FUNCTIONAL INTERACTIONS BETWEEN THE DISCOIDIN DOMAIN RECEPTOR 1 AND β1 INTEGRINS

by

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A thesis submitted in conformity with the requirements for the degree of

Master of Science

Graduate Department of Dentistry

University of Toronto

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Abstract

The rate limiting step of phagocytosis is the binding of collagen to specific receptors, which include β1 integrins and the discoidin domain receptor 1 (DDR1). While these two receptors may interact, the functional nature of these interactions is not defined. We examined the effects of DDR1 over-expression on β1 integrin function and determined that DDR1 over-expression enhanced cell attachment through β1 integrins. These data are consistent with data showing that DDR1 over-expression enhanced cell-surface, but not total, β1 integrin expression and activation. As shown by experiments with endoglycosidase H, DDR1 over-expression increased glycosylation of the β1 integrin subunit. Collectively these data indicate that DDR1 enhances β1 integrin interactions with fibrillar collagen, possibly by affecting the processing and trafficking of β1 integrins to the cell surface. Our data provide insight into the mechanisms by which fibrotic conditions such as cyclosporine A-induced gingival overgrowth are regulated.
Acknowledgments

My Master of Science has been the most challenging, most tiring, but by far, the most rewarding two years of my life. However, I cannot take sole credit for the completion of my degree, as this work would not have been possible if not for the support of so many wonderful individuals. First and foremost, I would like to thank Dr. McCulloch for taking a chance and allowing me to work in his lab. His immense intelligence and passion for science have been an inspiration, and his guidance and support, invaluable. I have felt it a privilege from day one to be working under his supervision. I am more than grateful to Dr. Moriarty for being endlessly generous with her time and for always providing me with valuable advice and direction. I am thankful to my committee member Dr. Bendeck for her more than helpful suggestions. Thank you to Wilson Lee for always making me feel like the funniest person alive by laughing at my jokes, no matter how bad they were, and of course, for the endless hours he has put in to help make my thesis complete. For much of the contents of this work I must also thank Stephen Spano, working with him was always a blast. I would like to say thanks to Carol Laschinger for teaching me how to make the perfect western blot and to my lab-mates Hamid Mohammadi, Dhaarmini Rajshankar, Qin Wang, Vanessa Pinto, Dominik Fritz, Ilana Talior and Pam Arora for their advice, friendship and fun times in the lab. I would like to thank Andrew Peters for inspiring me to not be afraid to pursue science, for keeping me motivated, and for his constant support and encouragement. Last but not least, I would like to thank my family. My grandparents Grete and Peter for their financial, and of course emotional investment in my future, and to my brother PJ and parents Gail and Herb for their unconditional love and support.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACER2</td>
<td>Alkaline ceramidase 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division control protein 42 homolog</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>DDR1 &amp; -2</td>
<td>Discoidin Domain receptor 1 and 2</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GnT</td>
<td>N-acetylglicosamine-glycosyltransferase</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HGF</td>
<td>Human gingival fibroblast</td>
</tr>
<tr>
<td>IG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus kinase 2</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>LAMP-2</td>
<td>Lysosomal associated membrane protein 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated kinase</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney epithelial cell line</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositide 3-kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecil sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>STAT5</td>
<td>Signal transducer and activator of transcription 5</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of metalloproteinase</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection fluorescence</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNT</td>
<td>Tris-HCL, NaCl, Tween</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor associated factor 6</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar-type H+-adenosine triphosphatase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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Chapter 1

Literature Review

I. Soft Connective Tissue

I. A. Structure and Function of the Extracellular Matrix

The extracellular matrix (ECM) is the major structural component of connective tissues (Alberts, 2002) providing support and attachment for organs, ligaments, fascia and other specialized components of organs and tissues (Hynes, 2009). The ECM acts to support and orient layers of cells at the basement membrane and in the interstitium provides a substrate for cell migration. ECM molecules are ligands for cell adhesion and signaling through cell surface receptors. The mechanical properties of the ECM regulate many aspects of cell behavior (Hynes, 2009). Further, the ECM is essential for the differentiation, proliferation, survival, polarity, and migration of cells with which it is in contact (Alberts, 2002; Hynes, 2009; Kim et al., 2011; Woessner, 1993).

Mammalian genomes encode hundreds of ECM proteins, including collagens, laminin, fibronectin, proteoglycans and glycosaminoglycans (Alberts, 2002; Hynes, 2009; Kinane, 2000; Woessner, 1993). Variations in the number, organization, composition and distribution of ECM proteins contribute to the distinctive structure and function of connective tissues (Hynes, 2009). The most abundant component of the ECM is the family of collagens (Eckes et al., 1999; Kim et al., 2011; Kinane, 2000; Perez-Tamayo, 1978), which together endow the ECM with impressive structural and information-processing properties.
I. A. i. Collagens

Collagens are a family of proteins with at least 29 known members (Kim et al., 2011). Type I collagen makes up approximately 80% of the global extracellular matrix in mammals (Eckes et al., 1999) although minor collagens such as type II, III, V and XI are also present in defined tissues and in special circumstances such as in development and wound healing (Eckes et al., 1999; Eyre, 2004; Kim et al., 2011; Narayanan and Page, 1983). All collagens share a common structural motif of helical fibrils that are formed by three protein subunits. The primary function of collagens is to act as the structural support for connective tissues; collagens also act as binding partners for other ECM proteins (Kim et al., 2011).

Interstitial collagens consist of three α chains composed of approximately 1000 residues with repeating Gly-X-Y triplets, where X and Y are most commonly proline and hydroxyproline, respectively (Chung et al., 2004). Due to the high imino-acid content and the tri-peptide unit repeats, each α chain assumes a left-handed helical conformation, producing a 95 kDa procollagen α chain subunit. The three left-handed α chains intertwine with one another to form a right-handed collagen fibril which then bind together with other collagen fibrils to form the collagen fiber (Chung et al., 2004; Messent et al., 1998; Meyer and Morgenstern, 2003). The triple-helical conformation of collagen makes interstitial collagens resistant to most proteinases (Chung et al., 2004). In vertebrates, intracellular, lysosomal hydrolases known as cathepsins and extracellular collagenases known as matrix metalloproteinases (MMPs), cleave the triple-helical structure of collagens to enable degradation and remodeling of the ECM in health, development and disease (Chung et al., 2004; Messent et al., 1998).
I. B. Cells of the Soft Connective Tissue

Connective tissue cells comprise a very large group of cells in soft and mineralizing tissues and include for example, fibroblasts, chondrocytes, adipocytes, osteoblasts, osteocytes, odontoblasts and smooth muscle cells, all of which are specialized for the secretion and degradation of collagens and other ECM proteins (Alberts, 2002). Fibroblasts in particular are the cells responsible for the maintenance and organization of soft connective tissues (Grinnell, 2003). Previously, fibroblasts were believed to only synthesize the supporting ECM framework for other cell types. More recently they are reported to function in a wide range of immune and inflammatory responses (Fries et al., 1994; Williams, 1998).

In response to cytokines, fibroblasts are able to modify their production of ECM components, which allow them to respond and cooperate with cells in complex responses such as wound healing. Intercellular communication between fibroblasts and cells of the immune system occurs frequently and contributes to the maintenance of homeostasis in mammalian tissues. For example, in chronic inflammatory diseases, perturbations of fibroblast function can lead to the development of disabling fibrotic disorders (Williams, 1998).

II. Homeostasis

Connective tissue remodeling results in a change in shape and/or structure of tissues whereas turnover involves the degradation and replacement of ECM proteins with no alteration in tissue structure. In both cases there is no impairment of tissue function. The functional differences between turnover and remodeling are relatively minor and the two terms are often used
interchangeably. Notably, in both processes, the synthesis and degradation of ECM must be kept in a steady state to maintain normal tissue form and function (Ten Cate and Deporter, 1974).

II. A. Function

Homeostasis involves a dynamic, functional balance between the production and breakdown of ECM components (Knowles et al., 1991). In soft connective tissues, fibroblasts contribute to tissue homeostasis and maintain equilibrium by balancing synthesis and degradation of collagen in physiological remodeling (Knowles et al., 1991). In normal, healthy tissues, remodeling is necessary for growth, development and for tissue repair in various pathological conditions including inflammatory diseases (Cox and Erler, 2011; Knowles et al., 1991).

II. B. Regulatory Mechanisms in Collagen Homeostasis

The mechanisms that regulate collagen homeostasis are not completely defined. Currently, two major routes for collagen degradation have been identified: an extracellular and an intracellular pathway (Knowles et al., 1991). In soft connective tissues, three separate extracellular pathways for ECM degradation have been identified: the MMP pathway, the plasmin pathway, and the pathway involving polymorphonuclear leukocyte-derived serine proteinases (Birkedal-Hansen et al., 1993). The polymorphonuclear leukocyte serine protease pathway mediates degradation of the ECM at neutral pH (Brower and Harpel, 1982) through the release of serine proteases, neutrophil elastase and cathepsin G (Birkedal-Hansen et al., 1993; Pham, 2006). These proteases are able to cleave various proteins including collagens, laminin, fibronectin and proteoglycans (Birkedal-Hansen et al., 1993).
Plasminogen, the precursor to plasmin, is found at high levels circulating in lymph and interstitial fluids (Birkedal-Hansen et al., 1993). This broad spectrum enzyme plays an important role in matrix remodeling, whereby activated plasmin rapidly cleaves Lys-Arg peptide bonds exposed on the surface of a wide range of ECM macromolecules including fibrin and fibronectin (Birkedal-Hansen et al., 1993). Although type I and II collagens cannot be cleaved by plasmins, these enzymes can activate MMPs (Davis et al., 2001) and thereby increase the degradation rates of collagen and other ECM molecules by MMPs.

II. B. i. Matrix Metalloproteinases

MMPs are zinc-dependent endopeptidases that can be activated at neutral pH. These enzymes are secreted in a pro-form and are typically activated near the cell surface by other activated MMPs or by serine proteinases. Their activity is tightly regulated by tissue inhibitors of metalloproteinases (TIMPs) (Davis et al., 2001; Hwang et al., 2009; Messent et al., 1998) and by cell receptors such as β1 integrins (Eckes et al., 1999). The MMP family consists of at least 23 known members, three of which are interstitial collagenases (Haas et al., 2000). MMP-1, -8 and -13 are the only three known members of this family that can cleave and degrade triple helical collagen fibrils, generating fragments approximately one quarter and three quarters of the total length of the native molecule (Chung et al., 2004; Eyre, 2004; Messent et al., 1998). These fragments denature at temperatures below physiological body temperatures, thereby allowing normally insoluble collagen to denature into gelatin (Eyre, 2004; Messent et al., 1998). Gelatin in turn is degraded further by gelatinases such as MMP-2 and MMP-9.

The role of collagenases is not just simply to degrade the collagen matrix; these enzymes also function to control cell behavior during tissue remodeling. Collagenase cleavage fragments alter
cellular activity by expressing cryptic biological functions (Chung et al., 2004) such as the promotion of epithelial cell migration during wound healing (Zhao et al., 1999) and the enhancement of tumor necrosis factor-α (TNF-α) processing and release (Birkedal-Hansen, 1995; Santibanez-Gallerani et al., 2000).

**III. Disorders of Homeostasis**

**III. A. Mechanisms of Fibrosis**

When the balance between the synthesis and breakdown of collagen is disrupted, disorders of the ECM such as fibrosis may develop. Inflammatory disease of the skin, liver, kidney, heart, gingiva and lung can all be attributed to overabundance and deregulated structural organization of ECM proteins, whether through reduced degradation, increased synthesis or inappropriate molecular organization (Fries et al., 1994). For example in osteoarthritis there is a shift towards increased proteolytic damage by MMPs that lead to the destruction of joint cartilages (Eyre, 2004). Similarly in periodontal tissues, the gingival crevicular fluid of patients with progressive periodontitis exhibits much higher active collagenase activity compared with healthy and gingivitis patients, emphasizing the role of active collagenase in destructive periodontitis (Kinane, 2000; Overall et al., 1991). Collagenase disrupts the fibrous meshwork of the ECM and increases matrix porosity (Berrier and Yamada, 2007), facilitating infiltration by inflammatory cells, as is seen in early stages of gingivitis (Kinane, 2000).

