THE EFFECTS OF DOUBLE STRANDED OLIGODEOXYNUCLEOTIDE ON COLLAGEN PRODUCTION IN A WOUND HEALING MODEL

A Thesis in
Laboratory Animal Medicine
By
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Abstract

Type I collagen is an integral part of dermis, granulation tissue and scar. Collagen deposition is promoted by TGF-β1, a member of a family of pro-fibrotic cytokines, which promotes fibroblast chemotaxis, proliferation, transformation into myofibroblasts, and the synthesis of collagen. We hypothesize that inhibiting the transcription of the COL1A1 gene, type I collagen will be suppressed early in the wound healing process. In this study, local injection of a double stranded oligodeoxynucleotide (dsODN) decoy containing the TGF-β regulatory element found in the distal promoter of the COL1A1 gene, is evaluated in a rat model of wound healing. The effect of reducing the synthesis of type I collagen on wound healing is evaluated by the analysis of type I collagen, type III collagen, and extra domain A (ED-A) fibronectin synthesis. Analysis of fibroblast proliferation, and angiogenesis as evaluated by vascular endothelium was performed. Fluorescent microscopy was used to assess granulation tissue responses to dsODN uptake by the target cells fibroblasts and myofibroblasts. Dot Blot analysis of EDA-fibronectin levels to type I and type III collagen levels were compared in implants treated with 10 nM dsODN, type I (p=0.02) and type III (p=0.01). Collagen levels were significantly decreased compared to controls. Treatments with 3 nM and 1 nM dsODN did not produce significant changes in the levels of type I, type III collagen or fibronectin. Compared to scramble dsODN decoy therapy, histology of decoy treated implants revealed increased cellular density due to decreased connective tissue deposition. When treated implants were stained with Sirius red and viewed under
polarized light, little collagen appeared between fibroblasts in treated implants. These collagen fiber bundles demonstrated a fine green birefringence, consistent with immature collagen fibers. Fluorescently labeled dsODN was found in the nuclei of both fibroblasts and myofibroblasts. In conclusion, dsODN therapy was successful at decreasing type I collagen and also had effects on type III collagen in a wound healing model. Histology revealed increased cell counts corresponding to areas of decreased collagen content.
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<tr>
<td>6-FAM</td>
<td>6-carboxy fluorescein</td>
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<tr>
<td>BAPN</td>
<td>Beta-aminopropionitrile</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>COL1A1</td>
<td>collagen, type I, alpha 1 gene</td>
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<tr>
<td>COL1A2</td>
<td>collagen, type I, alpha 2 gene</td>
</tr>
<tr>
<td>COL3A1</td>
<td>collagen, type III, alpha 1 gene</td>
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<tr>
<td>DAM HRP</td>
<td>donkey anti mouse horseradish peroxidase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsODN</td>
<td>double stranded oligodeoxynucleotide</td>
</tr>
<tr>
<td>ED-A</td>
<td>extra domain A</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethyl alcohol</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>kg</td>
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<td>KGF-2</td>
<td>Keratinocyte growth factor-2</td>
</tr>
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<td>mg</td>
<td>milligram</td>
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</tbody>
</table>
min  minute
ml  milliliter
mM  milimolar
nm  nanometer
nM  nanomolar
ODN  Oligodeoxynucleotide
PAGE  polyacrylamide gel electrophoresis
PBS  Phosphate buffered saline
PBST  Phosphate buffered saline with 0.001% Tween 20
PDGF  platelet derived growth factor
PVA  poly vinyl alcohol
rpm  revolutions per min
sec  seconds
SCID  severe combined immunodeficiency
SMA  smooth muscle actin
Smad  Small Mothers Against Decapentaplegic
TGF-β  transforming growth factor β
TIMP-1  Tissue inhibitor of metalloproteinase 1
µg  microgram
µl  microliter
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Chapter 1

Introduction

Wound healing is a complex process involving a variety of cells. Generally it proceeds in 3 overlapping phases: lag, proliferation, and remodeling. After disruption of the skin, hemostasis and inflammation occurs, which comprise the lag or inflammatory phase of repair. The body's first reaction to injury is to prevent further bleeding by clot formation. Vascular injury cause the vessels to vasoconstrict, and the endothelium and platelets activate the intrinsic coagulation cascade. The clot is a fibrin matrix serving as a scaffold for neutrophils, monocytes, fibroblasts, and endothelial cells migration. Neutrophils and macrophages accumulate in the dead space. Neutrophils clear away bacteria and other microbes and are replaced by macrophages which remove necrotic cells and debris. Macrophages generate nitrous oxide and cytokines that mediate angiogenesis and fibroplasia.

The proliferative phase of repair consists of re-epithelization and granulation tissue deposition. This phase is the anabolic phase of repair. Epithelialization starts soon after wounding and is stimulated by inflammatory cytokines IL-1, IL-6, KGF-2, epidermal growth factor, and transforming growth factor alpha (Diegelmann & Evans, 2004) (Broughton, Janis, & Attinger, 2006a). The normal layers of epidermis are rapidly restored if the basement membrane is intact (Broughton, Janis, & Attinger, 2006c). If the basement membrane is disrupted, the wound must rely on fibroblasts to form a provisional matrix on which the epidermis re-establishes itself. Macrophages recruit fibroblasts to the repair site by secreting transforming growth factor-β (TGF-β) and platelet derived
growth factor (PDGF). As fibroblasts migrate into the wound and become activated, they proliferate and synthesize proteins. TGF-β1 is also released increasing fibroblast production of collagen, proteoglycans, and fibronectin, components of the extracellular matrix (Diegelmann & Evans, 2004). As TGF-β continues to increase in the wound, it directs continued extra cellular matrix production and decreases its degredation. Fibroblasts under the influence of TGF- β increase production of collagen, increase tissue inhibitors of metalloproteinase, and decrease production of matrix metalloproteinase. TGF-β, extra domain A (ED-A) cellular fibronectin, and mechano-tension of the wound cause fibroblasts to differentiate into myofibroblasts (Gabbiani, 2003) (Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002). The exact mechanism of ED-A fibronectin in the transformation process in not known but the theory is the binding of ED-A fibronectin to a cell surface receptor or an extracellular matrix component is needed for permissive signaling (Gabbiani, 2003). Fibroblasts will also secrete keratinocyte growth factors 1 and 2 along with IL-6 which stimulate neighboring keratinocytes to migrate into the wound area, proliferate, and differentiate into epidermis(Diegelmann & Evans, 2004).

The remodeling phase of wound healing can last up to 1 year(Broughton et al., 2006c; Broughton et al., 2006a). Collagen deposition goes through a reorganization process to increase wound strength. Collagen, the major protein found in the human body, is regarded as the material responsible for providing strength in many tissues including skin and is (Broughton et al., 2006a; Deodhar
& Rana, 1997). There are 23 types of collagen. Type I and type III collagens are in the highest concentration in a healing wound.

Role of TGF-β In Wound Healing

TGF-β is a member of a family of potent cytokines known as the TGF-β superfamily. This superfamily includes inhibins, activin, anti-müllerian hormone, bone morphogenetic protein, decapentaplegic and Vg-1 (Roberts, 2002) (Burt, 1992; Chin, Boyle, Parsons, & Coman, 2004; Miyazono, 2000; Roberts, 1999). There are 3 isoforms of TGF-β, TGF-β1, TGF-β2, and TGF-β3. TGF-β1 promotes fibroblast chemotaxis, proliferation, transformation into myofibroblasts, and the synthesis of connective tissue components (Grouf, Throm, Balestrini, Bush, & Billiar, 2007) (Coker et al., 1997) (Santana, Saxena, Noble, Gold, & Marshall, 1995). Persistent exposure to TGF-β1 promotes excess scaring characterized by connective tissue containing type I collagen and ED-A fibronectin (Meisler et al., 1995) (Ling & Robinson, 2002).

