF treatment, cells were washed and treated with 50 μg/ml of bovine decorin or equimolar amount of recombinant human decorin for different time periods (1-30 min) or with increasing concentrations of decorin (0-100 μg/ml) for 10 min before fixation. Relative fluorescence intensity resulting from ROS formation was visualized using Nikon E600 D-Eclipse Laser Confocal Microscope, images were captured using EZ C1 2.3 software and quantitated from at least three parallel experiments as above.

To assess the intracellular source of decorin-induced ROS formation, fibroblasts were pretreated at 37°C for 3 h with or without the following drugs: DPI (40 μM) that binds covalently to a polypeptide of the plasma membrane to inhibit NADPH oxidase function (Li and Trush, 1998) or apocynin (40 μM) that impairs the assembly and translocation of NADPH oxidase membrane subunits (Stolk et al., 1994); rotenone (5 μM) that inhibits mitochondrial activity by blocking its respiratory chain complex I and disrupting electron transport (Teeter et al., 1969); allopurinol (50 μM) that inhibits xanthine oxidase (Watts et al., 1965); L-NAME (50 μM), an analog of arginine that inhibits nitrogen oxide production (McCall et al., 1991). To block ROS formation, cells were treated with 10, 50 or 100 mM NAC or 40 μM ebselen in PBS+ at 37°C for 2 h. DCF was added to the cells at the second hour of the ROS-scavengers treatment. Cells were then treated with decorin as above. Control samples were treated with corresponding
amounts of the drug diluents. The doses of ROS inhibitors (NAC and ebselen) and ROS source inhibitors (apocynin, allopurinol, DPI, L-NAME and rotenone) are in the range of the lowest dose that have been previously used to prevent ROS formation in endothelial cells and are about 10-fold higher than have been reported to inhibit 50% of the enzyme activity (Ali et al., 2004; Felty, 2006). Cells were then treated with DCF followed by treatment with decorin in the presence or absence of the drugs as above. For a positive control, oxidative activity was stimulated in a set of samples by a treatment with 100 μM H₂O₂ instead of decorin. Relative DCF fluorescence was quantitated as above.

5.3.8 Inhibition of cell surface receptors, signaling molecules or internalization pathways

To assess the mechanisms of decorin cell surface binding, internalization, signaling or ROS formation, fibroblasts were pretreated with or without various reagents at 37°C. Starting concentrations of the drugs and inhibitors were selected based on the information in the literature or manufacturer recommendations and after testing different dilutions of the inhibitors the highest concentration that gave the maximum inhibitory effect without toxicity were used for the experiments (data not shown). For the experiments, the following reagents or antibodies were used for pretreatment of the cells before decorin treatment: anti-IGF1R antibody (αIR3; 50 μg/ml) or AG1024 (10 μM) for 2 h to block ligand binding or phosphorylation of IGF1R, respectively; anti-EGFR antibody (Ab-3; 10 μg/ml) or AG1478 (20 μM) for 2 h to block ligand binding or phosphorylation of EGFR, respectively; RAP (80 μg/ml) for 2 h to block decorin binding to extracellular domain of LRP-1 (Herz et al., 1991; Brandan et al., 2006); sucrose (0.25, 0.5 or 1 M) for 1 h to block clathrin mediated endocytosis pathway (Heuser and Anderson, 1989); filipin (0.5, 1 or 2 μg/ml) for 2 h to inhibit caveolae-mediated endocytosis (Rothberg et al., 1990); everolimus (10 μg/ml for 2 h) or rapamycin (4 μg/ml for 6 h) to inhibit mTOR activation (Neuhaus et al., 2001); anti-α2β1 (BHA2.1; 10 μg/ml; Phillips and Bonassar, 2005) or anti-αv (L230; 20 μg/ml; Houghton et al., 1982) antibody for 1 h to inhibit ligand binding to corresponding integrin receptors; fucoidan (10 μg/ml) for 1 h to inhibit ligand binding to SR-A (Krieger and Herz, 1994; Santiago Garcia et al., 2003); U0126 (10 μM), SB415286 (30 μM) or LY294002 (50 μM) for 2 h to inhibit MEK1/2, glycogen synthase-3 kinase (GSK-3) or PI3K phosphorylation, respectively; monensin (100 μM) to block recycling endosome function (Mollenhauer et al., 1990) and/or cell membrane-permeable cysteine protease inhibitor E64d (20
μM) for 60 min to block degradation of lysosomal cargo (Murachi, 1985). For the treatment, all drugs were dissolved in DMEM to the appropriate final concentration. After pretreatment, cells were treated with decorin as above in the presence of the inhibitors and processed for the analyses as described above. Control samples were treated with corresponding concentrations of appropriate IgG or drug diluents.

5.3.9 Western blotting experiments

Fibroblasts were cultured in 35 mm cell culture plates as above for 24 h followed by incubation in serum-free DMEM for 24 h before treatment with decorin with or without the drugs for different periods of time as above. Positive control samples were treated with 10 ng/ml of EGF (to induce EGFR phosphorylation), 100 ng/ml of PDGF (to induce LRP-1 phosphorylation) or 10 ng/ml IGF (to induce IGF1R phosphorylation) instead of decorin (not shown). At various time points after treatment, cellular proteins were extracted with standard sodium dodecyl sulphate (SDS) sample buffer (2% SDS, 1% glycerol, 7% 0.2 M Na phosphate, pH 7.0, 0.003% bromphenol blue). Equal volumes of samples were boiled in the presence of 2-mercaptoethanol (5% final concentration) and electrophoresed on 7.5% SDS-polyacrylamide gels and then transferred to Hybond-ECL nitrocellulose membrane. The following primary antibodies were used for immunoblotting: anti-mTOR (1:100), anti-p-mTOR (1:100 dilution), anti-ERK1/2 (1:500), anti-p-ERK1/2 (1:500), anti-p-EGFR (1:200), anti-p-LRP-1 (1:200), anti-p-IGFR (1:100), anti-p-p70S6K (1:500) and anti-β-actin (1:10,000). Peroxidase-conjugated IgGs were used as secondary antibodies. Detection was performed using ECL™ chemiluminescence reagent and Kodak X-ray film. The band intensities were quantified using the ImageJ software. The experiments were performed at least three times with similar results.

5.3.10 siRNA transfections

Fibroblasts were cultured in their regular growth medium for 48 h. For siRNA transfection, Lipofectamine 2000 was added to Opti-MEM I medium and incubated at room temperature for 5 min. Similarly, siRNA was mixed with Opti-MEM I medium and kept at room temperature for 5 min. Both solutions were then mixed together and incubated at room temperature to allow complex formation. After 20 min, the mixture was diluted in Opti-MEM I medium and added to the cell cultures. The final concentration of clathrin and LRP-1 siRNA was 25 nM and 50 nM,
respectively. Control samples were treated with corresponding amounts of scrambled control siRNA. After 4 h at 37°C, FBS (10% final concentration) was added to the medium. After overnight cell culture, the medium was replaced with DMEM containing 10% FBS. After 24, the above procedures were repeated for the second transfection, and the cells cultured for another 48 h as above. The cells were then harvested for RNA extraction (see below). For experiments, the cells were trypsinized 24 h after the second transfection and seeded on glass coverslips in DMEM containing 10% FBS, treated with decorin and/or DCF and processed for immunostaining as above.

5.3.11 RNA isolation and real-time quantitative reverse transcription-PCR
Total RNA was extracted using the NucleoSpin RNA II kit and digested with DNase according to the manufacturer’s instructions. RNA integrity was checked by agarose gel electrophoresis and concentration measured by spectrophotometry at 260 nm. Total RNA (1 μg) was reverse-transcribed (RT) with oligo (dT) primers using iScript Select cDNA Synthesis Kit according to the manufacturer’s instructions. The following primers were designed and verified by using PerlPrimer software (Version 1.1.9; www.bioinfo.rpi.edu/applications/mfold/): β-actin (NM_001101): (f) 5'-CTGTGGCATCCACGAAAC-3', (r) 5'-CAGACAGCACTGTGTGG-3'; ALG9 (asparagine-linked glycosylation 9 homolog; BC009255; Kidd et al., 2007): (f) 5'-GAATGACCAGAATCTAGAAGAGCCA-3', (r) 5'-TCTCATGGTGTCATAAATCCACTAAA-3'; Clathrin heavy chain (BC051800): (f) 5'-TTCCGTCTTGCTCAGATGTG-3', (r) 5'-GCCACGATCTGATAGTAGTTG-3'; LRP-1 (NM_002332): (f) 5'-ACGCCCTCTGACGTGGTCC-3', (r) 5'-CATTTCCTTCGCAGTCATGGGTTG-3'. Primer specificity was confirmed by BLASTn software. For the PCR amplification reaction, 5 μl of diluted RT products were mixed with 10 μl of 2x Qi SYBR Green I Supermix. The RT products were used in a concentration that gave C_T values that were within the range of the standard curve. Primer concentrations were: 10 pM for ALG9, 15 pM for β-actin and 7.5 pM for clathrin and LRP-1 in a final reaction volume of 20 μl. Efficiency of target amplification was optimized up to 95% for each primer set using a 10-fold dilution series of cDNA while the standard curve was made. Real-time PCR amplification was performed on the MiniOpticon Real-Time System (Bio-Rad) using the program of 3 min at 95°C, followed by 35 cycles of 15 s at 94°C, 20 s at 55°C, 20 s at 72°C and completed with a melt curve analysis from 50°C to 90°C (2s for each
1°C). Amplification reaction was conducted for the clathrin and LRP-1 genes with both β-actin and ALG9 as reference genes and replicated three times in each experiment for each sample. The data were analyzed using Gene Expression Analysis for iCycler iQ Real-Time PCR Detection System software (Bio-Rad).

