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**Author:**

Kim, D; Pattamatta, U; Kelly, E; Healey, PR; Carnt, N; Zoellner, H; White, AJR

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# Inhibitory Effects of Angiotensin II Receptor Blockade on Human Tenon Fibroblast Migration and Reactive Oxygen Species Production in Cell Culture

Duri Kim<sup>1</sup>, Ushasree Pattamatta<sup>1</sup>, Elizabeth Kelly<sup>2</sup>, Paul R. Healey<sup>1,3,4</sup>, Nicole Carnt<sup>1</sup>, Hans Zoellner<sup>2</sup>, and Andrew J. R. White<sup>1,3,4</sup>

<sup>1</sup> Centre for Vision Research, the Westmead Institute for Medical Research, Sydney Medical School, University of Sydney, New South Wales, Australia

<sup>2</sup> The Cellular and Molecular Pathology Research Unit, Department of Oral Pathology, Faculty of Dentistry, University of Sydney, Westmead Hospital, New South Wales, Australia

<sup>3</sup> Department of Ophthalmology, Westmead Hospital, Sydney, New South Wales, Australia

<sup>4</sup> Save Sight Institute, Sydney Medical School, University of Sydney, New South Wales, Australia

**Correspondence:** Duri Kim, Lv6, Centre of Vision Research, the Westmead Institute for Medical Research, 176 Hawkesbury Rd, Westmead, New South Wales, Australia 2145. email: dkim5474@uni.sydney.edu.au

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**Purpose:** We investigate the effect of angiotensin receptor blockade on the migration of human Tenon fibroblasts (HTF), using irbesartan, an angiotensin II receptor type 1 (AT1R) blocker (ARB) as a potential antifibrotic agent in glaucoma filtration surgery.

**Methods:** Confluent HTF cultures were scratched with a 1 mL pipette tip and treated with either irbesartan (10, 50, and 100 µg/mL) or angiotensin II (2 µg/mL). The extent of HTF migration up to 30 hours, and cell number and morphology at 72 hours was evaluated. To assess the effect on reactive oxygen species (ROS) level, HTF were treated with either irbesartan (10 µg/mL) or angiotensin II (2 µg/mL) for 24 hours after scratching, and then stained with dihydroethidium (DHE) before evaluation by confocal microscopy.

**Results:** Irbesartan inhibited HTF migration by 50% to 70% compared to controls ( $P < 0.05$ ). Levels of ROS were almost completely attenuated by irbesartan (DHE fluorescence intensity of 5.68E-09) ( $P < 0.05$ ). Irbesartan reduced cell numbers by 50% and induced morphologic changes with loss of pseudopods ( $P < 0.05$ ). Conversely, angiotensin II increased cell numbers up to 4-fold while retaining cell viability.

**Conclusions:** Irbesartan inhibited HTF migration and ROS production. It also reduced cell numbers and altered HTF morphology. Angiotensin II increased cell number without altering morphology. This initial study warrants future investigations for further potential antifibrotic effects of this drug.

**Translational Relevance:** This in vitro study focused on investigations of irbesartan's effects on HTF migration, ROS production, as well as HTF cell numbers and morphology. It suggests a potential therapeutic strategy worth further exploration with a view towards postoperative wound healing modulation in glaucoma filtration surgery.

## Introduction

Glaucoma is the leading cause of irreversible blindness and the second most common identifiable cause of blindness worldwide.<sup>1</sup> Current glaucoma treatment aims to reduce intraocular pressure as the

principal modifiable risk factor for the disease. This includes fistularizing surgeries, such as trabeculectomy and tube implantation, which drain aqueous fluid into the lower pressure subconjunctival space. These generally are described by the term "glaucoma filtration surgery."

In trabeculectomy, the gold standard glaucoma

surgery, the postoperative wound healing response is the main determinant of resistance to aqueous egress and, therefore, surgical success.<sup>2</sup> Subconjunctival fibrosis and scar formation is a major cause of surgical failure.<sup>3</sup> Antifibrosis agents, such as mitomycin C (MMC) or 5-Fluorouracil (5-FU), are administered commonly to improve prognosis by retarding the postoperative wound healing process. These antimetabolites exert their antifibrotic effects through inhibition of fibroblast proliferation and induction of apoptosis.<sup>4</sup> However, MCC or 5-FU can have serious complications, such as excessive inhibition of wound healing and collateral tissue damage.<sup>5</sup> This can contribute to adverse outcomes, such as hypotony, late-onset bleb leakage, endophthalmitis, and blebititis.<sup>6</sup> Mitomycin resistance also can occur, which may lead to surgical failure despite its use.<sup>7</sup> It would be advantageous to find an alternative way to modulate postglaucoma filtration surgery wound healing, without such complications.

