Aldose Reductase Inhibition Prevents Development of Posterior Capsular Opacification in an In Vivo Model of Cataract Surgery

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PURPOSE. Cataract surgery is a procedure by which the lens fiber cell mass is removed from its capsular bag and replaced with a synthetic intraocular lens. Postoperatively, remnant lens epithelial cells can undergo an aberrant wound healing response characterized by an epithelial-to-mesenchymal transition (EMT), leading to posterior capsular opacification (PCO). Aldose reductase (AR) inhibition has been shown to decrease EMT markers in cell culture models. In this study, we aim to demonstrate that AR inhibition can attenuate induction of EMT markers in an in vivo model of cataract surgery.

METHODS. A modified extracapsular lens extraction (ECLE) was performed on C57BL/6 wildtype, AR overexpression (AR-Tg), and AR knockout mice. Immunofluorescent staining for the myofibroblast marker α-smooth muscle actin (α-SMA), epithelial marker E-cadherin, and lens fiber cell markers αA-crystallin and Aquaporin 0 was used to characterize postoperative PCO. Quantitative reverse transcription PCR (qRT-PCR) was employed to quantify postoperative changes in α-SMA, vimentin, fibronectin, and E-cadherin. In a separate experiment, the AR inhibitor Sorbinil was applied postoperatively and qRT-PCR was used to assess changes in EMT markers.

RESULTS. Genetic AR knockout reduced ECLE-induced upregulation of α-SMA and downregulation of E-cadherin. These immunofluorescent changes were mirrored quantitatively in changes in mRNA levels. Similarly, Sorbinil blocked characteristic postoperative EMT changes in AR-Tg mice. Interestingly, genetic AR knockout did not prevent postoperative induction of the lens fiber cell markers αA-crystallin and Aquaporin 0.

CONCLUSIONS. AR inhibition prevents the postoperative changes in EMT markers characteristic of PCO yet preserves the postoperative induction of lens fiber cell markers.

Keywords: aldose reductase, EMT, PCO, cataractogenesis

Posterter capsular opacification (PCO), or secondary cataract, is a common complication of cataract surgery, occurring in up to 20% to 30% of patients postoperatively.1 Cataract surgery involves removal of a majority of the fiber cell mass of the lens held within the lens capsular bag and subsequent implantation of a synthetic intraocular lens (IOL) to restore visual clarity. While cataract extraction eliminates most lens tissue, some lens epithelial cells (LECs) inevitably remain attached to the inner surface of the capsular bag. In response to the trauma of cataract surgery, these residual LECs undergo an aberrant wound healing response characterized by proliferation, transdifferentiation to a myofibroblast phenotype, and migration toward the posterior capsule, where they cause wrinkling and fibrosis of the normally smooth capsule and consequent disruption of visual axis clarity.2 Improvements in surgical tools and techniques, together with the introduction of new IOL materials and structural design, have lowered but not eliminated the incidence of PCO worldwide.3 To date no inhibitors have been approved to block the onset and progression of PCO at the cellular level.

Transforming growth factor-β (TGF-β) is considered a central regulator of PCO through its role in inducing epithelial-to-mesenchymal transition (EMT) of LECs.2 One of the key TGF-β signaling transduction pathways involves SMAD proteins,4–6 which convey information from the TGF-β receptor, leading to the formation of spindle-like myofibroblast cells7 and expression of EMT markers, including α-smooth muscle actin (α-SMA), fibronectin, and vimentin.8–12 This important signaling link in ocular tissues has been demonstrated through experiments showing cataract induction in lenses cultured in the presence of TGF-β.13 Furthermore, inhibiting TGF-β/SMAD signaling abrogates EMT and therefore PCO.14

Recent studies have suggested that aldose reductase (AR) also plays a critical role in the development of PCO. AR is well known for its role in the pathogenesis of various secondary complications of diabetes mellitus through its enzymatic function in converting glucose to sorbitol in the polyol
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pathway. As a key enzyme in the polyol pathway, AR is also thought to contribute to formation of reactive oxygen species (ROS) via three mechanisms: depletion of antioxidant molecules like NADPH and GSH, generation of NADH (which can lead to superoxide anion formation), and increased production of potent nonenzymatic glycation agents, which can increase ROS levels by creating advanced glycation end-products. ROS in ocular tissues have been shown to cause LEC growth and a PCO-like phenotype. Furthermore, various antioxidant agents, like caffeic acid and retinoic acid, have shown the potential to prevent this phenotype. Importantly, AR inhibition has been shown to suppress ROS production in a culture model. This demonstrated an important regulatory relationship between AR and PCO. Further substantiated by studies demonstrating that AR overexpression leads to induction of EMT and PCO biomarkers and that AR inhibition can decrease these markers. Indeed, AR has been hypothesized as a druggable target for prevention of cataracts and oxidative damage in diabetes.