5.4 RESULTS

5.4.1 Decorin interacts with fibroblast cell surface via its core protein and gets rapidly internalized

In order to study the interactions of decorin with human gingival fibroblasts, we treated the cells with 50 µg/ml of bovine skin decorin and analyzed its binding and internalization over time by immunostaining with an antibody that recognizes only bovine decorin. Decorin was first allowed to bind to cell membranes at 4°C to prevent internalization, and the unbound molecules were then removed by washing. At this point (time 0), decorin localized abundantly at the cell membranes but no intracellular decorin was detected (Figure 5.1A). Within 2 min after transferring the cells into 37°C, decorin had accumulated into distinct clusters on the cell membranes but very little decorin was found intracellularly (Figure 5.1A). Within 5 min, the amount of cell surface-associated decorin was strongly reduced as compared to time 0 as determined by immunostaining (Figure 5.1A) or by FACS analysis (Figure 5.1B). However, intracellular decorin was strongly increased (Figure 5.1A). Within 30 min, almost all cell surface decorin had disappeared and the intracellular amount had decreased as compared to earlier time points. After 60 min, very little decorin was found on the cell surface or intracellularly by immunostaining (Figure 5.1A). Decorin internalization was also concentration dependent as increasing concentrations of decorin caused increased accumulation of decorin inside the cells (Figure 5.1C). Similar results were obtained using three other human gingival fibroblast strains (data not shown). To find out whether glycosylation of the decorin molecule was required for the interaction with cells, we treated fibroblasts with 50 µg/ml of bovine skin (dermatan sulphate proteoglycan) or cartilage (chondroitin sulphate proteoglycan) decorin, or with equimolar amount of recombinant human decorin produced in Sf21 cells or with recombinant decorin GST-fusion protein encompassing the LRR1-12 as above. All decorin preparations bound to cell surface (not shown) and were internalized effectively within 15 min (Figure 5.1D). Similar results were also obtained using bovine skin decorin pretreated with chondroitinase ABC to
remove glycosaminoglycan chains and with recombinant human decorin produced in human 293 kidney cells (not shown). Collectively, the results indicate that decorin binds through its core protein to the human gingival fibroblast cell surface and is then rapidly internalized.

5.4.2 Decorin associates with IGF1R, EGFR and LRP-1 at the fibroblast cell surface

Previous studies have shown that decorin core protein can directly bind to IGF1R, EGFR, LRP-1 or α2β1 integrin or interact with SR-A expressed in different cell types (Moscatello et al., 1998; Guidetti et al., 2002; Santigo-Garcia et al., 2003; Schonherr et al., 2005; Brandan et al., 2006; Cabello-Verrugio, 2007). However, it is not known whether decorin can interact with all these receptors in a single cell type. To this end, we first examined whether decorin colocalized with any of these receptors on the cell surface by double immunostaining of nonpermeabilized cells. Fibroblasts were incubated with decorin at 4°C to allow cell surface binding but prevent internalization and then transferred to 37°C for 2 min to allow receptor clustering as a first step for decorin internalization. Results showed that decorin induced clustering of IGF1R, EGFR and LRP-1 on the cell surface and colocalized to these clusters (Figure 5.2A). No colocalization of decorin with SR-A or α2β1 integrin was noted (not shown). In order to further analyze the interaction of decorin with cell surface receptors, we blocked receptor-ligand interactions and examined decorin binding to the cell surface by immunostaining (Figure 5.2B). We also quantitated cell surface-bound decorin by image analysis (Figure 5.2C). Compared to control fibroblasts treated with decorin only, function blocking antibodies that block ligand binding to IGF1R (50 μg/ml) or EGFR (50 μg/ml) or RAP (80 μg/ml) that blocks ligand binding to LRP-1 reduced decorin staining intensity at the cell surface significantly (by about 65%, 23% and 35%, respectively; Figure 5.2C). Combined treatment with all three inhibitors reduced decorin binding by about 75% (Figure 5.2C). Pretreatment of cells with fucoaidan (10 μg/ml) or polyinosinic acid (100 μg/ml) that block ligand binding to SR-A or with function blocking antibodies against αν (L230, 20 μg/ml), α2β1 (BHA2.1, 10 μg/ml) or β1 (Mab13, 100 μg/ml) integrins had no effect (data not shown). These findings indicate that decorin associates at the same time with IGF1R, EGFR and LRP-1 on the cell surface of human gingival fibroblasts.
5.4.3 Decorin induces signaling through EGFR, IGF1R and LRP-1 in fibroblasts

In order to find out whether decorin treatment induced phosphorylation of the cytoplasmic domains of IGF1R, EGFR or LRP-1 in human gingival fibroblasts, we analyzed extracts from the cells treated with decorin as above by immunoblotting. The results showed that decorin induced phosphorylation of EGFR, IGF1R and LRP-1 within 1 min after treatment (Figure 5.2D). Peak activation for EGFR and IGF1R occurred at 5 min and for LRP-1 at 10 min. EGFR, IGF1R and LRP-1 can induce downstream signaling through MEK1/2 and its downstream effector ERK1/2 of the MAPK pathway and/or through PI3K/Akt pathway and its downstream target mTOR (Ma et al., 2002; Cianpolillo et al., 2005; Foulstone et al., 2005 Hu et al., 2006; LoPiccolo et al., 2007; Roberts and Der, 2007). Therefore, we analyzed whether decorin induced phosphorylation of ERK1/2 or mTOR in fibroblasts. Results showed that peak phosphorylation of mTOR (Figure 5.2E) and p70S6K (Figure 5.2F), a downstream effector of mTOR signaling, were induced within 10 min after decorin treatment. However, no apparent changes occurred in ERK1/2 phosphorylation (Figure 5.2G). In order to find out, which receptors were involved in mTOR activation, we pretreated the cells with or without RAP to block ligand-binding to LRP-1, or with AG1024 or AG1478 that block phosphorylation of IGF1R or EGFR, respectively, before decorin treatment and analyzed the phosphorylation of mTOR by immunoblotting as above. All three inhibitors significantly suppressed decorin-induced mTOR phosphorylation after 10 min (Figure 5.2H). Inhibition of LRP-1 expression by siRNA reduced its expression by about 75% and inhibited decorin-induced mTOR phosphorylation similar to RAP while control siRNA had no effect (data not shown).

5.4.4 Decorin induces formation of ROS

Reactive oxygen species (ROS) include singlet oxygen, superoxide ions, hydrogen peroxide and hydroxyl radicals that are natural byproducts of oxygen metabolism but they can also be formed as a result of receptor-mediated signaling events. In high concentration, they can cause oxidative damage to nucleic acids, proteins and lipids and in low concentration, they are powerful signaling molecules regulating gene expression, cell growth, survival and endocytosis (Hancock et al., 2001). The mTOR pathways has been previously linked to induction of ROS formation (Kim et al., 2005). To investigate whether decorin induced ROS formation, gingival fibroblasts were pretreated with DCF and then treated with 50 µg/ml of bovine decorin, and induction of
DCF fluorescence as an indicator of ROS formation was measured over time. No detectable ROS formation occurred in untreated cells. However, decorin induced within 5 min an increase in DCF fluorescence that peaked at 10 min and returned to control level after 20 min (Figure 5.3A and C). The response was also concentration dependent with significantly increased DCF fluorescence with 20-100 μg/ml of decorin after 10 min (Figure 5.3B and D). Similar results were obtained using two other human gingival fibroblast cell strains (not shown).

5.4.5 Decorin-induced ROS formation is mediated by IGF1R, EGFR and LRP-1
To determine which cell surface receptors were involved in decorin induced ROS formation, we pretreated fibroblasts with the function-blocking antibodies against IGF1R or EGFR, or with AG1024, AG1478 or RAP or a combination of the inhibitors and analyzed ROS formation as above. Parallel control samples were treated with appropriate non-immune IgG, fucoidan (to block SR-A) or with function blocking antibodies against αv or α2β1 integrins that do not appear to mediate decorin-cell interactions in human gingival fibroblasts. Compared to cells with no decorin treatment, 50 μg/ml of bovine decorin or equimolar amount of recombinant human decorin produced in S/21 cells potently induced ROS formation within 10 min (Figure 5.3E). Inhibition of EGFR, IGF1R or LRP-1 significantly reduced ROS formation (by about 40%, 60% or 50%, respectively) while inhibition of SR-A or αv or α2β1 integrins had no effect. A combined treatment with EGFR, IGF1R and LRP-1 inhibitors reduced ROS formation by about 75% (Figure 5.3E). Thus, decorin-induced ROS formation was mainly mediated by IGF1R, EGFR and LRP-1.

5.4.6 PI3K and mTOR regulate decorin-induced ROS formation
To find out whether decorin-induced ROS formation was downstream or upstream from decorin-induced mTOR activation, we pretreated fibroblasts with the ROS scavenger NAC (100 mM) before decorin treatment and analyzed mTOR phosphorylation by immunoblotting. Optimal concentration of NAC was chosen from preliminary experiments where it completely blocked decorin-induced ROS formation (see Figure 5.8A and C). Pretreatment with NAC had no effect on decorin-induced mTOR phosphorylation (Figure 5.3F), indicating that decorin-induced ROS formation is downstream from mTOR activation. To confirm this, we pretreated cells with rapamycin or everolimus that are inhibitors against mTOR pathway. PI3K can be activated by
receptor tyrosine kinases and by cell adhesion receptors and is an upstream activator of mTOR (LoPiccolo et al., 2007). Therefore, we also pretreated parallel samples with the PI3K inhibitor LY294002. Another set of samples was also pretreated with an inhibitor (SB415286) against GSK-3 pathway that is a downstream target of mTOR (Zhang et al., 2006) or with the MEK1/2 inhibitor U0126. Results showed that rapamycin and everolimus significantly reduced decorin-induced ROS formation by about 75% while LY294002 reduced it by about 50%. U0126 and SB415286 had no effect on decorin-induced ROS formation (Figure 5.3G). The findings indicate that decorin-induced ROS formation is mainly mediated by PI3K and mTOR.

