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Altering equine corneal fibroblast differentiation through Smad gene transfer

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Abstract

Objective—To explore the impact of equine corneal fibroblast (ECF) to myofibroblast (ECM) differentiation by altering the expression of the Smad genes either individually or in combination. Specifically, we sought to examine the ECF differentiation after (a) silencing of Smad 2, 3 and 4 pro-fibrotic genes individually and (b) over-expression of anti-fibrotic Smad 7 gene and in a combination with pro- and anti-fibrotic Smad genes.

Methods—ECF primary cultures were generated as previously described. ECFs were transfected with individual plasmids which silenced gene expression of either Smad2, 3, or 4 or in combination with a plasmid over-expressing Smad7 using Lipofectamine 2000™ or Lipofectamine BLOCK-iT™. Smad-transfected clones were then exposed to TGF-β1 to induce differentiation to myofibroblasts. Immunofluorescence and qRT-PCR techniques quantified levels of ECF differentiation to ECM by measuring alpha smooth muscle actin, a known marker of ECM transdifferentiation.

Results—Silencing of individual Smad2, 3, or 4 genes or overexpression of Smad7 showed significant inhibition of ECF transdifferentiation (73–83% reduction). Silencing of Smad2 showed the greatest inhibition of ECF transdifferentiation in (a) and was therefore utilized for the combination gene transfer testing. The combination gene transfer consisting of Smad7 overexpression and Smad2 silencing attenuated ECF differentiation significantly, however the level was not significant compared to the overexpression of Smad7 individually.

Conclusions—Using gene transfer technology involving pro-fibrotic Smad silencing, anti-fibrotic Smad over-expression or its combination is a novel strategy to control TGF-β1 mediated fibrosis in equine fibroblasts. Combination gene therapy was not better than single gene therapy in this study.

Keywords

Smad; cornea; fibrosis; gene transfer; equine; fibroblast

Introduction

Horses due to their prominent globe position, globe size and other environmental factors are at high risk for developing corneal ulceration (1, 2). Once ulcerated, corneal disease is often perpetuated through secondary infection of either bacterial or fungal origin (3–7). Once resolved, the cornea is often left with a scar that leads to a reduction in the visual field. Corneal scar formation is a recognized problem afflicting not only horses, but in many of our domestic animal species (8–13), as well as people (14). This loss of corneal transparency can result in a horse being unable to perform its duties such as racing, jumping, or trail riding and can prove to be dangerous to both itself and his/her handler (8).

Due to the unique anatomical structure of the cornea, corneal wound repair is unlike wound repair found in the rest of the body (15). Corneal scar formation in mammals is a complex cascade that is initiated by a break in the corneal epithelium and involves the transformation of quiescent keratinocytes to activated fibroblasts, alterations in the extracellular matrix, increased secretion of matrix metalloproteinases (MMPs), and altered gene expression most notably the Smad family of genes (16).

The Smad family of genes consists of the pro-fibrotic genes Smad2, 3 (receptor-activated Smads), Smad4 (common-partner Smad), and the anti-fibrotic or inhibitory gene Smad7. These genes become activated once corneal stroma is exposed to transforming growth factor beta-1 (TGF- β 1), one of the most potent activators of fibrosis (17–19), after a break in corneal epithelium (15). The activation of the TGF- β 1 mediated fibrotic pathway is perpetuated through the Smad signaling pathway once TGF- β 1 binds to a corneal keratocyte membrane's serine/threonine receptor. This in turn activates Smad2 and Smad3 to heterodimeric complexes that couple with a common mediator Smad4, and this complex then travels to the nucleus resulting in transcription of target genes which results in an alteration in extracellular matrix components (20, 21). Smad7 competitively binds to the TGF- β receptor inhibiting TGF- β signal transduction and thereby altering the response of Smad2 and Smad3 (22).

