INHIBITION OF COLLAGEN FIBRILLOGENESIS UPON SECRETION OF EXTRACELLULAR DOMAINS OF DDR1 AND DDR2 BY CELLS

A Thesis

Presented in Partial Fulfillment of the Requirements for
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* * * * *

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ABSTRACT

Discoidin Domain Receptors (DDRs) are unique members of the Receptor Tyrosine Kinases (RTKs) family. Most RTKs are activated by soluble proteins present in bodily fluids, however DDRs are activated by the extracellular matrix (ECM) protein, collagen. Collagen fiber assembly is a tightly controlled process affecting many physiological processes. DDR expression and activation plays a crucial role in collagen assembly, ECM remodeling, cell adhesion and proliferation.

Our earlier studies established a unique mechanism of collagen regulation by DDR2 extracellular domain (ECD). This work further investigates how DDR1 ECD and DDR2 ECD affects collagen fibrillogenesis. Mouse osteoblast cell lines stably or transiently over-expressing DDR1 or DDR2 ECD were utilized. Transmission electron microscopy, fluorescence microscopy, and hydroxyproline assays demonstrated that DDR ECD expression reduced the rate and quantity of collagen deposition and significantly altered fiber morphology. Collectively, our studies advanced our understanding of DDRs as powerful regulators of collagen deposition in the ECM.
Dedicated to my children;
James, Elizabeth, Cassidy and Spencer.
I would like to express my deep gratitude to all those who supported me in this endeavor. A special thanks to my advisor, Dr. Gunjan Agarwal, whose encouragement and guidance was paramount. I appreciate her willingness to take a part-time graduate student into her lab and am grateful for her stimulating suggestions and considerable advice. I am deeply indebted to Angie Blissett as she was instrumental in this work. She conducted the hydroxyproline assay experiments, collected the TEM images and provided the associated results cited in this thesis (Figures 7, 8, 9, and 11). She readily shared her knowledge of cellular biology and taught me every lab technique I learned. I would also like to thank Dr. Mark Ruegsegger, for his counsel and support throughout my BME sojourn.

Working full-time throughout my studies did not make me the typical graduate student, yet everyone in the Agarwal lab was wonderful to work with and easily accommodated my odd hours and schedule. I would like to thank the other members of the Agarwal lab who helped make this journey an interesting and fruitful one: Michelle Nauerth, who was always willing to discuss ideas and alternatives on many a long weekend, and Cosmin Mihai and Lalitha Sivakumar who also provided support throughout.
Lastly, I would like to express my appreciation to those who were my foundation; my parents, who set excellent examples for me in reaching my aspirations; my brother, who was both supportive and encouraging; Lynn and Sue, who were always there for me; Sharyn, who supported both my academic and spiritual aspirations; and Peter for being a sounding board along the way.
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CHAPTER 1

INTRODUCTION

1.1 TYROSINE KINASE RECEPTORS

Receptor Tyrosine Kinases (RTKs) are transmembrane receptors which are important regulators of normal cellular processes, including proliferation, differentiation, migration, cell survival, and angiogenesis.\(^1\) RTKs are recognized to have a critical role in the advancement of many human diseases including cardiovascular diseases, diabetes and cancer.\(^2\) When RTK regulation is disrupted due to mutations or receptor over-expression, it can lead to growth and progression of certain cancers. Many of the known human RTKs are proto-oncogenes.\(^3\)

In the human genome, ninety unique tyrosine kinase genes have been identified. Fifty-eight of these tyrosine kinase genes encode RTK proteins and have been further classified into twenty subfamilies, as can be seen in Table 1. Also notable, is that mouse orthologs can be identified for nearly all of the human RTKs,\(^2\) including Discoidin Domain Receptors 1 and 2 which are the focus of this work.

Typical ligands for RTKs are known to be polypeptide hormones, growth factors and cytokines. It is understood that for most RTKs, ligand binding leads to receptor dimerization followed by phosphorylation of specific tyrosine residues in the receptor’s
intracellular kinase domain which leads to receptor activation and downstream signaling events. However although active research by several groups aims to understand the intracellular signaling induced by RTKs, very little is understood about the functional role(s) of isoforms of RTKs which lack the kinase domain. These kinase dead isoforms can arise due to receptor ecto-domain shedding and/or alternative splicing and often preserve the capacity to bind to their ligands.

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Table 1. Overview of Human Tyrosine Kinase Families and Literature.2
1.2 DISCOIDIN DOMAIN RECEPTORS

**EXPRESSION AND FUNCTIONAL SIGNIFICANCE:** Discoidin Domain Receptors (DDRs) are unique members of the family of RTKs. Whereas most RTKs are activated by soluble proteins present in the blood plasma or other bodily fluids, DDRs are activated by the extracellular matrix (ECM) protein, collagen in its indigenous triple helix form. It has been shown that DDR1 is activated by collagen types I, II, III, IV, and VIII, while DDR2 is activated by fibrillar collagen types I, II, III and X.4,5

DDR1 and DDR2 are expressed in many different mammalian organs and cell types. DDR2 is found in mesenchymal cells while DDR1 is primarily expressed in epithelial cells of lung, kidney, colon and brain. Both DDR receptors are involved in cell interactions with the ECM and once activated control cell adhesion, cell proliferation, and ECM remodeling. DDR2 has been found to regulate proliferation of chondrocytes, hepatic stellate cells and fibroblasts. It has been determined that DDR1 is critical for arterial wound repair, cerebellar granule differentiation and mammary gland development.6

DDR1 and DDR2 have also demonstrated differences with respect to collagen degradation and levels of protein expression. Independent studies using human SMCs over-expressing DDR1 or DDR2 show induced matrix metalloproteinase (MMP) 1 at both mRNA and protein levels, with only DDR2 enhancing MMP2 activation.7 The level of DDR1 expression has been determined to be significantly higher in keloids than in normal fibroblasts or hypertropic scars. However, the slightly higher level of DDR2 expression observed in keloids as opposed to normal fibroblasts was deemed to be statistically insignificant.8
DDRs have been linked to a number of human diseases. These proteins have been shown to be over-expressed in several carcinomas; leukemia,9 lymphoma,10 lung cancer,11 hepatic cancer,12 prostate cancer,13 and glioma.14 It has been determined that DDR1 is up-regulated in the malignant cells of breast, brain, colon, ovarian, lung and esophageal cancers, while DDR2 has been found to be present in the stromal cells encircling the malignant cells.15 In addition to being atypically expressed in numerous malignancies, DDrs have been found to be regulated in lymphangioleiomyomatosis, rheumatoid arthritis, osteoarthritis16 and atherosclerosis.7,17 Animal studies done with DDR1-null mice have demonstrated a reduction of atherosclerotic aortic lesions18 and decreases in the histologic lesions of glomerular fibrosis and inflammation in renal fibrosis.19