Recently, we showed that TGF-β-mediated EMT and LEC migration can be disrupted through inhibition of AR in a cell culture model. This demonstrated an important regulatory relationship between AR and TGF-β signaling and suggested that AR inhibition could be a possible therapeutic strategy against PCO development. To further explore AR as a drug target for PCO prevention, it is important to demonstrate similar findings with an in vivo mouse model of cataract surgery. This facilitates more complete modeling of PCO characteristics and the ocular inflammatory response. In this study, we demonstrate that inhibition of AR, both pharmacologically and via genetic knockdown, can attenuate the induction of EMT markers in an in vivo model of cataract surgery.

METHODS

Transgenic Mice

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Colorado Institutional Animal Care and Use Committee. C57BL/6 wild-type (WT) mice were acquired from The Jackson Laboratory (Bar Harbor, ME, USA). The Par40 strain of AR transgenic mice (AR-Tg) and the AR knockout strain (ARKO) were generated as previously published techniques. Adult mice were anesthetized with 80 mg/kg ketamine and 5 mg/kg Xylazine. One eye of each mouse was dilated using several drops of topical phenylephrine and tropicamide. A 1- to 1.5-mm central corneal incision was made using a disposable ophthalmic knife. Following reimplantation of the anterior chamber with an ophthalmic viscoelastic agent, a similarly sized incision was made in the anterior capsule. A viscoelastic cannula was used to instill saline into the capsular space to hydrodissect the lens fiber mass away from the capsule. Angled jeweler forceps were used to remove the lens mass by applying gentle pressure near the equator of the eye. After the lens mass was expelled, careful irrigation of the capsule was performed to remove any residual lens material (in particular, the lens cortex). A viscoelastic agent was then injected into the capsular and anterior chamber to reimplant the eye and maintain its structural integrity postoperatively. The corneal incision was closed using 10-0 nylon sutures. Animals were euthanized 5 days postoperatively and lenses removed from both the surgical eye as well as the contralateral eye, which served as an experimental control. For analysis, whole eyes from ECLE experiments were preserved for immunofluorescence. In some cases, capsular bags were microdissected from the eye and used for isolation of RNA for PCR measurements. Surgery, euthanasia, and dissection were performed in parallel for each experiment to ensure consistency and comparability. Furthermore, the surgeon was blinded to the genetic strain of each mouse during operations. For experiments involving treatment with Sorbinil ([(6S)-6-Fluoro-2,3-dihydro-spiro[4H-1-benzopyran-4,4’-imidazolidine]-2’,5’-dione), mice were injected intraperitoneally with 10 mg/kg at the time of surgery followed by twice daily injections on postoperative days 1 through 5. Sorbinil was provided by Pfizer Central Research (Groton, CT, USA). Vehicle controls contained 1X phosphate buffered saline.

Immunofluorescence

Postoperative eyes were fixed in 4% paraformaldehyde for 30 minutes, then transferred to 1X phosphate buffered saline solutions of increasing sucrose concentration (10% for four hours, 20% for four hours, and 30% overnight). Tissues were then embedded in optimal cutting temperature (OCT) media (Tissue Tek, Torrance, CA, USA) and frozen to −80°C before being cut into 10-μm thick sections using a cryostat (Microm HM550; Thermo Fisher Scientific, Waltham, MA, USA). Sections were placed onto glass slides, fixed in acetone at −20°C, and blocked in 5% bovine serum albumin with 0.4% Triton X-100 for 30 minutes at room temperature. Samples were incubated with primary antibodies as outlined in the Table below. After washing slides three times with 1X phosphate buffered saline, samples were incubated with a 1:200 dilution of species appropriate secondary anti-IgG conjugated to AlexaFluor 488 (Thermo Fisher Scientific) for 30 minutes at room temperature. Slides were washed three times, covered with antifade mounting media (Vector Laboratories, Burlingame, CA, USA) containing 4’,6-diamidino-2-phenylindole (DAPD), and covered-slipped. Experiments were repeated at least three times on each mouse strain using appropriate internal controls for staining. The slides were imaged using a confocal microscope (Eclipse Ti; Nikon Corp., Tokyo, Japan). All processing was performed in parallel for each experiment/figure. Furthermore, all imaging parameters (including laser intensity and brightness) were held constant within each experiment. All immunofluorescence images present in this study came from the same set of mice, with different stains applied to nearly consecutive tissue sections.