5.4.7 Decorin endocytosis is mediated by the clathrin-pathway

Our findings indicated that after associating with fibroblast cell surface decorin is effectively internalized. Clathrin and caveolae-mediated endocytosis pathways are two major mechanisms for endocytosis of extracellular molecules and cell surface receptors and are important regulators of matrix turnover and receptor signaling (Polo and Di Fiore, 2006). To investigate the mechanisms of decorin internalization in gingival fibroblasts we examined intracellular localization of decorin using double immunostaining of decorin with clathrin or caveolin-1. Cells were first incubated with 50 μg/ml of bovine decorin at 4°C to allow cell surface binding and prevent internalization. The unbound decorin was then removed by washing before inducing decorin internalization by transferring the cells into 37°C for different periods of time. During 5-30 min after transferring the cells to 37°C, most intracellular decorin colocalized with clathrin (Figure 5.4A, arrows). We treated parallel cultures with fluorescently labeled transferrin, a specific marker for clathrin-mediated endocytosis pathway, together with decorin and analyzed colocalization after decorin immunostaining. Strong colocalization of decorin with intracellular transferrin was noted in the intracellular vesicles (Figure 5.4A, arrows). Double immunofluorescence staining of decorin and caveolin-1 showed only occasional colocalization (Figure 5.4A, arrowheads). In order to provide functional evidence about the role of clathrin-mediated pathway in decorin internalization, we treated cells with different concentration of sucrose to block clathrin-mediated endocytosis. Treatment of cells with 0.25, 0.5 or 1 M sucrose blocked endocytosis of transferrin by 48%, 54% and 83%, respectively (Figure 5.4B and C) and decorin by 42%, 63% and 86%, respectively (Figure 5.4B and D) after 10 min at 37°C. We also treated fibroblasts with clathrin or control siRNA and analyzed decorin endocytosis as above.
Clathrin siRNA down regulated clathrin expression by about 91% as compared to control cells (Figure 5.4F) and it significantly reduced transferrin (by 42%) and decorin (by 45%) internalization (Figure 4E, G and H).

As some decorin also colocalized with caveolin-1 in endosomes we also studied the role of this pathway in decorin internalization in more detail. When fibroblasts were treated with 0.5, 1 or 2 μg/ml filipin, an inhibitor of caveolae-mediated endocytosis, immunoreactivity for internalized cholera toxin B, a marker for the caveolae-mediated endocytosis pathway, was reduced by 9%, 42% and 83%, respectively (Figure 5.5A and C). Same concentrations of filipin reduced decorin endocytosis by 5%, 22% and 27%, respectively, but the differences to control samples did not reach statistical significance (Figure 5.5A and B). Endocytosis of transferrin was not affected by filipin at any concentration used (Figure 5.5A and D). Taken together, decorin internalization in gingival fibroblasts is mediated mostly by the clathrin pathway.

5.4.8 Endocytosed decorin is delivered to recycling and lysosomal degradation

To further characterize the fate of internalized decorin, we treated cells with 50 μg/ml of bovine decorin as above and colocalized decorin with different endosomal vesicles by double immunostaining. Cargo from clathrin-coated vesicles usually enters first to the Rab5-expressing early endosomes followed by transportation to late endosomes expressing Rab7 and/or to lysosomes expressing Lamp-1 for degradation or to Rab11 expressing recycling endosomes (Maxfield and McGraw, 2004). At 5 and 15 min after transferring the cells into 37°C, decorin colocalized abundantly with Rab5 positive early endosomes (Figure 5.6A, arrows). No colocalization of decorin with early endosomes was observed after 30 and 60 min. While a small portion of internalized decorin localized to Rab11 positive recycling endosomes after 5 min, most of it colocalized with recycling endosomes after 15 to 30 min. We could not detect colocalization of decorin within Rab7 positive late endosomes (data not shown) or lysosomes (Figure 5.6A, arrowheads), suggesting that decorin may have been rapidly degraded in late endosomes and lysosomes or that it was completely directed to recycling endosomes. To better understand the fate of internalized decorin, we treated fibroblasts with monensin (100 μM) that inhibits recycling endosome function. As compared with untreated control cells, monensin treatment led to 84% decrease in the amount of intracellular decorin (Figure 5.6B and C), suggesting that inhibition of decorin recycling had rerouted decorin to intracellular degradation.
in late endosomes or lysosomes. Inhibition of lysosomal enzyme activity by pretreatment of cells with a cathepsin inhibitor E64d (20 μM) resulted in significantly increased (about 1.5-fold) intracellular decorin accumulation after 45 min (Figure 5.6B and C). Thus, in the presence of functional recycling machinery, a portion of endocytosed decorin was degraded in late endosomes and/or lysosomes. Cells treated with a combination of both monensin and E64d showed significantly reduced intracellular immunoreactivity of decorin as compared with untreated cells. However, decorin internalization was 3-fold higher than in cells treated with monensin only (p<0.01; Figure 5.6B and C). Taken together, these results indicate that a portion of endocytosed decorin is destined to recycling and a portion to late endosomes and/or lysosomes where it is rapidly degraded.

5.4.9 Decorin endocytosis depends on decorin-induced EGFR, IGF1R and LRP-1 signaling

Our findings showed that decorin associated with IGF1R, EGFR and LRP-1 in human gingival fibroblasts. To characterize the role of these receptors in decorin endocytosis, we pretreated fibroblasts with inhibitors against these receptors followed by treatment with 50 μg/ml of bovine decorin and quantitation of internalized decorin. After 15 min, the amount of internalized decorin was significantly reduced after treatment with function blocking antibodies against EGFR (by about 45%) or IGF1R (by about 40%) or with LRP-1 inhibitor RAP (by about 50%). Combination of all three inhibitors caused inhibition of decorin internalization by about 65% (Figure 5.7A and B). Pretreatment of cells with the SR-A inhibitors fucoidan or polyinosinic acid or with function blocking antibodies against αv, α2β1 or β1 integrins had no effect (data not shown). Thus, interaction of decorin with EGFR, IGF1R and LRP-1 regulates decorin endocytosis in human gingival fibroblasts.

In order to find out whether decorin-induced signaling regulates decorin endocytosis, we pretreated the cells with AG1478 to block EGFR signaling, AG1024 to block IGF1R signaling, LY294004 to block PI3K, rapamycin or everolimus to block mTOR, U0126 to block MEK1/2 or SB415286 to block GSK-3 pathway and analyzed decorin endocytosis as above. All inhibitors, with the exception of the MEK1/2 and GSK-3 inhibitors, significantly inhibited decorin endocytosis. Inhibitors to mTOR and AG1024 had the greatest effect (about 70% inhibition of
decorin internalization) while AG1478 and LY294002 had a smaller inhibitory effect (about 40% inhibition of decorin internalization; Figure 5.7A and C).

5.4.10 Decorin colocalizes mostly with LRP-1 and to a lesser degree with EGFR but not with IGF1R in endosomes

In order to find out which receptor delivers decorin to endosomes, we performed double immunostaining of internalized decorin and EGFR, IGF1R or LRP-1 at various time points after treatment with bovine decorin (50 μg/ml). Most internalized decorin colocalized with LRP-1 in endosomes at 15 min (Figure 5.7D). At this time point, some colocalization was also found for decorin and EGFR while there was no endosomal colocalization for decorin and IGF1R at any time point (Figure 5.7D and data not shown). Taken together, our data suggest that interaction of decorin with IGF1R, EGFR and LRP-1 induces signaling that involves PI3K and mTOR and regulates decorin internalization mediated mostly by LRP-1 and to a lesser degree by EGFR.

5.4.11 Decorin-induced ROS formation is required for decorin endocytosis

To address the question whether decorin-induced ROS formation is involved in regulation of decorin endocytosis, fibroblasts were treated with different concentrations of the ROS inhibitor NAC before decorin treatment, and the amount of endocytosed decorin was quantitated as above. DCF fluorescence as a measure of decorin-induced ROS formation was analyzed in similarly treated parallel cultures. NAC caused a significant concentration-dependent inhibition of decorin-induced ROS formation and decorin endocytosis while endocytosis of transferrin was not affected (Figure 5.8A and C-E). Similar results were obtained by using another ROS inhibitor, a hydroperoxide scavenger ebselen (40 μM; Figure 5.8B, F and G). Blocking of decorin endocytosis with clathrin siRNA treatment had no effect on decorin-induced ROS formation, confirming that ROS formation is upstream of clathrin-mediated decorin endocytosis (not shown).