Conversely, collagen degradation may be inhibited in various fibrotic disorders. An *in vitro* study which examined collagen-coated bead internalization by human gingival fibroblasts noted a large reduction in bead internalization in cells obtained from fibrotic lesions compared with
cells from normal tissues (McCulloch and Knowles, 1993). Methylglyoxal has been detected in increased concentrations in the gingival crevicular fluids of patients with chronic periodontitis infections (Kashket et al., 2003). This glucose metabolite has been shown to block the binding step of phagocytosis though modifications of the Gly-Phe-Hyp-Gly-Glu-Arg binding motif in collagen (Chong et al., 2007). Methylglyoxal can also be found at high levels in patients with diabetes where individuals often suffer from cardiac fibrosis. Recent data suggest that methylglyoxal not only inhibits cell-matrix attachments but also promotes the development of intercellular adhesions and myofibroblast differentiation (Yuen et al., 2010).

Excessive production of matrix proteins is also associated with several diseases and fibrotic conditions (Diegelmann and Evans, 2004). In radiation-induced fibrosis, type I collagen production is increased 14-fold compared with healthy tissues (Remy et al., 1991). Similarly, in fibroblast cultures from individuals with progressive systemic scleroderma, there was a marked increase in type I and II collagen synthesis (Krieg et al., 1985). In oral sub-mucous fibrosis, a potential mechanism for increased collagen production has been suggested whereby transforming growth factor β (TGF-β)-induces synthesis of connective tissue growth factor-CCN2, which in turn is mediated by c-Jun NH2-terminal kinase (JNK), activin receptor-like kinase 5, and the p38 mitogen activated kinase (MAPK) (Chang et al., 2012).

**III. B. Drug-Induced Gingival Overgrowth**

The gingiva and periodontal ligament are excellent models for the study of ECM homeostasis in soft connective tissues because they exhibit a remarkably rapid rate of collagen turnover (Sodek, 1977). Further, drug-induced gingival overgrowth offers a good example of the complexities of homeostatic imbalance when small perturbations of collagen turnover occur as a result of certain
drugs that affect collagen remodeling. Drug-induced gingival overgrowth is associated with three different classes of drugs: phenytoin, an antiepileptic; cyclosporin A (CsA), an immunosuppressant; and the calcium channel blockers nifedipine and diltiazem (Kataoka et al., 2005). The severity and prevalence of drug-induced overgrowth differs between medications and there are also very large inter-patient variations of overgrowth in response to drugs. What is most perplexing about these disorders is that the pathological mechanisms of gingival overgrowth are not well-defined. Currently, these disorders appear to be induced by disruptions in collagen homeostasis (Kataoka et al., 2005).

**III. B. i. Cyclosporin A-Induced Gingival Overgrowth**

In the context of CsA-induced gingival overgrowth (Diagram 1), several studies have described potential mechanisms by which gingival fibrosis is induced. It has been proposed that MMPs, specifically those that degrade collagen and gelatin, and TIMPs, are implicated in CsA-induced fibrosis (Gagliano et al., 2004; Hyland et al., 2003; Murai et al., 2011; Tipton et al., 1991). Currently, the literature to support this view is inconclusive. MMP levels are reported as being either unchanged (Gagliano et al., 2004; Murai et al., 2011; Tipton et al., 1991) or decreased (Hyland et al., 2003) in patients who take CsA. Similarly, TIMP levels have been reported as unchanged (Gagliano et al., 2004; Hyland et al., 2003) or increased (Tipton et al., 1991) by CsA.

In fibroblasts, CsA inhibits the release of calcium ions from intracellular stores, such as the endoplasmic reticulum (Arora et al., 2001) and uptake of calcium ions by mitochondria (Gagliano et al., 2004), thereby blocking integrin activation and inhibiting collagen phagocytosis. Further, the lysosomal enzymes cathepsins B and L, which digest collagen intracellularly, have been identified as possible targets for inhibition by CsA. Recently it has been shown that CsA
impairs cathepsin B and L activity and down-regulates their synthesis (Murai et al., 2011; Omori et al., 2007). Although the exact mechanisms of drug-induced fibrosis are unknown, collagen degradation by fibroblasts in the gingiva is clearly inhibited (Arora et al., 2001; Chan et al., 2007).

Image courtesy of: http://www.exodontia.info/Drug-Induced_Gingival_Hyperplasia.html

IV. Phagocytosis

IV. A. Mechanism of Action

The intracellular or phagocytic pathway of collagen degradation is distinct from the extracellular, MMP-dependent pathway. In certain tissues such as the periodontium, MMPs are thought to be responsible for most ECM degradation that occurs in inflammatory lesions (Sodek and Overall, 1988) whereas phagocytosis by fibroblasts is believed to be responsible for the majority of collagen turnover in healthy tissues (Segal et al., 2001; Ten Cate and Deporter, 1974). Phagocytosis is a receptor-driven process that is dependent on remodeling of the actin
cytoskeleton and shares some of the cytoskeletal regulatory components involved in cell migration and cell adhesion (Arora et al., 2008; Segal et al., 2001).

As outlined in Diagram 2, the process of collagen degradation can be broken down into five major stages that involve: A) the initial recognition of the fibril by membrane-bound collagen receptors; B) partial digestion of collagen by cell-surface MMPs; C) envelopment of the collagen fibril by the phagocytic cup, and subsequent internalization into the phagosome; D) fusion of the phagosome with the lysosome to form the phagolysosome, followed by a rapid decrease in pH to allow for E) lysosomal digestion by cysteine proteases, such as cathepsins B and L (Everts et al., 1996; Yajima, 1986).

Diagram 2. Intracellular pathway of collagen degradation: Phagocytosis

Image courtesy of: Dr. C.A.G. McCulloch
Internalization of the collagen fibril in phagocytosis is mediated by the initial binding of collagen by cognate receptors such as the α2β1 integrin (Arora et al., 2000). Further, in addition to the importance of the α1β1 integrin in the binding step of phagocytosis, this adhesion receptor may also regulate the internalization step (Lee et al., 1996). During phagocytosis, actin assembly and integrin activation are coordinated through activation of members of the Rho family of small guanosine triphosphatases (GTPase) in response to extracellular signals including collagen-binding (Arora et al., 2008; Cougoule et al., 2004; Del Pozo et al., 2002). The small GTPase Rap1 is known to activate β1 integrins (Bos, 2005; de Bruyn et al., 2002) and is also required for collagen phagocytosis. Activation and recruitment of Rap1 to collagen binding sites are dependent on non-muscle myosin II-A filament assembly (Arora et al., 2008), which is implicated in cytoskeletal regulation and phagocytosis (Kang et al., 2002). Following phagocytosis, the α2 integrin subunit is rapidly recycled or synthesized (Lee et al., 1996). While little is known of the regulation of collagen degradation by phagocytosis, this process is thought to be stimulated by TGF-β (van der Zee et al., 1995).

As indicated above, digestion of phagocytosed particles in lysosomal compartments is largely dependent on the activity of cysteine proteinases such as cathepsin B (Everts et al., 1996). These enzymes cleave collagens in helical and non-helical regions that are distinct from MMP cleavage sites and cleavage requires an acidic environment with an optimal pH range of 4.0-5.0 (Burleigh et al., 1974). Acidification of the phagolysosome is necessary for collagen degradation but not for the fusion events that lead to phagosomal maturation (Arora et al., 2000). Phagosomes normally fuse with lysosomes downstream of collagen binding and internalization, which lead to the production of phagolysosomes, wherein the ingested materials are degraded (Oh and Swanson, 1996; Saftig and Klumperman, 2009). In collagen-containing vacuoles, the abundance
of lysosomal associated membrane protein 2 (LAMP-2), a late endosomal maturation marker that is enriched in endosomes, peaks at 120 minutes after internalization of collagen-coated beads, indicating that maximal fusion of the phagosome and lysosome occurs at about 120 minutes after initial collagen binding (Arora et al., 2000). The phagolysosome is generally considered the endpoint of the phagocytic pathway; however, there is evidence suggesting that the phagolysosome continues to undergo maturational changes after phagosome-lysosome fusion and that internalized particles can be further fragmented (Oh and Swanson, 1996). Notably, the vacuolar-type H⁺-adenosine triphosphatase (V-ATPase) inhibitor bafilomycin A₁ affects transit of proteins from early to late endosomes (Clague et al., 1994) and late endosomes to lysosomes (van Weert et al., 1995), suggesting that phagosomal maturation is a distinct pathway from endosomal maturation.

V. Receptors

V. A. Collagen binding: The rate limiting step in matrix remodeling

The cellular recognition and binding to localized domains on collagen fibrils are significant early events in the phagocytic pathway. Collagen binding is the rate-limiting step in phagocytic degradation of collagen by fibroblasts (Chong et al., 2007; Knowles et al., 1991). Previous work has demonstrated that in gingival fibroblasts the rate of phagocytosis of fibronectin- (McKeown et al., 1990) and collagen-coated (Knowles et al., 1991) latex beads is dependent on attachment to cell-surface receptors. Recognition and attachment systems in fibroblasts include cell surface receptors with high avidity for collagen (Knowles et al., 1991) such as integrins, specifically the α2β1 integrin, which is the principal adhesion receptor for type I fibrillar collagen (Chong et al., 2007; Dickeson et al., 1999)
Many collagen receptors have been identified in mammalian tissues including integrin heterodimers that comprise β1 integrins, discoidin domain receptors, glycoprotein VI, leukocyte-associated immunoglobulin (IG)-like receptor 1, and the mannose receptor family which includes endo180 and urokinase receptors (Leitinger and Hohenester, 2007). The β1 integrins are the best defined and most studied mammalian receptors for cell adhesion to collagen (Hynes, 2002).

V. B. β1 Integrins

V. B. i. Structure and Function

The term integrin first came into use in the 1980’s to describe cell surface receptors that “integrated” the cytoskeleton of one cell with that of an extracellular matrix protein (Schnapp, 2006). Integrins are heterodimeric cell adhesion receptors consisting of two, non-covalently associated α and β subunits. There have been 18 α and 8 β subunits identified in mammals, which pair to form a total of 24 integrins. The β1 subunit in particular, pairs with 12 different α subunits (Gu and Taniguchi, 2004; Schnapp, 2006; Sun et al., 2009). Each pairing of an α subunit with the β1 integrin leads to the formation of receptors with generally specific and non-redundant functions. Indeed, the specific α and β integrin subunits contribute to ligand specificity (Hynes, 2002) as outlined in Diagram 3. Aside from the β4 integrins, all β subunits have a large extracellular domain, a single pass trans-membrane domain and a short cytoplasmic tail (Gu and Taniguchi, 2004; Schnapp, 2006). The extracellular, N-terminal domains of α and β subunits associate to form the integrin headpiece, which contains the ECM binding site (Gu and Taniguchi, 2004). The C-terminal cytoplasmic region links to the actin cytoskeleton through binding to proteins such as talin, filamin, and vinculin, and also mediates interactions with cytoplasmic signaling molecules (Critchley, 2000; Gu and Taniguchi, 2004). Ligation of actin
filaments to integrins regulates the mobility of collagen receptors, thereby enhancing the recruitment and increasing the interaction of integrins with collagen during cell spreading (Arora et al., 2003).

Diagram 3. The β1 integrin family of extracellular matrix receptors.

Image courtesy of: (Lal et al., 2009)

The integrin family of cell adhesion receptors is essential for development, cell proliferation and wound healing (Gu and Taniguchi, 2004; Schnapp, 2006). The functional activity of integrin receptors are affected by subtle changes in the cellular environment, post-translational modifications, their organization and orientation at biological membranes (Alberts, 2002), and the presence of divalent cations, such as Ca$^{2+}$ and Mg$^{2+}$ (Schnapp, 2006). These various molecules and modifications to integrins, promote or suppress ligand binding, change ligand specificity, or stabilize integrin structure (Schnapp, 2006). For instance, Mg$^{2+}$ promotes cell adhesion whereas ligand affinity may be decreased by Ca$^{2+}$ or increased by Mn$^{2+}$ (Schnapp, 2006).
V. B. ii. Integrin Signaling and Regulation of Activity

In addition to their roles in enabling cell adhesion to the ECM, integrins also act as trans-membrane linkages that connect the ECM to the cytoskeleton (Hynes, 2002; Hynes, 2009). The cytoplasmic tails of integrins do not contain any intrinsic catalytic activity but instead act as organizing centers and scaffolding molecules for other signaling components and cytoskeletal proteins (Schnapp, 2006). The linkage provided by integrins facilitates the triggering of a large variety of signal transduction events that occur in a bidirectional mode. Through inside-out signaling, intracellular signals control cell motility and cell-ECM or cell-cell adhesion, whereas outside-in signals relay extracellular messages into cells (Hynes, 2009; van der Flier and Sonnenberg, 2001). Many integrins when bound by ligand can activate the focal adhesion kinase (FAK), which subsequently activates a number of classical signaling pathways such as MAPKs, phosphatidylinositol 3 kinase (PI3K), and protein kinase C (Schnapp, 2006). These signals regulate cell behavior including proliferation, survival, polarity, migration and differentiation (Hynes, 2009; Schnapp, 2006).