A traditional view of the renin angiotensin system (RAS) is limited to its endocrine physiologic role controlling blood pressure, electrolytes, and fluid homeostasis. However, significant evidence over the last decade has revealed locally intrinsic tissue-specific roles for the RAS, including roles in wound healing, fibrosis, and inflammation, well beyond its historic role as a circulating system.<sup>8,9</sup> The principal effector of the RAS, angiotensin II, has been shown to have proinflammatory and profibrotic properties in a number of organs, including the liver,<sup>10</sup> kidney,<sup>11</sup> cardiac muscle,<sup>12</sup> lung,<sup>13</sup> and skin,<sup>14</sup> mediated via the angiotensin receptor 1 (AT1R).<sup>15,16</sup>

Interestingly the RAS, especially its product angiotensin II, has been shown to have multifaceted roles in ocular pathology and physiology. The RAS contributes to several ocular pathologies, including uveitis,<sup>17-19</sup> macular degeneration,<sup>18-20</sup> diabetic retinopathy,<sup>18-20</sup> glaucoma,<sup>18-20</sup> and inflammatory disorders<sup>17,21-23</sup> in which angiotensin II has been shown to enhance vascular permeability. This is mostly via induction of chemokines and adhesion molecules, together with recruitment of inflammatory cells.<sup>19</sup>

Ocular physiologic roles for the RAS and angiotensin II, include influencing the eye's vasculature,<sup>24,25</sup> aqueous humor outflow regulation, and IOP control,<sup>26,27</sup> This is consistent with recent findings establishing the presence of all RAS components in the human eye,<sup>28</sup> including AT1R, with localized RAS elements in clinically relevant components of the eye, including the conjunctiva and sclera, validated at mRNA and protein levels.<sup>29</sup>

While exploration of ocular tissue-specific elements of the RAS, and implications of this in wound healing remain at an early stage, there are recent reports demonstrating higher expression of AT1R, AT2R, and angiotensin II in rabbit Tenon fibroblasts after trabeculectomy,<sup>30</sup> as well as potential therapeutic avenues created by the RAS in the eye.<sup>31,32</sup>

Losartan, the first approved AT1R blocker attenuates scar formation after trabeculectomy in vivo and in vitro, possibly via inhibitory effects on proliferation, migration, trans-differentiation and extracellular matrix deposition.<sup>31</sup> Olmesartan, another angiotensin receptor blocker (ARB), also inhibits fibroblast proliferation in vivo and in vitro.<sup>32</sup>

Consistent with these reports, we hypothesized that the RAS is involved in postoperative wound healing in the human eye, and that its blockade may modulate fibroblast activity in the context of postoperative wound healing in glaucoma filtration surgery. Therefore, based on recently established roles of the RAS beyond the cardiovascular and renal systems,<sup>10-14</sup> the focus of this study was to explore the possible role of the RAS, in modulation of wound healing in the eye.

Currently, eight ARBs are clinically available with United States Food and Drug Association (FDA) approval. Common molecular structures of biphenyl-tetrazol and imidazol groups of these drug classes have selective inhibitory effects on AT1 receptor blocker though its mimicry of angiotensin II. Small changes in chemical structures, for example the replacement of the chloride group in losartan with cyclopentyl for irbesartan, yield notable differences in their pharmacokinetics, pharmacodynamics, and molecular effects.<sup>33</sup>