Multiple studies have been performed on this group of genes examining the fibrotic effects after altering Smad7 expression (21, 23, 24). These studies have shown that down regulation of Smad2 and Smad3 occur when Smad7 is artificially expressed. To the authors' knowledge, combination gene therapy in which the pro-fibrotic arm of the Smad family was inhibited, while simultaneously upregulating the anti-fibrotic Smad7 has never before been examined in equine veterinary medicine. Therefore, we sought to determine the degree of fibrotic change as identified by the transformation of equine corneal fibroblasts (ECFs) to equine corneal myofibroblasts (ECMs) by both inhibiting and overexpressing different genes within the Smad family. This study aimed specifically to (a) examine the effects of Smad2, 3, and 4 gene silencing individually, Smad 7 gene overexpression on ECF transdifferentiation in response to TGF- β 1, and to identify the greatest potential of pro-fibrotic Smad gene silencing on ECF differentiation, and (b) compare the level of inhibition of ECF transdifferentiation to ECM by individual vs combination gene transfer of pro- and anti-fibrotic Smads. It was our hypothesis that Smad gene transfer in combination would

yield a greater anti-fibrotic effect than individual Smad gene transfer in equine corneal fibroblast in an *in vitro* model of corneal fibrosis.

Methods

Generation of cell cultures

Primary cultures of ECFs and ECMs were generated as previously described (25). Briefly, all corneal sections were harvested from healthy research horses being euthanized for reasons unrelated to this study. Prior to euthanasia, slit lamp biomicroscopy was performed by a board-certified veterinary ophthalmologist (EAG) to ensure that all horses were free of anterior segment disease. Immediately following euthanasia, 6 mm full thickness corneal buttons were harvested aseptically and immediately transported to the lab for further processing. All corneal buttons were washed with modified eagle media (Life technologies, Carlsbad CA) and the corneal epithelium and endothelium were aseptically removed by scraping with a #10 bard parker blade (Aspen Surgical, Caledonia MI). The corneal buttons were then sub-sectioned into 4 equal sized pieces and placed into a 100 × 20 mm tissue culture plate (Fisher Scientific, Pittsburg PA) and supplemented with MEM media containing 10% fetal bovine serum, penicillin, streptomycin, fungizone, and ciprofloxacin. All culture plates were incubated at 37°C in a humidified CO₂ chamber. Once 90% confluence was achieved (2–4 weeks) of the primary ECF monolayer, all corneal stromal sections were removed and the ECFs were then trypsinized for use in all other phases of this study. ECFs were counted prior to plating and a volume of 7.5×10^4 was used. ECMs were achieved for all phases of this study by supplying ECFs with MEM media supplemented with 5 ng/ml TGF-β1. Both a negative control group (cultured ECFs not exposed to TGF-β1) and a positive control group (ECFs exposed to TGF-β1 only) were utilized as standards for all analytical testing.

Gene Transfections

All RNA interference (RNAi) oligos (Table 1) were prevalidated through a previous experiment in our laboratory (16). Furthermore, the DNA sequences of RNAi in all plasmids were verified from the Pubmed gene database to ensure a complete matching with the equine genome. The RNAi oligos were cloned into pcDNA 6.2 miR RNAi commercial vector (Life technologies, Carlsbad CA). The Lipofectamine 2000™ BLOCK-iT™ transfection kit (Invitrogen Corporation, Carlsbad CA) was utilized for all transfections according to manufacturer's instructions. In summary, all ECF cell cultures were 90% confluent at the time of transfection. 24 hours prior to transfection, ECF cell cultures were supplemented with serum and antibiotic free DMEM (Dulbecco's modified Eagle's medium). The desired amount of the siRNA/RNAi plasmid and 10 µl of Lipofectamine 2000™ were combined and incubated at room temperature for 5 minutes, after which this combination was aliquoted to ECF cultures individually. During the transfection process and for the first 4 hours afterwards, only serum free DMEM media was utilized. After 4 hours, MEM media mixed as previously stated with TGF-β1 was utilized until the termination of the experiment at 72 hours. Prior to performing the transfection utilizing Smad plasmids, we validated the transfection efficiency of Lipofectamine 2000™ using the plasmid pcDNA3.1-m-cherry.

Results of our studies demonstrated transfection efficiency of Lipofectamine between 80–85% (Figure 2-F).