**STRUCTURAL CHARACTERISTICS:** DDR1 and DDR2 are transmembrane receptors comprised of five structural regions. There are two cytoplasmic regions: a C-terminal catalytic tyrosine kinase domain and a large juxtamembrane region. This is followed by a transmembrane region, and two extracellular regions: a “stalk” region consisting of a sequence of approximately 220 amino acids to DDrs, and an N-terminal ~150 amino acid discoidin homology (DS) domain (Figure 1a).15,20

Five DDR1 isoforms, DDR1a-e, exist in vivo and have been identified through alternative splicing of the DDR1 gene. Figure 1a contains a schematic representation of these isoforms.15,21 While the isoforms DDR1a to c are known to regulate cell signaling, the functional role of the kinase dead isoforms of DDR1, namely DDR1d and DDR1e is as yet unknown. The extracellular domain (ECD) of DDR1 is also known to be shed as a soluble protein after collagen stimulation15 (Figure 1b). Although the variants
for DDR2 have not yet been characterized there is evidence substantiating they exist.

Protein species for DDR2 have been detected in cultured human cells at molecular weights of 130, 90, 50 and 45 kDa, as well as two transcripts at 9.5 and 4.5 kb. Multiple transcripts for DDR2 have been identified for both cancerous and normal cell lines.7,15,16,22

Figure 1. a) Schematic representation of the different isoforms of DDR1. The panel shows the predicted length, molecular weight, and overall structure of the different DDR1 isoforms.21 b) A model for ligand-induced DDR1 ECD shedding. (1) DDR1 is predominantly present as 125 kDa full-length protein in the absence of collagen. (2) Collagen stimulation induces DDR1 autophosphorylation. Subsequently, the DDR1 sheddase is activated. (3) Membrane-anchored truncated DDR1 remains as tyrosine-phosphorylated 62 kDa protein after ectodomain shedding.23
1.3 COLLAGEN

Collagen, the ligand for DDR1 and DDR2 is the most abundant ECM protein found in vertebrates. Collagen comprises over half the protein content in mammals and is the major protein of connective tissue, cartilage and bone. Collagen is a fibrous structural protein produced primarily by fibroblasts and composed of repeating triple helix strands. Collagen molecules occur as diverse fibrillar, microfibrillar or network suprastructures which serve as scaffolds for the attachment of other macromolecular complexes. These suprastructures are assembled from a family of gene products called α-chains. There are 28 different types of collagens discovered in vertebrates which use 43 unique α-chains in their assembly. As can be seen in Figure 2, these collagen types can be categorized into five groups: transmembrane, fibril-forming, fibril-associated containing interrupted triples helices, beaded filament, and anchoring fibril network-forming collagens.

Figure 2. A summary of the structural models of the N- and C- noncollagenous domains of various classes of collagen suprastructures.
Collagen types I, II, III, and IV comprise ninety percent of the collagen present in the human body and of these, type I is the most abundant. Type I collagen is present in most bodily tissues except cartilaginous tissue and brain. It is a fibril-forming collagen packed into an organized overlapping bundle known as collagen fibrils. These fibers are arranged in various combinations and concentrations in different tissues to provide a mixture of diverse tissue properties. Because of its dominance in the human body and the fact that other collagen types possess structural similarities to collagen type I, collagen type I is the most widely studied collagen.15

1.4 COLLAGEN SYNTHESIS AND DEGRADATION

PROCOLLAGEN PRODUCTION AND ASSEMBLY: Type 1 collagen is secreted from cells as a soluble procollagen molecule comprised of three α chains, two α1 and one α2, in a triple helical format. As these α-chains are synthesized in the cell they undergo hydroxylation of the proline and lysine residues, followed by glycosylation at the hydroxylysyl residues. Peptidyl proline hydroxylation stimulates changes in the peptide backbone that lead to triple helix formation of the procollagen molecule. Once formed and secreted from the cell, the condensed triple helix configuration is very stable, resisting further hydroxylation by proteases such as trypsin, pepsin or chymotrypsin.15

COLLAGEN FIBRILLOGENESIS: Conversion of procollagen to collagen fibrils is known as fibrillogenesis, a process occurring external to the cell. Once procollagen is secreted into the extracellular matrix, the N- and C-polypeptides are cleaved by procollagen proteinases and the triple helical collagen molecules self-associate into fibrils. The term fibril is used to denote the initial collagen polymers and
the mature, longer, thicker polymers are known as fibers. This process is denoted in Figure 3. The collagen fibrillogenesis is considered to be a process tightly controlled by the cells mainly through collagen binding proteins such as decorin, fibronectin, lumican etc. However, the role of membrane anchored vs. secreted collagen binding proteins in regulating collagen fibrillogenesis is not completely clear.

Figure 3. Collagen biosynthesis consisting of procollagen secretion by cells into 300nm triple helical domain. Upon secretion the N- and C- propeptides are cleaved by procollagen proteinases, resulting in spontaneous self-assembly of the collagen molecules into fibrils.
**COLLAGEN DEGRADATION:** Three molecular pathways have been established for collagen degradation in physiological and pathological tissue-remodeling processes. The best understood pathway involves the collagenases, membrane-associated matrix metalloproteinases (MMPs), which cleave collagens within the extracellular or pericellular environment. MMPs are proteolytic peptidases which can be functionally classified into four groupings: collagenases, gelatinases, stromelysins, and membrane-types. MMP1, a collagenase, cleaves the α-chains of type I, II and III collagen. The generated fragments are thermally unstable and become substrates for MMP2 and MMP9 causing further degradation of the fragments.

A second degradation pathway is cathepsin K-mediated specifically for osteoclast-mediated bone resorption. This degradation takes place in the acidic environment between the osteoclast and the bone interface.