As shown in Diagram 4, integrins exist in different conformational states but at rest, most integrins exist in a low affinity state (Schnapp, 2006). In response to ligand binding, signaling pathways are activated that cause a conformational change in integrins to allow high affinity binding (Calderwood, 2004). This change in integrin conformation is known as affinity modulation (Schnapp, 2006). Ligand binding may also be strengthened through avidity modulation, which involves clustering of individual integrin molecules at a discrete, ligand binding site within the plasma membrane (Calderwood, 2004; Schnapp, 2006).
Diagram 4. β1 integrin activation in response to collagen binding.
Image courtesy of: (Niland and Eble, 2012)

V. B. iii. Glycosylation

Glycosylation is one of the most common types of post-translational modifications (Hagglund et al., 2007) and indeed the majority of secreted and cell surface proteins are glycosylated (Gu and Taniguchi, 2004). Glycosylation denotes a class of structurally diverse modifications that comprise O and N-linked glycans with carbohydrate functional groups ranging from monosaccharides to large, branched oligosaccharide chains (Gu and Taniguchi, 2004; Hagglund et al., 2007). Carbohydrates on the cell surface are frequently the first molecules to be
encountered and recognized by other cells, antibodies and invading viruses and bacteria. Accordingly these molecules contribute to a variety of interactions between the cell and the extracellular environment (Gu and Taniguchi, 2004).

β1 integrins are glycosylated by N-glycans (Gu and Taniguchi, 2004). The β1 integrin subunit is initially synthesized as an 87 kDa polypeptide core and partially glycosylated in the endoplasmic reticulum (ER) to form the immature β1 (β1 precursor) (Sun et al., 2009). The β1 precursor is transported to the Golgi complex where it is further glycosylated to produce the mature β1 (Akiyama et al., 1989; Bellis, 2004; Sun et al., 2009). This conversion from the precursor to the mature form is denoted as the maturation of β1 integrins (Sun et al., 2009). Once the mature β1 pairs with an α subunit, the heterodimer is transported to the cell surface (Sun et al., 2009).

While it is unknown how this process is regulated, the β1 maturation process represents a mechanism by which the function of β1 integrins may be controlled (Gu and Taniguchi, 2004; Sun et al., 2009). The adhesion of integrins to specific peptide sequences of a particular ligand is based on binding to α and β receptor subunits; however, strength of binding may be modulated by glycosylation (Zheng et al., 1994). For example, cell-ECM or cell-cell adhesion and other cellular processes mediated by integrins may be modulated by β1 maturation (Gu and Taniguchi, 2004; Sun et al., 2009). Zheng et al. (1994) showed that N-glycosylation is necessary for the association of the α5 and β1 subunits and for normal binding of the adhesion receptor to fibronectin. TGF-β accelerates β1 maturation, thereby increasing cell surface levels of β1 integrins and enhancing cell adhesion (Bellis et al., 1999). Talin and lipoprotein receptor-related protein-1 are required for the proper maturation process of the β1 integrin (Salicioni et al., 2004; Sun et al., 2009). Sphingosine that is generated in the Golgi complex through the action of the human alkaline ceramidase 2 (ACER2), but not other ceramidases, inhibits β1 integrin
maturation, leading to defects in cell-ECM adhesion (Sun et al., 2009). In contrast, RNA interference-mediated inhibition of ACER2 promotes β1 maturation and cell adhesion (Sun et al., 2009).

V. C. DDR1

V. C. i. Structure and function

The discoidin domain receptors (DDRs) are a more recently discovered family of non-integrin-type collagen receptors. Two gene products have been identified in mammals so far, and these include DDR1 and DDR2, which constitute a subfamily of tyrosine kinase receptors (RTK) (Leitinger, 2011; Vogel, 1999; Vogel et al., 1997). Both DDRs are different from other RTKs in that they are activated only by binding to collagens in their native triple-helical form (Vogel et al., 1997). DDR1 in particular is activated by all collagens tested so far, including collagens type I-VI and VIII (Leitinger, 2011). Further, while RTK activation of most cell surface receptors is generally very rapid, tyrosine phosphorylation of DDR is delayed, peaking at 90 minutes and lasting upwards of 18 hours after cell attachment to collagen (Vogel et al., 1997).

DDR1 functions as a collagen sensor and is activated by several types of collagen, subsequently triggering ECM degradation and turnover (Franco et al., 2002). In smooth muscles cells, the absence of DDR1 is associated with decreased cell proliferation, migration, and MMP and collagen synthesis (Franco et al., 2002). DDR1 and non-muscle myosin IIA interact to regulate adhesive contacts with collagen since DDR1 promotes cells spreading and is important for the assembly of non-muscle myosin IIA heavy chain into filaments on cells plated on collagen (Huang et al., 2009).
DDR1 and -2 contain an extracellular discoidin domain that is composed of approximately 160 amino acids and contains two discoidin domains, which are members of the coagulation factor V/VII type C superfamily (Ferguson, 2012). No other receptor tyrosine kinases contain extracellular discoidin domains, but these domains are found in a number of other proteins involved in cell adhesion (Ferguson, 2012). The extracellular region shares homology with a region identified in the protein discoidin I from the slime mold *Dictyostelium discoideum*, where it functions as galactose-binding lectin (Matsuyama et al., 2003).

Recombinant preparations of DDR1 have been used to identify loops 1 and 3 as the sites of ligand binding and receptor activation (Vogel et al., 2006). The juxtamembrane regions of DDRs are much longer than other RTKs and it has been speculated that similar to several growth factor receptors or Eph receptor subfamilies, this region has an auto-inhibitory function that accounts for the delayed kinetics of DDR1 phosphorylation (Vogel, 1999).

Unlike other RTKs whereby ligand binding precedes receptor dimerization, it is thought that DDR1 exists as pre-formed dimers on the cell surface. Collagen binding activates signaling cascades by altering the conformation of these dimers instead of by inducing receptor oligomerization (Lemmon and Schlessinger, 2010). Recent reviews present conflicting notions regarding the role of DDR1 cell surface dimers. According to Valiathan et al. (2012), DDR1 receptor dimerization of the ectodomains is a requirement for collagen binding. To support this view, ligand-independent DDR1 dimers were found to originate within the biosynthetic pathway and to be stable at the cell surface even in the absence of collagen (Abdulhussein et al., 2008; Mihai et al., 2009; Noordeen et al., 2006). Conversely, Ferguson et al. (2012) presented evidence indicating that short triple-helical collagen peptides are sufficient to activate DDR1, suggesting
that receptor clustering induced by collagen is not an essential aspect of DDR1 activation (Ferguson, 2012).

**V. C. ii. DDR1 Isoforms**

DDR1 maps to human chromosome 6 and is composed of 17 exons, which are alternatively spliced to produce five transcript variants, giving rise to five different isoforms (Valiathan et al., 2012). Conversely, DDR2 has 19 exons, which encode for only a single transcript (Valiathan et al., 2012). The DDR1 α- and β-receptor isoforms lack 37 and 6 amino acids respectively, whereas the c-isoform is the longest at 919 residues. DDR1d is truncated and is missing the entire kinase region. DDR1e is “kinase dead” and lacks sections of the juxtamembrane region and the ATP binding site (Alves et al., 2001; Playford et al., 1996; Valiathan et al., 2012). The most commonly expressed isoforms are DDR1 α and β. The relative expression ratios of these isoforms and their post-translational modifications appear to be controlled by complex but poorly understood regulatory mechanisms (Vogel et al., 2006).
Diagram 5. Structure of DDR1 transcript variants and isoforms.

Image courtesy of: (Alves et al., 2001)

V. C. iii. DDR1 Signaling Pathways

The activation of DDR1 appears to regulate a wide range of processes, suggesting that DDR1 may exert its effects through several pathways (Ruiz and Jarai, 2011). However, in general, the downstream signaling pathways of DDR1 are not well defined (Vogel, 1999). It has been proposed that DDR1 interacts with several cofactors including ShcA, Nck2, SHP-2, signal transducer and activator of transcription 5 (STAT5), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and p38 MAPK (Ruiz and Jarai, 2011). Src regulates DDR1 activation and downstream signaling through ERK1/2 and influences cell migration (Lu et al., 2011).
Downstream signaling of DDR is dependent on which DDR isoform is expressed. For instance, upon collagen-induced tyrosine phosphorylation, DDR1b associates with the phosphotyrosine-binding domain of the ShcA adaptor protein through its LLXNPXY motif (Vogel et al., 1997). DDR1b in macrophages activates the TNF receptor associated factor 6 (TRAF6) complex, triggering the p38 mitogen activated protein kinase and NF-κB pathways (Abdulhussein et al., 2004; Baumgartner et al., 1998; Vogel et al., 1997). Conversely, fibroblast-growth factor receptor substrate-2 binds to the juxtamembrane region of DDR1a (Vogel, 2001). SHP-2 is necessary for suppression of STAT1 and STAT3 tyrosine phosphorylation, cell migration and branching tubulogenesis induced by DDR1 a- or b- isoforms (Wang et al., 2006).

V. C. iv. DDR1 and Fibrosis

DDR1 has been implicated in various fibrotic conditions as indicated by experiments using knock-out mice and DDR1 silencing and over-expression systems. Flamant and colleagues (Flamant et al., 2006) reported reduced fibrosis and immune cell infiltration in DDR1 knock-out mice compared with wild-type animals, which exhibited increased fibrosis and macrophage and leukocyte infiltration and increased microalbumin, an indicator of renal disease. Similarly, Gross and co-workers (Gross et al., 2010) demonstrated that loss of DDR1 expression in mouse kidney delayed renal fibrosis and inflammation by reducing TGF-β and connective tissue growth factor. Compared with wild-type controls, DDR1 knock-out mice exhibit reduced collagen levels, myofibroblast differentiation and immune cell infiltration in bleomycin-induced lung fibrosis (Avivi-Green et al., 2006). The absence of DDR1 decreased smooth muscle cell proliferation and collagen synthesis, thereby reducing intimal thickening of the arterial wall (Franco et al., 2002). In cirrhotic liver, transcriptional profiling using DNA array and RT-PCR have detected elevated
DDR1 mRNA expression by more than two-fold, which is manifest in hepatocytes, leukocytes and biliary epithelial cells (Song et al., 2011). In the same study it was found that there was increased abundance of soluble and membrane-associated DDR1 fragments of varying size, which were detected in cirrhotic liver but not in liver. Further, increased DDR1a expression affected cell behavior in hepatocytes, which included elevations of cell adhesion and immobilization of cells on type I collagen and fibronectin (Song et al., 2011).

**VI. DDR1 Interactions**

**VI. A. DDR1 and Metalloproteinases**

As indicated above, cell attachment to type I collagen induces DDR1 phosphorylation and activation as well as the production of a C-terminal fragment of DDR1 (Vogel, 2002). The generation of the C-terminal fragment can be blocked by metalloproteinase inhibitors, indicating that DDR1 might represent a novel target for limited proteolysis by a family of enzymes known as disintegrin metalloproteinases or secretases (Slack et al., 2006; Vogel, 2002). Two inhibitors of transporter-associated-with-antigen-processing subunit 1 and TIMP-3 caused dose-dependent reductions in collagen-induced shedding of the DDR1 ectodomain but did not block generation or phosphorylation of the C-terminal fragments. These data suggest that liberation of the DDR1 C-terminal fragments may be mediated by a distinct collagen-regulated protease (Dejmek et al., 2003; Franco et al., 2002). Conceivably, DDR1 ectodomain shedding may be part of an inhibitory feedback loop that serves to regulate the ability of DDR1 to promote adhesion and migration of cells on collagen (Slack et al., 2006).
DDR1 is widely expressed in fast-growing invasive tumors of breast (Johnson et al., 1993), ovary (Laval et al., 1994), esophagus (Nemoto et al., 1997) and brain (Ram et al., 2006). Hepatocellular carcinoma cells over-expressing either DDR1 a- or b-isoforms showed a significant increase in MMP-2 and -9 expression, indicating that increased tumor invasiveness may be linked to increased DDR1 and may be mediated by MMP-2 or -9 (Park et al., 2007). To support this finding, Hou and co-workers (Hou et al., 2001) found that MMP-2 and -9 levels were down-regulated in DDR1-null smooth muscle cells. Ruiz and Jari (2011) showed that in normal human lung fibroblasts, type I collagen is able to induce DDR1 and MMP-10 expression through integrin-independent activation of DDR2. These authors also found that type I collagen-induced DDR1 gene expression requires recruitment of phospho-Janus kinase 2 (JAK2) to DDR2 and extracellular signal regulated kinase (ERK) 1/2 activation, and involves the recruitment of the nuclear factor PEA3 to the DDR1 promoter region (Ruiz and Jari, 2011).