TGF-β has a wide range of effects on the immune system. In vitro studies have shown that TGF-β is involved with proliferation, activities, and differentiation of lymphocytes, dendritic cells, and macrophages. TGF-β affects both B and T cells by inhibiting their proliferation, while stimulating their entrance into apoptosis (Moustakas, Pardali, Gaal, & Heldin, 2002). This serves as a means of immunosupression and regulation of the immune system. Mice engineered with knockout of the TGF-β1 gene develop a multifocal inflammation with severe infiltration of lymphocytes and macrophages into most major organ systems at 6
weeks (Crowe, Doetschman, & Greenhalgh, 2000). These TGF-β1 deficient mice also have signs of autoimmune disease in multiple organ systems (Letterio et al., 1996). Analysis of these mice show an increased adhesion of leukocytes to the vascular endothelium and major histocompatibility complex levels are increased at the start of inflammation (Letterio et al., 1996). They attributed the phenotype of these mice to a loss of TGF-β1 anti-proliferative effect on lymphocytes. TGF-β1 /- and severe combined immunodeficiency homozygous (Scid /-) mice were created to evaluate the effects of TGF-β1 on wound healing without the severe immune response associated with removal of the TGF-β1 gene. These mice showed a delay of about 1 week in all phases of healing compared to Scid /- mice with the wild type TGF-β1 gene (Crowe et al., 2000). This model shows the importance of allowing TGF-β1 to function normally in wound repair.

Type I Collagen

Trauma initiates wound repair and the deposition of granulation tissue, which is rich in type I collagens. Type I collagen has 2 α1(I) chains and 1 α2(I) chain stabilized in a triple helix. The COL1A1 and COL1A2 genes reside on differing chromosomes but their expression is potentially coordinated by similar CIS elements in their 5’-flanking region (Cutroneo, 2003). Similar CIS elements include AP1, SP1, YB-1b but differ in the transacting factor initiating transcription (Dhalla, Ririe, Swamynathan, Weber, & Guntaka, 1998). The TGF-β1 receptor advances type 1 collagen expression by promoting the transcription of proα2 (I) gene [COL 1A2] through the Smad signaling pathway (Cutroneo & Ehrlich, 2006)
The Smad signaling pathway is the classic signal transduction pathway for TGF-β family members. The TGF-β receptors and Smad signaling pathway is responsible for a variety of effects on fibroblasts including collagen production and differentiation into myofibroblasts (Evans, Tian, Steadman, & Phillips, 2003). TGF-β binds to the TGF-β1 receptor in the cell membrane, which in turn forms a heterodimer with the TGF-β1 receptor initiating activation of the Smad regulatory proteins. The TGF-β1 receptor protein has serine/threonine kinase activity phosphorylating Smads 2 and 3. Smads 2 and 3 then couple with Co-Smad 4 and the complex translocates into the nucleus. Once in the nucleus, this group is directed to DNA targets by specific inhibitors and activators to promote gene transcription. Increased expression of Smads 2, 3, and 4 lead to increases in collagen production (Zhang, Ou, Inagaki, Greenwel, & Ramirez, 2000). Smads 6 and 7 belong to a group of inhibitory proteins that antagonize receptor mediated Smads. Smad 7 negatively regulates Smad 2, preventing the Smad 2, 3 phosphorylation from occurring, effectively down regulating the fibrotic signal from TGF-β1 stimulation (Tahashi et al., 2002). The transcription of COL1A1 gene is through the TGF-β1 activating protein (Cutroneo, 2006). Similar to the Smad signaling pathway responsible for the initiation of transcription of the COL2A1 gene, the TGF-β activator protein complex is phosphorylated by the TGF-β1 and TGF-β1 receptor heterodimer. The TGF-β activator protein complex then goes to the nucleus where it directly binds to the TGF-β CIS element in the distal promoter region of the COL1A1 gene (Cutroneo & Chiu, 2000). This specific TGF-β CIS element makes an effective target for dsODN decoy therapy.
DsODN decoy therapy relies on targeting a specific gene and DNA sequence to block gene expression. The more specific the sequence greater the likely hood the dsODN molecule will only have specific effects on the desired target. If the DNA sequence is shared by multiple genes, the dsODN decoy could bind multiple trans-acting factors potentially affecting transcription of non-targeted and un-wanted genes. Type III collagen is composed of a homotrimer of α1(III) chains. The COL3A1 gene is responsible for the production of type III procollagen. Initiation of transcription is activated by the TGF-β1, Smad 2,3,4 complex similar to the initiation of transcription of the COL1A2 gene (Verrecchia, Chu, & Mauviel, 2001).

TGF-β1 increases ED-A Fibronectin by a different pathway compared to type I and type III collagen. Along with the Smad signaling pathway, TGF-β also activates c-Jun N-Terminal Kinase (JNK) a member of the mitogen activated protein (MAP) kinase family (Kyriakis & Avruch, 1996). Hocevar et al, determined TGF-β stimulates fibronectin synthesis through a c-Jun N-terminal kinase dependent pathway. They also show TGF-β’s effects to be independent of Smad 4 which is an important co-Smad in type I collagen transcription (Hocevar, Brown, & Howe, 1999).

Members of the Smad signaling pathway are directly involved in the initiation of the COL1A2 gene transcription but are also involved in other genes. An example is the cooperation of Smad 3/4 with Runx1/AML1 and Runx/AML2 binding the germ line IgCα gene promoter sequence to induce transcription leading to IgA class switching (Feng & Derynck, 2005). By targeting one of the
Smad proteins it would affect a variety of processes in the cell not just the production of collagen.

Overview of Hypertrophic Scarring

The body’s response to trauma and tissue loss is repair through the restoration of connective tissue. Regeneration of tissue identical to tissue lost is ultimately the best possible outcome. The repair process can be inappropriate resulting in excessive scar formation. This scar tissue can form hypertrophic scars resulting in cosmetic and functional defects to the skin (Ariyan, Enriquez, & Krizek, 1978; Slemp & Kirschner, 2006). Scar tissue can inhibit normal mobility of joints and disrupt locomotion. In excess scarring, myofibroblast populations persist, a rich vasculature remains and exuberant immature disoriented collagen fibers are common (Ehrlich, 1998).

Incidence of Hypertrophic Scarring

Any damage to the deep dermal tissue including ear piercing, vaccinations, abrasions, surgery and burns can lead to hypertrophic scarring. A severe clinical presentation occurs in burn patients, where the closure of the wound leads to excess scar deposition, scar contracture and loss of functional motion. Rates of hypertrophic scarring in burn victims ranges from 67-91% (Bombaro et al., 2003) (Leventhal, Furr, & Reiter, 2006) Hypertrophic scarring also occurs in surgical patients undergoing routine procedures. Post surgical hypertrophic scarring can occur in 40-70% of patients. (Leventhal et al., 2006) There is an increased
incidence of hypertrophic scar formation when surgery is performed over or around joints as commonly done in orthopaedic procedures.

Hypertrophic scars commonly occur after wound closure and may progress or regress. Chest, ears, cheeks, shoulders, upper arms and upper back are areas predisposed to excess scarring (Alster & Tanzi, 2003). Lesions are raised above the surface of the skin and often have abnormal pigmentation. The scar tissue is cosmetically inappropriate and rarely regains normal pigmentation, texture, and hair re-growth. Symptoms such as pain, itching and burning, along with restriction of motion are commonly associated with hypertrophic scars.

Leventhal et al, (Douglas Leventhal, MD; Maxwell Furr, BS; David Reiter, MD, DMD: Arch Facial Plast Surg. 2006;8:362-368) conducted a literature search identifying 70 published clinical treatments of hypertrophic scarring. Their meta-analysis revealed a 70% chance of improvement with treatment but only a 60% mean amount of improvement. They also found no statistical difference in any of the treatment modalities. Treatments of abnormal scar include surgery to excise the scar, injection with steroids, radiation therapy, cryotherapy, laser therapy and bleomycin injection (Hudson & Renshaw, 2006; Saray & Güleç, 2005; Whang et al., 2006; Leventhal et al., 2006). Surgical intervention with procedures designed to release the scar tension and transposition of healthy skin are attempted to regain function in affected limbs (Hudson & Renshaw, 2006; Motamed, Hasanpoor, Moosavizadeh, & Arasteh, 2006). Excision and primary repair of scar tissue is commonly performed in plastic surgery. Z-plasty is one of the most commonly used surgical techniques used to remove scar tissue with minimum
contraction and reduce tension across the incision (Suzuki et al., 1998). Z-plasty involves the creation of two triangular flaps of equal dimension that are then transposed. This technique has been expanded upon to include double opposing V-Y-Z plasty to correct more serious contractures (Tan, Atik, & Ergen, 2006). It is also possible to transpose a pedicle flap into an area where scar tissue has been removed. The ultimate goal is to reduce the amount of connective tissue produced by fibroblasts and degrade existing connective tissue in the lesion. Most of the aforementioned treatments involve significant discomfort to patients or have potential unwanted side effects. The ability to limit scar tissue formation in a focal area with little side effects would be of great value to patients.