The third pathway is intracellular and is facilitated by the binding of collagen fibrils to precise cell surface receptors (β1 integrins or urokinase plasminogen activator receptor-associated protein), then cellular uptake, delivery to lysosomes, and proteolytic degradation of the acid-denatured collagen by cathepsins.

**1.5 REGULATION OF COLLAGEN BY DDRs**

Thus far DDRs are known to regulate collagen in two distinct ways: up-regulation of MMP1, MMP2, and MMP9 is triggered by prolonged tyrosine phosphorylation of the DDR kinase domain. Secondly the activation of DDR1 or DDR2 in human or mouse cell lines is known to modulate collagen synthesis. DDR1 activation was shown to suppress mRNA for collagen α1-chains for collagens type I and III and up-regulate
collagen VIII (α1). DDR2 activation resulted in up-regulation of collagen VIII (α1) and
down-regulation of collagen VI (α2).\textsuperscript{30} Further, in vivo and in vitro studies have revealed
increased mRNA levels for procollagen alpha1(I) and alpha1(III) in DDR1 knock-out
mice, suggesting an enhancement of matrix synthesis in the absence of DDR1.\textsuperscript{18}

Recently a novel mechanism of collagen regulation by DDRs has been established
by the Agarwal laboratory. This involves direct inhibition of collagen fibrillogenesis by
interaction of the DDR1 and DDR2 ECD with collagen type 1. They have reported,
through in vitro assays with DDR1-Fc or DDR2-Fc fusion proteins (which contained only
the ECD of DDR1 or DDR2), that the DDR ECD regulates collagen fibrillogenesis.
DDR ECD interaction with collagen was found to lock collagen molecules in an
incomplete fibrillar state, both in vitro and on surfaces of cells over-expressing
DDR1.\textsuperscript{16,29} Presence of DDR1 or DDR2 ECD resulted in altered morphology of collagen
type 1 fibers (\textbf{Figure 4}).\textsuperscript{28}
Figure 4. TEM images showing collagen fiber morphology present in the presence of DDR2. At low magnification of 7000x (first row), no apparent difference in fiber morphology is observed for all 3 samples. The periodic banded structure in collagen fibers in samples with collagen alone (PBS) or collagen with a control protein (TrkB) can be seen at a magnification of 30,000x or higher (rows 2 and 3). Fibers formed in the presence of DDR2 lack this native periodic banded structure of collagen.28

More extensively, this group has demonstrated how expression of cell-surface anchored, kinase dead DDR2 (DDR2-/KD) affects fibrillogenesis of collagen endogenously secreted by the cells. They created a stably transfected cell line expressing DDR2-/KD, which also endogenously secretes collagen. It was demonstrated that DDR2-/KD alters collagen fiber morphology (Figure 5), slows the rate of collagen fibrillogenesis, and leads to reduced collagen deposition.16 While these results show that
cell surface anchored collagen binding proteins like DDR2 can influence collagen fibrillogenesis, the role of secreted proteins present in the ECM is not completely understood.

**Figure 5.** Over-expression of DDR2 kinase dead (KD3) inhibits native banded structure of collagen fibers in the ECM. The native nontransfected cells show well-formed collagen fibers with banded structure. In contrast, stable cell lines expressing DDR2 kinase dead presented weak or no banding structure.16
1.6 PURPOSE OF THE CURRENT RESEARCH

SPECIFIC HYPOTHESES TO TEST: The intent of this research is to seek further understanding of the role DDRs play in the assembly process of collagen. Studies will focus on DDR1 and DDR2 ECD to ascertain how soluble ECM proteins like DDR ECD affect collagen fibrillogenesis. Our hypothesis is: DDR ECDs will exhibit inhibition of collagen fibrillogenesis and alterations to collagen fiber morphology. Comparisons and contrasts will be made between previous results uncovered using membrane anchored DDR2 and our findings in this work using DDR1 ECD and DDR2 ECD.

METHODS USED: We will employ a variety of biochemical and biophysical techniques along with quantitative analysis of data. Experiments will be conducted using fluorescent microscopy, electron microscopy, fiber analysis and hydroxyproline assays on cell samples.
CHAPTER 2

MATERIALS AND METHODS

2.1 CREATION OF EXPRESSION CONSTRUCTS FOR SOLUBLE DDR ECD

An expression plasmid encoding the ECD of DDR1 or DDR2 was generated using the full-length mouse DDR1-myc and DDR2-myc constructs obtained from Regeneron Pharmaceuticals, Tarrytown, NY (23). The coding regions of the DDR1 ECD (amino acid Met-1 to Ser-412) and DDR2 ECD (amino acids Met-1 through Ile-400) were amplified by polymerase chain reaction utilizing the Pfu TURBO polymerase (Stratagene, La Jolla, CA) and the following primers:

**DDR1-ECD:**
Forward: 5’-GAAGGATGGGGACAGGGACCCTC-3’
Reverse: 5’-GCTCCCCTCCGCCTTGCCCAC-3’

**DDR2 ECD:**
Forward: 5’-AGGATGATCCCGATTCCCAGA-3’
Reverse: 5’-GATCCGAGTGTTGCTATCATCAAC-3’

*Figure 6* shows a representation of the DDR ECD constructs which lead to the expression of truncated DDR1 and DDR2 proteins (*Figure 6b* and *6d*). While
representations of the full-length receptors, maintaining the extracellular, transmembrane and juxtamembrane regions are shown in Figure 6a and 6c.

Figure 6: Schematic depiction of DDR1c and DDR2 proteins. The numbers designate the sequence of amino acids in our recombinant proteins. a) V5-tagged full-length mouse DDR1 protein. Discoidin domain (ECD), transmembrane (TM), juxtamembrane (JM) and tyrosine kinase domains are designated. b) DDR1 ECD construct, ending at coding region Ser-412. c) V5-tagged full-length mouse DDR2 protein, with ECD, TM, JM and tyrosine kinase domains shown. d) DDR2 ECD construct, ending at coding region Ile-400.