Quantitative (q)RT-PCR

Following dissection of capsular bags from postsurgical and control eyes, RNA was isolated (RNeasy Microarray Tissue Mini Kit; Qiagen, Austin, TX, USA) according to the manufacturer’s protocol. Complementary DNA was synthesized using a CDNA synthesis kit for qRT-PCR (iScript Advance; Bio-Rad Laboratories, Hercules, CA, USA). For qRT-PCR, we used a SYBR green supermix (iTaq Universal SYBR Green Supermix; Bio-Rad Laboratories) according to the manufacturer’s instructions on a qRT-PCR detection system (CFX Connect; Bio-Rad Laboratories). The primers used in this study (Integrated DNA Technologies, Coralville, IA, USA) were α-SMA (forward 5’-CTGTATAGGGTTTCTGTTGA-3’, reverse 5’-GAGCTCGAAMCTGCCTGAC-3’), fibronectin (forward 5’-TTTCGGTGGAGCACCTGGA-3’, reverse 5’-GCTATCCATTCCTCCTGAC-3’), vimentin (forward 5’-TCAACATGTCGTCGATGTT-3’, reverse 5’-ATCAGCTCACCAACAGCAG-3’), and E-cadherin.
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TABLE. List of Primary Antibodies Used in This Study

| Antibody   | Dilution | Incubation     | Product #, Company                 |
|------------|----------|----------------|-----------------------------------|
| α-SMA      | 1:500    | 1 hour at room temperature | ab32575, Abcam (Cambridge, MA, USA) |
| E-Cadherin | 1:50     | Overnight at 4°C | 4065, Cell Signaling (Danvers, MA, USA) |
| z-A-Crystallin | 1:200 | 1 hour at room temperature | ADI-SPA-221, Enzo (Farmingdale, NY, USA) |
| Aquaporin 0 | 1:200    | Overnight at 4°C | H-44, Santa Cruz (Dallas, TX, USA) |

(Forward 5'-AGTTCGTTTCTTTGTTCTCTGAG-3'; reverse 5'-GAGCTGTCTACCAAAGTGAGC-3'). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control (forward 5'-CTGGGAGAACATGCCAAGTA-3'; reverse 5'-TGTTGCGTGAGCTGCTATTCA-3'). Statistical analysis was performed with graphing software (GraphPad Prism; GraphPad Software, Inc., La Jolla, CA, USA) using the 2^(-ΔΔCt) method. Data were analyzed by ANOVA with Tukey's post hoc test. Asterisks refer to P values according to the following: *P < 0.05, **P < 0.01, and ***P < 0.001.

RESULTS

Genetic Knockout of AR Reduces ECLE-Induced Upregulation of the Myofibroblast Marker α-SMA

Previous research in lens epithelial cell cultures has demonstrated a link between AR inhibition and the attenuation of TGF-β2 induced expression of EMT and fibrosis markers (α-SMA, fibronectin, vimentin). However, it is unclear whether this inhibition can lead to observable changes in protein expression or gross morphology in the in vivo situation. To test whether AR inhibition can prevent EMT and therefore PCO in an animal model, we used immunofluorescence staining to examine eyes of wild-type (WT), AR knockout (ARKO), and AR overexpression (AR-Tg) mice 5 days after extracapsular lens extraction surgery (ECLE). Immediately after surgery, a thin layer of anterior lens epithelial cells is present lining the anterior capsule bag in any strain, with only a thin layer of anterior epithelial cells present (A, C, E). Five days after surgery, all strains show α-SMA expression. However, ARKO (B) has appreciably less expression overall as compared to WT (D) and AR-Tg (F), with α-SMA being confined to a relatively thin layer along the capsular border. WT (D) has a much thicker layer of α-SMA deposition along the capsular border, but still lacks α-SMA expression connecting the anterior and posterior capsule. Unlike ARKO and WT, AR-Tg (F) displays robust α-SMA expression spanning the entire capsular bag space from the posterior capsule to the anterior capsule. All panels show α-SMA expression (green) along with nuclei (DAPI, blue). Dotted line traces the anterior capsule; dashed line traces the posterior capsule. Arrows denote α-SMA depictions along the anterior capsule, while arrows denote α-SMA deposits along the posterior capsule. Scale bar: 50 μm.