In this study, we chose to study the effect of irbesartan. Irbesartan is an AT1-specific competitive antagonist with a much greater affinity for the AT1 receptor than for the AT2 receptor of more than 8500-fold with no agonist activity.<sup>34</sup> It has highest bioavailability with an average absolute value of 60% to 80% among its drug class with no food interaction.<sup>35</sup> Pharmacokinetically, it does not require biotransformation to an active metabolite.<sup>35</sup> Due to its cyclopentyl group, it has higher affinity to the AT1 receptor with an IC<sub>50</sub> of 1.3 nmol/L compared to an IC<sub>50</sub> of 20 nmol/L for losartan, and a lower K<sub>d</sub> of approximately 2 nM compared to 10 nM for losartan.<sup>33,36</sup> Irbesartan is highly efficacious and results in a high 24-hour mean systolic blood pressure reduction effect equivalent to that of olmesartan.<sup>37</sup> Of special relevance to the current study is that

irbesartan also has additional wound healing molecular effects of significance, including potential anti-inflammatory and antioxidant effects as shown in recent studies.<sup>38–41</sup>

In this *in vitro* study, we investigated the effect of irbesartan on HTF with angiotensin II as a positive control. This initial study is to provide a basis for further study with a view to its potential role as a therapy to modify wound healing in glaucoma filtration surgery.

## Methods

### Human Tenon's Fibroblast Culture

Primary human Tenon's fibroblast (HTF) cell lines from Tenon's capsule biopsies of patients during cataract surgery at Westmead Hospital (New South Wales, Australia), were propagated and stored in liquid nitrogen as described previously.<sup>7</sup> This previously described method provides fibroblast cultures of high purity. This was confirmed from observation of the spindle morphology in cells. The tenets of the Declarations of Helsinki were observed. Informed consent was obtained and approval from the Westmead Hospital Human Research Ethics Committee was obtained.

HTFs were thawed and cultured in complete M199 media containing: 10% bovine calf serum (BCS); the antibiotics penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (2.5 µg/mL); and 0.4 mM of L-glutamine (M199, BCS, antibiotics, anti-fungal and L-glutamine, all from Gibco; Thermo Fisher Scientific, Waltham, MA). Experiments were conducted with HTF in passage number 4, at 37°C, under 5% CO<sub>2</sub>, and 100% humidity.

### Drug Preparation

Irbesartan (Sigma-Aldrich Corp, St. Louis, MO) and human angiotensin II (Sigma-Aldrich Corp) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Corp) and prepared as a concentrated stock. DMSO was used as a vehicle for the purpose of concentrated stock preparation for experiments without any effects on HTFs. For this study, the drugs were diluted further with culture medium to achieve the concentrations desired by the experimental design. The final concentrations of DMSO in experimental solutions were 0.04% for 10 µg/mL irbesartan, 0.2% for 50 µg/mL irbesartan, 0.4% for 100 µg/mL irbesartan, and 0.2% for 2 µg/mL angiotensin.

### Fibroblast Migration Assay

HTFs were seeded onto 24-well plates in M199 containing 10% BCS and antibiotics. At near confluence, HTF cultures were scratched with a 1 mL pipette tip and treated with either: irbesartan at a range of concentrations (10, 50, and 100 µg/mL), angiotensin (2 µg/mL), or media as control. HTFs were photographed through a microscope at a magnification of ×40 (Olympus CK2, Tokyo, Japan) and Scopphoto camera (Scopetek, Hangzhou, China) at 0, 3, 6, 24, and 30 hours after scratch. All experiments were performed in sextuplet ( $n = 6$ ) replicate cell wells. We determined 0 hour as the start point and 30 hours as the endpoint due to estimated imminent complete scratch closure in some HTF groups. Images then were analyzed with ImageJ (National Institutes of Health [NIH], Bethesda, MD), Adobe Photoshop (Adobe Systems, San Jose, CA), Microsoft Office (Microsoft, Redmond, WA), and Prism (Graphpad Software, La Jolla, CA). The average distance migrated by HTF at increasing times following scratching was calculated by comparison of the denuded areas remaining relative to the zero time point per each cell well.