The resulting PCR products (DDR1 ECD: 1241 bp and DDR2 ECD: 1203 bp) were subjected to Taq polymerase to include 3’ A-overhangs in the PCR product for enabling ligation immediately into the pcDNA3.1/V5-His-TOPO vector using the Top10 chemically competent cells from Invitrogen. Recombinant clones were identified by restriction analysis using the double digest with Age1 and Kpn1 (for DDR1 ECD) and Kpn1 and EcoRV (for DDR2 ECD). The authenticity (i.e. correct orientation and in

15
frame with the V5 coding region) of the resulting clones was verified by
dideoxynucleotide sequencing.

**TRANSIENT TRANSFECTIONS USING DDR1/DDR2 CONSTRUCTS:**
Mouse osteoblast cells (ATCC), MC3T3-E1, subgroup-4 were grown to 60% to 80%
confluency and transiently transfected with mammalian expression plasmids. Fugene 6
(Roche Diagnostics) was used as the transfection reagent. Transient transfections were
done multiple times to confirm reproducibility, each time allowing for 24 to 36 hours of
transfection. To verify the expression of DDR1 ECD and DDR2 ECD protein tagged with
V5 epitope, cells were transfected with the DDR1 ECD and DDR2 ECD expression
constructs (described above) using FuGene 6 transfection reagent (Roche, Basel,
Switzerland). After 36 hours of transfection the culture media was collected and the cells
were lysed. The conditioned media and the cell lysates were subjected to SDS-PAGE
followed by Western blotting onto nitrocellulose membranes. The membranes were
probed with anti-V5 primary mouse monoclonal antibodies (Invitrogen) (1:1000) and
imaged using enhanced chemiluminescence (Amersham Biosciences) after incubation
with anti-mouse IgG horseradish peroxidase. As a control expression vector encoding
full-length DDR1-V5 protein was also used. The recombinant proteins tagged with the 14
amino acid V5 epitope: -Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr31
could be identified using the V5 antibody.

**CREATION OF STABLE CELL LINES:** The pcDNA3.1 vector used to
generate expression plasmids for DDR ECD also encodes the antibiotic resistance gene
gentamicin which helped us identify cells which are transfected and express the DDR ECD.
Mouse osteoblast cells (ATCC) MC3T3-E1, subgroup-4 were seeded (60 –80% confluent)
on two 100 mm dishes in MEM-α with 10% (v/v) fetal bovine serum and 1% Streptomyosin (Gibco). One set of MC3T3 cells was transfected with DDR1 ECD expression construct (1.085 μg/ml) using FuGene 6 from Roche Diagnostics. The second set of MC3T3 cells was transfected with DDR2 ECD expression construct (1.095 μg/ml) using Fugene 6. Thirty hours after transfection, the cells were incubated with media containing 475 μg/ml of geneticin. After 12 to 14 days of culture in media containing geneticin, cells from the surviving DDR1 (or DDR2) ECD colonies were transferred to 35 mm dishes. Each 35 mm plate consisted of cells from only a single surviving colony. These cells were cultured and monitored over a period of 28 to 38 days. Thereafter dishes with adequate cell confluence and morphology were selected from each group (DDR1 ECD or DDR2 ECD) for verification of protein expression with western blotting. Nine dishes of prospective DDR1 ECD colonies and nine dishes of prospective DDR2 ECD were selected for this testing. The cells were serum starved for 24 to 48 hours and then lysed. Both the serum and lysates were analyzed using SDS PAGE and Western blotting with an anti-V5 antibody (Invitrogen). One DDR1 ECD stable cell line (ECD1) and one DDR2 ECD stable cell line (ECD2) was selected based on expression of DDR1 or DDR2 ECD proteins and healthy cell morphology. Collagen expression was also verified for the selected cell lines using Western blotting and hydroxyproline assays.

2.2 TRANSMISSION ELECTRON MICROSCOPY

Samples for transmission electron microscopy (TEM) were prepared for both DDR1 ECD and DDR2 ECD stable cell lines as well as for non-transfected cells, which served as the control group. The cells were cultured on Thermanox plastic coverslips
(Nalge Nunc International, New York) placed in 35 mm dishes. Samples were treated with 25 μg/ml of ascorbic acid as ascorbate is required for prolyl hydroxylation of collagen and thereby induce cross-linking and collagen fiber deposition in the extracellular matrix. The samples were incubated for one, two, three and four week periods before being fixed. The cells were fixed by using 4% (v/v) glutaraldehyde overnight, 1% osmium tetroxide for 1 hour, and en bloc staining with saturated aqueous uranyl acetate for 1 hour. A graded ethanol series (30%–100%) was used to dehydrate the samples at which point they were embedded in an epoxy resin. After polymerization, the resin disk was obtained and the 35 mm dishes discarded. The coverslip was rapidly inserted into liquid nitrogen to remove it from the resin disk. The resin disk was cut in half and liquid resin was used to glue the two halves with the cell sides facing. This combined resin disk was then cut into rectangular segments for sectioning.

Sectioning was done using a Leica Ultracut UCT ultramicrotome (Leica-Microsystems Wien, Austria). 200-mesh grids were used to pick up the ~80 nm thick sections, which were then stained with Reynolds's lead citrate. Transmission Electron Microscopy was used to examine the sections with a Zeiss EM 900 TEM (Carl-Zeiss SMT, Peabody, New York) operating at 80 kV. An Olympus SIS Megaview III camera (Lakewood, Colorado), was used to obtain digital micrographs at magnifications ranging from 7000x to 85,000x.

2.3 TEM IMAGE ANALYSIS

Collagen fiber analysis was performed using Image J software (NIH) to measure the diameter of collagen fibers in the ECD of the TEM images. Collagen fiber diameters
were measured on cross-sectional or longitudinal images of collagen fibers in the TEM micrographs using magnifications of 50,000x or 85,000x. For each specimen type, at least two independent cell samples were made and processed. At least two TEM grids were made from each sample, and numerous regions on each grid were imaged. 200 fiber diameters were captured for each specimen type, and a statistical analysis conducted to provide to average diameter, standard deviation and frequency distribution. The morphology of collagen fibers was also analyzed in TEM images to identify presence or absence of D-periodicity in collagen fibers.

2.4 HYDROXYPROLINE (HP) ASSAY

Hydroxyproline assays were performed to determine the collagen content in the extra cellular matrix. Quantification of HP content was completed on three cell specimens: DDR1 ECD stably transfected cells, DDR2 ECD stably transfected cells and non-transfected cells for 7 and 14 days of culture. Cell samples were prepared in triplicate, with and without ascorbic acid, and HP content in the adhered cellular layer was quantified.