FIGURE 1. Expression of the myofibroblast marker α-SMA 0 hours and 5 days after cataract surgery in ARKO (A, B), WT (C, D), and AR-Tg (E, F) mice. Immediately after surgery, no α-SMA expression is seen within the remnant capsular bag in any strain, with only a thin layer of anterior lens epithelial cells present (A, C, E). Five days after surgery, all strains show α-SMA expression. However, ARKO (B) has appreciably less expression overall as compared to WT (D) and AR-Tg (F), with α-SMA being confined to a relatively thin layer along the capsular border. WT (D) has a much thicker layer of α-SMA deposition along the capsular border, but still lacks α-SMA expression connecting the anterior and posterior capsule. Unlike ARKO and WT, AR-Tg (F) displays robust α-SMA expression spanning the entire capsular bag space from the posterior capsule to the anterior capsule. All panels show α-SMA expression (green) along with nuclei (DAPI, blue). Dotted line traces the anterior capsule; dashed line traces the posterior capsule. Arrows denote α-SMA depictions along the anterior capsule, while arrows denote α-SMA deposits along the posterior capsule. Scale bar: 50 μm.

Genetic Knockout of AR Reduces ECLE-Induced Downregulation of the Epithelial Marker E-Cadherin

Concomitant with induction of myofibroblast markers like α-SMA, EMT is characterized by down-regulation of epithelial markers such as E-cadherin. In a similar experiment as above, we performed immunostaining directed at E-cadherin. Immediately postoperatively, an E-cadherin positive layer of anterior lens epithelial cells is present lining the anterior capsule (Figs. 2A, 2C, 2E). Five days postoperatively, ARKO mice displayed persistent yet mildly attenuated E-cadherin expression in a thin cell layer lining the anterior and posterior capsule (Fig. 2B). WT mice, on the other hand, displayed markedly decreased E-cadherin expression postoperatively, with only faint expression in select areas along the capsular border (Fig. 2D). By contrast, postoperative AR-Tg capsules showed even greater loss of E-cadherin expression in the cellular mass filling the capsular bag (Fig. 2F), displaying essentially background levels of staining. These data demonstrated that our mock cataract surgery procedure caused downregulation of E-cadherin even in the absence of AR inhibition. In AR-Tg mice, the residual E-cadherin staining was notably less robust compared to WT mice (D vs. F), indicating that AR expression is required for the induction of EMT and fibrosis markers postoperatively. These findings support the hypothesis that AR is a key regulator of EMT and fibrosis in ECLE, and suggest potential therapeutic targets for the prevention of PCO.

FIGURE 2. Expression of the lens epithelial cell marker E-cadherin 0 hours and 5 days after cataract surgery in ARKO (A, B), WT (C, D), and AR-Tg (E, F) mice. Immediately after surgery, E-cadherin expression is present in all strains (A, C, E). Two days postoperatively, E-cadherin expression is barely detectable in all strains except AR-Tg (D). Five days postoperatively, AR-Tg mice show very faint expression of E-cadherin in the posterior capsule (F). By contrast, E-cadherin expression is largely absent in WT mice (D) and ARKO mice (B). All panels show E-cadherin expression (green) along with nuclei (DAPI, blue). Dotted line traces the anterior capsule; dashed line traces the posterior capsule. Scale bar: 50 μm.
Genetic Knockout of AR Prevents Characteristic EMT Changes in mRNA Levels Induced by Cataract Surgery