DsODN Therapy

The dsODN decoy is a therapeutic approach for regulating gene transcription. ODN decoys are a class of anti-genes that regulate gene transcription. Transfection of dsODN decoys corresponding to the cis-sequence results in the attenuation of authentic cis-trans interaction, leading to the removal of the trans-factor binding to the endogenous cis-element with subsequent modulation of gene expression. One of the principle problems with ODN therapy relates to its sensitivity to nucleases, where unmodified ODN decoys have a short half-life. ODN can be rapidly degraded by endo- and exonucleases in body fluids and blood. A commonly used class of ODN is phosphorothioate oligodeoxynucleotides containing sulfur in place of one of the non-bridging
oxygens in the phosphodiester bond between adjoining nucleosides. These modified ODN have greater stability by being more resistant to nuclease digestion, increasing its half life (Phillips & Zhang, 2000). Double stranded ODN have increased activity within the cell and are subject to less degradation than single stranded ODN. The use of double stranded oligodeoxynucleotide decoy (dsODN) specific to the COL1A1 promoter region showed a greater reduction in collagen production than single stranded ODN (Cutroneo & Boros, 2002).

Decoy therapy is not only a strategy for an anti-gene therapy, but it is a powerful tool for investigating the consequences for endogenous gene regulation. Administration of NFK-β decoy ODNs in arthritic joints of rats from collagen-induced arthritis, led to an amelioration of the condition (Tomita et al., 1999). The intramuscular injection of ODN decoy to the NFK- binding site inhibits weight loss in mice tumor models. (Kawamura et al., 1999). Other examples of decoy therapies include preventing neointimal thickening in cardiovascular disease (Morishita, Aoki, & Kaneda, 2001), and arrested tumor growth in cancer (Ahn et al., 2003).

Preliminary Models and Data
Schistosoma mansoni is a trematode parasite affecting 2 billion people worldwide and 300 million people suffer severe morbidity as a result of being infected with the parasite (Child, 2006). Schistosomiasis induces hepatic and intestinal fibrosis in murine models of disease. Schistosomiasis is the fibrotic response to the parasite eggs in the hepatic tissue. These eggs induce a T
lymphocyte mediated granulomatous inflammation with chronic hepatic fibrosis. This granuloma model develops excessive deposition of collagen and fibronectin. Infected mouse livers show elevated levels of TGF-β1. Boros and coworkers (2005) systemically treated infected mice with 800mg of dsODN, containing the TGF-β regulatory element acting as a promoter inhibitor, over 4 days. COL1A1, as well as TGF-β1 and TIMP-1 and type III procollagen were suppressed(Boros et al., 2005). This study determined that dsODN decoy therapy in an in vivo models inhibited type I and type III collagen synthesis in chronic liver fibrosis.

Ehrlich et al, (2003 unpublished data) placed polyvinyl alcohol (PVA) sponge implants in subcutaneous pockets in the backs of rats. Implants were treated with 10nM dsODN and 9 days post implantation the sponge implants were harvested. Histological and type I collagen analysis was performed on the implants. By H&E staining, increased cell density and decreased space separating cells was identified in the dsODN decoy treated sponges. Sirius red staining was performed and PVA sponges were evaluated microscopically using polarized light. Granulation tissue in dsODN treated sponges granulation tissue had less intense birefringence suggesting less mature collagen fibers were layed down.
Chapter 2

Hypothesis

Gene decoy therapy represents one of several evolving techniques to affect the production of collagen by inhibiting transcription of a specific gene. Inhibition of type I collagen synthesis by blocking the transcription of the pro $\alpha_1$(I) gene [COL1A1] through the TGF-$\beta$1 activation protein pathway has the potential to affect the quantity and quality of granulation tissue formation. The effects of inhibiting type I collagen synthesis are unknown but may prove effective in treating abnormal scars.

We postulate that treatment with dsODN decoy therapy will alter both the quality and quantity of granulation tissue deposited in an in vivo rat wound healing model. The amount of type I collagen deposited in rat PVA sponge implants will be decreased and the biochemical and histological character of granulation tissue in dsODN decoy treated animals will be altered compared to control animals.

The specific aims of this investigation are:

1. Determine the minimal dose of dsODN decoy therapy that optimally inhibits type I collagen deposition while not affecting type III collagen or EDA fibronectin synthesis.

2. Identify dsODN uptake and cellular phenotypes in an in vivo model of wound healing, using fluorescent labeled dsODN with fluorescence microscopy.
Chapter 3

Materials and Methods

The experimental protocol was approved by The Penn State Hershey Institutional Animal Care and Use Committee, and animal care was provided according to the guidelines set forth in The Guide for the Care and Use of Laboratory Animals, (National Research Council), under the supervision of veterinarians in the Department of Comparative Medicine, College of Medicine, The Pennsylvania State University. A total of 26 male, 6 week old, approximately 175g, Sprague Dawley rats (Charles River Labs, Wilmington, MA,) were individually housed during quarantine and maintained throughout the study. Housing conditions included a with a 12 hr light:dark photoperiod and temperature and humidity in the room were maintained at 68°F ± 2°F and 50% ± 20%, respectively. Water and rodent diet, (Harlan Teklad, Madison, WI) were provided ad libitum.

ODN Ordering

Unpurified decoy and scramble ODN was produced by the Macromolecular Core Facility (Penn State Hershey College of Medicine, Hershey PA). See Figure 1 for exact sense and antisense sequences. The single stranded ODN was synthesized on a Polygen GmbH (Langen, Germany) synthesizer and was dried and stored at -70°C until annealed. Purified ODN was purchased from Operon Biotechnologies Inc. (Huntsville, AL). High performance liquid chromatography (HPLC) was used to purify ODN to remove truncated failure sequences created during the synthesis of the molecules. These
truncated sequences may have unwanted effects on both targeted and non-targeted gene sequences. A purity level equal to 85% or greater pure nucleotides was achieved after HPLC. A portion of the purified nucleotides was ordered with a fluorescent tag to allow identification of the location of the molecules within cells. 6-carboxy fluorescein (6-FAM) was added to the 5’ end of the sense strand of both decoy and scramble ODN to facilitate identification of ODN via fluorescence microscopy inside cells. 6-FAM is a carboxyfluorescein derivative that absorbs light at 495 nm wavelength and fluoresces at 520 nm wavelength. It is commonly used to label oligonucleotides.

Annealing ODN

Oligodeoxynucleotides (ODN) were annealed by placing equal nanomolar amounts of sense and antisense strands into a 1.7 ml microcentrifuge tube filled with 500ul of sterile Dulbecco’s phosphate buffered saline (PBS). The strands were heated to 95°C for 4 min in a temperature controlled heat block, allowed to cool to room temperature for 45 min, then aliquoted into 0.2ml doses. Individual doses were frozen and stored at -70°C until use.

PVA Sponge Implants

Sponges were cut from a sheet of poly vinyl alcohol (PVA) material 0.3cm x 20 cm x 35 cm) with a 1.2 cm diameter circular punch. The inner diameter of 0.5 cm was removed with a second punch to create a doughnut shaped disc. Plastic backing material of 1.2 cm diameter was attached to one side of the
sponge with No Tape™ silicon adhesive (Vapon Inc., West Caldwell, NJ). All sponges were allowed to dry and then weighed to the nearest 0.01 gram.

Prior to surgery all animals were anesthetized with 30-50 mg/kg ketamine (Ketaset® Fort Dodge/Wyeth, Madison NJ) and 5-10 mg/kg xylazine (Rompun® Bayer, Shawnee Mission, KS) given via intraperitoneal injection with a 27 gauge needle. Skin on the dorsum of the animal was clipped (6x6cm) and cleaned with Nolvasan® Surgical Scrub (2% Chlorhexidine) (Fort Dodge/Wyeth) followed by 70% isopropyl alcohol rinse. All surgical instruments were steam sterilized prior to use. Using aseptic technique and a #15 scalpel blade, a 2cm long full thickness skin incision was made parallel to the spine. The incision was centered over the thoracolumbar junction of the spine as determined by manual palpation. A minimal amount of blunt dissection of subcutaneous tissues was required to create a pocket for sponge implantation. The sponge implants were sterilized by boiling them in distilled water for 5 mins. Sponges were blotted dry to remove excess water and allowed to cool prior to implantation. Two PVA sponges were inserted subcutaneously, one on either side of the spine at the level of the thoracolumbar junction. Three stainless steel staples were used to close the incision. The rat was allowed to recover from anesthesia and returned to individual housing.