Gene expression and quantification

Following the termination of the experiment period, RNA was extracted from cells of all groups using a RNeasy kit (Qiagen, Valencia CA) in accordance with manufacturer's instructions, and reverse transcribed to cDNA as previously reported (9). Samples were then stored at -80°C until analyzed by real-time qPCRs (qRT-PCR), performed utilizing a StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad CA). At analysis, a 20 μl reaction mixture was composed out of 2 μl cDNA, 2 μl forward primer (200 nM), 2 μl of reverse primer (200 nM), and 10 μl of 2 $\times$ SYBR green super mix (Bio-Rad Laboratories, Hercules CA). This reaction mixture was run at universal cycle (95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 60 sec) as recommended by manufacturer's instructions. Forward and reverse primer sequences for both α -SMA and β -actin (housekeeping gene) were utilized as previously validated for ECF cells (9, 26) (Table 2).

Immunofluorescence

Cells were fixed in 4% fresh paraformaldehyde in preparation for immunofluorescence staining for α -SMA. Incubation of cells with 5% bovine serum albumin at room temperature for 30 minutes was performed, after which mouse monoclonal α -SMA antibody (Dako, Carpinteria CA) at a 1:200 dilution was added to all samples for 90 minutes. Alexa 488 goat anti-mouse IgG secondary antibody was then added at a 1:500 dilution and incubated for 1 hour. The samples were then washed with HEPES buffer, mounted with vectashield mounting media containing DAPI (Vector Laboratories, Burlingame CA) and photographed with a Leica fluorescent microscope (Leica DM 400B) equipped with a digital camera (SpotCam RT, Sterling Heights MI).

Statistical Analysis

Data for immunohistochemistry was quantified by assessing the number of α SMA and DAPI-positive cells in 10 randomly selected non-overlapping fields at 100 $\times$ magnification under Leica Fluorescent microscope (Leica) by evaluators masked to the treatment group (AS, RT). Results for all values were expressed as a mean value $\pm$ standard deviation. Statistical analysis of qRT-PCR data was performed using a one-way analysis of variance (ANOVA) followed by a Tukey's test. A value of $P = 0.05$ was considered significant.

Results

Effect of individual Smad gene silencing on the expression of α SMA mRNA

To investigate the effect of individual Smad gene silencing on ECF differentiation expression of α SMA was quantified with qRT-PCR. Figure-1 shows relative α SMA mRNA expression in the ECFs transfected with naked vector and grown in the absence of TGF- β 1 (negative control), in the presence of TGF- β 1 (positive control), and ECFs transfected with Smad2, 3, or 4 RNAi vector grown in the presence of TGF- β 1. Treatment of ECFs with TGF- β 1 significantly increased α SMA mRNA expression as compared to negative control (7-fold $\pm$ 0.43; $P < 0.01$). Additionally, ECF cells transfected with Smad2, 3, or 4 RNAi

clones grown in the presence of TGF- β 1 demonstrated significant decrease in α SMA mRNA levels compared to the control (4.5–5.5-fold $\pm$ 0.53; $P < 0.01$). However, no significant differences in the inhibition of α SMA mRNA among individual Smad2, 3 and 4 groups were observed.

Effect of individual Smad gene silencing on the expression of α SMA protein

The effect of individual Smad gene silencing on ECF differentiation to ECM transfected with Smad2, 3 or 4 RNAi plasmid, grown in the presence or absence of TGF β 1, and expression of α SMA protein, was quantified using immunofluorescence. The knockdown of Smad2, 3 or 4 significantly attenuated TGF β 1-mediated increase in α SMA ($P < 0.001$) (Figure 2; Part A). ECFs transfected with a naked plasmid and grown in the absence of TGF- β 1 demonstrated 3–5% level of α SMA+ cells (negative control; Figure-2; Part A–A). ECF cells transfected with a naked plasmid and grown in the presence of TGF- β 1 demonstrated 95–98% level of α SMA+ cells (positive control; Figure-2; Part A–B). The inhibition of α SMA+ cells by Smad2 RNAi was 80% (Figure-2; Part A–C), Smad3 RNAi was 78% (Figure-2; Part A–D) and Smad4 RNAi was 73% (Figure-2; Part A–E). The decrease in the α SMA+ cells among the Smad2/3/4 silencing was not statistically significant (Figure-2; Part B, $P = 0.8324$). Due to the Smad2 RNAi having the highest inhibition of α SMA cells, this RNAi was selected for testing in combination with Smad7 over-expression for the remainder of the experiment.