VI. B. DDR1 and β1 Integrins

DDR1 and β1 integrins are two completely different types of collagen receptors. DDR1 can be activated in the presence of β1 integrin blocking antibodies, indicating that DDRs can participate in signaling responses independent of integrins (Vogel et al., 2000). Notably, some of the downstream signaling pathways activated by DDR1 appear to intersect with β1 integrin-activated pathways (Valiathan et al., 2012).

In MDCK cells, DDR1 blocks integrin→FAK→Cdc42-mediated cell spreading (Yeh et al., 2009) and integrin-STAT1/3-mediated cell migration (Wang et al., 2006). A recent study by Suh and Han (2011) examined the effect of collagen I on mouse embryonic stem cell self-renewal and related signal pathways. They found that binding of type I collagen to β1 integrins activated
integrin-linked kinase, Notch and Gli-1. In contrast, type I collagen binding to DDR1 activated Ras, PI3K/Akt and ERK. Both activated Gli-1 and ERK enhance Bmi-1 expression, which leads to cell cycle progression and increased cell proliferation (Suh and Han, 2011).

Coordinated DDR1 and β1 integrin signaling can induce upregulation of N-cadherin expression, cell scattering and epithelial to mesenchymal transition in response to collagen I in pancreatic cancer cells (Shintani et al., 2008). In this experimental system, DDR1 activates Pyk2 while integrin β1 activates FAK. The signaling scaffold protein p130Cas, which binds to FAK and Pyk2, also binds DDR1 in pancreatic cancer cells. The Pyk/FAK-p130Cas complex activates JNK1, which then upregulates N-cadherin through c-Jun, leading to cell scattering (Shintani et al., 2008). Based on these examples, it is conceivable that DDR1 and β1 integrins may play cooperating or antagonizing roles in their interactions with type I collagen and the cellular processes that they control. Currently it is not known how these two different receptor systems interact to control cell adhesion to collagen and their potential involvement in the generation of fibrotic lesions.
Statement of the Problem

Collagen remodeling by fibroblasts is crucial for physiological matrix turnover and is disrupted in inflammation, fibrosis and malignancy. In physiological conditions, the balance between secretion and degradation of the collagen matrix is maintained. However, disruptions of collagen phagocytosis and the resultant imbalances of matrix homeostasis have important clinical consequences in various tissues, specifically the gingiva in which the rate of collagen turnover is one of the fastest of any connective tissue. The rate limiting step of phagocytosis is the binding of fibrillar collagen to specific receptors, which include β1 integrins and the discoidin domain receptor 1 (DDR1). The receptor tyrosine kinase, DDR1 is endogenously expressed in fibroblasts along with the β1 integrin. Previous data suggest that these two receptors interact, but, the functional nature of these interactions has not been well defined. One of the central steps in phagocytosis, which determines tissue homeostasis is collagen binding to cells. My preliminary data indicates that DDR1 over-expression in fibroblasts enhances collagen binding through β1 integrins. Currently, the mechanism by which this interaction is enhanced is not well defined.

Hypothesis

The Discoidin Domain Receptor 1 enhances β1 integrin binding to type I collagen.

Objectives

1. Determine whether DDR1 expression affects collagen remodeling.
2. Examine whether DDR1 regulates cell adhesions.
3. Determine the role of DDR1 in mediating β1 integrin cell surface expression and activation.
4. Assess a potential role for DDR1 in regulating β1 integrin glycosylation.
5. Examine whether DDR1 plays a role in drug-induced fibrosis of gingival connective tissues.
Chapter 2

Introduction

Homeostasis of connective tissue in many organ systems is maintained through balanced synthesis and degradation of matrix proteins but is disrupted in inflammatory and fibrotic diseases. A critical, defining process that contributes to connective tissue homeostasis is collagen degradation, which in physiological remodeling is mediated by collagen phagocytosis (Everts et al., 1996). Collagen phagocytosis by fibroblasts is a receptor-driven process in which cellular recognition and binding to localized domains on collagen fibrils are crucial regulatory events in the phagocytic pathway (Chong et al., 2007; Knowles et al., 1991). Collagen recognition and attachment systems in fibroblasts include cell surface receptors with high affinity for collagen such as integrins (Knowles et al., 1991), specifically the α2β1 integrin, which is an important adhesion receptor for type I fibrillar collagen (Chong et al., 2007; Dickeson et al., 1999). The α2β1 integrin is also an important regulatory molecule for the binding step of collagen phagocytosis (Arora et al., 2000; Lee et al., 1996).

The functional activity of β1 integrin receptors is affected by a broad range of regulatory molecules and processes including the concentration of divalent cations such as Ca$^{2+}$ and Mg$^{2+}$ (Schnapp, 2006), collagen structure and folding, and the clustering, allosteric modifications, post-translational modifications, organization and arrangement of integrins at cell membranes (Alberts, 2002). N-linked glycosylation in particular, is a poorly defined, post-translational regulatory mechanism for control of β1 integrin function (Bellis, 2004). Variations of β1 integrin glycosylation may influence receptor conformation (Bellis, 2004) surface expression (Akiyama et al., 1989; Hotchin and Watt, 1992), and receptor-mediated functional activity including cell
adhesion and spreading on collagen (von Lampe et al., 1993). Diskin et al., 2009) Since β1 integrin binding is affected by differences of glycosylation, it is likely that the downstream signaling processes that regulate cell adhesion are also affected, which includes the recruitment of actin binding proteins such as talin, paxillin and vinculin to focal adhesion complexes (Critchley, 2000; Keselowsky et al., 2004). While variations of normal glycosylation of the β1 integrin have been identified in tumor cells (Bellis, 2004), the role of integrin glycosylation in the regulation of collagen adhesion and phagocytic function has not been described.

In addition to fibrillar collagen-binding integrins, discoidin domain receptors (DDRs) are a separate family of collagen receptors that exhibit tyrosine kinase activity after ligand binding (Leitinger, 2011) but differ from other receptor tyrosine kinases in that collagens are the primary ligand for DDR activation (Vogel et al., 1997). DDR1 in particular is activated by many types of collagens and appears to act as a sensor that triggers ECM degradation and turnover (Franco et al., 2002; Leitinger, 2011). The biological importance of DDR1 in physiological matrix turnover is supported by experiments using genetic disruption that manifest as a variety of fibrotic conditions of kidney (Flamant et al., 2006; Gross et al., 2010), liver (Song et al., 2011), and lung (Avivi-Green et al., 2006). Consequently, normal expression of DDR1 may be important for connective tissue homeostasis.

DDR1 is tyrosine phosphorylated and activated by cell binding to collagen even in the presence of β1 integrin blocking antibodies, indicating that DDR1 can participate in signaling responses independent of β1 integrins (Vogel et al., 2000). Curiously, downstream signaling pathways activated by DDR1 can also intersect with β1 integrin-activated pathways (Valiathan et al., 2012). For example, activation of DDR1 inhibits integrin→FAK→Cdc42-mediated cell spreading (Yeh et al., 2009) and integrin→STAT1/3-mediated cell migration (Wang et al., 2006).
After stimulation with type I collagen, β1 integrin activates Gli-1 whereas DDR1 activation stimulates ERK; combined activation of these two proteins enhances Bmi-1, which drives cell proliferation (Suh and Han, 2011). In pancreatic cancer cells, coordinated DDR1 and β1 integrin signals can induce N-cadherin trafficking to the cell membrane, cell scattering and epithelial to mesenchymal transition in response to type I collagen (Shintani et al., 2008). Collectively these data indicate that β1 integrin and DDR1 may interact to regulate adhesion to collagen. However, the nature, locus and post-translational modifications that are involved in these potential regulatory processes are not defined and the functional interactions between DDRs and integrins in control of collagen phagocytosis are not understood. We have examined here the association and functional relationships between DDR1 and β1 integrin and the potential role of DDR1 in modulating collagen adhesions through changes in post-translational modifications of β1 integrin.
Materials & Methods

Reagents

Rabbit monoclonal anti-DDR1 (D1G6) XP® antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal anti-DDR1 (C-20, sc-532) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-integrin β1, (cytosolic), mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 6C5), and mouse monoclonal anti-paxillin (5H11) antibody were obtained from Millipore (Billerica, MA, USA). Mouse monoclonal anti-vinculin (hVIN-1), mouse monoclonal anti-talin (8d4), jararhagin snake venom from Bothrops jararaca, cyclosporin A from Tolypocladium inflatum, dimethyl sulphoxide Hybri-Max™, and protease inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rat monoclonal anti-mouse CD29 (9EG7) antibody, hamster monoclonal anti-mouse CD29 (HM β1-1), purified rat monoclonal anti-mouse CD29 (KMI6), and type I rat tail collagen were purchased from BD Biosciences (Mississauga, ON, Canada). Rabbit monoclonal anti-vimentin (EPR3776) was purchased from Epitomics (Burlingame, CA, USA). FITC-conjugated streptavidin and Cy3-conjugated streptavidin were purchased from Cedarlane Labs (Burlington, ON, Canada). Mouse monoclonal (2A 8F4) secondary antibody to rat IgG2a-heavy chain (biotin) was purchased from Abcam (Cambridge MA, USA). FITC mouse monoclonal anti-rat IgG2a (MRG2a-83), Alexa Fluor® 647 Armenian hamster IgG anti-mouse CD49b (HMα2) were purchased from BioLegend (San Diego, CA, USA). Goat anti-mouse and goat anti-rabbit IgG (H + L)-HRP conjugates were purchased from Bio-Rad (Hercules, CA, USA). Fluoresbrite® Microparticles (1 μm crimson and FITC beads) were purchased from Polysciences (Warrington, PA, USA). Endoglycosidase-H was purchased from Roche.
(Mannheim, Germany). Versene solution was purchased from Invitrogen (Grand Island, NY, USA).

**Cell Culture**

Mouse NIH-3T3 and NIH-3T3-stably transfected with DDR1 (b-isoform) were provided by Wolfgang Vogel (University of Toronto, Toronto, Canada). Integrin β1-deficient GD25 cells were provided by Dr. Reinhard Fässler (Max-Planck Institute for Biochemistry, Munich, Germany). Cells were cultured at 37°C in complete DME medium containing 10% fetal bovine serum and antibiotics (Penicillin G 124 units/mL, Gentamicin SO₄, 50 μg/mL, Fungizone 0.25 μg/mL). Cells were maintained in a humidified incubator under 95% air and 5% CO2 conditions, and were passaged with 0.05% trypsin with 0.53 mM EDTA (Gibco, Burlington, ON).

**Immunoblotting**

Whole cell extracts were prepared on ice by rinsing with cold Ca²⁺- and Mg²⁺-free PBS before lysis with 1% TNT buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail, 200 mM NaVO₃, 20 mg/mL PMSF). The collected lysates were kept on ice and sheared with a 27 gauge needle 4-5 times prior to constant rotation in TNT at 4°C for 30 minutes. The homogenate was centrifuged at 14,000 g for 10 minutes at 4°C and the supernatant was retained for biochemical analysis of protein content via bicinchoninic acid (BCA) analysis. Equal amounts of protein (10 μg) were separated on 9% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to membranes and probed with appropriate antibodies. Immunoblots were quantified by scanning densitometry and ImageJ software.
Flow Cytometry

Cells were seeded onto collagen coated (1 mg/mL type I rat tail) or uncoated tissue culture plastic for indicated lengths of time. To measure β1 integrin activation, cells were immunostained with the neoepitope antibody, 9EG7 (Lenter et al., 1993), that recognizes activated β1 integrin. Prior to immunostaining, cells were quickly (~20 sec) harvested in ice-cold Versene and fixed in ice-cold Versene containing 1% paraformaldehyde, a procedure that preserves the integrin activation state in attached fibroblasts (Kim et al., 2010a; Kim et al., 2010b). Bound 9EG7 was stained with FITC-conjugated anti-rat IgG2a antibody and fluorescence of single cells was analyzed by flow cytometry (Beckman-Coulter). To measure total β1 integrin surface expression cells which were not fixed or permeabilized were immunostained with KMI6 antibody and fluorescence was measured using flow cytometry. To measure α2 integrin surface expression cells which were not fixed or permeabilized were immunostained with CD49b antibody and fluorescence was measured using flow cytometry.