Individual doses of ODN were brought to room temperature 30 mins prior to animal treatment. Treatments were administered once daily on the morning of days 3,4,5,6 post sponge implant. Twenty five gauge needles were placed on 1ml syringes and 0.2ml of air were drawn into the syringe prior to loading with 0.1
ml ODN. This ensured there was no ODN left in the syringe hub or needle. Animals were manually restrained for treatment. The needle was advanced through the skin until contact was made with the plastic backing in the middle of the implant and then slightly withdrawn prior to injecting. Animals did not show any adverse signs or need for pain medications after treatments were performed and were returned to their cages.

Ten days after surgical sponge implantation all animals were sacrificed via an intraperitoneal injection of 0.5ml of a concentrated (240mg/ml) pentobarbital solution (Sleepaway® Fort Dodge/Wyeth). Cervical dislocation was used as a secondary method of euthanasia. A razor blade was used incise the skin creating a wide margin around the sponge implants. The capsule surrounding each implant was carefully dissected away using forceps and sharp tonotomy scissors. Once removed, all tissue not directly part of the capsule was trimmed away. Implants were weighed to the nearest 0.01 g on a digital balance (AB104-S/FACT, Mettler Toledo) and weights recorded. The tissue effacing the plastic implant backing was trimmed away to facilitate sponge removal. A razor blade separated the sponge with intact capsule from the plastic backing material. The sponge was bisected into two equal halves. One half was used for biochemical analysis, placed into a 2 ml microcentrifuge tube with 1ml of 500 mM chilled acetic acid. Tonotomy scissors mascerated the tissue in the solution to facilitate collagen extraction. An additional 0.5 ml of acetic acid was added with 0.15 mls of a 100 mcg/ml pepsin (Sigma Chemical Co., St. Louis MO). Acetic acid and pepsin were used to optimize the extraction of collagen while digesting some of
the fibronectin. There was a sufficient amount of fibronectin available for dot blot analysis and we had a similar fibronectin response in all samples. Each sample was then centrifuged for 10 sec and stored at 4°C for 24 hrs.

Biochemical Analysis

Implant samples for biochemical analysis were incubated for 24 hrs at 4°C. All samples were centrifuged for 5 mins at 5000 rpm to separate tissue from soluble protein supernatant. One ml of supernatant was removed from each sample and placed in a microcentrifuge tube. Samples were kept on ice during handling. Each sample was titrated to a basic pH of 12 by adding 0.300ml of 2 M and vortexing for 10 seconds. pH was assessed by using pH paper (Hydrion Papers, Micro Essential Laboratory) to ensure uniformity of samples. All samples were stored at 4°C.

Dot Blot Dilution Determination

The first dot blot assays produced a significant background rendering them useless for densitometry readings. (Figure 2A) Multiple attempts at repeating the assays continued to yield useless results. Bio Rad was contacted as equipment failure was thought to be the most likely cause. New silicone gaskets were tried as well as increasing the background blocking time prior to antibody incubation. Finally multiple dilutions to reduce the amount of background present on the autoradiographs was tried. It was found a dilution of 1:10 or 1:100 created optimal blots (Figure 2B) for densitometric readings. Once the dilution factor was determined the same dilution was used for type I collagen,
type III collagen, fibronectin, and total protein analysis. The need to dilute the samples up to 100 times illustrates the sensitivity of the assay.

Samples were diluted to a 1:10 or 1:100 concentration by combining 0.1 ml of sample with 0.9 ml of sterile water or 0.01 ml sample with 0.99 ml of sterile water respectively. The diluted samples were vortexed for 10 sec and returned to an ice bucket. This created the stock solution used for both the dot blot and protein assay. A nitrocellulose membrane was soaked in 1x PBS for at least 10 min prior to use. Samples for the dot blot apparatus (Bio Dot, BioRad) were created by combining 0.02 ml of sample with 0.198 ml of 1x PBS per well and then vortexed for 10 sec. The apparatus was assembled with the nitrocellulose membrane and the wells were washed with PBS and vacuum applied to remove the PBS. Samples were vortexed prior to loading and 2 wells were loaded with each sample. The apparatus sat for 2 hrs to allow for gravity drainage. After 2 hrs, suction was applied to empty all wells and then each well was washed with PBS. With suction on, the apparatus was disassembled and the nitrocellulose membrane removed and cut into appropriate sized sections. The membrane then was dried overnight at 4°C.

PBS with 0.001% Tween 20 (PBST) was prepared for the background blocking and antibody dilution. Nitrocellulose sections were placed into a dried milk solution for 1 hr. Primary antibody solutions at a 1:10,000 dilution were prepared using collagen I (Sigma Chemical Co, St. Louis, MO), collagen III (Sigma Chemical Co), and EDA Fibronectin (Sigma Chemical Co) antibodies. Nitrocellulose membranes were incubated in primary antibody 1 hr, thoroughly
washed with PBST, and placed into secondary antibody. The secondary antibody is donkey anti mouse horseradish peroxidase (DAM HRP) (Sigma Chemical Co), was diluted 1:100,000 with PBST. The nitrocellulose membrane was incubated with a secondary antibody for 1 hr and then washed with PBST. The nitrocellulose membrane was dried and treated with Femto Chemiluminescent (Pierce) for 5 min and then exposed to radiographic film (Kodak). Radiographic films were developed with an automatic processor.

Auto radiographs were scanned using a Linotype-Hell (Heidelberg CPS Ltd, Cheltenham, UK) scanner and images were imported into Adobe Photoshop®. Densitometry readings of the auto radiographs were performed using Quantiscan software (Biosoft, Cambridge, UK). All data was then imported into a Microsoft Excel® spreadsheet for basic analysis.

Individual samples for protein content was determined by the BCA™ protein assay kit (Pierce Biotechnology). The BCA protein assay is a highly sensitive colormetric reaction capable of quantifying protein concentrations from 20-2,000 µg/ml. Protein fractions equal to and larger than tripeptide fragments produce a colorometric change that is evaluated. A 96 well plate (Becton Dickinson) was used to analyze samples of non-soluble protein lysate compared against a standard. Bovine serum albumin was used as a standard and paired 20 µl samples were placed in the first 2 wells in the top left of the plate. Paired 20 µl unknown samples were pipetted in to wells along the top and 5th row of the plate. Ten µl of distilled water was pipetted into the remaining wells. Using a clean pipette tip, 10 µl of standard was withdrawn from the first well and mixed
with distilled water in the well immediately below. Pipetting and mixing of 10 µl of
diluted sample was continued to create a serial dilution. Each unknown sample
repeated the dilution procedure 3 times to create a total of 4 wells for analysis. A
total volume of 200 µl per well of 1:50 mixture of Reagent B (4% cupric sulfate) to
Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and
sodium tartrate in 0.1 M sodium hydroxide) was calculated and mixed. After
pipetting 200 µl of the Reagent A:Reagent B mixture into each well the plate
was incubated at 37°C for 30 min. The assay was read at 562 nm by MRX™
Revelation absorbance reader. (Dynex Technologies, Inc., Chantilly, VA)

Histology

The second half of the implant was further divided into 2 equal samples
representing one quarter of the total implant. One quarter of the implant was
placed in 3 mls of buffered formalin for histopathology. The formalin fixed implant
was embedded in paraffin and sectioned for hematoxalyn and eosin staining as
well as Sirius red staining. Sirius red staining was performed on 5 µm thick
formalin fixed paraffin embedded tissue sections. Slides were de-paraffinized by
immersing the slides xylene 3-5 mins, xylene 3-5 mins, 100% EtOH 2 mins,
100% EtOH 2 min, 95% EtOH 2 min, 70% EtOH 2 min, and rinsed with tap water
3-5 min. After de-paraffinizing the slides, they were treated overnight by
immersion in Bouins fixative. Bouins fixative is composed of 750ml picric acid
solution (saturated aqueous solution), 250 ml 37-49% formaldehyde, 50ml glacial
acetic acid. Slides were immersed for 20 min in 0.1% Sirius Red (Rowley
Biochemical Institute) in saturated aqueous picric acid solution. After treating with Sirius Red, slides are washed 4 times for 1 min each with distilled water to remove unbound dye. Just before coverslipping, slides were dehydrated by immersing them in 95% EtOH 2 min, 95% EtOH 2 min, 100% EtOH 2 min, 100% EtOH 2 min, xylene 2 min, xylene 2 min, xylene 2 min, and xylene 2 min. Finally the slides were coverslipped with Permount mounting media (ProSciTech, Australia).