Comparison of single and 2-gene combination therapy on the expression of α SMA mRNA

Figure 3 shows qRT-PCR quantification of single and 2-gene combination therapy on ECF differentiation. ECF cultures transfected with naked vector, Smad7 alone or Smad7 in combination with Smad2-RNAi, grown in $\pm$ TGF- β 1, and used for α SMA mRNA quantification are represented in Figure 3. Naked vector transfected ECF cultures grown in the presence of TGF- β 1 showed significantly higher levels of α SMA mRNA compared to the cultures grown in the absence of TGF- β 1 (7.2-fold $\pm$ 0.44; $P < 0.01$). Both, single (Smad7 alone) and 2-gene combination (Smad7 and Smad2-RNAi) gene transfer yielded nearly a 6-fold decrease in the α SMA mRNA levels compared to naked vector transfected cultures grown in the presence of TGF- β 1 ($P = 0.01$). The 2-gene combination showed a slightly higher decrease in α SMA mRNA levels compared to Smad7 alone. However, this difference was not statistically significant ($P = 0.369$). Table 3 demonstrates the effect of Smad gene transfer on TGF-induced α SMA mRNA levels.

Comparison of single and 2-gene combination therapy on the ECF differentiation

Figure 4 shows quantification α SMA protein in ECFs transfected with naked vector, Smad7 alone, or Smad7-and-Smad2-RNAi cultures grown in $\pm$ TGF- β 1. As expected, ECFs transfected with naked vector grown in the absence of TGF- β 1 showed few α SMA+ cells (Figure-4A) whereas when grown in the presence of TGF- β 1 demonstrated 92–96% α SMA + cells (Figure-4B). Both, *single* (Smad7 overexpression; Figure-4C) and *2-gene combination* (Smad2 silencing and Smad7 overexpression; Figure-4D) gene transfer significantly decreased ECF differentiation, exhibiting 83–85% less α SMA+ cells ($P < 0.001$) compared to ECFs transfected with naked vector and grown in the presence of TGF- β 1.

However, combination gene transfer did not have significantly lower α SMA+ cells when compared to Smad7 alone (Figure 5, $P=0.73$)

Discussion

Corneal repair due to injury and/or disease is a multi-step process that utilizes a variety of different cytokines and pro-fibrotic genes to make the necessary changes within a corneal fibroblast resulting in the transdifferentiation to a myofibroblast. Transdifferentiated myofibroblasts result in a corneal opacity (scar) which results in visual defects (10). Gene therapy is a budding field in veterinary medicine. Our group has previously reported the anti-fibrotic effects of a variety of different potential topical therapeutics in both equine and canine cell lines (9, 11, 13, 27, 28). This paper differs from our previous reports as we aimed to target specific genes in the fibrotic cascade versus utilizing agents with broader spectrum anti-fibrotic actions.

This investigation supports the conclusion that Smad7 is the gene with the most promise for targeted anti-fibrotic gene therapy in equine ophthalmology. A significant decrease in the severity of fibrotic change when Smad7 is specifically expressed occurs across a variety of different species and tissues; for example, Smad7 has previously been reported to decrease corneal haze after photorefractive surgery in a rat (29), and in the kidney (30), liver (31), and lungs of other animal models (32). These findings support the idea that its role within the cytoplasm of the cell is potentially overriding the fibrotic effects of other Smad genes. Smad7 becomes activated within the cornea due to the expression of TGF- β (29). Once activated, Smad7 blocks the TGF- β dependent phosphorylation of Smad2 and Smad3, thereby preventing their coupling with Smad4 and subsequent translocation into the cell nucleus (19, 33). Based on our research this negative feedback loop, once overexpressed, can significantly reduce the severity of fibrosis in ECFs.