To assess the HP content in each sample, first a cell proliferation assay was used to quantify the cell population of each sample using Calcein-Am (BioChemika 17783). The cell samples were washed in PBS, followed by the addition of 2 μl of 1 mM Calcein-AM stock solution to each well. The plates were carefully swirled to mix the Calcein-AM and then incubated for 30 minutes at room temperature. A fluorescent plate reader was used to identify the fluorescence signal using Cytofluor II software (Global Medical Instrumentation, Ramsey, MN) at an excitation wavelength of 485 nm and emission at
530 nm. After the cell count was done for each well, cell density was normalized with respect to the lowest cell count value measured for each time point. This was done to reduce discrepancies due to variation in growth rate of the samples.

Thereafter the adherent cell layers were scraped and pipetted into individual eppendorf tubes. The samples were brought to a volume of 50 μl with a final concentration of 4N sodium hydroxide and autoclaved for 20 minutes at 120 degrees Celcius. Next 450 μl of chloromine T reagent was added to each sample and underwent incubation at room temperature for 25 minutes. This was followed by mixing 500 μl of Ehrlich's reagent into each sample and incubating them at 65°C for 20 minutes. Absorbance of the samples was measured at 560 nm using a Beckman DU730 spectrophotometer. A standard curve of HP was devised ranging from 0.5 μg/ml to 10 μg/ml. The amount of HP in each sample was obtained using calibration against this standard curve. Collagen content was estimated by considering that hydroxyproline comprises 12.5% of collagen fibers.16

2.5 FLUORESCENCE MICROSCOPY

Fluorescence microscopy was used to ascertain the rate of collagen fibrillogenesis by native vs. DDR ECD expressing cells for exogenously added fluorescently labeled collagen. Native cells of two types were used: 3T3 mouse osteoblast cells (ATCC), MC3T3-E1, subgroup-4 and rat aortic smooth muscle cells (ATCC), A7r5 SMC. The native cells and transiently or stably transfected 3T3 cells expressing either DDR1 ECD, DDR2 ECD, full length DDR1 or kinase dead DDR1 were seeded on 1% (w/v) Poly-L-lysine coated glass coverslips to facilitate better adherence. Thereafter, cells were
incubated with collagen type 1 labeled with Fluorescein Isothiocyanate (FITC) from Sigma Chemicals, Missouri, at a final concentration of 1 μg/ml. At the appropriate timepoint selected for the experiment (30 min, 1 hr, 3 hr, etc), the cells were washed and fixed with 2% formalin (Fisher Scientific, Kalamazoo, Michigan). After 30 minutes of fixation at room temperature, the cells were washed and incubated for 20 to 30 minutes at 4 degrees Celsius with 4',6-diamidino-2-phenylindole (DAPI) stain. Glass coverslips were then mounted onto microscope slides using ProLong Gold antifade reagent (Invitrogen Molecular Probes P36934).

The slides were examined with 20x and 63x objectives on a Zeiss Axiovert 200 microscope. An EXFO mercury lamp was used to excite the sample and appropriate filter cubes were used for fluorochrome observation: YFP-2427A (Semrock) was used to observe FITC and filter set # 49 (Zeiss) was used for observance of DAPI nuclear stain.

2.6 PHASE CONTRAST MICROSCOPY

Phase contrast microscopy was used to examine cell morphology of MC3T3 native cells and stably transfected MC3T3 cells expressing either DDR1 ECD or DDR2 ECD. Cells were grown on glass bottom dishes and imaged using phase contrast microscopy with a 20x objective on the Zeiss Axiovert microscope.
CHAPTER 3

RESULTS

3.1 CHARACTERIZATION OF STABLE CELL LINES

In order to ascertain the effects of over-expressed DDR1 and DDR2 ECDs on endogenously formed collagen, we used mouse osteoblast cell line E1-3T3. Based on previous studies\(^{15-16,28-29}\), these cells endogenously secrete and form well-defined collagen fibers in the ECM over a period of one to several weeks. 3T3 cell lines stably transfected with DDR1 ECD or DDR2 ECD expression constructs were created to ensure long term secretion of these proteins in the ECM. Of the many stable cell lines generated, two were selected. Selection was done on the basis of healthy cell morphology and expression level of the DDR ECD proteins. The presence of DDR1 ECD and DDR2 ECD in our stable cell lines was determined using western blotting. Figure 7 displays these results, confirming protein expression in the conditioned media obtained from these samples (Figure 7a) and analogous morphology between the native and transfected cell lines (Figure 7b).
Figure 7. a) Verifications of DDR1 and DDR2 ECD in conditioned media of stable cell lines. Western blots were performed on the media collected from each cell line as indicated using anti-V5 antibodies. Presence of DDR1 ECD and DDR2 ECD was observed at their expected molecular weights of ~64 kDa as a single band (for DDR1 ECD) and as a doublet band for DDR2 ECD. b) Cell morphology of nontransfected and stably transfected 3T3 cell lines. Similar cell shape and structure is present in native, DDR1 ECD and DDR2 ECD cell samples. Images shown were collected using phase contrast microscopy at 20x magnification.

3.2 DDR1 AND DDR2 ECD DISRUPT STRUCTURE OF COLLAGEN FIBERS

To determine if secretion of DDR1 and DDR2 ECD by the cells affects the structure of collagen fibers endogenously assembled by the cells, we employed ultrastructural morphological analysis using TEM of cell samples. Figure 8 shows the
collagen fiber morphology of native, DDR1 ECD stable and DDR2 ECD stable cell lines at weeks 1, 2, 3 and 4.

Figure 8: TEM images showing ultrastructure of collagen fibers assembled in the ECM of 3T3 cells. Nontransfected samples show well-formed collagen fibers with defined D-periodic structure. This banded structure of collagen fibers is hindered in DDR1 ECD and DDR2 ECD stable cell lines for all time points.
Collagen fibers formed in the ECM of nontransfected cells had well-defined banded structures exhibiting D-periodicity. Over 100 measurements were taken of the darkened bands of nontransfected samples establishing a D-periodicity average of 61 nm +/- 5 nm across nontransfected samples across weeks 1 through 3. This is in line with previous results from other studies. In the samples for both stable cell lines, ultrastructural banding of collagen is hindered. Fibers in the ECM of the stable cell lines are poorly formed, lacking D-periodicity.