5.4.12 Decorin-induced ROS formation and endocytosis depend on mitochondrial electron transport and NADPH oxidase activity

After establishing that decorin-induced ROS formation was critical for decorin endocytosis, we wanted to find out the intracellular source of decorin-induced ROS formation. To this end, we
pretreated cells with inhibitors against different intracellular sources of ROS. Electron transport system in mitochondria is one of the major sources of ROS production, and this pathway can be blocked by rotenone (Teeter et al., 1969; Hancock et al., 2001). In addition, cell membrane-associated NADPH oxidase can be activated by PI3K and catalyses ROS formation in various cells, including fibroblasts, and its activity can be blocked with DPI or apocynin (Stolk et al., 1994; Li and Trush, 1998; Hancock et al., 2001). Xanthine oxidoreductase catalyses the oxidation of hypoxantine to xantine leading to the production of ROS, and this reaction can be inhibited by allopurinol (Watts et al., 1965; Hancock et al., 2001). Xanthine oxidoreductase also produces nitrose oxide (NO\(^{•}\)), which can potentially contribute to the oxidation of DCF (Rao et al., 1992; Hancock et al., 2001). Nitrose oxide synthase can also be involved in production of NO\(^{•}\) and it can be inhibited with L-NAME (McCall et al., 1991; Hancock et al., 2001). The ROS inhibitor ebselen decreased decorin-induced ROS formation and decorin endocytosis by about 90% as compared to cells treated with decorin only (Figure 5.8B, F and G). DPI, apocynin and rotenone also significantly reduced decorin-induced ROS formation and endocytosis while allopurinol and L-NAME did not have significant effects (Figure 5.8B, F and G). These results indicate that mitochondrial electron transport and NADPH oxidase are the major sources of decorin-induced ROS formation and regulate decorin endocytosis in human gingival fibroblasts.

5.5 DISCUSSION

In the present study, we showed that decorin associates through its core protein with three distinct cell surface receptors in human gingival fibroblasts, namely EGFR, IGF1R and LRP-1. These interactions induced rapid tyrosine phosphorylation of the respective receptor cytoplasmic domains and downstream signaling that involves mTOR and its target p70S6K as well as PI3K, NADPH oxidase and mitochondrial electron transport-dependent formation of ROS. This signaling pathway regulated decorin endocytosis and lysosomal degradation mainly through the clathrin pathway.

The finding linking decorin-induced mTOR activation to ROS formation that regulates decorin endocytosis is particularly novel. In high concentrations, ROS cause cell and tissue damage, while when generated intracellularly in a controlled manner in small quantities, ROS, especially superoxide (O\(^{2−}\)), and hydrogen peroxide (H\(_2\)O\(_2\)), are powerful regulators of cell signaling, gene expression, growth and survival and exert their effect by oxidizing their target
molecules. In normal cells, intracellular ROS formation can be triggered by several stimuli, including certain growth factors, cytokines and integrins and is further modulated by downstream signaling events (Terada, 2006). In fibroblasts and other nonphagocytic cells, the primary source of intracellular ROS generation is the membrane-localized NADPH oxidase but mitochondrial electron transport chain and xanthine oxidoreductase can also be involved (H Hancock et al., 2001; Valko et al., 2007). By using inhibitors against the above pathways, our findings indicate that decorin-induced ROS generation requires NADPH oxidase, PI3K and mitochondrial electron transport activity while xanthine oxidoreductase is not involved.

Similar to the present study, the PI3K/mTOR pathway regulates ROS formation in certain transformed cells (Kim et al., 2005). Recently, a genome wide analysis using high throughput RNA interference strongly linked PI3K and mTOR pathway to regulation of clathrin-mediated endocytosis also in HeLa cells (Pelkmans et al., 2005). In Drosophila melanogaster, TOR interacts with Hsc70-4, a regulator of clathrin dynamics (Henning et al., 2006). However, it is not known whether TOR-dependent ROS formation is involved in this process. Previously, mTOR has been linked to the regulation of mitochondrial metabolism (Schieke et al., 2006) but it is not known whether it may regulate also other sources of ROS formation. How ROS regulate endocytosis is also unclear. However, oxidant activity, especially NADPH oxidase, regulates cell membrane dynamics and actin cytoskeleton, which are important players in clathrin-mediated endocytosis (Terada, 2006; Ungewickell and Hinrichsen, 2007). In epidermal cells, ROS formation is also critical for clathrin-mediated endocytosis of the keratinocyte growth factor receptor (Belleudi et al., 2006).

There is biochemical evidence that decorin core protein binds to EGFR in various cells that express high levels of EGFR, to IGF1R in kidney fibroblasts and endothelial cells and to LRP-1 in myoblasts (Iozzo et al., 1999; Santra et al., 2002; Schonherr et al., 2005; Brandan et al., 2006; Schaefer et al., 2007). Our study provides evidence that decorin interacts with all these receptors in human gingival fibroblasts, as decorin caused rapid clustering of these receptors and colocalized with them at the cell surface. Furthermore, inhibitors that block ligand binding to these receptors significantly reduced decorin binding to the cell surface. Decorin causes clustering of EGFR into large supramolecular complexes also in A431 cells (Zhu et al., 2005). This property of decorin could be attributed to its existence as dimers in physiological conditions, potentially allowing multiple interactions at the same time (Scott et al., 2003; Scott et
al., 2004). It remains to be shown whether decorin caused accumulation of IGF1R, EGFR and LRP-1 to the same clusters or signaling complexes.

Interestingly, decorin-induced signaling from EGFR, IGF1R and LRP-1 converged to the same pathway, as inhibition of signaling from each of the receptors separately significantly reduced decorin-induced mTOR phosphorylation and ROS formation. In kidney fibroblasts and endothelial cells, decorin induces IGF1R-mediated activation of PI3K, PKB/Akt and their downstream target mTOR but interestingly EGFR-mediated signaling does not appear to be involved (Schonherr et al., 2001; Schonherr et al., 2005; Schaefer et al., 2007). There is evidence, however, that EGFR is capable to regulate mTOR signaling also in certain transformed cells (Nomura et al., 2003). In myoblasts, decorin-induced signaling through LRP-1 also involves PI3K (Cabello-Verrugio and Brandan, 2007) but the role of other receptors or downstream targets have remained unexplored. Interestingly, blocking of decorin binding or signaling through all of these receptors at the same time inhibited decorin cell surface binding, decorin-induced ROS formation and internalization maximally by about 70-75% suggesting that also other receptors or signaling pathways are involved. ROS generation itself, however, is critically important for decorin endocytosis as evidenced by 90% inhibition of decorin internalization by ROS inhibitors.

Decorin colocalized mostly with LRP-1 and to a lesser degree with EGFR in endosomes. However, we did not find any endosomal colocalization of decorin with IGF1R although this receptor was present in endosomes. Therefore, decorin may be delivered to endosomes by LRP-1 and EGFR while signaling through all of these three receptors can trigger decorin endocytosis. Our findings also showed that decorin internalization occurred mainly via the clathrin-mediated pathway and delivered decorin partially to recycling endosomes and partially to lysosomal degradation. We can not completely rule out that caveolae pathway or another endocytosis pathway may also be involved to a smaller extent as endocytosed decorin occasionally colocalized with caveolin-1, and filipin, an inhibitor of caveolae endocytosis that may also affect other related endocytosis pathways (Marsh and Helenius, 2006), slightly reduced decorin internalization. This is similar to a previous study using human skin fibroblasts (Feugaing et al., 2007). In both skin (Feugaing et al., 2007) and gingival fibroblasts, blocking of EGFR signaling also reduced decorin endocytosis. However, unlike in the present study, inhibition of IGF1R did not have any effect on decorin endocytosis in skin fibroblasts (Feugaing et al., 2007). In contrast
to skin and gingival fibroblasts, decorin endocytosis was blocked by inhibition of caveolae pathway in A431 cells (Csordaz et al., 2000; Zhu et al., 2005). Thus, the mechanism of decorin endocytosis and signaling appears to be cell type dependent.

While decorin is widely expressed in the extracellular matrix of many different tissues and cells, it is somewhat surprising that different cells interact with it using different receptors and that the signaling events induced by decorin appear cell type specific. Other findings in the present study provide further evidence for the cell specificity. For instance, platelets or macrophages interact with decorin using α2β1 integrin or SR-A, respectively (Guidetti et al., 2002; Santiago-Garcia et al., 2003), while these receptors were not involved in gingival fibroblasts. Furthermore, unlike in tumor or sarcoma cells (Moscatello et al., 1998), decorin did not induce EGFR-mediated ERK1/2 phosphorylation in human gingival fibroblasts. Transformed cells express over ten times more EGFR compared to human gingival fibroblasts (Modeer and Andersson, 1990; Csordaz et al., 2000), and cellular response to decorin may depend on the type and number of decorin receptors they express. Furthermore, specificity and outcome of different signaling pathways, including ROS signaling, depend on the cellular context (Hancock et al., 2001; Shaul and Seger, 2007).

Decorin turnover by endocytosis may be an important mechanism to regulate decorin abundance in the extracellular matrix, and this has biological significance, as decorin regulates collagen fibrillogenesis and tissue integrity (Ameye and Young, 2002). It also binds and inactivates TGF-β and sequesters it to the extracellular matrix (Hildebrand et al., 1994). These functions may be important in tissue turnover during development, tissue homeostasis and wound healing. In addition, reduced abundance of decorin is associated with tumor growth and tissue fibrosis, and both of these processes can be reversed by ectopic decorin expression or administration of exogenous decorin (Kolb et al., 2001; Tralhao et al., 2003). Therefore, suppression of decorin endocytosis by ROS inhibitors may be an attractive target to promote accumulation of decorin in the extracellular matrix. As ROS are powerful signaling molecules, it is likely that decorin regulates also other cell functions through this pathway.