While the experiments above provide qualitative evidence that AR levels correlate positively with induction of EMT markers associated with PCO from a histologic perspective, we sought to confirm these results with quantitative measures of mRNA transcripts of EMT-related genes. To achieve this aim, we isolated RNA from postoperative capsular bags as well as contralateral capsular bags produced by ECLE at the time of euthanasia. In both WT and AR-Tg mice, we observed the expected changes in mRNA levels of informative genes associated with postoperative EMT. For example, capsular bags of WT and AR-Tg lenses contained markedly elevated transcript levels for $\alpha$-SMA (Fig. 3A), fibronectin (Fig. 3B), and vimentin (Fig. 3C). The increase in AR-Tg fibronectin (Fig. 3B) did not reach statistical significance due to elevated baseline expression, consistent with our prior work with upstream EMT signaling mediators. As expected, transcript levels for the epithelial marker E-cadherin were reduced in WT and AR-Tg lenses (Fig. 3D). Interestingly, E-cadherin reduction was more rapid and marked in AR-Tg than WT (which did not reach statistical significance 5 days postoperatively), suggesting a more robust EMT reaction. In contrast, in ARKO mice no significant changes in gene transcript levels of key markers of EMT were noted following ECLE in AR-deficient lenses (see ARKO in Figs. 3A–D). Thus, our surgical model induces a quantifiable change in EMT-related gene expression patterns that can be prevented through genetic knockout of AR.

Genetic Knockout of AR Does Not Prevent the Postoperative Induction of the Lens Fiber Cell Marker $\alpha$-A-Crystallin

It is thought that incidental surgical trauma during cataract surgery leads to proliferation of remnant LECs along the posterior capsule, leading to two distinct histologic types of PCO: fibrotic PCO and pearl-type PCO. Fibrotic PCO results
from an EMT response in the remnant epithelial cells, which become myofibroblasts and express factors like α-SMA (as described above). Pearl-type PCO, by contrast, results from differentiation of epithelial cells into immature fiber cells, which are rich in crystallin proteins. Interestingly, both histologic variants can exist simultaneously within the same capsule and appear to come from a common response to cataract surgery. Recently, experiments in cell culture have demonstrated that the known inducer of EMT and fibrotic PCO, TGF-β, is also a key inducer of the lens fiber cell response and pearl-type PCO. With the data above demonstrating that AR inhibition can suppress post-cataract EMT and fibrotic PCO, we were motivated to investigate the influence of AR on expression level of the pearl-type PCO response by immunostaining for the fiber cell markers α-crystallin (Fig. 5) and Aquaporin 0 (AQP0, Fig. 6). Prior to surgery, α-crystallin is known to be present diffusely throughout the lens in all mouse strains (data not shown). Immediately following surgery, all strains exhibit α-crystallin positivity in the remnant lenticular tissue, specifically the anterior lens epithelium (Figs. 5A, 5C, 5E). In both WT (Fig. 5D) and AR-Tg (Fig. 5F), abundant α-crystallin is distributed throughout the capsular bag. In fact, the α-crystallin distribution mirrors the α-SMA distribution we observed (Figs. 1D, 1F), consistent with the notion that both the fibrotic and pearl-type postoperative responses share a common trigger and coexist in the same capsule. Interestingly, ARKO mice demonstrated a preserved pearl-type postoperative reaction, as evidenced by abundant α-crystallin expression diffusely throughout the capsular bag (Fig. 5B). Immediate postoperative capsules contained little to no AQP0 (Figs. 6A, 6C, 6E), while all strains exhibited AQP0 positivity at 5 days (Figs. 6B, 6D, 6F). ARKO capsules displayed the most robust response (Fig. 6B), with AQP0 staining occupying a greater portion of the capsule compared to WT (Fig. 6D) and AR-Tg (Fig. 6F). In light of our observed minimal postoperative α-SMA expression in ARKO mice (Fig. 1B), this suggests that low levels of AR are associated with a reduced EMT and fibrotic PCO response, while at the same time preserving the pearl-type fiber cell response.