### Dihydroethidium Reactive Oxygen Species Assay

To determine the effect of irbesartan on reactive oxygen species (ROS) production by HTF, HTFs were seeded on gelatin-coated cover slips in each well and cultured to confluence in complete media M199 containing 10% BCS and antibiotics in a 37°C, 5% CO<sub>2</sub> incubator. After aspiration of the media, HTFs were scratched with a 1 mL pipette tip and treated with either irbesartan 10 µg/mL, angiotensin 2 µg/mL or vehicle for 24 hours. This experiment was performed in triplicate ( $n = 3$ ) replicate cell wells. The concentration of 10 µg/mL irbesartan was used as the minimum effective dose determined from the scratch assay and by previous studies.<sup>42</sup> HTFs then were stained with 10 µM dihydroethidium (DHE; Sigma-Aldrich Corp) for 5 minutes. After washing with phosphate-buffered saline (PBS; Gibco, Thermo Fisher Scientific), coverslips were transferred onto glass slides and subsequently photographed under the same standardized intensity settings on three filter channels with a confocal microscope (Olympus FV1000). Three images were taken from different sites in each well at ×20 magnification. Fluorescence intensity of DHE was analyzed with ImageJ.



## HTF Cell Counts and Cellular Morphology Analysis

At 30 hours after scratch, supernatants were collected and replaced with M199 media containing 10% BCS and antibiotics without additional treatments of irbesartan or angiotensin. HTFs were incubated in 37°C, 5% CO<sub>2</sub> for a further 42 hours and observed at 72 hours after scratch. Images were taken with a microscope at the magnification of ×40 as described for migration assays above. Quadruplicate ( $n = 4$ ) manual cell counts were performed of all cells in the photomicrographs of the middle half of the images alongside the scratch with ImageJ and Adobe Photoshop. Cell count data were expressed in units of cells per visual area, and means and standard deviations accordingly. Only adherent HTFs were counted. Cell morphology was analyzed by calculating circularity (Circularity =  $4 \pi \times \text{area} \div (\text{perimeter})^2$ ).

## Statistical Analysis

Statistical analysis and graphic output were performed using Prism and Excel software. Bonferroni corrected Student's *t*-test was used for comparisons of multiple groups against a reference, such as control. *P* values less than 0.05 were considered statistically significant.

## Results

### Irbesartan Inhibited HTF Migration

Irbesartan inhibited HTF migration such that at 10, 50, and 100 µg/mL, irbesartan reduced scratch closure by 69%, 65%, and 52% relative to controls at 30 hours (Bonferroni corrected unpaired *t*-test  $P < 0.05$ ; Fig. 1). All experiments were performed in sextuplicate ( $n = 6$ ) cell wells; however, for angiotensin and control groups, five culture wells were analyzed for each because of unfavorable scratch orientation. There was no statistically significant effect of angiotensin on migration. We determined 10 µg/mL to be the minimum effective dose of irbesartan in this experimental model, so this was used as the dose of irbesartan in the ROS assay described below.<sup>42</sup>

### Irbesartan Reduced ROS Levels

ROS production detected by DHE fluorescence was observed readily, with irbesartan-treated HTFs almost completely attenuating fluorescence compared

to controls (Bonferroni corrected *t*-test,  $P < 0.05$ ). The angiotensin-treated group did not display any statistically significant change (Fig. 2).

### Effects of Irbesartan and Angiotensin on HTF Cell Number and Morphology at 72 Hours After Scratch

At 72 hours after scratch, irbesartan reduced HTF cell numbers and there was morphologic change with increased circularity, whereas angiotensin II-treated HTFs exhibited higher cell numbers with retained viability. Figure 3 shows representative images of HTFs, graphs of HTF circularity, and HTF cell numbers.