5.6 Acknowledgements

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Figure 5.1: Cell surface binding and internalization of decorin in fibroblasts.
Figure 5.1: Cell surface binding and internalization of decorin in fibroblasts. A: Fibroblasts grown on coverslips were treated with bovine cartilage decorin (DCN; 50 μg/ml) at 4°C for 20 min to allow DCN binding to cell membranes, cells were then washed to remove unbound DCN and incubated at 37°C to induce DCN internalization followed by fixation after different periods of time. Immunofluorescence staining was performed using a monoclonal anti-bovine DCN antibody. In order to localize cell surface-associated DCN, a set of cells were left non-permeabilized before staining. In order to localize intracellular DCN, another set of cells were permeabilized with 0.5% Tx-100 before staining. Magnification bar: 10 μm. B: Fibroblasts were treated with DCN (50 μg/ml) as above at 4°C for 20 min to allow cell surface binding of DCN, washed to remove unbound DCN and then fixed (time 0) or transferred to 37°C for 5 or 30 min to induce DCN internalization before fixation. After immunostaining of cell surface-associated DCN as above, the relative fluorescence intensity was quantitated by FACS analysis. C: Fibroblasts grown on coverslips were treated with increasing concentrations of DCN as above at 4°C for 20 min, washed to remove unbound DCN, transferred to 37°C for 15 min to allow DCN internalization and permeabilized and immunostained to analyze internalized DCN as in A. D: Fibroblasts grown on coverslips were treated with 50 μg/ml of DCN from bovine skin or cartilage (bDCN) or with equimolar amount of recombinant human DCN produced in S/21 cells or with recombinant human DCN GST-fusion protein encompassing the LRR1-12 (rh-DCN) expressed and purified from E. coli for 15 min as in A, permeabilized and immunostained for internalized decorin using anti-bovine or anti-human decorin antibodies as above.
Figure 5.2: Interaction of decorin with EGFR, IGF1R and LRP-1 and decorin-induced signaling.
Figure 5.2: Interaction of decorin with EGFR, IGF1R and LRP-1 and decorin-induced signaling. A: For double immunofluorescence staining, fibroblasts grown on coverslips were treated with bovine cartilage decorin (DCN; 50 μg/ml) at 4°C for 20 min, transferred to 37°C for 2 min, fixed and immunostained for cell surface-associated DCN and IGF1R, EGFR or LRP-1. The cell surface receptors were also localized in a set of cells that were treated as above except that they were not incubated with DCN. Arrowheads indicate cell surface clusters of IGF1R, EGFR or LRP-1 induced after DCN treatment. Arrows indicate colocalization of DCN with IGF1R, EGFR or LRP-1 at the cell surface clusters. B: Fibroblasts were pretreated with or without function-blocking antibodies against IGF1R (50 μg/ml), EGFR (50 μg/ml) or with RAP (80 μg/ml) for inhibition of LRP-1 ligand binding or with combination of all three receptor inhibitors at 37°C for 60 min. Cells were then incubated with DCN (50 μg/ml) together with the inhibitors for another 20 min at 4°C followed by an incubation at 37°C for 2 min. Cells were then fixed and immunostained for cell surface DCN. C: Staining intensity of DCN at the cell surface was quantitated by image analysis. D-G: To determine whether DCN induces phosphorylation of IGF1R, EGFR or LRP-1 or downstream targets ERK1/2, mTOR or p70S6K, cells were treated with DCN (50 μg/ml) for different periods of time at 37°C. Phosphorylation of target proteins were analyzed by immunoblotting using corresponding total proteins or β-actin as loading controls. H: Cells were pretreated with or without AG1478 (20 μM), AG1024 (10 μM) or RAP (80 μg/ml) before treatment with DCN for 10 min and phosphorylation of mTOR was analyzed by immunoblotting as above. Band intensities were quantitated relative to total mTOR expression in the same experiment. The results are expressed as mean +/- s.d. from three parallel experiments. Statistical comparisons were performed relative to DCN only treated samples. *: p < 0.05; **: p < 0.01.
Figure 5.3: IGFIR, EGFR and LRP-1 mediate decorin-induced ROS formation.
Figure 5.3: IGF1R, EGFR and LRP-1 mediate decorin-induced ROS formation.

A and C: Fibroblasts grown on coverslips were pretreated with 10 μM DCF and then treated with bovine cartilage decorin (DCN; 50 μg/ml) at 37°C for different periods of time, fixed and analyzed for relative DCF fluorescence intensity as an indicator of ROS formation. B and D: To determine whether decorin-induced ROS formation is concentration dependent, DCF-treated cells were incubated with or without increasing concentrations of DCN at 37°C for 10 min, and relative DCF fluorescence intensity quantitated as above. E: To study the role of cell surface receptors in DCN-induced ROS formation, fibroblasts were pretreated with DCF (10 μM) with or without AG1478 (20 μM), anti-EGFR antibody (50 μg/ml), AG1024 (10 μM), anti-IGF1R antibody (50 μg/ml), RAP (80 μg/ml) or with a combination of anti-EGFR and anti-IGF1R antibodies and RAP for 2 h. A set of parallel samples was pretreated with fucoidan (10 μg/ml) or with function blocking antibodies against α2β1 (10 μg/ml) or αv integrin (20 μg/ml) or with appropriate non-immune IgG (50 μg/ml). Cells were then treated with DCN (50 μg/ml) at 37°C for 10 min. Relative DCF fluorescence intensity was compared with cells treated with 50 μg/ml DCN or equimolar amount recombinant human DCN (rh-DCN) expressed and purified from E. coli only. F: To investigate whether decorin-induced ROS formation is up or downstream of mTOR activation, fibroblasts were pretreated with or without the ROS inhibitor NAC (100 mM) for 2 h followed by a 10 min treatment with DCN from bovine cartilage (50 μg/ml) at 37°C. Protein-extracts from treated and control cells were subjected to immunoblotting using antibodies against phosphorylated and total mTOR. G: To further study involvement of different signaling pathways in DCN-induced ROS formation, cells were pretreated with or without rapamycin (4 μg/ml), everolimus (10 μM), LY294002 (100 μM), U0126 (10 μM) or SB415286 (30 μM) followed by DCF and DCN treatment as above and analyzed for relative DCF fluorescence intensity. Statistical comparisons were performed relative to DCN only treated samples. *: p < 0.05; **: p < 0.01.
Figure 5.4: Decorin endocytosis is mainly mediated by the clathrin pathway.

A: To characterize decorin endocytosis pathway, cells grown on coverslips were incubated with bovine cartilage decorin (DCN; 50 µg/ml) for 20 min at 4°C, washed to remove unbound DCN, immediately fixed (time 0) or transferred to 37°C for indicated periods of time to allow DCN endocytosis before fixation. Parallel samples were treated with rhodamine-conjugated transferrin (TF; 5 µg/ml) as above. Cells were then permeabilized, and double immunostaining of internalized DCN with clathrin, caveolin-1 or TF was performed. Arrows indicate colocalization of internalized DCN with clathrin, TF or caveolin-1. B-D: To provide functional evidence for clathrin-mediated endocytosis of DCN, fibroblasts were pretreated with or without increasing concentrations of sucrose to inhibit clathrin-mediated endocytosis followed by a treatment with DCN or TF for 10 min as above and the relative fluorescence intensity of internalized TF or DCN were quantitated. E-H: Fibroblasts pretreated with clathrin or control siRNA (25 nM) were treated with DCN or TF for 10 min as above and analyzed for relative intracellular TF or DCN immunofluorescence intensity as above. Down regulation of clathrin expression was confirmed by qRT-PCR. *: p < 0.05; **: p < 0.01.
**Figure 5.5:** Role of caveolae pathway in decorin endocytosis.

A-C: To block the caveolae pathway, fibroblasts were pretreated with or without 0.5, 1 or 2 μg/ml filipin for 60 min followed by incubation with bovine cartilage decorin (DCN; 50 μg/ml) or FITC-conjugated-CTB (2 μg/ml) at 37°C for 10 min. After fixation, permeabilization and immunostaining for DCN, relative fluorescence intensity of internalized DCN or CTB was assessed. A and D: To confirm that filipin treatment did not affect the clathrin pathway, cells treated with filipin as above were treated with 5 μg/ml rhodamine-conjugated transferrin (TF) and fluorescence intensity of internalized transferrin was assessed after incubation at 37°C for 10 min. *: p < 0.05.
Figure 5.6: Internalized decorin is targeted to recycling endosomes and lysosomal degradation.

A: To analyze localization of decorin to different endosomal compartments after internalization, fibroblasts were treated with bovine cartilage decorin (DCN; 50 µg/ml) at 37°C for different periods of time, and double immunostaining of DCN with early endosomes (Rab5), recycling endosomes (Rab11) or lysosomes (Lamp-1) was performed. Arrows and arrowheads indicate colocalization of DCN with Rab5 or Rab11 expressing endosomes, respectively. B and C: Fibroblasts were pretreated with monensin (100 µM), E64d (20 µM) or with a combination of both inhibitors for 60 min followed by a 45 min incubation with DCN (50 µg/ml) at 37°C. Cells were fixed, permeabilized and immunostained for DCN. The relative amount of intracellular decorin was quantitated by measuring immunofluorescence intensity. Inserts in B show higher magnification images. Statistical comparisons were performed relative to decorin only treated samples. **: p < 0.01.
Figure 5.7: Role of EGFR, IGF1R and LRP-1 signaling in decorin endocytosis.
A, B and C: To investigate the role of decorin (DCN) binding to cell surface receptors in DCN endocytosis, fibroblasts were pretreated with or without function blocking antibodies against EGFR (50 μg/ml), IGF1R (50 μg/ml) or with RAP (80 μg/ml) or with a combination of all three receptor inhibitors for 2 h. To investigate whether DCN-induced signaling regulates endocytosis, cells were pretreated with or without AG1478 (20 μM), AG1024 (10 μM), LY294002 (100 μM), U0126 (10 μM), SB415286 (30 μM) or everolimus (10 μM) for 2 h or with rapamycin (4 μg/ml) for 6 h. Cells were then incubated with bovine cartilage DCN (50 μg/ml) for 10 min at 37°C and processed for decorin immunostaining and quantitation of relative intracellular DCN immunofluorescence intensity. D: To find out which receptor delivers DCN to endosomes, fibroblasts were treated with DCN (50 μg/ml) at 37°C for different time periods and colocalization of internalized DCN with EGFR, IGF1R or LRP-1 was analyzed by double immunostaining of permeablized cells. Arrows indicate intracellular colocalization of DCN with LRP-1 or EGFR. Statistical comparisons were performed relative to DCN only treated samples. *: p < 0.05; **: p < 0.01.
Figure 5.8: Decorin-induced ROS formation is required for decorin endocytosis.
Figure 5.8: Decorin-induced ROS formation is required for decorin endocytosis. A, C, D and E: Fibroblasts were pretreated with DCF (10 μM) and with or without increasing concentrations of ROS-scavenger NAC. Cells were then treated for 10 min with bovine cartilage decorin (DCN; 50 μg/ml) at 37°C before fixation and analysis of relative DCF fluorescence intensity. Parallel samples were treated with NAC as above without DCF treatment followed by an incubation with DCN (50 μg/ml) or rhodamine-conjugated transferrin (TF; 5 μg/ml) at 37°C for 10 min. After immunostaining for DCN, relative fluorescence intensity of internalized DCN or transferrin was analyzed. B, F and G: In order to analyze the intracellular sources of DCN induced ROS formation and their role in DCN endocytosis, fibroblasts were pretreated with DCF and with or without DPI (40 μM), apocynin (40 μM), rotenone (5 μM), allopurinol (50 μM) or L-NAME (50 μM) followed by DCN treatment and quantitation of ROS formation and intracellular DCN fluorescence intensity as above. DCN endocytosis was also examined following pretreatment of fibroblasts with another ROS-inhibitor ebselen (40 μM) as above. Magnification bar = 20 μm. Statistical comparisons were performed relative to DCN only treated samples. *: p < 0.05; **: p < 0.01.
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Chapter 6: General discussion, conclusion and future directions.
6.1 General discussion