3.3 DDR ECDs AFFECT COLLAGEN FIBER DIAMETER

Fiber diameter analysis was conducted on the collagen fibers present in the ECM for both nontransfected and DDR ECD stable cell lines. Figure 9 shows a comparison of fiber diameters after two weeks for each of the three cell lines. The TEM images clearly show the larger fiber diameter of the native cells when compared to the cell lines over-expressing DDR1 or DDR2 ECDs, where fiber diameters were significantly smaller with a more malformed appearance. While nontransfected cells exhibited an average fiber diameter of 55 nm, the average fiber diameter for DDR1 and DDR2 ECD cell samples was 23 and 26 nm respectively. This disparity in fiber diameter results between native cells and cells over-expressing the proteins was consistent with other time points as well (Figure 10). DDR1 ECD samples had an average fiber diameter in the range of 23.1 nm to 27.6 nm across the four week timepoints, while DDR2 ECD samples exhibited a slightly higher fiber diameter ranging from 23.8 nm to 28.4 nm. The nontransfected cell samples were consistently larger in all cases, producing fiber diameters with averages between 45nm to 55nm across the four week timepoints, confirming that DDR1 ECD and
DDR2 ECD expression in the ECM reduces collagen fiber diameter. The standard deviations of average fiber diameter for both DDR1 and DDR2 ECD samples were comparable at 6 nm after two weeks of culture and ranged from 5.3 to 7.5 nm across all samples. Standard deviation was observed to be slightly higher in the nontransfected cell samples, 9 nm after two weeks of culture and reaching up to 10 nm deviation after four weeks.
Figure 9. Collagen fiber diameter is affected by over-expression of soluble DDR1 and DDR2 ECDs in the ECM. The average diameters for both DDR1 ECD and DDR2 ECD are notably smaller than the nontransfected samples. In the histogram plots, $d$ is the average diameter (nm) obtained by measuring 200 fibers from each sample type. The measurements and TEM images shown were on cells cultured for two weeks.
To assess how DDR1 and DDR2 ECD affect the growth rate of collagen fiber diameters, we analyzed the collagen fiber diameter from samples at weeks 1, 2, 3 and 4. **Figure 10** shows that both the DDR1 and DDR2 ECD cell lines inhibited collagen fiber diameter growth with little to no increase in average fiber diameter over a four week period. The nontransfected cell line had an average fiber diameter nearly twice that in the cell lines with soluble DDR ECD in the ECM.

**Figure 10:** Over-expression of soluble DDR1 and DDR2 ECDs in the ECM diminishes the growth rate of collagen fiber diameters. The cells over-expressing DDR1 ECD (ECD1) and DDR2 ECD (ECD2) proteins had little to no increase in fiber diameter throughout the four week period. The nontransfected cell line possessed an average fiber diameter 40 to 60% higher than ECD1 and ECD2 cell lines at all timepoints. Analysis was conducted for n=200 fibers for each time point for each sample type.
3.4 DDR ECD STABLES DECREASE COLLAGEN DEPOSITION IN ECM

Our microscopic analysis (Figure 8) indicated suppression of fibrillar collagen content in cells over-expressing DDR1 or DDR2 ECD. To ascertain and quantify the total collagen content in the adherent ECM, we carried out the hydroxyproline (HP) assay, a biochemical technique used for routine measurement of collagen content in tissue specimens.28,34

Native cells or DDR1 and DDR2 ECD stable cell lines were cultured in the presence and absence of ascorbic acid for 1 and 2 weeks. For each cell line HP samples were made from the adhered cell layer from cells grown with and without ascorbic acid (Figure 11). Little difference is evident in the samples prepared without ascorbic acid. At day 7, samples over-expressing DDR1 ECD exhibited 18% of collagen content compared to nontransfected cells and at day 14, 52%. Cells over-expressing DDR2 ECD displayed only 3% of collagen content for day 7 compared to nontransfected cells and after 14 days, 57%.
Figure 11. Collagen content in nontransfected vs. DDR ECD transfected cells. HP (-AA) is the HP concentration of cells adhered to the ECM grown in the absence of ascorbic acid. Δ HP is the difference in HP content between samples grown with and without ascorbic acid. In the absence of ascorbic acid, there is negligible difference in the quantity of HP present across the samples. Differences in Δ HP demonstrate collagen content is reduced in cell lines over-expressing DDR1 ECD or DDR2 ECD. Nontransfected cells clearly displayed higher collagen content after both 7 and 14 days of culture.

3.5 DDR ECDs INHIBIT THE KINETICS OF COLLAGEN FIBRILLOGENESIS

To ensure that the inhibition of collagen fibrillogenesis observed in the DDR ECD stable cell lines was due to interactions caused by the over abundance of DDR1 ECD or DDR2 ECD proteins rather than less collagen being secreted by these cells, fluorescent microscopy was used. Native cells and cells transiently or stably transfected with DDR1 ECD, or DDR2 ECD, were cultured and then stimulated at various timepoints with exogenously added monomeric collagen I labeled with fluorescein isothiocyanate (FITC). The nontransfected cells show evidence of increased fiber formation as early as 1 hour after collagen stimulation with continued growth through the 12 hour period. During
this same period, there is negligible or very little fiber formation in the stably transfected DDR ECDs, with the transient transfections showing a bit more collagen fiber formation with time than the stable transfections (Figures 12 and 13).

**Figure 12.** DDR1 ECD and DDR2 ECD impedes exogenous collagen fibrillogenesis. FITC-labeled collagen was added to native and DDR ECD transient and stable cell samples and incubated for 30 min, 1 hr, 3 hr, 6 hr, and 12 hr periods. Nontransfected cell samples show collagen fiber formation beginning around 1 hour and continuing throughout the 12 hour period. Transiently transfected cells expressing DDR1 and DDR2 ECD demonstrate inhibited fiber formation. Stably transfected DDR1 and DDR2 ECD samples also show a similar inhibition of collagen fiber formation. Images were collected using fluorescent microscopy at 20x magnification.
Figure 13. DDR1 ECD and DDR2 ECD impedes exogenous collagen fibrillogenesis. As in Figure 11, FITC-labeled collagen was added to native and DDR ECD transient and stable cell samples and incubated for 30 min, 1 hr, 3 hr, 6 hr, and 12 hr periods. No notable fiber formation is evident at the 30 minute incubation period even for native cell samples. Growth in polymeric collagen fiber formation is seen from 1 hour through the 12 hour period for the native cells. Some fiber formation occurs at the 6 and 12 hour timepoints of the transient DDR1 and DDR2 ECD cell samples with little to no formation occurring in the stable ECD constructs. Images were collected using fluorescent microscopy at 63x magnification.