Bead Binding

To measure matrix protein binding to cells, carboxylate-modified fluorescent polystyrene beads (1 μm diameter; excitation/emission: 505/515 nm and excitation/emission: 625/664 nm) were coated with either type I fibrillar collagen (3.66 mg/mL; polymerization induced by incubation of beads at pH 7.4) or fibronectin (10 μg/mL), or with BSA (0.66 mg/mL) as a non-specific binding control. Cells were seeded overnight onto tissue culture plastic. Collagen- or fibronectin-coated FITC beads were loaded onto the dorsal surface of cells together with BSA-coated crimson beads (12 of each bead type/cell, unless otherwise indicated) and incubated for 1 hour at 37°C, unless otherwise specified. After incubation, unbound beads were washed with Ca²⁺- and Mg²⁺- free
PBS prior to being trypsinized for preparation of single cell suspensions. Cells were pelleted and resuspended in PBS and mean bead binding was analyzed using flow cytometry (Knowles et al., 1991).

In an experiment of similar design, cells were seeded overnight onto tissue culture treated 8-well chamber slides (20,000 cells/well). Collagen-coated FITC beads were loaded onto the dorsal surface of cells together with BSA-coated crimson beads (60 of each bead type/cell) and incubated for 1 hour at 37°C in the presence or absence of β1 integrin blocking antibody (CD29; 62.5 μg/mL) or pre-immune serum as a control. As an indicator of the efficacy of the CD29-blocking antibody activity, cells rounded up but were not completely lifted from the culture dish prior to termination of the experiment. Following bead binding cells were gently rinsed 1x with PBS to remove non-adherent and loosely attached beads and blocking antibody, and fixed with 4% paraformaldehyde for 20 minutes at room temperature. After removal of the fixative, nuclei were stained with DAPI. By fluorescence microscopy, three separate fields of view were randomly selected and a minimum of 20 DAPI-stained nuclei and associated beads were counted. The mean ± S.E.M. of the percentage of the cell population with bound beads was calculated.

**Gel Contraction Assays**

Type 1 rat tail collagen (1.36 mg/mL) was added to a solution consisting of DMEM, 0.24 M NaHCO₃, FBS, 10X antibiotic, and 0.1 N NaOH. Cells were added to the solution to produce a final concentration of 150,000 cells/mL. Aliquots of the gel-cell solution (0.2 mL) were pipetted onto the center of each well of a 24 well non-tissue culture plate, ensuring that no gel contacted the side of the well. The cell-gel solution was allowed to polymerize for 5 mins at room
temperature before adding 1 mL of growth medium to each well. We used a floating gel contraction assay to measure collagen remodeling by cells (Nakagawa et al., 1989); after polymerization, gels containing cells were released from the base of the dish before incubation at 37°C. Measurements were made of the collagen gel diameters with a dissecting microscope and an interocular grid at time 0 hrs and approximately every 10-12 hrs for a total of 72 hours. To study collagen contraction by cells, an attached gel contraction assay was used (Nakagawa et al., 1989). After collagen polymerization, gels containing cells were incubated at 37°C for 3 days before releasing from the base of the dish with a pipette. Measurements of gel diameter were made at 0 min and every 30 mins after until contraction stopped. Data obtained from three separate experiments were plotted and linear regression was used to estimate the rate of collagen contraction. The mean slope for each experiment was determined by best fit of the linear regression.

**Cell Migration Assay**

Cells in monolayers were seeded onto type I rat tail collagen-coated plates (0.1 μg/mL) (Chou et al., 1996). A scratch in the monolayer was created using a 200 μl pipette tip to study migration into the denuded space (Coomber and Gotlieb, 1990; Zahm et al., 1997). Phase-contrast images were obtained at regular intervals after scratching until the cell denuded area was closed by cell migration. Images obtained in the same field of focus were compared to quantify cell migration rate.

**Immunostaining**

Cells were plated overnight on type I fibrillar collagen-coated (1 mg/mL collagen) (Chou et al., 1996) glass coverslips (MatTek dishes; MatTek Corp., Ashland, MA, USA) to enable spreading
and formation of focal adhesions. Cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100 in PBS, and blocked for 1 hr in 0.2% BSA in PBS. Cells were incubated with primary antibody and FITC-conjugated secondary antibody for 1 hr each. Immunostained cells were visualized by total internal reflection fluorescence (TIRF) microscopy (Leica, Heidelberg, Germany; 100 x oil immersion lens). Focal adhesion proteins in contact with the collagen substrate (optical penetration depth <110 nm) were analyzed. The mean number of focal adhesions in the periphery (< 5 μm from the cell membrane) and in the cell body (> 5 μm from the cell membrane) were quantified with Leica MetaMorph® Microscopy Automation & Image Analysis Software (20 cells/condition; Leica Microsystems., Wetzlar, Germany).

**Integrin Cleavage Experiments**

Cells in monolayers were grown to 90% confluence on tissue culture plastic or non-tissue culture plastic coated with polymerized type I collagen (Chou et al., 1996). Cells were treated with 10 μg/mL of jararhagin in DMEM growth medium for 1 hr at 37°C (Klein et al., 2011). After 1 hr cells were immediately placed on ice and the jararhagin-containing medium was removed. Cells were prepared by rinsing with cold Ca²⁺- and Mg²⁺- free PBS before lysis in 1% TNT buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail, 200 mM NaVO₃, 20 mg/mL PMSF). Protein concentrations were measured using a BCA assay prior to immunoblot analysis.

**Endo-H Deglycosylation Experiments**

Cells in monolayers were grown to 90% confluence on uncoated tissue culture plastic or non-tissue culture plastic coated with 200 μg/mL of type I rat tail collagen neutralized to pH 7.5.
Cells were rinsed with cold Ca\(^{2+}\)-, Mg\(^{2+}\)- free PBS prior to lysis with extraction buffer (1% Triton X-100, 0.125% Tween-20, 0.5% deoxycholate, 50mM HEPES, 0.5M NaCl pH 7.5, protease inhibitor cocktail, 200 mM NaVO₃, 20 mg/mL PMSF). Protein concentrations were measured using a BCA assay and 10 μg/μL of glycoprotein sample were combined with 5X reaction buffer (250 mM sodium phosphate buffer), denaturation solution (0.02% SDS, 0.1 M β-ME), PMSF and protease inhibitors. Samples were heated at 100°C for 3 min and endoglycosidase-H was added to attain a final enzyme concentration of 0.25mU/μL, followed by incubation at 37°C for 24h. The reaction was terminated by ice-cold acetone/trichloroacetic acid precipitation. The pelleted samples were air dried prior to the addition of sample loading buffer and subsequent immunoblot analysis (Akiyama et al., 1989; Salicioni et al., 2004).

**Statistics**

For all data, mean and standard errors of means were computed. Where appropriate, comparisons between two samples were made by Student’s t-test (unpaired) with statistical significance set at a type I error rate of p<0.05. All experiments were performed in triplicate unless otherwise stated.
Results

Cell characterization

We examined the role of DDR1 in regulating β1 integrin interactions with collagen in cultured cells. NIH3T3 wild-type and DDR1 (b isoform) over-expressing fibroblasts, and mouse GD25 cells derived from differentiated ES cells that carry a null mutation in both β1 integrin alleles were examined. Prior to experiments cell lysates were prepared and immunoblotted for total DDR1 and β1 integrin proteins. DDR1 was readily detected in NIH3T3 wild-type and GD25 cells (Fig. 1A). β1 integrins were expressed in NIH3T3 wild-type and DDR1 over-expressing cells, but not in GD25 cells (Fig. 1B). Immunoblots of GAPDH protein were used as a loading control.

Collagen binding to β1 integrins is enhanced by DDR1 over-expression

We first determined whether DDR1 over-expression affects cell adhesion to collagen. Wild-type 3T3 and DDR1 over-expressing cells, or GD25 cells, were plated overnight on tissue-culture plastic. Collagen-coated FITC fluorescent polystyrene beads (1 μm, 12 beads/ cell) were loaded onto the dorsal surface of cells and incubated for 1 hour prior to analysis by flow cytometry to determine the percentage of the population with bound beads. Binding of BSA-coated crimson beads was measured simultaneously as a non-specific binding control. Collagen-coated bead binding to DDR1 over-expressing cells was enhanced by 4.23-fold (p < 0.001) and 5.18-fold (p < 0.001) compared with wild-type and GD25 cells, respectively (Fig. 2A). In an experiment of similar design, we incubated wild-type or over-expressing cells with collagen-coated beads for 2, 4 or 24 hours prior to analysis by flow cytometry. Collagen-coated bead binding to DDR1 over-
expressing cells was enhanced by 1.54-fold at 2 hrs (p < 0.01), 1.74-fold at 4 hrs (p < 0.0001) and 1.39-fold at 24 hrs (p < 0.0001) compared with wild-type cells (Fig. 2B).

We conducted experiments to determine whether the increased collagen binding observed in cells over-expressing DDR1 was due in part to the additive adhesive effect of high levels of DDR1 expression. Since the primary ligand of DDR1 is fibrillar collagen (Vogel et al., 1997), we coated beads with fibronectin (McKeown et al., 1990) to examine whether the increased collagen binding was due to enhanced β1 integrin adhesion independent of the α integrin subunits. Notably, an important adhesion receptor for fibronectin is the α5β1 integrin (Zhang et al., 1993). Fibronectin-coated beads were loaded onto to the dorsal surface of cells and incubated for 1 hour prior to analysis of bead binding. Similar to the data for collagen bead binding, fibronectin-coated bead binding to DDR1 over-expressing cells was enhanced by 1.56-fold compared with wild-type cells (p < 0.01) (Fig. 2C).

To determine the relative contribution of binding that was attributable to DDR1 over-expression versus enhancement of β1 integrin activity, cells were plated overnight in 8-well chamber slides. Binding of collagen-coated FITC beads (1 μm diameter; 60 beads/cell) in the presence of pre-immune serum or CD29-blocking antibody (62.5 μg/mL) was analyzed after 1 hour incubation. The mean percentage of cells with bound beads was determined by counting beads and DAPI-stained nuclei. Compared with cells treated with pre-immune serum (controls), collagen coated-bead binding to cells was reduced by 2.42-fold in wild-type cells (p < 0.001) and by 1.32-fold in DDR1 over-expressing cells (p < 0.0001) treated with CD29-blocking antibody. BSA-coated crimson beads (1 μm diameter; 60 beads/cell) were measured simultaneously as non-specific binding controls (Fig. 2D).
Since α2β1 is the major integrin receptor for type I fibrillar collagen (Arora et al., 2000; Lee et al., 1996), we wanted to determine whether DDR1 enhances cell surface expression of the α2 integrin subunit. Total surface α2 integrin expression of cells plated overnight on tissue culture plastic was measured by immunostaining with CD49b antibody and flow cytometry. Analysis of cell staining indicated that expression of total cell surface α2 integrins was enhanced by 1.91-fold (p < 0.0001) in DDR1 over-expressing cells compared with wild-type cells (Fig. 2E).

**DDR1 over-expression enhances collagen remodeling and contraction**

We determined the effect of DDR1 over-expression on remodeling and contraction of collagen gels in two related assays. First, collagen remodeling was measured using a floating gel contraction assay (Nakagawa et al., 1989), which examines the ability of cells in compliant gels to extrude fluid from the gels and reorganize the collagen fibrils through migratory and reorganizational activities. For this assay cells were incubated in collagen gels during polymerization and the gels were then floated in growth media. Gel contraction was measured over 3 days. DDR1 over-expression enhanced floating collagen gel contraction by 3.99-fold (p < 0.01) compared with wild-type cells and increased gel contraction by 15.08-fold (p < 0.01) compared with GD25 cells (Fig 3A).