Previous studies have discussed about expression and localization of SLRPs in different tissues, including periodontal ligament, skin, cornea, lung and kidney in different animal and human models (Oksala et al., 1995; Zhang et al., 1995; Cheng et al., 1999; Qian et al., 2004). There is biochemical evidence that gingival fibroblasts produce several proteoglycans, including decorin, biglycan and fibromodulin of the SLRP family in culture (Larjava et al., 1988; Larjava et al., 1992; Häkkinen et al., 1996), but little is known about the role of SLRPs in gingival wound healing. Gingival tissue shares common characteristics with early fetal skin. For example, gingiva displays faster extracellular matrix (ECM) turnover and wound closure and regeneration with no or minimal scar formation (Sciubba et al., 1978; Rowat and Squier, 1986). Thus, gingival tissue may provide a model to study wound regeneration. We wanted to investigate the role of SLRPs decorin, biglycan, fibromodulin, and lumican in gingival wound regeneration because these molecules bind to collagen and regulate collagen fibrilogenesis in different tissues. In addition, SLRPs regulate key cell functions involved in wound healing, including cell adhesion, migration, proliferation, gene expression, and activity of TGF-β (Hocking et al., 1998; Penc et al., 1998; Stander et al., 1999; Kresse and Schonherr, 2001). Increased activity of TGF-β leading to accumulation of excess collagen and other components of the ECM is the hallmark of fibrosis and scar formation (Cutroneo, 2003). Decorin, biglycan, and fibromodulin can interact and inhibit TGF-β activity and alleviate fibrosis (Hildebrand et al., 1994; Kolb et al., 2001a; 2001b). To this end, we first studied the localization of SLRPs in normal human gingiva and during wound healing. We hypothesized that changes in the localization and/or abundance of SLRPs regulate wound healing at different time points. Our preliminary unpublished findings also indicated that decorin interacted at the cell surface with the major collagen endocytosis receptor Endo180. Therefore, we also studied localization of this receptor during wound healing. Finally, our findings provided evidence that gingival cells may interact with decorin in vivo. Therefore, we studied the interaction of decorin with gingival fibroblast in more detail.

**Localization of SLRPs in ECM and cells in gingiva** - In gingiva, SLRPs localized to ECM and to certain cells. In the ECM, decorin was most abundant in the papillary connective tissue and localized to collagen fibers that appeared to be organized in thinner network-like structures than in the deeper connective tissue. In contrast, biglycan, fibromodulin and lumican were more abundant in the deep connective tissue that showed slightly thicker and denser
collagen organization. These data suggest that SLRPs interact with collagen in normal gingiva and may regulate collagen fibrilogenesis and collagen remodeling in a site-specific manner.

During wound healing, biglycan immunoreactivity was first observed after 7 days in wound connective tissue where the first signs of collagen deposition were observed. At days 14 and 28 after wounding, biglycan localized strongly to collagen fibers throughout the wound connective tissue while strongest association of decorin with collagen fibers occurred at 14-60 days post-wounding in subepithelial areas. These results suggest that decorin and biglycan collaborate to regulate collagen fibrilogenesis and organization especially during active collagen remodeling from 14 days post-wounding. While ECM-associated fibromodulin did not show notable changes during wound healing, lumican was significantly down-regulated in the ECM over time (Fig. 6.1). It is possible that this significant reduction of lumican abundance leads to reduced accumulation of collagen and less scaring in gingiva. Although no clinical signs of scar formation was observed at 60 days post-wounding, collagen organization and cell density in some areas in the deep wound connective tissue was not completely normalized. To confirm complete normalization of the SLRP abundance and collagen organization wounds collected at later time points than 60 days need to be studied. Interestingly, mice deficient in decorin, fibromodulin, lumican or both lumican and fibromodulin showed abnormal collagen fibril and fibril bundle morphology in the periodontal ligament (Matheson et al. 2005) suggesting the critical role of SLRPs in collagen fibrilogenesis and normalization of structure and organization of collagen also during wound healing.

SLRPs also associated with distinct connective tissue cells in gingiva and the immunoreactivity of SLRPs strongly increased in fibroblasts, myofibroblasts, pericytes, macrophages, endothelial and epithelial cells at certain time points during wound healing (Fig. 6.1). Localization of SLRPs to distinct cells suggests that SLRPs are produced by these cells, endocytosed by these cells or that they are present in the pericellular matrix or at the cell surface. SLRPs present in the pericellular matrix may influence interaction of cells with other extracellular molecules while cell surface-associated SLRPs may interact with cell membrane receptors and regulate cell signaling. Thus, SLRPs may regulate distinct cell functions during wound healing, including re-epithelialization, angiogenesis and ECM remodeling.

Re-epithelialization - Basal epithelium is comprised of keratinocytes that differentiate to give rise to the upper layers. Distinct localization of biglycan, fibromodulin, and lumican in
basement membrane zone and basal epithelial cells in unwounded gingiva suggests that these molecules may provide a distinct niche for basal epithelial cells and regulate epithelial cell differentiation. Previous studies have reported role of SLRPs in cell differentiation. For example, biglycan modulates osteoblast differentiation and matrix mineralization (Parisuthiman et al., 2005) and markedly stimulates the growth and differentiation of monocyctic cells from haemopoietic stem cells of the rat bone marrow (Tomoyasu et al., 1998). Lumican expression has been correlated with the differentiation of human osteosarcoma cells (Nikitovic et al., 2008).

During wound healing, restoration of epidermis occurs via re-epithelialization that is initiated by keratinocyte activation and migration. Spatiotemporal expression of probiglycan, fibromodulin and lumican in migrating and wound epithelium overtime suggests that SLRPs are involved in re-epithelialization of gingival wounds. Studies in lumican-deficient mice suggested that lumican expressed by injured corneal epithelium promotes keratinocyte cell adhesion and migration (Sakia et al., 2000) and re-epithelialization (Sakia et al., 2000; Yeh et al., 2005). Biglycan induces signaling by the small GTPases RhoA and Racl resulting in fibroblasts migration in lung (Tufvesson and Westergren-Thorsson, 2003) but nothing is known about its function in epithelial cells during wound healing. In addition, the role of fibromodulin in re-epithelialization warrants further studies.

Angiogenesis - Angiogenesis is a critical process in growth and development as well as in wound healing and involves the growth of new blood vessels from pre-existing ones and migration of endothelial cells into the wound bed (Burri et al., 2004). During gingival wound healing, endothelial cells started to emerge in the granulation tissue at day 3. Compared to normal unwounded gingiva, the number of blood vessels peaked at 7 and 14 days and normalized at 60 days post-wounding. Strong immunoreactivity of prodecorin, probiglycan and fibromodulin localized to pericytes and endothelial cells when formation of new blood vessels was underway. This suggests that SLRPs are involved in neovascularization during wound healing. Endothelial cells express decorin during angiogenesis in vitro and in vivo (Jarvelainen et al., 1991; 1992). Previous studies of the role of decorin in angiogenesis have shown conflicting results. Decorin deficiency led to impaired angiogenesis in injured mouse cornea (Schönherr et al., 2004). However, mice deficient in decorin showed accelerated revascularization in cutaneous wound healing (Järveläinen et al., 2006). In addition, decorin appears to suppress tumor cell-mediated angiogenesis (Grant et al., 2002). The role of biglycan and fibromodulin in neovascularization
during wound healing is unclear. Neovascularization did not significantly change in fibromodulin- or biglycan-deficient animals (Schönherr et al., 2004). However, increased expression of biglycan was found in migrating endothelial cells in a cell monolayer wounding model *in vitro* (Kinsella et al., 1997). In addition, biglycan enhances vascular smooth muscle cell proliferation and migration in mouse coronary arteries (Shimizu-Hirota et al., 2004). We observed strong immunoreactivity of lumican in endothelial cells and pericytes at later time points of wound healing when less neovascularization is required suggesting that lumican may slow down formation of blood vessels. This is supported by findings in mice showing that lumican inhibits angiogenesis (Albig et al., 2007).