Slides for Hematoxylin and Eosin staining were de-paraffinized by immersing the slides xylene 4 mins, xylene 4 mins, 100% EtOH 2 mins, 100% EtOH 2 min, 95% EtOH 2 min, 70% EtOH 2 min, and rinsed with tap water 3-5 min. Slides were immersed in Harris' Hematoxylin for 4 mins then rinsed with running tap water. The slides were then dipped 5 times in ammonia water and rinsed for 15 min in running tap water. Eosin-Phloxine was applied for 2 min then rinsed. Just before coverslipping, sides were dehydrated by immersing them in 95% EtOH 2 min, 95% EtOH 2 min, 100% EtOH2 min, 100% EtOH 2 min, xylene 2 min, xylene 2 min, and xylene 2 min.

The other quarter was subdivided and placed into a cryosectioning mold and it was filled with mounting medium (Tissue-Tek Sakurea Finetechical Co. Ltd, Tokoyo, Japan). Samples were stored in a -70°C freezer until sectioned. Frozen samples were cryosectioned at 8 µm thickness for immunohistochemistry and fluorescent microscopy.

Fluorescent Histology

Cryosectioned implants from the 10 nM purified ODN treatment were chosen at random for immunohistochemical staining for Von Willebrands Factor
(Factor VIII) (Sigma Aldrich Co) and α-smooth muscle actin (SMA) (Sigma Aldrich Co). Slides were defrosted from storage at -70°C and allowed to dry for 10 min. Implant sections were fixed with 4% para-formaldehyde for 10 min. and rinsed with cytoskeletal buffer. Cytoskeletal buffer was created by adding 137 mM NaCl, 5 mM KCl, 1.1 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4 mM NaHCO₃, 2 mM MgCl₂, 3.3 mM Glucose, 2 mM EGTA and 5 mM Pipes (Fisher Scientific) (Dave, Banducci, Graham, Allison, & Ehrlich, 2001). Cytoskeletal buffer helps maintain the microtubule skeleton of cells during immunohistochemistry. Triton X100 0.1% was applied and rinsed prior to background blocking of samples. Triton X100 is a non-ionic detergent used to permeabilize cell membrane to allow antibody intracellular uptake. Sections to be stained with Factor VIII were blocked with 5% goat serum for 1 hr to reduce background staining. Sections to be stained with α-SMA were background blocked with 5% donkey serum for 1 hr. The slides were rinsed and primary antibodies diluted 1:200, were added to each slide. After 1 hr incubation with primary antibody, the samples were rinsed with cytoskeletal buffer and secondary antibodies applied. The secondary antibody for Factor VIII was rhodamine labeled goat anti mouse IgG diluted 1:200 along with alexa (green) phalloidin diluted 1:200. For α-SMA samples, the secondary antibody was rhodamine labeled donkey anti mouse IgG diluted 1:200. All secondary antibodies were rinsed with cytoskeletal buffer and nuclear stain 4’,6-diamidino-2-phenylindole (DAPI) applied for 5 mins. DAPI binds to DNA to identify cell nuclei, staining them blue. After rinsing the slides, cover slips were
attached with GEL/MOUNT™ (biØmeda corp, CA). Fluorescence microscopy was used to visually identify α-SMA microfilaments and Factor VIII.

6-FAM Animals
To investigate the uptake of dsODN by fibroblasts and myofibroblasts in vivo, 6-FAM labeled dsODN were injected into 2 separate animal implants. PVA implants were placed subcutaneously in 2 rats as previously described. At 9 days one rat had, 10 nM of 6-FAM labeled decoy ODN injected into both sponges implants. The other animal had 10nM of 6-FAM labeled scramble ODN injected into both sponge implants. Implants were harvested on day 10 for cryosectioning and staining with rhodamine α-SMA and DAPI. Implant sections were kept in limited light conditions to preserve the fluorescence of the 6-FAM label. After cryosectioning the slides were stored at -70°C until used. Immunohistochemistry was performed as previously described but all samples were blocked with 5% donkey serum and slides from both decoy ODN and scramble ODN were stained with rhodamine α-SMA and DAPI. Fluorescent microscopy to identify 6-FAM ODN uptake in fibroblast and myofibroblast nuclei as well as general distribution of ODN in cell cytoplasm.

Cell Counts
Quantitative cell counts were performed on H&E stained sections of sponge implants to determine if there were differences in cellularity attributed to dsODN treatment. Five cellular areas within each sponge matrix along the inner
border of the capsule were randomly chosen for counting. Using a 250x magnification and a standard grid, the number of cells inside 4 squares were counted per area. This yielded approximately 35-55 cells per 4 boxes for a total of 200-300 cells per sample.

Statistical analysis
Comparison of both type I and type III collagen to fibronectin/total protein and cell counts were analyzed by performing a standard unpaired Student’s T-test using Minitab® (Minitab Inc, State College, PA) software package. All graphs were created in Microsoft Excel® (Microsoft Inc, Redmond, WA). Statistical significance was set at p<0.05.
Figure 1. Exact Nucleotide Sequence of Decoy and Scramble dsODN

DECOY

SENSE STRAND

ANTI-SENSE STRAND

SCRAMBLE

SENSE STRAND

ANTI-SENSE STRAND

Figure 1. Exact sense and antisense base pair sequences for dsODN
Figure 2. Pre and Post Dot Blot Dilution

2A: Auto Radiograph with Concentrated Sample type I collagen

2B: Auto Radiograph after dilution of sample 1:10 and 1:100 type I collagen
Chapter 4
Results

Type I and III Collagen
The ratio of type I collagen and type III collagen was compared to a loading standard of fibronectin divided by total protein. Purified 10 nM decoy dsODN implants showed type I collagen was 46% (p=0.021) and type III collagen was 52% (p=0.013) of scramble treated implants. (Figures 3A,3B) Non-purified 10nM dsODN type I collagen was 56% (p=0.011) but type III collagen was 90% (p=0.073) when compared to similarly treated scramble treated implants. (Figures 4A,4B) Type I collagen in 3nM purified dsODN treated implants was 64% (p=0.18) of scramble treated implants(Figures 4A) while type III (p=0.54) collagen was increased 121% (Figure 4B). 1nM purified dsODN treated implants type I collagen was 76% of scramble treated implants (p=0.047). (Figure 6A)

Fibronectin is a glycoprotein widely distributed in extra cellular matrix and increased levels are in granulation tissue. Both ED-A and ED-B splice segments are found in cellular fibronectin. TGF-β preferentially increases ED-A fibronectin in in vitro cultured fibroblasts (Balza, Borsi, Allemanni, & Zardi, 1988).

Colormetric reactions of the Pierce BCA Protein assay is dependent on the number of peptide bonds and the presence of four particular amino acids (cysteine, cysteine, tryptophan, and tyrosine). The BCA protein assay does not accurately measure collagen due to decreased numbers of cysteine, tryptophan, and tyrosine residues present in collagen.

By dividing the fibronectin by the amount of total protein present we were able to normalize the differences in sample protein concentrations loaded on a
per volume basis. There was a statistically significant increase in the fibronectin/total protein ratios in non-purified 10nM 155% (p=0.008), purified 10nM 211% (p=0.014), and purified 1nM 138% (p=0.006) decoy dsODN treated implant groups compared to scramble dsODN treated implant groups. (Figures 3D, 4D, 6D) The fibronectin/total protein ratio in the 3 nM treated implant group was increased 138% (p=0.62) but was not statistically different compared to scramble treated dsODN. (Figure 5D)

Total protein content was decreased significantly decrease in 10 nM purified dsODN (56% of scramble treated implants, p=0.015) and non-purified 10 nM dsODN (64% of scramble treated implants, p=0.039). The 1 nM and 3 nM treated implants did not show statistically significant decreases in total protein.

Histology
Fluorescent microscopy was used to determine the uptake of dsODN in an in vivo model of wound healing. Both fibroblasts and myofibroblasts were positive for 6-FAM labeled dsODN uptake 24 hrs after administration. Labeled dsODN (green) were observed both within the cell cytoplasm and nuclei of fibroblasts and myofibroblasts. (Figure 8A) The DAPI stained nuclei had a definite visual color change when green 6-FAM dsODN was present in the nuclei compared to surrounding cells. The nuclei had an aqua color resulting from a combination of the blue DAPI nucleus and the green of the 6-FAM dsODN decoy. 6-FAM scramble and decoy dsODN were identified primarily in the nucleus and cell cytoplasm of fibroblasts compared to myofibroblasts. (Figure 11A)
H&E stained sections of 10 nM purified dsODN (p=0.004) decoy treated sponge implants and 10 nM non-purified dsODN (p=0.0002) treated implants showed a statistically significant higher cell count compared to scramble treated implants. The collagen in these areas was not linearly arranged and there was less present. Fibroblast nuclei did not align in a linear orientation (Figure 9).