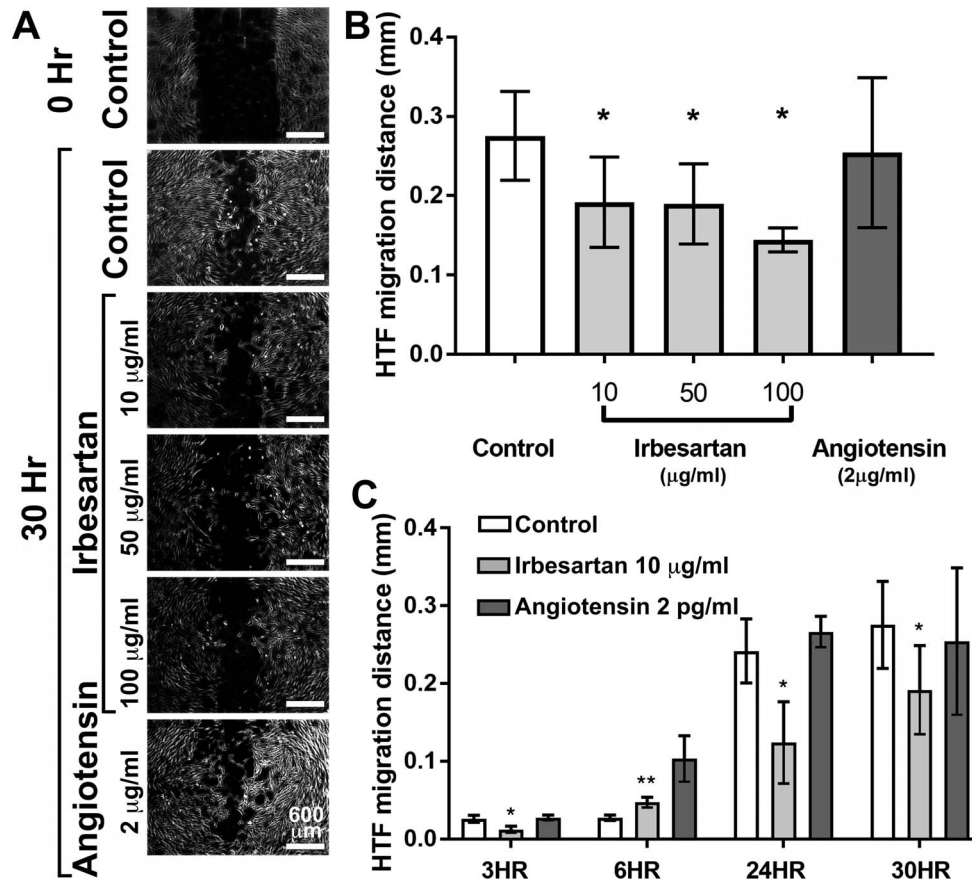
Angiotensin II-treated HTFs had the typical appearance of fibroblasts with a circularity measure of 0.33 (Bonferroni corrected *t*-test,  $P < 0.0001$ ). However, irbesartan-treated HTFs displayed altered appearance with loss of dendritic pseudopod processes and increased circularity to 0.61 (Bonferroni corrected *t*-test,  $P < 0.0001$ ). Control groups exhibited circularity of 0.18 with retained pseudopod processes. For HTF circularity analysis, 100 µg/mL irbesartan was used to determine the effect of the drug on HTF morphology, rather than trying to determine the minimum effective dosage.

The number of surviving adherent cells revealed clear contrasts among the groups. Compared to control groups with  $364.5 \pm 41.10$  (SD) cells per visual area, irbesartan (10 µg/mL) had a near halved cell number to  $215.8 \pm 46.96$  (SD) cells per visual area. Conversely, the angiotensin-treated HTFs cell number was  $1622 \pm 345.0$  (SD) cells per visual area, an almost 4-fold increase compared to the control group. Using a Bonferroni corrected *t*-test, all three groups were different from each other, with statistically significant *P* values of less than 0.05.

## Discussion

HTF migration is a crucial step in wound healing, which involves tissue contraction at the bleb site after trabeculectomy.<sup>43</sup> In this *in vitro* study, irbesartan exhibited an antimigratory effect that, if seen *in vivo*, would be expected to be antifibrotic. This may have clinical translational relevance in ophthalmology and medicine.<sup>11</sup>

Additionally, irbesartan significantly modulated ROS formation to almost complete attenuation. Reduced superoxide (O<sub>2</sub><sup>-</sup>) formation signifies an important role of this drug class. ROS, such as



**Figure 1.** Photomicrographs and histogram of the effect of irbesartan on HTF migration in the scratch assay. (A) Representative photomicrographs are shown for each culture condition studied at 0 and 30 hours after scratch. There was reduced scratch closure in irbesartan-treated HTF groups compared to controls. (B) As demonstrated in the histogram shown, irbesartan reduced the HTF migration to approximately 50% to 60% compared to the control group. (C) The histogram shows reduced HTF migration in the irbesartan group compared to controls at the increasing time points. Error bars: represent the standard deviation on all graphs. \* $P < 0.05$ , \*\* $P < 0.005$ .

hydrogen peroxide ( $H_2O_2$ ) and  $O_2^-$  have well established roles in differing aspects of wound healing,<sup>44</sup> including inflammation,<sup>45</sup> fibroblast migration, proliferation,<sup>46</sup> and angiogenesis.<sup>47</sup>

The effect of irbesartan in reducing  $O_2^-$  formation may have potential to be adapted for therapeutic use in other ocular pathologies. The already catalogued presence of the RAS in the eye<sup>28</sup> involving multiple ocular pathologies supports this suggestion.