**ECM remodeling** - Decorin, fibromodulin and lumican have been shown to protect collagen from degradation by collagenases (Geng et al., 2006) providing a potential mechanism by which they may modulate proteolytic collagen turnover during wound healing (McGaw and Ten Cate, 1983; Steffensen et al., 2001). Interestingly, we observed strong colocalization of decorin with collagen from 14-60 days post-wounding when wound tissue remodeling is underway suggesting that decorin regulates wound strength and integrity by inhibition of newly formed collagen degradation. While fibromodulin abundance did not change at ECM during remodeling phase, it is not clear whether increased presences of decorin compensated down-regulation of lumican during wound healing in this process.

Matrix turnover during wound healing can also occur by endocytosis and phagocytosis (Zhao et al., 1999; Madsen et al., 2007). Previous studies have described turnover mechanisms by which collagens, decorin and biglycan were internalized by receptor mediated endocytosis or phagocytosis in fibroblasts (Hausser et al., 1992; Everts et al., 1996; Wienke et al., 2003). Fibroblasts are considered to be the major cell type responsible for the maintenance and turnover of the connective tissue ECM. Type I collagen binds to the surface of fibroblast via $\alpha_2\beta_1$ integrin resulting in collagen phagocytosis (Goldberg, 1982; Goldberg and Burgeson, 1982). Interestingly, decorin can potently inhibit collagen phagocytosis by fibroblasts by a mechanism that does not appear to involve inhibition of integrin function (Bhide et al., 2005). Our preliminary unpublished findings indicated that decorin interacted with the major collagen endocytosis receptor Endo180. Therefore, it is possible that interaction of decorin with Endo180 may modulate collagen binding to Endo180 and regulate cellular uptake of collagen during tissue
remodeling. Therefore, we studied localization of this receptor during wound healing in relation to decorin.

**Localization of Endo180 during gingival wound healing** - Our double immunostaining results showed that during gingival wound healing prodecorin and Endo180 colocalized in fibroblasts (Fig. 6.2). In addition, during wound healing, the immunoreactivity of Endo180 and prodecorin appeared to follow the same trends in myofibroblasts, endothelial cells and pericytes but not in macrophages (Fig 6.2). Thus, these two molecules may interact during wound healing. Further studies are required to characterize interaction of Endo180 with decorin and the outcome in wound healing.

During wound healing, localization of Endo180 was temporally regulated in connective tissue cells and epithelium. For example, while we found distinct localization of Endo180 in blood vessels of normal unwounded gingiva, endothelial cells in the small and/or new forming blood vessels and pericytes showed little or no expression of Endo180 during wound healing. However, at later time points the large and well-differentiated blood vessels strongly expressed Endo180 (Fig 6.2). Previous studies have also reported that in the placenta, the larger blood vessels expressed Endo180, while the smaller ones were negative (Sheikh et al., 2000). Currently, the role of Endo180 in vascularization is not known but our findings suggest that Endo180 may be more involved in the later development or maintenance of the blood vessels. In addition, the up-regulation of Endo180 expression in tumor endothelium suggests a role for Endo180 in tumor angiogenesis (St. Croix et al., 2000).

Endo180 regulates directional sensing and migration of cells (Sturges et al., 2003). In certain cells, Endo180 associates with uPAR and uPA to form a trimolecular complex (Behrendt et al., 2000). Binding of uPA to uPAR initiates focalized plasminogen activation and promotes proteolytic degradation of the fibrin clot and facilitates keratinocyte migration in wound healing (Romer et al., 1996). During gingival wound healing, Endo180 localized in basal epithelial cells and its expression was increased in migrating keratinocytes. Thus, Endo180 may be involved in keratinocytes migration during re-epithelialization by the above mechanisms. Interestingly, strong immunoreactivity of Endo180 in the basal and suprabasal wound epithelium during wound healing coincided with gradual increase in the thickness and maturation, suggesting a role for Endo180 also in the differentiation of new epithelium.
Endo180 is one of the key molecules mediating ECM turnover and remodeling. Endo180 may regulate these processes by different mechanisms. For example, studies showed reduced collagen uptake and turnover in Endo180 gene knockout mice (East et al., 2003; Engelholm et al., 2003; Curino et al., 2005). Endo180 was strongly localized to fibroblasts during wound healing particularly from 14-28 days post-wounding when connective tissue remodeling and collagen uptake and internalization potentially occurs. This suggests a role of Endo180 in matrix turnover during gingival wound healing. We also observed strong upregulation of Endo180 in myofibroblasts after 14 days (Fig. 6.2). This coincided with wound contraction and alignment of collagen fibrils in the wound ECM. Wound contraction is achieved by myofibroblasts that attach to collagen by integrin-type collagen receptors and contract the collagen network. Endo180 is involved in Rho-ROCK-based contractile signaling and Endo180 containing endosomes have been shown to regulate contractile signals at the site of adhesion turnover (Sturge et al., 2006) providing a possible mechanism by which it may be involved in wound contraction.

Growth factors such as TGF-β1, EGF or PDGF strongly up-regulated the expression of Endo180 in human gingival fibroblasts suggesting that these growth factors play a key role in the regulation of Endo180 expression in vivo. TGF-β and PDGF play critical roles in matrix production, re-epithelialization and fibroplasia (Werner and Grose 2002; Niessen et al., 2001). EGF produced by fibroblasts is involved also in keratinocyte migration and re-epithelialization (Wenczak et al., 1992; Yu et al., 1994). Reports indicate a significant increase in the concentrations of TGF-β1, and PDGF within one week after injury while EGF appears to increase at later time points (Watelet et al., 2002a, 2002b). Interestingly, we observed increased immunoreactivity of Endo180 in wound epithelium from 7-28 days post-wounding suggesting that growth factors may regulate re-epithelialization at least in part by modulating Endo180 expression during wound healing.

**TGF-βs in gingival wound healing** - Accumulation of abnormally organized collagen fibers as a response to increased activity of TGF-β1 relative to TGF-β3 is one of the major characteristics of scar formation (Shah et al., 1995; Ferguson and O’Kane, 2004). Evidence indicate that exogenously added TGF-β3 to wounds reduces skin scar formation (Shah et al., 1995). However, the role of TGF-β in re-epithelialization is not quite clear.

In gingival wound healing, TGF-β1 and TGF-β3 showed strong coexpression in wound epithelium overtime but the biological significance of this finding remains to be shown.
instance, endogenously increased latent TGF-β1 expression in basal keratinocytes in transgenic mice resulted to accelerated wound closure in partial-thickness wounds by promoting keratinocyte migration and re-epithelialization (Tredget et al., 2005; Reynolds et al., 2005) while in full-thickness wounds these mice showed delayed re-epithelialization (Chan et al., 2002). There is also conflicting data suggesting that altered TGF-β signaling either prevents or enhances re-epithelialization process. For example, overexpression of Smad2 in cutaneous wounds in mice resulted in defective keratinocytes migration and delayed healing (Hosokawa et al., 2005) while lacking type II TGF-β receptor resulted in increased proliferation of keratinocytes and accelerated dermal re-epithelialization (Amendt et al., 2002). Remarkably, either overexpression of Smad3 or lacking Smad3 both result to accelerated wound re-epithelialization (Roberts et al., 2003; Kloeters et al., 2007). TGF-β3 has been shown to protect keratinocytes against experimentally induced cell death in vitro and in vivo (Li et al., 1999) and may serve for similar function in gingival wound healing.

During wound healing, particularly after 60 days, TGF-β3 showed relatively higher immunoreactivity in wound connective tissue cells as compared to TGF-β1 (Fig. 6.1). Thus, increased abundance of TGF-β3 relative to TGF-β1 may contribute to reduced scar formation in human gingival wounds. It is not clear whether strong immunoreactivity of fibromodulin and biglycan in wound epithelium and of decorin, biglycan and fibromodulin in fibroblasts and ECM from 14 to 60 days post-wounding (Fig. 6.1) is linked to TGF-β1 retention. However, decorin, biglycan and fibromodulin have been shown to potently sequester TGF-β1 activity in vitro and in vivo (Hildebrand et al., 1994; Shah et al., 1994; 1995). Studies in a rat model of fetal wound healing suggest that fibromodulin may be a biologically relevant modulator of TGF-β1 activity during scar formation (Soo et al., 2000). Interestingly, adenoviral-mediated overexpression of fibromodulin and decorin decreased expression of TGF-β1 and TGF-β2 and reduced scar formation in incisional wounds (Stoff et al., 2007) or tissue fibrosis in lung (Kolb et al., 1994).

**Interaction of decorin with gingival fibroblasts** - We provided evidence that gingival cells may interact with SLRPs during wound healing as they colocalized with wound cells. Therefore, we studied the interaction of decorin, the best characterized member of the SLRPs family, with gingival fibroblast in more details. Decorin has high amino acid sequence homology with other SLRPs and may thus share biological activities with other members of the SLRP family. Fibroblasts play critical role in ECM turnover and during wound healing and studying
the interaction of decorin with gingival fibroblasts may provide valuable information about the role of SLRPs in wound healing. We showed that decorin binds to IGF1R, EGFR and LRP-1 in gingival fibroblasts and induced clustering of these receptors at the cell surface. Findings of structural and biophysical properties of decorin showed that it exists in solution as highly stable dimers (Scott et al., 2003; 2004). Decorin dimers possess large contact areas that are associated with high binding affinities (Scott et al., 2003). Therefore, decorin might be able to exploit its dimeric nature to bridge different ligands and bind to several cell surface receptors and aggregate them to form clusters. Previous studies have shown that receptor clustering is critical for their signaling function (Hato et al., 1998; Strasser et al., 2004). It is also required for matrix turnover mediated by endocytosis (Schekman and Singer, 1976).