Collagen gel contraction that is attributable to cell-mediated contractile behavior was measured using an anchored gel contraction assay (Chung et al., 2004). Cells were incubated in collagen gels that were then attached to the bottom of a 12-well plate. After 3 days of attachment cells build up considerable tension in the gels. After release of the gels from the base of the dish, gel contraction was measured over several hours. We determined that DDR1 over-expression
enhanced anchored collagen gel contraction by 3.55-fold (p < 0.01) compared with wild-type cells and by 3.97-fold (p < 0.01) compared with GD25 cells (Fig. 3B).

**DDR1 over-expression and cell migration on collagen**

We examined whether DDR1 over-expression impacts cell migration using an *in vitro* scratch assay. Wild-type or DDR1 over-expressing cells were grown to confluence on a collagen-coated substrate prior to scratching and differential interference contrast images of cells were obtained. Representative images obtained at 0 hours or 12 hours after scratching showed cell migration into the scratch and a reduction of the width of the denuded cell monolayer (Fig. 4A). Cells over-expressing DDR1 showed no significant difference in migration rate compared with wild type cells (Fig. 4B, C; p = 0.21).

**DDR1 over-expression enhances activated β1 integrin staining in focal adhesions**

DDR1 does not localize to focal adhesion contacts (Vogel et al., 2000), however we sought to determine whether over-expression of DDR1 influences the activation and recruitment of β1 integrins to focal contacts. Cells were plated overnight on collagen and stained with 9EG7, a neo-epitope antibody that binds to activated β1 integrins (Lenter et al., 1993) prior to analysis by TIRF microscopy. Representative images of 9EG7 staining indicate that DDR1 over-expressing cells contained more activated β1 integrins compared with wild-type cells (Fig. 5A). Analysis of the periphery of cells stained with 9EG7 by TIRF microscopy and subsequent computation of cell areas indicated that there was no significant difference in cell area between wild-type and DDR1 over-expressing cells (Fig. 5B; p = 0.48). Further analysis of focal adhesion staining showed that the number of activated β1 integrin-containing focal adhesions was enhanced by 1.75-fold (p < 0.001) in DDR1 over-expressing cells compared with wild-type cells. Further, the
length of 9EG7-stained focal adhesions was increased by 1.33-fold (p < 0.0001) in DDR1 over-expressing cells compared with wild-type cells (Fig. 5C). Focal adhesion area stained by 9EG7 was not significantly different between the two cell types (Fig. 5D; p = 0.064).

**DDR1 over-expression enhances talin staining in focal adhesions**

Talin is an adaptor protein recruited to focal contacts following the binding of β1 integrins to collagen (Craig and Johnson, 1996) and other matrix ligands. We measured talin expression in the focal adhesions of wild-type and DDR1 over-expressing cells plated overnight on collagen by immunostaining and TIRF microscopy. Consistent with the results obtained from activated β1 integrin staining, representative images indicate that DDR1 over-expressing cells exhibited more talin-stained focal adhesions compared with wild-type cells (Fig. 6A). Analysis of talin staining in the cell periphery indicated that the cell area of DDR1 over-expressing cells was slightly (0.82-fold) smaller (p < 0.05) compared with wild-type cells (Fig. 6B). Further, DDR1 over-expressing cells exhibited 2.54-fold (Fig. 6C; p < 0.0001) more focal adhesions, which were 1.27-fold (Fig. 6D; p < 0.0001) longer, and had an area which was 1.35-fold greater (Fig. 6E; p < 0.0001) compared with wild-type cells.

**DDR1 over-expression enhances paxillin staining in focal adhesions**

Paxillin is recruited to focal adhesions after talin (Jiang et al., 2003). Accordingly, we measured paxillin expression in focal adhesions in wild-type and DDR1 over-expressing cells. Immunostaining and analysis of images obtained by TIRF microscopy indicated that DDR1 over-expressing cells demonstrated more paxillin-containing focal adhesions than wild-type cells (Fig. 7A). Analysis of paxillin staining in the cell periphery indicated that the cell area measured by paxillin staining was 1.19-fold (p < 0.05) greater in DDR1 over-expressing cells compared
with wild-type cells (Fig. 7B). DDR1 over-expressing cells exhibited 3.12-fold (Fig. 7C; p < 0.0001) more focal adhesions, which were 1.28-fold (p < 0.0001; Fig. 7D) longer, and were 1.30-fold more numerous (Fig. 7E p < 0.0001) than wild-type cells.

**DDR1 over-expression enhances vinculin staining in focal adhesions**

Vinculin is an adaptor protein that links β1 integrins to the actin cytoskeleton and is recruited to focal adhesions after the recruitment of talin and paxillin (Craig and Johnson, 1996; Critchley, 2000). We measured vinculin immunostaining in cells plated overnight on collagen by immunofluorescence and TIRF microscopy. Representative images indicate that DDR1 over-expressing cells exhibited more vinculin containing focal adhesions than wild-type cells (Fig. 8A). Analysis of vinculin staining in the cell periphery indicated that there was no significant difference in total cell area between the wild-type and DDR1 over-expressing cells (Fig. 8B; p=0.78). Consistent with our data obtained from activated β1 integrin, talin and paxillin staining, the number of vinculin containing focal adhesions was 2.64-fold greater in DDR1 over-expressing cells compared with wild-type cells (Fig. 8C; p < 0.0001). Further, vinculin-stained focal adhesions in DDR1 over-expressing cells were 1.50-fold longer (Fig. 8D; p < 0.0001) and with a 1.66-fold larger area (Fig. 8E; p < 0.0001) compared with wild-type cells.

**DDR1 over-expression enhances total β1 integrin surface expression and activation**

We examined the effect of DDR1 over-expression on β1 integrin expression on the cell surface. Total surface β1 integrin expression of cells plated overnight on tissue culture plastic was measured by immunostaining with KMI6 antibody and flow cytometry. Analysis of cell staining indicated that expression of total cell surface β1 integrins was enhanced by 3.20-fold (p < 0.01)
in DDR1 over-expressing cells compared with wild-type cells (Fig. 9A). Previously we measured activated β1 integrin expression contained in focal adhesions, which are discrete sites associated with tight attachment to the collagen substrate (Critchley, 2000). By flow cytometry and immunostaining, we measured total activated β1 integrin expression on the entire cell surface. In cells plated overnight on type I collagen, analysis of total 9EG7 staining indicated that DDR1 over-expression increased total activated β1 integrins on the cell surface by 1.55-fold (p < 0.05) compared with wild-type cells (Fig. 9B). Analysis of 9EG7 staining of cells plated overnight on tissue culture plastic indicated that total activated β1 integrin surface expression was 1.32-fold (p < 0.001) greater in DDR1 over-expressing cells compared with wild-type cells (Fig. 9C). We deprived cells of attachment to matrix ligands by preparation of cell suspensions and intentional maintenance in suspension for 2 hours. Analysis of 9EG7 staining of cells in cells subjected to prolonged detachment from matrix proteins indicated that total activated β1 integrin surface expression was 1.62-fold (p < 0.01) greater in DDR1 over-expressing cells compared with wild-type cells (Fig. 9D).

**DDR1 over-expression enhances enzymatic cleavage of surface β1 integrins**

Cells plated on tissue culture plastic or collagen were treated with the disintegrin jararhagin to induce cleavage of β1 integrins at the cell surface (Klein et al., 2011). After treatment, whole cell lysates of wild-type and DDR1 over-expressing cells were immunoblotted for total β1 integrin protein expression (Fig. 10). Immunoblots of GAPDH protein were used to estimate equality of lane loading. Independent of the substrate, DDR1 over-expression increased cleavage of the β1 integrin subunit (indicated upper and lower molecular weight bands) after jararhagin.
**DDR1 over-expression alters post translational modifications of β1 integrins**

In all immunoblots examined we found a consistent difference of the apparent molecular mass of β1 integrins in wild-type cells compared with DDR1-over-expressing cells. In wild-type cells β1 integrins appeared as a single, 130 kDa band; occasionally we detected a faint, ~125 kDa band (Fig. 1B). In contrast, β1 integrins of DDR1 over-expressing cells consistently exhibited two discrete bands: a faint, upper band migrating at ~130 kDa, and a denser lower band migrating at ~125 kDa. We considered that this variation was due to differences in post-translational modifications of the β1 integrin subunit (Bellis, 2004) that might include variations of glycosylation. Accordingly, whole cell lysates of wild-type and DDR1 over-expressing cells plated on collagen or tissue culture plastic were treated with endoglycosidase-H and immunoblotted for the β1 integrin (Fig. 11). Immunoblots of GAPDH protein were used as a loading control. Independent of the substrate on which cells were plated, cells that over-expressed DDR1 exhibited increased glycosylation of the β1 integrin subunit, which is suggested by a shift of the lower molecular weight band (~125 kDa) to a band of ~110 kDa after treatment with endoglycosidase-H.
Discussion

The principal finding of this study is that DDR1 enhances β1 integrin interactions with fibrillar collagen. Over-expression of DDR1 increased cell surface β1 integrin expression and activation, and enhanced collagen binding to β1 integrins, collagen remodeling and reorganization, and the recruitment of integrins and the adaptor proteins, talin, paxillin and vinculin to focal adhesion complexes. Collectively, these findings offer novel insights into the role of DDR1 in regulating β1 integrin function, possibly involving post-translational modifications and trafficking of the β1 integrin to the cell surface.

Collagen binding to β1 integrins is enhanced by DDR1 over-expression

Collagen binding to receptors, in particular the α2β1 integrin, is the rate-limiting step in the phagocytic pathway of collagen degradation by fibroblasts (Arora et al., 2000). We measured fibrillar collagen binding using a collagen-coated bead binding assay (Knowles et al., 1991) and found that over a 24 hour time course, DDR1 over-expression enhanced collagen binding. While binding was increased for all time points measured, there was a relatively larger increase of collagen bead binding at early times after bead incubation in cells with DDR1 over-expression, indicating that DDR1 may, by itself, contribute to increased binding of collagen while also contributing to enhanced binding because of its impact on the β1 integrin. Notably, we found in the time courses that once this early increase of binding occurred, subsequent increases attributable to DDR1 over-expression were diminished. We conjecture that these subsequent increases of adhesion may be attributed to enhanced β1 integrin function. Indeed, since the primary ligand of DDR1 is fibrillar collagen (Leitinger and Hohenester, 2007) and as an important adhesion receptor for fibronectin is the α5β1 integrin (Hynes, 2002; Zhang et al.,
1993), we tested this conjecture using fibronectin-coated bead binding. We found that fibronectin bead binding in DDR1 over-expressing cells was enhanced by similar proportions to those observed at later time points in the time course experiment using collagen-coated beads. Further, when we inhibited β1 integrin function with blocking antibody, the reductions in collagen binding observed in DDR1 over-expressing cells closely matched the levels by which binding was initially increased. Together these observations indicate that DDR1 over-expression enhances collagen binding to β1 integrins independent of DDR1-mediated collagen adhesion.

**DDR1 over-expression enhances collagen remodeling and contraction**

Since we found that DDR1 over-expression enhanced collagen binding, we explored its role in β1 integrin-mediated remodeling and reorganization of collagen, processes which are necessary for the maintenance of connective tissue homeostasis (Leitinger, 2011). Data from floating collagen gel assays, which measure remodeling of collagen fibrils through migratory and reorganizational activities of cells (Grinnell, 2003), indicated that collagen gel contraction was enhanced in cells over-expressing DDR1. These findings indicate that DDR1 enhances β1 integrin functional interactions with fibrillar collagen, which is in contrast to earlier reports showing that DDR1 inhibits β1 integrin function (Yeh et al., 2009). The difference between the current data and the findings of Yeh and co-workers could be a reflection of the relatively higher levels of DDR1 expression that were obtained in our cells.

**DDR1 enhances activated β1 integrin and adaptor protein staining in focal adhesions**

DDR1 does not localize to focal adhesion contacts (Vogel et al., 2000). However, since collagen binding was enhanced by DDR1 over-expression, we sought to determine whether the activation
and recruitment of β1 integrins and the actin binding proteins talin, paxillin and vinculin to focal contacts, were also influenced. Temporal analyses of the recruitment of these proteins to cell adhesions show that after β1 integrin activation and ligand binding (Liu et al., 2000), talin (Craig and Johnson, 1996), paxillin (Jiang et al., 2003) and vinculin (Critchley, 2000) are recruited sequentially to focal complexes. These complexes link collagen-bound β1 integrins to the actin cytoskeleton and enable downstream signaling (Critchley, 2000; Liu et al., 2000). TIRF imaging indicated that the number, length and area of immunostained focal adhesion proteins were generally larger in cells over-expressing DDR1 than wild type cells. Further, the observed differences in the size and number of immunostained focal adhesions were increased sequentially with each subsequent protein that was recruited during the adhesion maturation process, consistent with our data showing that DDR1 promotes binding and interactions with collagen.