**DISCUSSION**

The world’s leading cause of visual impairment is cataracts, causing 10.8 million cases of blindness and an additional 35.1...
differentiation are triggered simultaneously in response to coexist in the same capsule, but that both EMT and lens fiber we demonstrate that not only do both of these processes patients develop PCO postoperatively 1 usually requiring PCO, 16 AR inhibition suppresses ROS production in ocular lens fiber cell markers. 38, 40, 50 Both fibrotic and pearl-type PCO characterized by expression of lens fiber cell markers, 40 TGF-β is a key regulator of PCO through its role in inducing EMT in LECs. 1, 2 The classic TGF-β signal transducers are SMAD proteins, 4–6, 48 which lead to the formation of myofibroblast cells, 9 expression of various EMT markers, 8–12 and the histologic phenotype of fibrotic PCO. 58 Critically, it is known that TGF-β signaling increases in LECs that remain after cataract surgery, 16, 47 which can, in turn, undergo an aberrant wound-healing response resulting in PCO. TGF-β is known to be a key regulator of PCO through its role in inducing EMT in LECs. 2 The classic TGF-β signal transducers are SMAD proteins, 4–6, 48 which lead to the formation of myofibroblast cells, 9 expression of various EMT markers, 8–12 and the histologic phenotype of fibrotic PCO. 58 Critically, it is known that TGF-β signaling increases in LECs that remain after cataract surgery, 16 representing a response to the mechanical trauma and injury of the procedure itself. TGF-β can also lead to another phenotype of PCO characterized by expression of lens fiber cell markers, 40 called pearl-type PCO, 38 through non-SMAD pathways. 49

Another key regulator of PCO is aldose reductase (AR). Studied in-depth for its role in the ocular complications of diabetes, AR contributes to PCO through the production of ROS. Experimentation into AR signaling has demonstrated that AR contributes to ROS production, 55 ROS contributes to PCO, 16 AR inhibition suppresses ROS production in ocular tissues, 20–22 AR overexpression can induce PCO, 25 and AR inhibition can decrease markers of PCO. 24 Recently, we connected this extensive body of AR research with the large body of knowledge about TGF-β by showing that AR inhibition prevents the markers of PCO by inhibiting TGF-β signaling. 21

This present work extends previous cell culture and in vitro experiments and demonstrates that AR inhibition in an animal model of cataract surgery can suppress the histologic phenotype of fibrotic PCO. Through immunofluorescence and PCR, we showed that overexpression of AR results in a more robust induction of EMT after model cataract surgery, while genetic or pharmacologic AR inhibition significantly suppressed the cardinal manifestations of fibrotic PCO. With most prior research on AR and PCO employing cell culture models, this present work validates the hypothesis that AR inhibition can prevent PCO by using an in vivo animal model of surgery to display suppression of fibrotic PCO on a histologic level. Importantly, we recognize the limitation of our model to obtain reliable protein quantification due to extremely small tissue samples. Instead, we have combined corroborating mRNA and histologic evidence to support our conclusion.

As noted above, the postoperative induction of TGF-β can also lead to a pearl-type PCO, characterized by expression of lens fiber cell markers. 58, 10, 50 Both fibrotic and pearl-type PCO can coexist in the same postoperative capsule. 50 In this work, we demonstrate that not only do both of these processes coexist in the same capsule, but that both EMT and lens fiber differentiation are triggered simultaneously in response to surgery (Figs. 1D, 2D, 5D, 6D). Furthermore, we show that while AR inhibition effectively inhibits EMT, it does not prevent fiber cell differentiation and expression of the fiber cell markers α-crystallin (Fig. 5D) and AQP0 (Fig. 6B). Taken together, these data represent the first example that this modified cataract surgical model can effectively trigger both types of PCO responses simultaneously and that AR inhibition can selectively inhibit EMT without abrogating fiber cell differentiation. It was recently shown that TGF-β triggers both pathways through different downstream pathways, including SMAD and non-SMAD cascades. 40, 51, 52 While we have demonstrated that AR inhibition prevented PCO and EMT markers through suppression of SMAD signaling, 21 AR activity has been shown to affect non-SMAD pathways as well. 53 Presumably, AR inhibition has less effect on those downstream pathways of TGF-β signaling that cause lens fiber cell differentiation, but this remains to be tested experimentally. Additionally, it is unknown whether AR inhibition can directly augment fiber cell differentiation or whether it simply shifts the biochemical equilibrium away from EMT, thereby increasing flux through the fiber cell pathway.

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