Similar changes were not noted in the 3 and 1 nM treated sponge implants. The areas immediately adjacent to the treated regions had more linearly arranged collagen bundles and fibroblast cell nuclei. There appears to be more collagen present around individual cell nuclei.

Sirius red staining was performed to evaluate collagen deposition in implants after treating with COL1A1 specific dsODN. Sirius red staining of 10nM purified dsODN decoy showed decreased collagen staining within the sponge implant. The collagen present had fine birefringent green fibrillar pattern indicative of immature type III collagen. (Figure 10A) Larger bundles of birefringent yellow and red mature type I collagen in the capsule bordered the areas of decreased collagen (Cuttle et al., 2005) (Li, Lei, & Gao, 2002) (Junqueira, Cossermelli, & Brentani, 1978) (Junqueira, Bignolas, & Brentani, 1979). The mature collagen was arranged in a linear fashion around the capsule. Blood vessels present in the inner layers of decoy treated implant capsules had little collagen surrounding them. The quality of the collagen was very fine fibrillar birefringent green collagen (Figure 10B). Blood vessels present in the 10nM purified dsODN scramble treated implants showed thick bundles of
birefringent red and yellow collagen encircling them (Figure 10C) which is what is expected.

Myofibroblasts express $\alpha$-SMA filaments differentiating them from fibroblasts. The majority of the cells in the 10nM purified dsODN decoy and scramble treated sponge implants were positive for $\alpha$-SMA and had an intense fluorescent response (Figure 11B). This is indicates the myofibroblast is the predominant cell type present in the outer and medial capsule of the implants. The inner sections of the sponge implants had no $\alpha$-SMA uptake indicating the cells were fibroblasts. We expected the most mature cells (myofibroblasts) to be on the outer region of the capsule and the most immature cells (fibroblasts) in the inner regions. DsODN did not affect cell phenotypes and maturation of fibroblasts into myofibroblasts. The maturation of fibroblasts to myofibroblasts is dependent on the Smad pathway and was unaffected by decoy dsODN (Evans et al., 2003).

VonWillebrands factor was assessed to determine vascular and capillary in growth into the implant. There was a predominance of small vessels present in 10nM purified dsODN decoy treated implants. Purified 10nM dsODN scramble treated implants had a fewer large vessels compared to decoy treated implants. The large vessels in the scramble treated implants had slightly more vWF present within their lumen. (Figure 12) There was no evidence of altered or displaced endothelial cell growth. Capillaries were not identified with this stain so it was impossible to say there was a difference in the capillary beds between the treated and untreated implants.
Figure 3. Operon 10 nM dsODN Treated Implants

Figure 3A: Type I collagen in decoy treated implants was 46% of scramble implants. (p=0.021)

Figure 3B: Type III collagen in decoy treated implants was 52% of scramble implants. (p=0.013)

Figure 3C: Total protein in decoy treated implants was 56% of scramble implants. p=(0.015)

Figure 3D: Fibronectin/total protein in decoy treated implants was 211% of scramble implants. (p=0.014)
Figure 4. Non-Purified 10 nM dsODN Treated Implants

**Figure 4A:** Type I collagen in decoy treated implants was 56% of scramble implants. (p=0.011)

**Figure 4B:** Type III collagen in decoy treated implants was 90% of scramble implants. (p=0.073)

**Figure 4C:** Total protein in decoy treated implants was 64% of scramble implants. (p=0.039)

**Figure 4D:** Fibronectin/total protein in decoy treated implants was 155% of scramble implants. (p=0.008)
Figure 5. Operon 3 nM Treated Implants

Figure 5A: Type I collagen in decoy treated implants was 64% of scramble implants. (p=0.18)

Figure 5B: Type III collagen in decoy treated implants was 121% of scramble implants. (p=0.54)

Figure 5C: Total protein in decoy treated implants was 74% of scramble implants. p=(0.27)

Figure 5D: Fibronectin/total protein in decoy treated implants was 113% of scramble implants. (p=0.62)
Figure 6. Operon 1 nM dsODN Treated Implants

Figure 6A: Type I collagen in decoy treated implants was 76% of scramble implants. (p=0.0087)

Figure 6B: Type III collagen in decoy treated implants was 76% of scramble implants. (p=0.67)

Figure 6C: Total protein in decoy treated implants was 98% of scramble implants. p=(0.90)

Figure 6D: Fibronectin/total protein in decoy treated implants was 138% of scramble implants. (p=0.006)
Figure 7. Cell Counts of dsODN Treated Implants Compared to Scramble

Figure 7A: Decoy treated implant cell counts were 142% of scramble implants. (p=0.004)

Figure 7B: Decoy treated implant cell counts were 96% of scramble implants. (p=0.67)

Figure 7A: Decoy treated implant cell counts were 125% of scramble implants. (p=0.0002)

Figure 7B: Decoy treated implant cell counts were 104% of scramble implants. (p=0.91)
Figure 8. 6-FAM Labeled dsODN Uptake by Fibroblasts *in vivo*

Figure 8. Fibroblast nuclei are stained blue with DAPI while fluorescently labeled (6-FAM) dsODN specific to the COL1A1 promoter region are green. Note the color change of the nuclei with dsODN uptake compared to surrounding cells with no uptake into the nucleus.

Figure 9. H&E Staining of dsODN 10 nM Operon Treated Sponge Implant

Figure 9. The inner most region of the sponge shows decreased collagen formation and less regular arrangement of cell nuclei and collagen bundles compared to the outer region of the capsule.
Figure 10. Sirius Red Staining of dsODN 10 nM Operon Treated Sponge Implant

Figure 10A. Operon 10 nM dsODN treated sponge implants show decreased collagen production compared to the outer capsule. The collagen is fine, birefringent green indicative of immature collagen.

Figure 10B. Operon 10 nM dsODN treated sponge implant showing a central vessel with decreased collagen production.
Figure 10C. Operon 10 nM scramble dsODN treated sponge with large vessels present in the outer capsule.

Figure 11. 6-FAM Labeled dsODN Uptake in vivo

Figure 11A. Fibroblast cell nuclei are stained blue with DAPI, α-Smooth Muscle Actin is stained red indicating myofibroblasts, and fluorescently labeled (6-FAM) dsODN are green in this image. The majority of the green dsODN is associated with fibroblasts (cells without smooth muscle actin).
Figure 11B: Fibroblast cell nuclei are stained blue with DAPI, α-Smooth Muscle Actin is stained red indicating myofibroblasts, and fluorescently labeled (6-FAM) dsODN are green in this image. The majority of cells are exhibiting α-Smooth Muscle Actin which is indicative of myofibroblasts.

Figure 12: VonWillebrands factor (red) in the lumen of a vessel in the capsule of 10 nM Operon dsODN treated sponge implant. Cell nuclei are stained blue with DAPI and vascular smooth muscle cells within a large vessel stained with smooth muscle actin (green).
Chapter 5

Discussion

Overview
Many therapies have been tried for controlling or reducing hypertrophic scarring. Oligodeoxynucleotide therapy represents a class of potential therapeutics for controlling connective tissue deposition. The dsODN specific to the promoter region of the COL1A1 gene decreases collagen production while not affecting cell phenotypes, thus offering a potential therapy for hypertrophic scars.