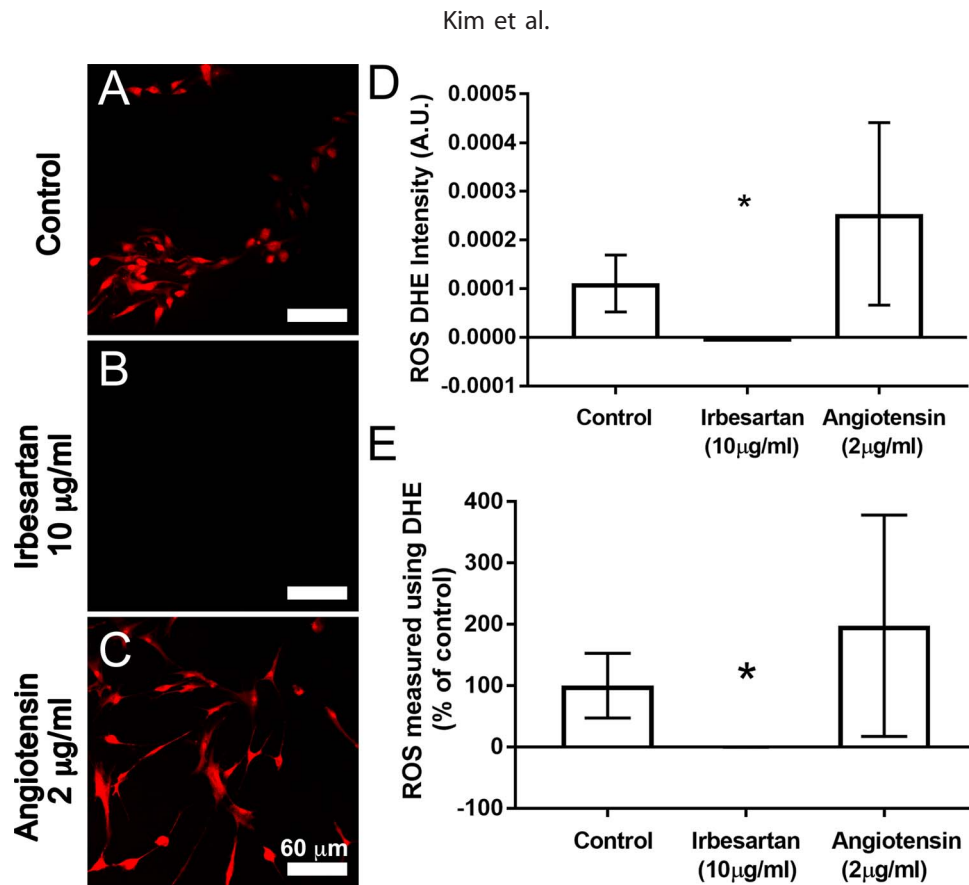
The proliferative and ROS inductive effect of angiotensin II seen in this study, supports a role in fibrosis, while the maintained fusiform morphology of HTF stimulated in this way also is consistent with a fibrotic phenotype. Conversely, the effects seen of irbesartan on morphology and ROS production suggests an anti-inflammatory and antifibrotic phenotype.

We interpret increased circularity with loss of dendritic processes in HTFs as denoting suppressed

cell adhesion, locomotion, and migration, which can have lasting effects in the tissue remodeling phase of wound healing, leading to suppressed scar formation.<sup>48</sup> Reduced or increased cell numbers may suggest potential effects of RAS modulation in HTFs on cell survival.

It is interesting to note that in this 72-hour assay, at 30 hours after scratch, the media was changed without additional treatment of either irbesartan or angiotensin II. Persistence of a response in the absence of continuous stimulation with the drug suggests a lasting effect of angiotensin receptor blockade after treatment.