EFFECT OF MEMBRANE ANCHORED VS. SOLUBLE DDR1 ECD ON COLLAGEN FIBRILLOGENESIS: Our laboratory has previously demonstrated that cells over-expressing full length DDR2, DDR1 or membrane anchored kinase dead DDR2 inhibit fibrillogenesis of exogenously added collagen. However no results were reported on membrane anchored kinase dead DDR1. To examine how membrane anchored vs. soluble DDR1 ECD affects fibrillogenesis of exogenously added collagen,
3T3 cells transiently transfected with three DDR1 isoforms; DDR1 full length (DDR1-FL), DDR1 kinase dead (DDR1-KD) and DDR1 ECD were used.

As seen in Figures 14 and 15, collagen fibrillogenesis is facilitated in nontransfected cells, with polymer fiber formation evident as early as after 1 hour of incubation. DDR1-FL and DDR1-KD cell samples hampered collagen fiber formation and exhibited speckled patterns arising from FITC collagen, most prominent around the cell nuclei. After 12 hours of incubation, DDR1-FL and DDR1-KD samples no longer showed the speckled patterns and fiber formation was detected albeit at a much lower level than untransfected cells. DDR1 ECD showed the least amount of collagen fiber formation throughout the incubation period. The speckled structures were not observed for cells expressing DDR1 ECD.
Figure 14. Transient transfections of DDR1 Full Length (FL), Kinase Dead (KD) and ECD constructs inhibit exogenous collagen fibrillogenesis. FITC-labeled collagen was added to native, DDR1-FL, DDR1-KD and DDR1 ECD expressing cell samples and incubated for 30 min, 1 hr, 3 hr, 6 hr, and 12 hr periods. DDR1-FL and DDR1-KD inhibited collagen fiber by generating speckled patterns around cell nuclei, most prominent after the 3 and 6 hour incubation periods. DDR1 ECD showed little evidence of collagen fiber formation and the speckled structures were absent in these samples. Images were collected using fluorescent microscopy at 20x magnification.
Figure 15. Transient tranfections of DDR1 Full Length(FL), Kinase Dead(KD) and ECD constructs inhibit exogenous collagen fibrillogenesis. FITC-labeled collagen was added to native, DDR1-FL, DDR1-KD and DDR1 ECD cell samples and incubated for 30 min, 1 hr, 3 hr, 6 hr, and 12 hr periods. DDR1-FL and DDR1-KD presented collagen fiber inhibition as speckled areas around cell nuclei, whereas DDR1 ECD showed the least pattern of polymeric fibers. Native cells showed consistent fiber formation starting around the 1 hour timepoint and continuing throughout the period. Images were collected using fluorescent microscopy at 63x magnification.

3.6 COLLAGEN FIBRILLOGENESIS BY SMCs

Mouse osteoblast 3T3 cells were used as the control group throughout the experiments conducted to ascertain affects of DDR1-KD, DDR1 ECD and DDR2 ECD on endogenous and exogenous collagen fibrillogenesis. To test if our assays work universally for other cell types, we analyzed collagen fibrillogenesis by A7r5 rat aortic smooth muscle cells (SMCs). Figure 16 displays nontransfected SMCs after incubation
with exogenously added FITC collagen for timepoints, ranging from 30 minutes through
12 hours. It is interesting to note that the length of collagen fibers formed in these
samples is considerably larger than that observed in osteoblasts (3T3).
Figure 16: Collagen Fibrillogenesis in nontransfected Smooth Muscle Cells. Collagen fiber formation is striking in native rat aorta SMCs. Growth begins around the 1 hour timepoint, similar to that seen in 3T3 mouse osteoblast cells, and continues fiber assembly resulting in longer fiber formation. Images shown were collected using fluorescent microscopy at 20x and 63x magnifications.
CHAPTER 4

DISCUSSION

4.1 EFFECT OF MEMBRANE ANCHORED VS. SOLUBLE DDR2 ECD ON COLLAGEN FIBRILLOGENESIS

The role of cell surface anchored vs. soluble collagen binding proteins in regulating collagen fibrillogenesis has been a topic of debate for several decades. The DDR receptors serve as a good model system to examine this effect.

Recent work in the Agarwal lab demonstrated that the cell-surface anchored DDR2 receptor, when lacking its kinase domain, still remains a regulating factor in collagen fibrillogenesis. These results signified that cell surface-anchored, collagen-binding proteins have the capacity to control collagen fibrillogenesis and alter fiber formation. This group had also previously demonstrated that DDR2 ECD binds to collagen resulting in inhibited collagen fibrillogenesis and altered morphology of collagen type 1 fibers in vitro. These results were arrived at using in vitro assays with DDR2-Fc fusion proteins, which consisted of the extracellular domain of mouse DDR2 fused with Fc (constant domain) of human immunoglobulin (IgG1).

In the current work, we examined how expression of soluble DDR2 ECD affects fibrillogenesis of collagen endogenously secreted by the cells. For this purpose we
created an osteoblast cell line stably expressing DDR2 ECD. Our results show that cells over-expressing DDR2 ECD yielded collagen fibers in the ECM with disrupted native banded structure and smaller fiber diameter, reduced deposition of collagen in the ECM, and impaired collagen fibrillogenesis exhibiting similarities to expression of cell-surface anchored kinase dead DDR2. However, some differences in the effect of DDR2-KD vs. DDR2 ECD were noted as discussed below.