Effects of dsODN on Type I and Type III Collagen
The ratio of type I collagen and type III collagen was compared to a loading standard of fibronectin divided by total protein. Only the purified 10 nM decoy dsODN treated implants showed a statistically significant decrease (p=0.021) in type I collagen and type III collagen (p=0.013) compared to scramble treated implants. DsODN decoy molecules targeted at the promoter region of the COL1A1 gene block type I collagen production in a mouse schistosomiasis model of liver fibrosis (Boros et al., 2005). Each animal was given a large dose (800mg) of dsODN injected directly into the liver or given intravenously. Utilizing RT-PCR to quantitate the amount of COL1A1 and COL1A3 gene expression, Boros et al, found a significant decrease in both COL1A1 and to a lesser extent COL3A1 gene expression. This finding correlated to our analysis of type I and type III collagen production in sponge implants treated with 10nM of purified decoy dsODN. In the Boros paper,
supression of both type I and type III collagen could have been attributed to the large doses of dsODN used. The binding of the dsODN to the COL1A1 TGF-β activator protein may have been saturated causing the additional dsODN to bind non-specifically to different TGF-β activator proteins. We hypothesized that at a lesser dose, the dsODN would bind specifically to the COL1A1 TGF-β activator protein. We expected to see a decrease in type I collagen production but not type III collagen production when using a smaller dose of dsODN. An explanation of this dual suppression can be attributed to similarities in the 5’ flanking regions of the COL1A1 and COL3A1 genes. Boros et al, did a search of the mouse genome comparing the consensus element of the dsODN and found partial homologous elements in the 5’ flanking region of the COL3A1 gene (Boros et al., 2005). The suppression of both type I and type III collagen by a low dose of dsODN suggests lack of specificity of the dsODN in blocking TGF-β activator proteins. Similar non-specific effects of oligodeoxynucleotides have been described in the literature. Fisher et al, compared the efficacy and specificity of an antisense oligodeoxynucleotide specific to a site flanking the start codon of the MDR1 message, which codes for P-glycoprotein found in the cell membrane, to the expression of 2059 other genes using DNA array technology. They found a marked reduction in the production of the MDR1 message and 37 other genes(Fisher, Ye, Sergueev, Fisher, & Shaw, 2002).
Purified dsODN vs Non-Purified dsODN

One complicating factor associated with the use of dsODN in research and its potential use in clinical medicine is the high cost associated with production of sequence specific ODN. We decided to compare the effects of non-purified ODN molecules produced in our facility to a commercially prepared HPLC purified ODN from Operon Biotechnologies Inc. (Huntsville, AL). HPLC and polyacrylamide gel electrophoresis (PAGE) are two common methods used to purify ODN prior to use. Purification removes any truncated sequences left over from the production of the ODN. The effects of these DNA fragments have not been determined but they may have detrimental non-specific effects inside the cell. The non-purified molecules had similar effects on type I collagen, total protein, and fibronectin/total protein as the purified dsODN. However only purified dsODN decreased type III collagen production. It is possible that truncated sequences could bind to the full length dsODN. This would prevent the dsODN from binding the trans-acting factors associated with promotion of transcription which will eliminate the decoy effects. The effects of non-purified dsODN were generally decreased compared to the purified dsODN which demonstrates an increased efficacy of purified molecules. We also did not see any detrimental effects of using the non-purified molecules in our experiment. DsODN uptake happens more rapidly with shorter sequences (Loke et al., 1989). It is possible the truncated sequences present in the non-purified dsODN were preferentially taken up before the larger intact sequence. This would allow the intact sequences more contact with inflammatory cells and proteases causing them to be broken down, effectively limiting their availability to the target cells.
Dose Suppression of Collagen

Traditionally dsODN has been used in relatively large quantities to investigate its effects upon select genes. Previous work in our laboratory suggested nanomolar quantities were active in wound healing models. We postulated there would be dose dependent reduction in type I collagen when comparing 1, 3, and 10 nM concentrations of purified dsODN. We did see a dose dependent reduction of type I collagen but only the 10 nM dose reached statistical significance. Type III collagen was also significantly decreased at this dosage. Neither the 1 nM or 3 nM doses showed statistically significant decreases in type I and type III collagen. The lack of a significant reduction in type I and type III collagen at the lower dosages may be directly related to dsODN availability to the fibroblasts. A factor potentially affecting bio-availability of dsODN is the presence of inflammatory cells and their ability to phagocytize molecules. With any in vivo model of wound healing neutrophils and macrophages have to be accounted for when considering delivery of a substance to specific cells. The center of the sponge is where the highest concentration of inflammatory cells exists. We made no attempt to quantify the loss of dsODN to the inflammatory cells but they must be considered as a potential source of nucleotide uptake and subsequent loss. Little research has been done to identify non-specific inflammatory cell uptake of oligodeoxynucleotides when in vivo models have been used. There are no published studies on this topic related to the COL1A1 promoter dsODN used in our experiment.
Total Protein Content

A statistically significant decrease in total protein content of the 10 nM purified dsODN (56% of scramble treated implants, p=0.015) and non-purified 10 nM dsODN (64% of scramble treated implants, p=0.039) was determined. The 1 nM and 3 nM treated implants did not show statistically significant decreases in total protein. Boros et al. determined non-collagen protein content actually increased in dsODN specific to the COL1A1 promoter region treated mice with liver fibrotic granulomas. The difference in the results are attributed to the differences in the methods for assaying non-collagen protein. We used the Pierce BCA protein assay, which is based upon the reduction of Cu$^{2+}$ to Cu$^{1+}$ by protein amino acid residues. The reaction of bicinchoninic acid reacting with Cu$^{1+}$ produces a product that has an absorbance peak at 540 nm. The Pierce BCA Protein assay is sensitive to peptide bonds and the presence of cysteine, cysteine, tryptophan and tyrosine amino acids. The BCA protein assay is not useful for quantifying collagen, because it has neither cysteine nor tryptophan and only 6 tyrosines per 1000 residues. Boros isolated liver fibrotic granulomas and studied them in short term organ culture. $^3$H-proline was added to the cultures and the amount of labeled proline incorporated into newly synthesized collagen was measured. The $^3$H-labeled proline not incorporated into the collagen fraction was assumed to be non-collagen protein. The method is excellent at measuring collagen synthesis but it is poor at measuring non-collagen proteins. Here dsODN addition had effects on the level of non-collagen protein synthesized. Other ODN studies showed the potential for specifically
targeted sequences to affect other genes (Fisher, Ye, Sergueev, Fisher, & Shaw, 2002). Boros, has identified promoter sequence homology with the COL3A1, TIMP-1, and TGF-β1 genes through a search of the mouse genome. What is unclear is if the search for targeted genes was limited to the 3 genes related to extracellular matrix synthesis or the whole mouse genome.

Histologic Evaluation of dsODN Treated Sponge Implants

In our sponge implant model dsODN was injected directly in the center of the sponge and then allowed to diffuse into the surrounding cells. Purified dsODN 10nM decoy produced the most pronounced histologic changes. Increased fibroblast cell nuclei arranged in an irregular pattern were observed in H& E stained sections. When these high cell density areas are evaluated with Sirius red staining there is little collagen and it is very fine immature collagen. The role of collagen synthesis in fibroblast proliferation has not been determined. However, it is possible the production and post-translational processing of collagen acts as a feedback control mechanism on fibroblast proliferation. Another possible explanation for the increased cellularity is COL1A1 specific dsODN increased fibroblast migration into the sponge matrix. Li et al, showed that fibroblast motility is initiated by the presence of collagen and increased by PDGF(Li et al., 2004). It is unlikely the increased motility is due to the decrease in collagen present in the area of increased cellularity given these findings but the dsODN may have other effects on cell motility.
Polyvinyl alcohol is used in many commercial applications including packaging materials, photosensitive coatings, ceramic materials, and fabrics. It has been used in a variety of wound healing models and is generally accepted to be inert. Since the distinct histologic changes were noted in the inner layer of the capsule extending into the inner matrix of the sponge there may be some adherence of dsODN to the PVA sponge. This would limit the diffusion of dsODN into the capsule and produce more profound local effects on cells associated with the sponge material.

Areas of decreased collagen and increased cellularity are bordered with distinct decreased fibroblast numbers and thick, mature collagen production. The larger bundles of mature collagen bordering the reduced collagen areas may represent a compensatory mechanism of myofibroblasts to produce additional collagen. An additional possibility is the cells surrounding areas of decreased collagen exhibit increased post translational processing of collagen into organized bundles.

Fibroblast vs Myofibroblast dsODN Uptake

In sponge implants, fluorescent imaging of fibroblast and myofibroblast dsODN uptake has shown more nucleotide to be associated with fibroblasts compared to myofibroblasts. This is of particular importance because the myofibroblast produces more type I collagen than fibroblasts. (Petrov, Fagard, & Lijnen, 2002; Agarwal, Britton, Alaseirlis, Li, & Wang, 2006). To maximally inhibit collagen production, oligodeoxynucleotide must be taken up by myofibroblasts. This uptake by myofibroblasts may be critical to inhibiting excessive scar
formation. However, there has not been any documentation of COL1A1 dsODN being preferentially taken up by fibroblasts over myofibroblasts.

There are several possibilities to explain our in vivo findings of preferential uptake by fibroblasts of COL1A1 dsODN compared to myofibroblasts. In our PVA sponge model the most mature cells (myofibroblasts) are found in the middle and outer layers of the capsule while the inner portion is populated with juvenile fibroblasts. By injecting into the center of the sponge, fibroblasts are preferentially exposed to the highest quantity of dsODN. The PVA sponge model relies on simple diffusion of the ODN throughout the sponge and surrounding tissue. Since the capsule (containing the highest concentration of myofibroblasts) forms at the outer edge of the. DsODN would be in contact with the fibroblast population for a greater length of time as the molecule diffuse through the capsule.