This initial study raises the clinically relevant possibility of a therapeutic method with a view to use irbesartan as an antifibrotic agent in glaucoma surgery. This drug class offers potential advantages in that it already has a well-established systemic safety



**Figure 2.** Confocal microscopic photographs and histogram demonstrating the effect of irbesartan on ROS levels in the ROS-DHE assay (A–C) Representative photographs via DHE fluorescent detection are shown for each group. Irbesartan attenuated the ROS levels compared to controls and angiotensin. (D–E) The histogram shows irbesartan significantly reduced the level of ROS levels to almost complete attenuation as opposed to angiotensin, which appeared to increase ROS levels. *Error bars:* Standard deviation on all graphs (\* $P < 0.05$  relative to control).

profile and is used widely with range of known therapeutic properties.

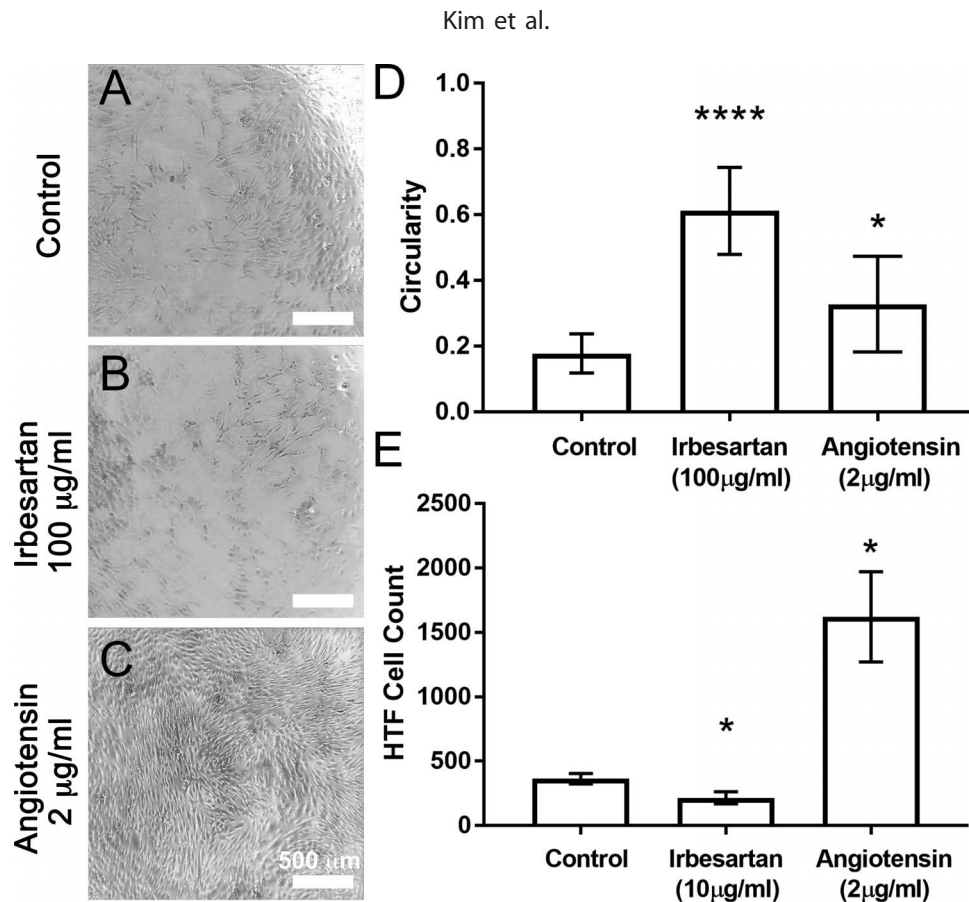
Our findings of this drug class are consistent with the results of recently published studies as we also demonstrated the inhibitory effect of angiotensin receptor blockade (AT1) on fibrosis in human Tenons.<sup>30–32</sup>

A previous study<sup>30</sup> established the promoting effect of Ang II on fibrosis in vivo after trabeculectomy in rabbits and in vitro using HTFs. Since then, Shi et al.<sup>31</sup> also showed that losartan attenuated scar formation in vivo and in vitro. In their study, losartan ( $10^{-5}$  M) decreased HTF cell proliferation compared to the control by 20.4%, migration by 48.3% at 24 hours, and transdifferentiation and ECM (extracellular matrix) synthesis by almost 2-fold.<sup>31</sup> Additionally, they also indicated that losartan (5 mg/mL) attenuated bleb scarring after trabeculectomy in rabbit eyes with a 77.8% inhibition compared to control.<sup>31</sup>