Glycosylation and/or absence of the GAG chain from the decorin molecules did not affect its interaction with the cells suggesting that interactions of decorin with IGF1R, EGFR and LRP-1 in fibroblasts is mediated by the core protein of the molecule. LRR6 of the decorin core protein has been shown to bind to EGFR (Santra et al., 2002). However, it is not known which LRR mediates binding of decorin to IGF1R and LRP-1. As simultaneous blocking of IGF1R, EGFR and LRP-1 did not completely inhibit binding of decorin to the cell surface, also other receptors that function as decorin receptors might also exist and remain to be characterized. Based on our preliminary data, Endo180 is one candidate molecule that may serve as a novel decorin receptor.

Interactions of decorin with fibroblasts induced tyrosine phosphorylation of IGF1R, EGFR and LRP-1 leading to formation of reactive oxygen species (ROS). ROS are potent signaling molecules and have been shown to regulate various cell functions including, gene expression and growth (Hancock et al., 2001). Thus, decorin may regulate expression of different genes involved in wound healing through induction of ROS. We showed that decorin-induced ROS formation was mediated by mTOR as separate inhibition of IGF1R, EGFR and LRP-1 significantly decreased decorin-induced mTOR phosphorylation and ROS formation. Formation of ROS has been linked to the mTOR pathway also in transformed cells (Kim et al., 2005). Our findings also indicate that ROS formation is necessary for decorin internalization by fibroblasts and NADPH oxidase and mitochondrial electron transport are major sources for decorin-induced ROS formation.
Our findings showed that after binding to the cell surface decorin was rapidly internalized. Therefore, we wanted to analyze whether interaction of decorin with EGFR, IGF1R and LRP-1 regulates decorin endocytosis. Our results indicated that fibroblasts endocytosed decorin mostly in association with LRP-1 and to a lesser degree with EGFR but not with IGF1R. These data suggest that decorin endocytosis is mediated by LRP-1 and EGFR while signaling through IGF1R, EGFR and LRP-1 regulates decorin internalization. In human skin fibroblasts, blocking of EGFR signaling significantly reduced decorin endocytosis (Feugaing et al., 2007). However, decorin did not associate with IGF1R in endothelial cells (Schonherr et al., 2005). LRP-1 is also involved in decorin internalization in myoblasts and CHO cells through clathrin-mediated endocytosis (Brandan et al., 2006).

We also identified clathrin-mediated endocytosis pathway as a major route for decorin internalization in gingival fibroblasts. Cargos from clathrin-coated vesicles usually enter first to the early endosomes (Rubino et al., 2000) followed by transportation to late endosomes expressing and/or to lysosomes for degradation or to recycling endosomes (de Renzis et al., 2002). In gingival fibroblasts, decorin was first delivered to early endosomes after which it was routed to recycling endosomes and lysosomes for degradation. Decorin is an antifibrotic molecule that binds to and inhibits activity of TGF-β (Hildebrand et al., 1994). Uptake and lysosomal degradation of decorin may decrease its abundance in the ECM. Of note, in tissue fibrosis or cancer stroma, decorin abundance was found to be significantly lower than in normal tissue. Interestingly, overexpression of decorin reverses tissue fibrosis or prevents tumor growth (Kolb et al., 2001; Tralhao et al., 2003). Therefore, drugs that reduce ROS formation and decorin endocytosis may increase presence of decorin in the ECM and help to alleviate fibrosis.

6.2 Summary and conclusions
In normal unwounded gingiva, SLRPs colocalized with type I collagen. During wound healing, SLRPs associated with new forming and mature collagen fibers. Together with evidence showing collagen abnormalities of SLRP gene knockout mice in periodontal tissues, these data suggest that SLRPs collaborate to regulate collagen fibrilogenesis in normal gingiva and during wound healing.

SLRPs associated with fibroblasts, myofibroblasts, endothelial cells, pericytes, macrophages and epithelial cells in normal unwounded gingiva. During wound healing SLRPs
were spatiotemporally regulated in distinct wound granulation and connective tissue cells and in epithelium. Given the role of SLRPs in critical cell functions such as cell adhesion, migration, and proliferation and activity of TGF-β, the findings suggest that SLRPs regulate wound re-epithelialization and connective tissue regeneration. Compared to TGF-β1, TGF-β3 showed higher immunoreactivity during wound healing particularly at later time points (Fig. 6.1) suggesting a role for TGF-β3 in the reduction of scaring in gingival tissue.

Endo180 colocalized with decorin in wound fibroblasts during gingival wound healing. The immunoreactivity of Endo180 and decorin appeared to follow the same trends in these cells during wound healing (Fig 6.2) suggesting that they may interact with each other during wound healing. During wound healing, immunoreactivity of Endo180 was spatiotemporally regulated in the differentiating wound epithelium and in subsets of wound myofibroblasts, pericytes, macrophages and endothelial cells (Fig. 6.2). Growth factors increased the expression of Endo180 in cultured human keratinocytes and fibroblasts suggesting they regulate Endo180 expression in vivo. Given that Endo180 regulates cell migration and collagen remodeling by fibroblasts, our findings suggest that Endo180 plays a role in re-epithelialization and connective tissue remodeling during wound healing.

Decorin associated with EGFR, IGF1R and LRP-1 in gingival fibroblast cell surface and induced rapid tyrosine phosphorylation of these receptors followed by NADPH oxidase- and mitochondrial electron transport-dependent formation of ROS (Fig. 6.3). ROS formation was mediated by decorin-induced activation of mTOR and p70S6K via interaction with EGFR, IGF1R and LRP-1 and was necessary for decorin endocytosis in fibroblasts. Decorin was internalized mainly by clathrin-mediated endocytosis leading to endosomal degradation and recycling of decorin (Fig. 6.3). This study provides a mechanism by which fibroblasts may regulate decorin abundance in the ECM. Inhibition of ROS leading to reduced decorin endocytosis may increase bioavailability of decorin in the ECM that may result in down-regulation of TGF-β activity and reduced scaring.

6.3 Future directions
In this study, we characterized expression and localization of SLRPs, TGF-βs and Endo180 in normal, unwounded gingiva and during human gingival wound healing as a model for non-scaring tissue. However, the role of SLRPs during skin wound healing that often results in scar
or hypertrophic scar formation is not completely clear. Therefore, characterization of protein and
gene expression levels of SLRPs relative to activity of TGF-β1 on tissue biopsies obtained from
skin and gingiva during wound healing would be needed. Creation of experimental skin wounds
in human may have ethical restrictions and/or subject limitations. Therefore, by using an
established and well-characterized pig wound model, it would be possible to compare gingival
wound regeneration with wound healing leading to scar (occurs in Yorkshire pigs) or
hypertrophic scar (occurs in Red Duroc pigs) formation in skin in the same animals. The pig skin
model resembles human skin wound healing due to similarities in relative thickness of the
dermis, epidermis and similar density of skin components and re-epithelialization process (Wang
et al., 2000). In order to demonstrate that increased expression of decorin may prevent scar
formation, decorin gene delivery technique using viral vectors or exogenously added decorin
could be used to increase decorin abundance in pig skin wound healing. The role of fibromodulin
or biglycan in the reduction of scaring could also be examined by inducing overexpression of
these SLRPs during skin wound healing. It is possible that marked reduction of lumican
abundance during gingival wound healing contributes to reduced scaring. To investigate this,
local administration of lumican-siRNA to down-regulate lumican expression during pig skin
wound healing and scar formation can be used.

Our preliminary findings also showed that decorin may interact with Endo180 in gingival
fibroblasts. During wound healing, Endo180 and decorin colocalized in fibroblasts and also
showed same pattern of immunoreactivity in pericytes and endothelial cells. However, it is not
clear whether decorin interacts with Endo180 in these cells and what is/are the outcome(s) of
these interactions. One possibility is that decorin bound to Endo180 may modulate interaction of
Endo180 with collagen and regulate collagen turnover. Therefore, further studies are required to
analyze the functional role of End180 / decorin interaction in collagen turnover in vitro and vivo.

We demonstrated that ROS formation was required for decorin endocytosis. Therefore,
drugs that inhibit ROS formation may be used to promote accumulation of decorin in the ECM
leading to reduced bioavailability of TGF-β and reduced scar formation. In addition, detailed
studies are required to elucidate the role of decorin-induced ROS formation in cell signaling. In
particular, TGF-β1 signaling pathway is appealing since it plays a central role in wound healing.
Therefore, further studies are required to investigate whether decorin regulates TGF-β signaling
pathway by mechanisms that involve decorin-induced ROS formation in gingival fibroblasts.
ROS potentially induce the expression of wide range of genes including genes that are involved in wound healing. However, it is unclear which genes are targeted by decorin-induced ROS formation. Genes that are involved in scar formation and tissue remodeling including type I collagen and MMPs and growth factors such as TGF-βs, PDGF, and CTGF are particularly molecules of interest. The data may provide valuable information to control wound healing and scar formation. Finally, the role of other SLRPs in the regulation of ROS formation needs to be identified.
and deep (b) granulation or wound connective tissue over time. Figure 6.1: Summary of relative immunoreactivity of SFRPs and TGF-βs in subepithelial (A)
Figure 6.2: Relative proportion of cells showing immunoreactivity for decorin and Endo180 during wound healing.

Proportion of cells showing immunoreactivity for prodecorin and Endo180 showed similar trend in αSMA expressing fibroblasts (A), pericytes (B), and capillary endothelial cells (C) but not in macrophages (D) during wound healing in gingiva.
Figure 6.3: Schematic model of decorin-induced signaling in human gingival fibroblasts.
6.4 References


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