**DDR1 over-expression and cell migration on collagen**

Cell migration is dependent upon cell-generated forces that facilitate the formation of stable attachments to the collagen matrix and that enable locomotion (Lauffenburger and Horwitz, 1996). However, if cell attachments are highly adherent, migration may be inhibited because the trailing edge of cells cannot detach from the substratum to enable forward translocation (Yuen et al., 2010). Since DDR1 over-expression enhanced collagen binding to β1 integrins, we examined whether DDR1 over-expression impacts cell migration using an *in vitro* scratch assay. Compared with wild type cells, cells over-expressing DDR1 showed no significant difference in migration rate. Notably, we found that when the cell area was measured using peripheral immunostaining for activated β1 integrin, vinculin, talin or paxillin, there were no marked differences attributable to DDR1 over-expression compared with wild type cells. As unidirectional migration is dependent on advancement of the cell periphery at the leading edge, the lack of effect of DDR1
over-expression on increasing expression of cell attachment proteins in peripherally-located attachments indicate that DDR1 does not affect all cell attachments equally across the ventral surface of the cell. In contrast, we found that cell adhesions were enlarged in the central part of cells over-expressing DDR1. We speculate that as DDR1 over-expression selectively enhances cell adhesion to collagen in the central part of cells, while not significantly affecting adhesion in the cell periphery, cell migration is not detectably affected by DDR1 over-expression.

**DDR1 over-expression enhances total β1 integrin surface expression and activation**

We found that DDR1 over-expression enhanced recruitment of activated β1 integrins and actin binding proteins in focal adhesions, which are discrete sites associated with tight cellular attachment to collagen substrates (Critchley, 2000). We followed up these analyses by examining β1 integrin expression on the whole cell surface. Analysis of suspended cells by flow cytometry indicated that expression of total and activated β1 integrins on the cell surface were increased in equal proportion in DDR1 over-expressing cells compared with wild-type cells. Curiously, β1 integrin activation did not appear to be affected by cell detachment from collagen, since cells that were maintained in prolonged suspension expressed comparable levels of cell surface activated β1 integrins as did cells that were quickly detached from collagen and immediately analyzed. These data indicate that DDR1 may promote recruitment of constitutively activated β1 integrin to the cell surface, independent of ligand binding. These findings are consistent with our observations that DDR1 over-expression enhances the adhesion of fibrillar collagen to β1 integrins. Notably, high levels of β1 integrin (Andrews et al., 2001; Klein et al., 1991) and DDR1 (Heinzelmann-Schwarz et al., 2004; Quan et al., 2011; Yang et al., 2010) expression have been implicated in the development of tumor cells and DDR1 expression levels are associated with
increased MMP-1, -2 -9 and -10 expression (Ferri et al., 2004; Park et al., 2007; Ruiz and Jarai, 2011), proteins which are expressed by cells that actively remodel the ECM. Accordingly we speculate that DDR1 may play a similar role in modulating β1 integrin expression and function to facilitate cell adhesion-dependent remodeling of the ECM.

**DDR1 regulation of trafficking and post-translational modification of β1 integrin**

We determined whether DDR1 affects β1 integrin trafficking to the cell surface by exploiting the susceptibility of cell surface β1 integrin to cleavage by the disintegrin jararhagin (Klein et al., 2011). Independent of whether cells were plated on collagen or tissue culture plastic, DDR1 over-expression increased cleavage of the β1 integrin subunit after treatment with jararhagin. This finding confirms our observation that DDR1 over-expression enhances cell surface β1 integrin expression. Further, in all immunoblots examined, we found consistent differences of the apparent molecular mass of β1 integrins in wild-type cells compared with DDR1-over-expressing cells. We considered that this variation may be due to differences in post-translational modifications of the β1 integrin subunit (Bellis, 2004; Diskin et al., 2009; Gu and Taniguchi, 2004), which may include variations of glycosylation. Independent of the substrate on which cells were plated, cells that over-expressed DDR1 exhibited increased N-linked glycosylation of the β1 integrin subunit. While glycosylation is a largely understudied regulatory mechanism for β1 integrin function (Bellis, 2004), our findings may explain earlier data. For example, variations of β1 integrin glycosylation have been implicated in regulation of integrin trafficking to the cell surface (Hotchin and Watt, 1992) as well as integrin-mediated cell functions including migration (Janik et al., 2010), adhesion (von Lampe et al., 1993; Zheng et al., 1994), and spreading (Diskin et al., 2009).
In summary, our findings indicate that DDR1 over-expression enhances fibrillar collagen binding to β1 integrins. The effect of DDR1 over-expression on β1 integrin cell surface expression and activation may be attributed to DDR1-regulated post-translational modifications of the β1 integrin. These modifications in turn may translate into enhanced adhesion formation, collagen remodeling and reorganization, processes that are essential for connective tissue homeostasis.
Figure Legends

**Figure 1. Cell characterization.** A) Whole cell lysates of mouse NIH3T3 wild-type or DDR1 (b isoform) over-expressing fibroblasts, or β1 integrin-null mouse GD25 cells were prepared and immunoblotted for total DDR1 expression. B) Immunoblotting for total β1 integrin protein expression in the same mouse cells described in Fig. 1A.

**Figure 2. Collagen binding to β1 integrins is enhanced by DDR1 over-expression.** A) Binding of collagen-coated FITC beads (1 μm diameter; 12 beads/cell) to cells was analyzed by flow cytometry after 1 hour incubation. Collagen coated-bead binding to DDR1 over-expressing cells was enhanced at 1 hour compared with either wild-type (p < 0.001) or GD25 cells (p < 0.001). Cell binding to BSA-coated crimson beads (1 μm; 12 beads/cell) was measured simultaneously as a non-specific binding control. Data represent mean±S.E.M. (n=3 independent experiments). B) Flow cytometric analysis to determine collagen-coated bead binding (1 μm diameter beads; 12 beads/cell) to DDR1 over-expressing cells. Binding was enhanced at 2 hrs (p < 0.01), 4 hrs (p < 0.001) and 24 hrs (p < 0.001) after incubation with beads compared with wild-type cells. Data represent mean ± S.E.M. (n = 3 independent experiments). C) Binding of fibronectin-coated beads (1 μm diameter; 12 beads/cell) to DDR1 over-expressing cells was enhanced compared with wild-type cells as indicated by analysis with flow cytometry (p < 0.01). Data represent mean ± S.E.M. (n = 3 independent experiments). D) Cells were plated overnight in 8-well chamber slides. Binding of collagen-coated FITC beads (1 μm; 60 beads/cell) in the presence of pre-immune serum or CD29 blocking antibody (62.5 µg/mL) was analyzed after 1 hour incubation. Mean percentage of cell population with bound beads was determined by
counting beads and DAPI-stained nuclei. Collagen coated-bead binding to cells was reduced by CD29 blocking antibody in wild-type (p < 0.001) and DDR1 over-expressing (p < 0.0001) cells. BSA-coated crimson beads (1 μm; 60 beads/cell) were measured simultaneously as non-specific binding controls. Three separate fields of view were randomly selected and a minimum of 20 DAPI-stained nuclei and associated beads were counted. Data represent mean ± S.E.M. (n = 4 independent experiments). E) Cells were plated overnight on tissue culture plastic. Total α2 integrin surface expression was measured by immunostaining of non-fixed and non-permeabilized cells with CD49b antibody, followed by flow cytometry. Analysis of cell staining indicated that DDR1 over-expression increased total α2 integrin expression on the cell surface compared with wild-type cells (p < 0.0001). Data represent mean ± S.E.M. of fluorescence channel number of cells stained with fluorescence-conjugated antibodies to (n = 3 independent experiments).

Figure 3. DDR1 over-expression enhances collagen remodeling and contraction.

A) Collagen remodeling was measured over 3 days using a floating gel contraction assay. DDR1 over-expression enhanced floating collagen gel contraction compared with wild-type cells (p < 0.01) or GD25 cells (p < 0.01). Data represent mean ± S.E.M. (n = 3 independent experiments).

B) Collagen contraction was measured using an anchored gel contraction assay. DDR1 over-expression significantly enhanced anchored collagen gel contraction compared with either wild-type (p < 0.01) or GD25 cells (p < 0.01). Data represent mean ± S.E.M. (n = 3 independent experiments).

Figure 4. DDR1 over-expression does not affect cell migration on collagen. A) Representative, differential interference contrast images of wild-type or DDR1 over-expressing
cells plated on collagen. Cells were examined in *in vitro* scratch assays at 0 hours and 12 hours after scratching. **B)** Best linear fit of reduction of scratch width (mm) over time. The reduction in scratch width was not significantly different between the wild-type and DDR1 over-expressing cells (*p* = 0.2098). **C)** Comparison of migration rates between wild-type and DDR1 over-expressing cells. There was no significant difference in migration of DDR1 over-expressing cells on collagen compared with wild-type cells (*p* = 0.2098). All data represent mean ± S.E.M. (*n* = 3 independent experiments).

**Figure 5.** *DDR1 over-expression enhances activated β1 integrin staining in focal adhesions.* **A)** Cells plated overnight on collagen were immunostained for activated β1 integrins using 9EG7 antibody and imaged by TIRF microscopy. Representative images indicate that DDR1 over-expressing cells expressed more activated β1 integrins compared with wild-type cells. Total cell area estimated from peripheral 9EG7 staining and 9EG7 staining in focal adhesions was analyzed in DDR1 over-expressing cells and wild-type cells. Data are indicated in following histograms. **B)** No significant difference in total cell area (*p* = 0.4807); **C)** increased number of activated β1 integrin-containing focal adhesions (*p* = 0.001); **D)** greater focal adhesion length (*p* < 0.0001); and **E)** no significant difference in focal adhesion area (*p* = 0.064). MetaMorph was used to quantify mean ± S.E.M. of cell area and focal adhesions in cells (*n* = 20 cells analyzed for each cell type).

**Figure 6.** *DDR1 over-expression enhances talin staining in focal adhesions.* **A)** Cells plated overnight on collagen were immunostained for talin and imaged by TIRF microscopy. Representative images indicate that DDR1 over-expressing cells contained more
talin-containing focal adhesions than wild-type cells. Total cell area estimated from peripheral talin staining and talin staining in focal adhesions was analyzed in DDR1 over-expressing cells and wild-type cells. Data indicate that in DDR1 over-expressing cells compared to wild type cells: **B)** total cell area was significantly greater (p < 0.05); **C)** there were increased numbers of talin-containing focal adhesions (p < 0.0001); **D)** focal adhesions were longer (p < 0.0001); and **E)** talin-stained focal adhesions exhibited increased area (p < 0.0001). MetaMorph was used to quantify mean ± S.E.M. of cell area and focal adhesions in cells (n = 20 cells analyzed for each cell type).

**Figure 7. DDR1 over-expression enhances paxillin staining in focal adhesions.**

**A)** Cells plated overnight on collagen were immunostained for paxillin and imaged by TIRF microscopy. Representative images indicate that DDR1 over-expressing cells contained more paxillin-stained focal adhesions than wild-type cells. Analysis of paxillin staining in the cell periphery for cell area and in focal adhesions indicated that for DDR1 over-expressing cells: **B)** total cell area was significantly greater (p < 0.05); **C)** there were increased numbers of paxillin-containing focal adhesions (p < 0.0001); **D)** focal adhesion lengths were longer (p < 0.0001); and **E)** paxillin-stained focal adhesion area was larger (p < 0.0001). MetaMorph was used to quantify mean ± S.E.M. of cell area and focal adhesions in cells (n = 20 cells analyzed for each cell type).

**Figure 8. DDR1 over-expression enhances vinculin staining in focal adhesions.**

**A)** Cells plated overnight on collagen were immunostained for vinculin and imaged by TIRF microscopy. Representative images indicate that DDR1 over-expressing cells contained more
vinculin-stained focal adhesions than wild-type cells. Analysis of peripheral vinculin staining and vinculin staining in focal adhesions in DDR1 over-expressing cells compared with wild type cells indicated: B) no significant difference in total cell area (p = 0.7783); C) increased number of focal adhesions (p < 0.0001); D) longer focal adhesions (p < 0.0001); and E) increased area of vinculin-stained focal adhesions (p < 0.0001). MetaMorph was used to quantify mean ± S.E.M. of cell area and focal adhesions (n = 20 cells analyzed for each cell type).