Olmesartan also was shown to exhibit dose-dependent inhibitory effects in HTF proliferation

with an 11% inhibition at 0.75 µmol/mL.<sup>32</sup> It also exerted antiscarring effects in vivo via a decrease in ECM remodeling in rabbit Tenon's capsule through an increased expression of tissue inhibitor of matrix metalloproteinases (TIMP)-1, TIMP-2, and decreased matrix metalloproteinases (MMP)-2 and proliferating cell nuclear antigen (PCNA).<sup>32</sup> While concordant, our study differs from other recently published studies in that we investigated irbesartan as the drug of choice. To our knowledge, this study is the first to investigate irbesartan as a potential antifibrotic drug in the eye.

In our study, it is interesting to note that irbesartan led to the larger reduction (%) in cell migration to 69% (10 µg/mL), 65% (50 µg/mL), and 52% (100 µg/mL) relative to controls at 30 hours ( $P < 0.05$ ), compared to losartan with 41.3% and 48.3% (at 12 and 24 hours with posttreatment;  $P < 0.001$ ) and 49.6% and 48.6% reduction (at 12 and 24 hours with pretreatment;  $P < 0.01$ ).<sup>31</sup> We speculated that this result may be due to irbesartan's higher affinity to the AT1 receptor as well as high efficacy.<sup>33,36,37</sup>



**Figure 3.** Photomicrographs and histograms showing the effect of irbesartan on HTF cell number per visual field and morphology. (A–C) Representative images at 72 hours after scratch revealed HTF with marked changes in cell morphology and number. Irbesartan-treated HTF had reduced number compared to control, whereas there was increased cell number to higher confluency in the angiotensin-treated group. (D) Histogram showing the quantified circularity in HTF controls, as well as for HTF treated with irbesartan and angiotensin. Irbesartan resulted in altered morphology towards a more circular or ellipsoid shape with relative loss of dendritic processes (\* $P < 0.05$ , \*\*\*\* $P < 0.001$ ). (E) Histogram showing HTF cell numbers in control, irbesartan, and angiotensin groups at 72 hours. Compared to controls, irbesartan reduced HTF number to almost half, whereas angiotensin-treated groups had an almost 4-fold increase in cell number. \* $P < 0.05$ . Error bars: standard deviation on all graphs.

Further, our in vitro study investigated anti-inflammatory and antioxidant effect as well as potential cellular effects of this drug additional to its major antimigratory effect. This is consistent with evidence suggestive of the role of tissue-specific RAS in inflammation and fibrosis.<sup>9</sup> This also is in line with irbesartan's anti-inflammatory, antioxidant and anti-fibrotic properties shown in other organs and tissues.<sup>39</sup> The inhibitory effect of irbesartan in inflammation, apoptosis, and fibrosis has been demonstrated in cardiovascular, renal, pulmonary, and gastrointestinal systems. Irbesartan is thought to exert these effects through AT1- dependent as well as nonindependent mechanisms involving peroxisome proliferator-activated receptor (PPAR)  $\gamma$  activation<sup>39</sup>; however, this effect is not yet studied in the eye or using ocular tissues. While the specific mechanisms of

irbesartan in wound healing are beyond the immediate scope of this study, it would be of value to investigate these mechanisms further in future work.

The effect of irbesartan on the ocular system has not had prior investigation, so that it is difficult to speculate on the possible ocular complications associated with this drug. We also feel it may be premature to compare the therapeutic benefit of this drug to MMC or 5-FU, based on the current initial study alone. Although current data are encouraging, and while it is widely accepted that ARBs are among the safest and best tolerated cardiovascular drugs approved for clinical use, further studies regarding safety for use in the ocular system are required. Extensive evidence of this drug and its safety in in vivo, in vitro, and in clinical studies would be of advantage in further studies using this drug for the



purpose of an antifibrotic therapeutic after trabeculectomy.

The effect of angiotensin receptor blockade in wound healing in HTF is an area of current interest and active research by a number of laboratories.<sup>31,32</sup> While our findings present evidence of a potentially valuable drug action of irbesartan, the mechanism for this is beyond the scope of the current study and requires further investigation. We believe further in vitro, in vivo, and clinical studies are justified.

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