Another explanation may be related to the mechanism of cell uptake of dsODN. Receptor mediated endocytosis is the most accepted theory of dsODN cellular uptake. The exact receptor type responsible for dsODN endocytosis in the membrane of fibroblasts is unknown. There is potential for this receptor to change or have a lower expression in the cell membrane when fibroblasts differentiate to myofibroblasts. This could account for increased uptake of dsODN in fibroblasts compared to myofibroblasts.

We are currently pursuing the topic of cellular uptake of dsODN with cell culture experiments that are not finished at this time. By determining the rate of uptake by fibroblasts and myofibroblasts and the effects on type I and type III
collagen we can better understand possible therapeutic uses of dsODN. This information will allow us to then begin designing cell specific methods of increasing dsODN uptake.

ODN Modifications Affecting Uptake

Uptake of ODN by target cells is inefficient and new methods of increasing the efficiency of ODN uptake are being developed. Liposome encapsulation is one technology used to facilitate the uptake of ODN into cells. Liposomes form spontaneous complexes with negatively charged nucleic acids and protect against enzymatic degradation of ODN. Traditional methods of transfection including electroporation and calcium phosphate precipitation can be toxic to cells. Liposomes present a less toxic option to increase ODN influx into target cells. Endocytosis is still the presumed mechanism of liposome encapsulated ODN uptake (Lappalainen, Miettinen, Kellokoski, Jaaskelainen, & Syrjanen, 1997). Lappalainen et al, found the nuclear membrane to be a barrier to penetration of liposome encapsulated nucleotides. This is important because eukaryotic gene transcription occurs primarily in the nucleus and many ODN are targeted to prevent a specific gene transcription. Microbubbles are another merging technology that is capable of delivering ODN to specific cells and tissues. Microbubbles are perfluorocarbon gasses trapped within lipid coatings which increase the stability and allows for delivery of dsODN(Unger, 2004). Ultrasound allows precise rupturing of the microbubbles in a targeted tissue. Hagg et al, demonstrated microbubbles loaded with androgen receptor decoy ODN increased transfection of ODN in prostate tumor cells by 49% compared to
untreated controls (Haag et al., 2006). Both liposomes and microbubble technology represent new technology to deliver ODN to targeted sites.

Fibroblast vs Myofibroblast dsODN Uptake

Our fluorescent imaging of fibroblast and myofibroblast dsODN uptake has shown more nucleotide to be associated with fibroblasts compared to myofibroblasts in vivo sponge implants. This is of particular importance because the myofibroblast produces more type I collagen than fibroblasts. (Petrov, Fagard, & Lijnen, 2002; Agarwal, Britton, Alaseirlis, Li, & Wang, 2006). To maximally inhibit collagen production, oligodeoxynucleotide must be taken up by myofibroblasts. This uptake by myofibroblasts may be critical to inhibiting excessive scar formation. There has not been any documentation of COL1A1 dsODN being preferentially taken up by fibroblasts over fibroblasts. We are currently pursuing this topic with cell culture experiments that are not finished at this time. We are attempting to compare the rate of uptake with effects on type I and type III collagen.
Chapter 6

Future Directions

Cell Culture

Human dermal fibroblasts derived from foreskin samples of pediatric patients were used to determine rate of 6-FAM tagged ODN uptake and duration within fibroblasts and myofibroblasts. Masur et al, demonstrated fibroblasts plated at low density (5 cells per mm2) produce a population of 70-80% myofibroblasts after 5-7 days incubation time. Fibroblasts plated at high density (500 cells per mm2) produce only 5-10% myofibroblasts (Masur, Dewal, Dinh, Erenburg, & Petridou, 1996). A coulter counter (Beckman Coulter) was used to identify the number of fibroblast cells in suspension prior to being plated into an 8 well chamber slide. Thirty two thousand cells per well were plated for the myofibroblast population and 300,000 cells per well for the fibroblast population. The wells were filled with 370 microliters of Dulbeccos Modified Eagles Medium (DMEM), placed into a 37°C incubator and monitored for cell growth. After 5 days the cells had reached a high degree of confluency which is consistent with myofibroblasts. The fibroblasts population was ready to be used 2 days after plating.

At the start of the experiment, DMEM was removed from each well and 200 µl of serum free media containing Beta-aminoproprionitrile (BAPN) 10 µg/ml and 50 µg/ml Vitamin C were added. The serum free media contained 50 mg of gentamycin and 10 mls of a 200 mM L-glutamine solution per 500mls of media.
One hr later, 5 µl of 6-FAM labeled dsODN was added to each well and cells were returned to the incubator. Fluorescence microscopy (Zeiss, Axiovert) was begun at 1, 4, 24, and 48 hrs post addition of 6-FAM dsODN.

Fluorescent combined with phase contrast microscopy imaging showed a progressive accumulation of dsODN within the cytoplasm and nucleus of myofibroblasts. At 1 hr dsODN was present in the media, cell cytoplasm, and nuclear accumulation was present. Four hrs post addition of dsODN showed more dsODN present within the cell cytoplasm and less free in the media. Nuclear accumulation was greater compared to the 1hr time point. Twenty four hrs post dsODN addition most dsODN was found within the cell cytoplasm and nucleus. The nucleus became distinct and nucleolus were visible. Forty eight hrs post dsODN achieved a maximum accumulation in the nucleus with scant amounts in the media and cytoplasm (Figure 13).

Time Lapse Imaging
Using a microinjector (Eppendorf, FemtoJet) a small amount of ODN was locally injected around a group of fibroblasts. Fluorescent microscopy time lapse recording was used to visualize dsODN uptake into the cell and nucleus. A computer script was written to capture an image every 5 sec for 1 hr. The script was stopped after 2 mins when no further changes were noted in nuclear uptake of ODN. Fibroblasts took up dsODN within seconds of exposure and achieved maximum nuclear uptake by 45 sec (Figure 14).
These findings corroborate our *in vivo* evidence of fibroblasts having increased uptake of dsODN compared to myofibroblasts. Additional research involving paired experiments to evaluate dsODN uptake of fibroblasts and myofibroblast is pending. We will use flow cytometry to obtain quantitative cell counts of fibroblasts and myofibroblasts uptake of 6-FAM labeled dsODN. By staining the nuclei with DAPI and the myofibroblasts with αSMA it is possible to count and separate the cell types using the flow cytometer located in the core facility at the medical center. Flow cytometry will not allow us to determine the presence of dsODN in the nucleus of cells so a separate experiment will be performed. After separating the cell types with flow cytometry we will then lyse the cells to obtain the nuclei. Flow cytometry may again be used to count the nuclei with fluorescent dsODN present.

In the histologic evaluation of 10 nM Operon dsODN treated sponge implants, the areas cell nuclei with reduced collagen content compared to outer areas of the capsule. Through literature searches have not identified a relationship between collagen production and cell proliferation. It is possible that decreasing fibroblast collagen production causes a compensatory mechanism whereby fibroblasts proliferate to restore collagen amounts. We will immunohistochemical stain for proliferating cell nuclear antigen which is a cell proliferation marker commonly used to determine actively replicating cells. It is expected the inner treated region of the sponge implant will exhibit increased staining in response to actively replicating fibroblasts. Another reason the cells may be producing less collagen is the dsODN could be inducing apoptosis.
Given previous research done by Boros, Cutroneo, and our preliminary cell culture results it is unlikely apoptosis is a cause of decreased collagen production but must be considered. Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) detects DNA fragmentation in apoptotic cells will be used to assess our dsODN treated sponge implants. Inner areas of the sponge implant will have little evidence of immunohistochemical uptake if the cells are rapidly dividing.
Figure 13. Time Course of 6-FAM Labeled dsODN Uptake In Myofibroblasts

Figure 13A: 1 hr post dsODN treatment

Figure 13B: 4 hrs post dsODN treatment

Figure 13C: 24 hrs post dsODN treatment treatment

Figure 13D: 48 hrs post dsODN treatment
Figure 14. Time Lapse Images of dsODN Uptake By Fibroblasts

Figure 14A: Prior to treatment with dsODN

Figure 14B: Time 0

Figure 14C: 5 sec post dsODN

Figure 14D: 10 sec post dsODN

Figure 14E: 15 sec post dsODN

Figure 14F: 20 sec post dsODN
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