The collagen fiber diameter in the DDR2 ECD stable cell samples (Figure 10) was smaller than DDR2-KD stable cell lines by 12 nm after full lateral growth. Although the difference in fiber diameter between the native cell lines used for these present and earlier studies was an average of 8 nm, the smaller diameter of the ECD2 fibers is still notable. DDR2 ECD produced fibers 55% of the diameter of the corresponding native cell line with DDR2-KD producing fibers 67% of the diameter of the corresponding native cell line. Secondly, the cells over-producing DDR2-KD constructs produced collagen fibers which continued to grow laterally (in fiber diameter) through three weeks of incubation. This was not the case with the cells over-expressing DDR2 ECD, which reached their steady state of collagen fiber diameter at the first week of incubation. Fiber banding morphology was weak in both DDR2-KD and ECD2 with no significant differences between the two.

The collagen content in the adherent ECM, as demonstrated by the HP assay results (Figure 11), was reduced in DDR2 ECD stable cell lines. In previous work done using cells over-expressing DDR2-KD constructs, the highest expressing DDR2-KD construct showed results similar to those observed with DDR ECDs. After 7 days incubation, the DDR2-KD exhibited 12% collagen content compared to the native cells,
and 52% after 14 days. Our current results suggest that DDR2 ECD demonstrates a similar inhibition of collagen content (57% after 14 days). However, given that soluble collagen content was not measured in the media, full collagen content cannot be ascertained to fully validate this comparison between the two expression constructs.

Taken together our observations suggest that DDR2 ECD present in soluble form in the ECM leads to more pronounced reduction of fiber lateral growth than membrane bound DDR2. We speculate this is due to soluble DDR2 ECD being distributed throughout the ECM and thus having more ability to affect collagen fiber formation even in ECM regions away from the proximity of the cell membrane. Thus the DDR2 ECD proteins are able to serve as a stronger inhibitor of collagen fibrillogenesis as compared to DDR2-KD membrane-bound proteins.

### 4.2 EFFECT OF DDR1 VS. DDR2 ECD ON COLLAGEN FIBRILLOGENESIS

Several groups have established that binding of DDR1 and DDR2 to collagen is controlled only by the DDR ECD. Previous investigations have revealed that DDR1 has a binding pattern distinct from DDR2. DDR2 was observed to have a specific binding location on individual collagen molecules, yet no such location could be determined for DDR1. DDR1 was found to bind at adjacent or overlapping collagen molecules and was absent for the most part on isolated, single collagen molecules. The Agarwal lab had also demonstrated that DDR1-Fc (consisting of DDR1 ECD only) also inhibits collagen fibrillogenesis in-vitro. Further transient transfection of full length DDR1 and DDR2 in cells was shown to inhibit collagen fibrillogenesis for collagen exogenously added to the cells by formation of large aggregates of collagen on their cell
surface instead of collagen fibril formation. To evaluate how DDR1 ECD affects collagen fibrillogenesis for cells endogenously secreting collagen, we carried out similar experiments and analysis as for DDR2 ECD.

Our results show that DDR1 ECD resulted in reduced collagen fibrillogenesis as evident by fiber diameter, fiber morphology, and collagen content when compared to native cells. DDR1 ECD produced fibers with an average lateral diameter smaller than that of DDR2 ECD but the difference was not considerable (26.4 nm versus 24.4 nm). Nor was any significant difference observed between the fiber growth rates between these two stable cell lines. Collagen fibrillogenesis inhibition as evidence through fluorescent microscopy also yielded similar results with no distinguishable differences between the two: all significant variations were with respect to native cell samples. Collagen content results showed the most prominent reduction in the DDR2 ECD stable cell line, only 3% of the collagen present in nontransfected cells after 7 days incubation, compared to 18% in DDR1 ECDs. However, after 14 days incubation the DDR2 ECD stable cell line exhibited 57% collagen content compared to native cells, with the DDR1 stables exhibiting 52%. Given the close results at day 14 of incubation, it is difficult to draw conclusions regarding the strength of collagen inhibition between DDR1 and DDR2 soluble ECDs without being able to ascertain the collagen content of the media in addition to the adhered cells and ECM.

These results may also provide insight into how the membrane anchored DDR1-/KD may affect collagen fibrillogenesis. Since we found that DDR2-/KD and DDR2-ECD exhibit a very similar affect on collagen fibrillogenesis, it is likely that DDR1-/KD
(and also full length DDR1) may have a similar effect on collagen fibrillogenesis and fiber morphology as DDR1 ECD.

4.3 PHYSIOLOGICAL IMPLICATIONS

Our results elucidate the effects of DDR1 and DDR2 ECDs on regulation of collagen in the ECM demonstrating significant physiological importance given the large number of normal tissues and malignant diseases in which DDRs are expressed.

It is known that through alternative splicing, DDR1 can exist in five distinct isoforms in vivo, DDR1a-e. Since our DDR1 ECD construct resembles the naturally occurring kinase-dead DDR1 splice variants, DDR1d and DDR1e, this work advocates the DDR1d and DDR1e isoforms have a functional role in collagen regulation. Characterizing the DDR1 and DDR2 isoforms that possess DDR ECD is important to further our understanding of the roles played by these proteins in health and disease.

Weakening of the collagen fibers caused by up-regulation of DDR isoforms containing the DDR ECD may enable cancer metastasis in the resultant supple tissue. With the ability to disrupt fibrous formation, DDRs may have the potential to slow progression of benign tumors. The influence of DDRs on the collagen present in diseases such as rheumatoid arthritis or atherosclerosis remains to be evaluated. It is thus critical to evaluate the expression levels of various DDR1 and DDR2 isoforms to understand ECM regulation for disease progression, drug-design or therapeutic applications.
4.4 FUTURE STUDIES

Given that DDRs are prominent in inflammatory diseases and several malignancies, extending research to determine which DDR isoforms are up-regulated and how expression of these isoforms is accomplished would be valuable information in better understanding the mechanisms of these diseases. This is not a small undertaking but a comparison of diseased cells to native cells over-expressing a specific DDR isoform may be a place to start.

To date our in vivo collagen fibrillogenesis investigations, both exogenous and endogenous, have used 3T3 osteoblasts cells. Research done using smooth muscle cells may provide more applicable information as to DDRs behavior in vascular diseases. Use of collagen type III and collagen type IV to stimulate DDRs may also provide novel insights with regard to wound healing due to the prevalence of these collagen types in granulation tissue and basement membrane respectively.
LIST OF REFERENCES