**Figure 9. DDR1 over-expression enhances total β1 integrin surface expression and activation.** A) Cells were plated overnight on tissue culture plastic. Total β1 integrin surface expression was measured by immunostaining non-fixed and non-permeabilized cells with KMI6 antibody, followed by flow cytometry. Analysis of cell staining indicated that DDR1 over-expression increased total β1 integrin expression on the cell surface compared with wild-type cells (p < 0.01). Total activated β1 integrin surface expression on cells was measured by immunostaining with 9EG7 antibody and flow cytometry of non-fixed and unpermeabilized cells. B) Analysis of cell staining indicated that in cells plated overnight on type I collagen, DDR1 over-expression significantly increased total activated β1 integrin surface expression compared with wild-type cells (p < 0.05). C) Analysis of cells plated overnight on tissue culture plastic indicated that total activated β1 integrin surface expression was significantly greater in DDR1 over-expressing cells compared with wild-type cells (p < 0.001). D) Analysis of 9EG7 staining of cells in suspension indicated that total activated β1 integrin surface expression was significantly greater in DDR1 over-expressing cells compared with wild-type cells (p < 0.01). All data represent mean ± S.E.M. (n = 3 independent experiments).
**Figure 10. DDR1 over-expression enhances enzymatic cleavage of β1 integrin at the cell surface.** Following 1 hr treatment with jararhagin to induce cleavage of surface β1 integrins, whole cell lysates of wild-type and DDR1 over-expressing cells plated on collagen or tissue culture plastic were prepared and immunoblotted for total β1 integrin protein and GAPDH expression. Independent of the substrate used for cell plating, DDR1 over-expression led to increased cleavage of the β1 integrin subunit (upper and lower molecular weight bands) following treatment with jararhagin. This is a representative image selected from 3 independent experiments.

**Figure 11. DDR1 over-expression alters post translational modification of β1 integrins.** Whole cell lysates of wild-type and DDR1 over-expressing cells plated on collagen or tissue culture plastic were treated with endoglycosidase H for 24 hours prior to immunoblotting for total β1 integrin protein and GAPDH expression. Independent of the substrate used for cell plating, DDR1 over-expression led to an increase in glycosylation of the β1 integrin subunit as indicated by the band shift of the lower molecular weight band (denoted by the arrows: ◀ glycosylated, ▲ deglycosylated) following treatment with endoglycosidase H. This is a representative image selected from 3 independent experiments.
Figure 1

A

[Image of Western blot with bands at 130 kDa labeled DDR1 and 36 kDa labeled GAPDH for 3T3 WT, 3T3 DDR1 OE, and GD25]

B

[Image of Western blot with bands at 130 kDa labeled β1 Integrin and 36 kDa labeled GAPDH for 3T3 WT, 3T3 DDR1 OE, and GD25]
Figure 2

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

D

![Graph D](image)

E

![Graph E](image)
Figure 3

A

Mean Slope of Floating Gel Diameter (µm/hr)

B

Mean Slope of Attached Gel Diameter (µm/hr)
Figure 4

A

0 hrs post scratch

12 hrs post scratch

DDR1 WT

DDR1 OE

B

Scratch Width (mm)

Time (hrs)

0 2 4 6 8 10 12

 DDR WT

 DDR OE

C

Mean Slope of Scratch Width (µm/hr)

DDR WT

DDR OE
Figure 5

A

B

Cell Area (µm²)

WT

OE

9EG7

9EG7

C

Number of Focal Adhesions

DDD WT

DDD OE

***

D

Focal Adhesion Length (µm)

DDD WT

DDD OE

****

E

Focal Adhesion Area (µm²)

DDD WT

DDD OE

****
Figure 6

A

WT
OE

B

Cell Area ($\mu$m$^2$)

0
2000
1000
3000
4000

DDR WT
DDR OE

C

Number of Focal Adhesions

0
100
200
300

DDR WT
DDR OE

D

Focal Adhesion Length ($\mu$m)

0
2
1
3

DDR WT
DDR OE

E

Focal Adhesion Area ($\mu$m$^2$)

0
1
2

DDR WT
DDR OE

***
****

*
Figure 7

A

WT

OE

Paxillin

Paxillin

B

Cell Area (µm²)

0

2000

1000

3000

4000

DDR WT

DDR OE

C

Number of Focal Adhesions

0

100

200

300

DDR WT

DDR OE

D

Focal Adhesion Length (µm)

0

1

2

3

DDR WT

DDR OE

E

Focal Adhesion Area (µm²)

0

1

2

3

DDR WT

DDR OE

***

****

***

***
Figure 8

A

Figure 8

B

C

D

E

***

****

********

64
Figure 10

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130 kDa → β1 integrin

36 kDa → GAPDH
Figure 11

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β1 integrin
▲ glycosylated
▼ deglycosylated

GAPDH

130 kDa →

36 kDa →
Future Directions and Conclusions

Our data provide new insights into the role of DDR1 in regulating β1 integrin maturation, trafficking and function. However, there are several unresolved issues. The current results focus on the role of DDR1 in collagen remodeling and adhesion using a DDR1 over-expression system. Although we have employed the use of β1 integrin blocking antibodies to examine collagen binding, a useful approach would be to analyze receptor-collagen interactions in cells with knockdown of β1 integrin expression and knockdown of DDR1 expression using the same approach. These experimental methods would be achieved with β1 integrin or DDR1-targeted siRNA knockdown and then conducting the collagen remodeling and adhesion assays described above. We expect that knockdown of β1 integrins and DDR1 would inhibit cell binding and matrix remodeling.

Partially glycosylated β1 integrin subunits form a pool within the ER (Akiyama et al., 1989). Although we found that DDR1 enhances glycosylation of β1 integrins, our data do not indicate the potential role of DDR1 in regulating trafficking of partially glycosylated β1 subunits from the ER to the Golgi. Processing of N-linked glycans is controlled by six different N-acetylglucosamine-glycosyltransferases (GnTs) located in the cis-Golgi and medial Golgi (Vagin et al., 2009). Conceivably, DDR1 is involved in the up-regulation of these enzymes. Accordingly, PCR analysis of GnT transcription may clarify how DDR1 regulates integrin maturation and cell surface expression. Further, since we found that DDR1 modulates β1 integrin function by increasing post-translational modifications, specifically through the addition of N-linked glycans, it would be useful to learn how integrin function is affected by altered expression of these oligosaccharides. Prior to experimental analysis of cell-collagen interactions, treatment
of cells with 1-deoxymannojirimycin, an inhibitor of α-mannosidase II, which prevents N-linked oligosaccharide processing (Gu and Taniguchi, 2004), would provide insight into the functional role that these glycans play in cell adhesion and collagen remodeling.

We analyzed cell surface expression of the α2 integrin, the primary α integrin for collagen binding. We found that DDR1 over-expression increased expression of the α2 subunit on the cell surface. Since we believe that DDR1 over-expression enhances collagen binding and remodeling through β1 integrins, we need to determine how DDR1 may regulate pairing of the β1 subunit with other α-subunits and their presentation on the cell surface. Further analysis by immunostaining other α-subunits that pair with β1 to form type I collagen receptors, such as α10 or α11 and for fibronectin, the α5 fibronectin subunit, by fluorescence microscopy or flow cytometry would be of value. We expect that DDR1 over-expression would enhance the pairing and surface expression of these other β1 integrins as well.

Finally, our data provide some insight into the processes that are important for connective tissue homeostasis. Analysis of our supplemental experiments indicated that subpopulations of HGFs treated with CsA exhibited reduced collagen-coated bead binding. Additionally, when cells over-expressing DDR1 were treated with CsA, we noted that collagen-coated bead binding and collagen remodeling were also reduced. Since DDR1 over-expression has been implicated in cancers and multiple fibrotic conditions, and since CsA-induced gingival overgrowth is a condition in which the collagen phagocytic pathway is disrupted, we expect that DDR1 expression would be increased in the subpopulations of cells that are sensitive to CsA. In studies using DDR1 knock-out animal models, we expect that these mice are protected against CsA-induced gingival overgrowth.
In conclusion, DDR1-regulated post-translational modifications of the β1 integrin subunit may regulate β1 integrin function in collagen remodeling and cellular adhesion. An in-depth understanding of how β1 integrin maturation and function are controlled by DDR1 could provide insight into how these important collagen receptors interact and may suggest novel strategies for treatment of fibrotic conditions.
Appendix

Cyclosporin A (CsA) is an immunosuppressant commonly used in organ transplantation to prevent rejection by lowering the activity of T cells (Chan et al., 2007). An adverse effect of CsA treatment is gingival overgrowth. In CsA-induced gingival overgrowth, collagen degradation by fibroblasts is inhibited, resulting in fibrosis (Arora et al., 2001; Chan et al., 2007). Since fibrillar collagen is the primary ligand for DDR1, we hypothesized that CsA perturbs DDR1 function. In the experiments described below, we examined the impact of CsA on DDR1 function in the context of collagen binding and remodeling.
Appendix Legends

Appendix 1. Collagen binding is reduced in HGF subpopulations and DDR1 over-expressing cells treated with CsA. A) Human gingival fibroblasts obtained from gingival explants from individual patients were plated overnight on tissue culture plastic in media containing CsA (10 μg/mL) or vehicle (DMSO). Binding of collagen-coated FITC beads (1μm; 12 beads/cell) to HGFs was analyzed after 1 hour incubation by flow cytometry. Collagen-coated bead binding to cells was reduced in subpopulations of cells treated with CsA (patient numbers 1, 2, 5 and 7: p<0.05, 0.01, 0.05, and 0.01, respectively) compared with untreated cells. B) Cells were plated overnight on tissue culture plastic in media containing CsA (10 μg/mL) or vehicle (DMSO). Binding of collagen-coated FITC beads (1μm; 12 beads/cell) was significantly reduced in DDR1 over-expressing cells (p<0.01) and GD25 cells (p<0.01) treated with CsA. All data represent mean±S.E.M. (n = 3 independent experiments).

Appendix 2. CsA treatment reduces collagen remodeling in DDR1 over-expressing cells. A) Cells inoculated in type I collagen gels (1.36 mg/mL, neutralized) containing collagen-coated FITC beads (50 beads/cell) were incubated overnight in medium containing CsA (10 μg/mL) or vehicle (DMSO). Collagen gels containing cells were dissolved by collagenase prior to analysis of bead binding by flow cytometry. Treatment with CsA reduced collagen-coated bead binding in DDR1 over-expressing cells (p<0.01). B) Collagen remodeling was measured using a floating gel contraction assay. Compared with untreated cells (vehicle, DMSO), contraction of type I collagen gels (1.36 mg/mL, neutralized) was reduced in DDR1 over-expressing (p < 0.05) and GD25 cells (p < 0.05) treated with CsA (10 μg/mL). C) Collagen
contraction was measured using an anchored gel contraction assay. CsA treatment had no effect on collagen contraction in all cell types analyzed. All data represent mean ± S.E.M. (n = 3 independent experiments).

**Appendix 3. CsA treatment does not affect cell migration on collagen.** Cell migration across collagen-coated plastic (1 mg/mL) was measured over 12 hours using a scratch wound assay. **A** ) Slope of scratch width (mm) over 12 hours. The reduction in scratch width was not significant in wild-type (p = 0.564) or DDR1 over-expressing (p = 0.545) cells treated with CsA (10 μg/mL) or vehicle (DMSO). **B** ) Comparison of migration rates in wild-type and DDR1 over-expressing cells treated with CsA. There was no significant difference in migration of wild-type (p = 0.564) or DDR1 over-expressing (p = 0.545) cells treated with CsA (10 μg/mL) or vehicle (DMSO). All data represent mean ± S.E.M. (n = 3 independent experiments).
Appendix 2

A

![Bar graph showing Mean Population with Bound Beads (%)]

- DDR WT
- DDR OE
- GD25

** (p < 0.01)

B

![Bar graph showing Mean Slope of Floating Gel Diameter (µm/hr)]

- DDR WT
- DDR OE
- GD25

* (p < 0.05)

C

![Bar graph showing Mean Slope of Attached Gel Diameter (µm/hr)]

- DDR WT
- DDR OE
- GD25

DMSO, CsA
Appendix 3

A

Scratch Width (mm)

Time (hrs)

B

Mean Slope of Scratch Width (µm/hr)

DMSO

CsA

DDR WT DMSO

DDR WT CsA

DDR OE DMSO

DDR OE CsA

DDR WT

DDR OE
References