It was noted in part (a) of this experiment that Smad2, 3 or 4 all had similar effects in the degree of α SMA reduction. While it was not surprising that inhibition of Smad2 & 3 had similar results as they both become activated in similar ways, it was interesting that inhibition of Smad4 did not have a more significant impact on the transdifferentiation of ECFs. Smad4 is considered a common-partner Smad, in that it aids in the delivery of phosphorylated Smad2 and Smad3 to the nucleus of the cell (34). Therefore, it is reasonable to speculate that its inhibition would have caused a more significant inhibition in fibrotic change, as Smad4 could theoretically block Smad2 and Smad3 in an indirect manner. The similar response noted between all groups in this study is likely due to TGF- β 1 activating Smad-independent pathways for eventual ECF transdifferentiation and thereby circumventing the Smad-dependent pathway of transdifferentiation. An alternative pathway that has been known to be activated by the TGF- β superfamily includes the p38 mitogen-activated protein kinase (MAPK) signaling pathway (35, 36). It has been shown that blocking this pathway can also reduce the transdifferentiation of mouse mammary cells and yet have little effect on the degree to which Smad2 is phosphorylated (37). A mouse model of renal fibrosis showed that both pathways are independent yet both play roles in the development of renal fibrosis (38). Therefore, we believe it is highly likely that an

alternative pathway became active in our study due to supplementation of TGF- β 1 to our cell cultures resulting in similar ECF to ECM transdifferentiation between groups.

An unexpected result of this experiment is that no significant benefit was detected when inhibition of the pro-fibrotic Smad2 gene was used in conjunction with over-expression of the anti-fibrotic Smad7 gene. A potential explanation as to why this finding occurred could be related to the fact that Smad7 prevents the TGF- β dependent phosphorylation of both Smad2 and Smad3. Since this phosphorylation process is needed to activate these pro-fibrotic Smad genes, it is likely that overexpression of the Smad7 gene in our *in vitro* model adversely affected this process. Therefore, the additional inhibition of the Smad2 gene in ECFs did not yield any significant anti-fibrotic benefit. This finding is consistent with other reports in the literature where similar results have been observed in a mouse model of myocardial fibrosis and rabbit corneal endothelial cells (39, 40).

It should be noted that our negative control group expressed SMA at a level of 3–5% in the absence of TGF- β . This finding is likely related to normal culture conditions in a tissue culture plate, which can cause fibroblasts to shift from one phenotype to another spontaneously. Regardless this level of SMA expression is consistent with other scientific reports involving equine fibroblasts in an *in vitro* setting (9, 11, 25).

While a variety of research has been done evaluating gene therapy utilizing Smad 7, only a few studies have evaluated corneal gene therapy with Smad 7 on an *in vivo* model. These two studies include using a lentiviral vector with Smad 7 in rats after photorefractive keratotomy (29) and a recent article from our laboratory utilizing an adeno-associated virus vector with Smad7 in a rabbit after photorefractive keratotomy (41). The results of both of these studies indicate that Smad7 was able to reduce the severity of corneal haze compared to controls in an *in vivo* model. These findings are important as they further support our *in vitro* results.

Relatively little information is present in the veterinary literature regarding gene therapy to the equine cornea (9, 12). To our knowledge, this is the first paper describing the effects of Smad gene expression in a large animal species. In summary, this study identifies increased Smad7 gene expression as a potential target for future *ex vivo* or *in vivo* corneal gene therapy experiments. Additionally, this paper identifies that an enhanced anti-fibrotic effect as determined in the degree of ECF to ECM transdifferentiation is not present when inhibition of a pro-fibrotic Smad gene is coupled with expression of the anti-fibrotic Smad7 gene in an *in vitro* model. Future studies are needed to determine if overexpression of Smad7 together with MAPK signaling inhibition offers improved anti-fibrotic activity as noted in non-ocular tissues (38) than Smad therapy alone.

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Figure 1.

Graphical representation of relative mRNA fold change of αSMA. Quantitative real time PCR demonstrated the effect of Smad2, 3 or Smad4 RNAi on the expression of αSMA between the negative control (-TGF-β1), positive control (+TGF-β1) and transfected with Smad2, Smad3, and Smad4 RNAi. There was a significant reduction between all Smad groups tested and the positive control. However, no significant reduction was observed among Smad2, Smad3, and Smad4 RNAi transfected. (Error bars=standard deviation, * P<0.05). All data were normalized with β-actin.

Figure 2.

Part A: Representative immunofluorescence images showing inhibition of α SMA⁺ cells after Smad2/3/4 gene silencing. The negative control ECF cultures ($-$ TGF- β 1) showed no α SMA staining (A) and positive control ECF cultures grown in the presence of TGF- β 1 (5 ng/ml) shows maximum α SMA staining (B). The knockdown of Smad2 (C), Smad3 (D) or Smad4 (E) markedly reduced TGF- β 1 mediated expression of α SMA. The transfection efficiency of Lipfectamine 2000TM utilizing pcDNA3.1 m-cherry represented in (F).

Part B: Shows the quantification of α SMA expression demonstrating a significant reduction in the α SMA⁺ cells with Smad2/3/4 silencing (* P <0.001). The DAPI-staining (blue) depicts that there was no significant difference in the total number of nuclei between control and transfected cultures.

Figure 3.

Graph representing relative fold change of αSMA mRNA as evaluated by qPCR between the negative control (–TGF-β1), positive control (+TGF-β1) and Smad2 RNAi+Smad7 and Smad7 alone. A significant reduction in αSMA mRNA between the positive control and Smad2 RNAi+Smad7 and Smad7 alone transfected ECFs was detected. No significant reduction was observed between Smad2 RNAi+Smad7 and Smad7 alone. All data was normalized with β-actin (Error bars = standard deviation, * P<0.005)

Figure 4.

Representative immunofluorescence images showing effect of combination of Smad2 RNAi + Smad7 and Smad7 alone on the expression of αSMA in ECFs. The negative control ECF cultures (-TGF-β1) showed no αSMA staining (A) and positive control ECF cultures grown in the presence of TGF-β1 (5 ng/ml) shows maximum αSMA staining (B). The ECFs transfected with Smad2 RNAi+Smad7(C) and Smad7 alone (D) demonstrate significant reduction in αSMA+ cells. The DAPI-staining (blue) depicts that there was no significant difference in the total number of nuclei between control and transfected cultures.

Figure 5.

Histogram demonstrating the effect of Smad2 RNAi+Smad7 and Smad7 alone on the expression of αSMA in ECFs. When ECFs were transfected with Smad2 RNAi+Smad7 and Smad7 alone, there was significant reduction of αSMA+ ECFs observed compared to the positive control (P=0.73).

Table 1

Nucleotide sequences of validated RNAi used in study.

Smad2 RNAi-validated (5' to 3')	GAGCAGACCTCTCTGAATTTGGTTTTGGCCACTGACTGACCAAATTCAGAGGTTCTGCT
Smad2 RNAi-validated (5' to 3')	GTGTAAAGGCCTGTTGTATCCCGTTTTGGCCACTGACTGACGGGATACAAGGCCTTTACA
Smad3 RNAi-validated (5' to 3')	GCACTGAGGCACTCTGCCGAAGAGTTTTGGCCACTGACTGACTCTTCGCAGTGCCTCAGTG
Smad3 RNAi-validated (5' to 3')	GTTCATCTGGTGGTCACTGGTTGTTTTGGCCACTGACTGACAACCAGTGCACCAGATGAA
Smad4 RNAi-validated (5' to 3')	GTTCCGACCAGCCACCTGAAGGTTTTGGCCACTGACTGACCTTCAGGTCTGGTCGGAA
Smad4 RNAi-validated (5' to 3')	GTTTCCGACCAGCCACCTGAAGGTTTTGGCCACTGACTGACCTTCAGGTCTGGTCGGAAA

Table 2

qRT-PCR primer sequences used.

α SMA Forward Primer Sequence	TGGGTGACGAAGCACAGAGC
α SMA Reverse Primer Sequence	CTTCAGGGGCAACACGAAGC
β -actin Forward Primer Sequence	CGGCTACAGCTTCACCACCA
β -actin Reverse Primer Sequence	CGGGCAGCTCGTAGCTCTTC

Table 3Effect of Smad gene transfer on TGF- β 1-induced α SMA mRNA level

Gene Transfer	Decrease in TGF- β 1-induced α SMA mRNA after Smad gene transfer	SEM	Significance
Smad-2 silencing	4.5 fold	0.53	P<0.001
Smad-3 silencing	5.1 fold	0.53	P<0.001
Smad-4 silencing	5.5 fold	0.53	P<0.001
Smad7 overexpression	5.8 fold	0.44	P=0.01
Smad-2 silencing + Smad-7 overexpression	6.0 fold	0.44	P=0.01