Published in final edited form as:
*Invest Ophthalmol Vis Sci*. 2008 December ; 49(12): 5466–5475. doi:10.1167/iovs.08-2374.

# Cell Cycle Arrest by Kynurenine in Lens Epithelial Cells

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## Abstract

**Purpose—**Indolemine 2,3-dioxygenase (IDO)-mediated oxidation of tryptophan produces kynurenines (KYNs), which may play a role in cataract formation. The molecular mechanisms by which KYNs cause cellular changes are poorly understood. The effects of KYNs on mouse lens epithelial cells by overexpression of human IDO were investigated.

**Methods—**Lens epithelial cells (mLECs) derived from human IDO-overexpressing hemizygous transgenic (hemTg) and wild-type (Wt) mice were used. IDO activity was measured by quantifying kynurenine (KYN) by HPLC. KYN-mediated protein modifications were detected by immunocytochemistry and measured by ELISA. Cell proliferation and apoptosis were measured with commercially available kits. Cell distribution between cell cycle phases was examined with flow cytometric analysis. Immunoprecipitation followed by LC/MS was used to identify kynurenine-modified proteins.

**Results—**mLECs derived from hemTg animals exhibited considerable IDO immunoreactivity and enzyme activity, which were barely detectable in Wt mLECs. KYN and KYN-mediated protein modification were detected in hemTg but not in Wt mLECs; the modified proteins were myosin II and $\alpha/\gamma$-actin. HemTg mLECs displayed reduced viability and proliferation. Cell cycle analysis of hemTg mLEC cultures showed approximately a twofold increase in cells at $G_2$/M or in both phases, relative to Wt mLECs. Blocking IDO activity with 1-methyl-$_{D,L}$-tryptophan in hemTg mLECs prevented KYN formation, KYN-mediated protein modification, and $G_2$/M arrest.

**Conclusions—**Excess IDO activity in mLECs results in KYN production, KYN-mediated modification of myosin II and $\alpha/\gamma$-actin, and cell cycle perturbation. Modification of myosin II and $\gamma$-actin by KYN may interfere with cytokinesis, leading to defective epithelial cell division and thus a decreased number of fiber cells.

Indoleamine 2,3-dioxygenase (IDO) catalyzes the oxidation of tryptophan to *N*-formylkynurenine (NFK), the first step in the kynurenine pathway. Through a series of reactions that involve intermediates such as kynurenine (KYN) and 3-OH kynurenine (3OHKYN), *N*-formylkynurenine eventually forms NAD.[1,2] 3OHKYN is glucosylated to form 3OHKYN-glucoside, which is a major kynurenine in the human lens.[3] IDO production

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Disclosure: **M. Mailanko**, None; **D. Smith**, None; **S. Howell**, None; **B. Wang**, None; **J.W. Jacobberger**, None; **T. Stefan**, None; **R.H. Nagaraj**, None

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is markedly increased during infection or inflammation and is induced by proinflammatory cytokines, including interferon (IFN)-$\gamma$.[4]

Several lines of evidence point to a role for IDO in suppressing cell proliferation by depleting the essential amino acid L-tryptophan. IDO has been implicated in the inhibition of propagation of viruses[5] and protozoan parasites[6] in eukaryotic cells and is also associated with inhibition of T-cell-mediated immune responses in tumor cells.[7,8] In the placenta, IDO expression inhibits maternal T-cell-mediated rejection of the allogeneic fetus.[9] IDO-expressing dendritic cells inhibit allogeneic T-cell proliferation in vitro.[10]

In the eye lens, IDO is expressed in the anterior epithelium.[11] Lens absorption of ultraviolet A light is attributable in part to KYNs. KYNs readily pass through cell membranes and may diffuse through the cortex into the lens nucleus. At physiological pH, KYNs undergo side-chain deamination to produce $\alpha$, $\beta$-unsaturated ketoalkenes,[12] which react with nucleophilic amino acids present in lens proteins and also with cysteinyl residues of glutathione[3,13-16] through Michael addition, to form covalent adducts. Such adducts are present in the nuclear regions of lenses in people more than 50 years of age,[17] but their concentrations are reduced in cataractous lenses from age-matched patients,[18] possibly due to further degradation. Recent studies have demonstrated the deleterious effects of such modifications on lens proteins, including $\alpha$-crystallin. For example, modification of His83 by deaminated KYN in $\alpha$B-crystallin reduces its chaperone function.[19] KYNs have been implicated in reactive oxygen species (ROS)-mediated crystallin modification, as they can spontaneously produce ROS in the presence of trace metal ions. Such reactions are implicated in cross-linking of crystallins,[20] suggesting that KYNs are responsible, in part, for chemical modification and cross-linking of proteins in aging and cataractous lenses.

KYNs also exert cytotoxic effects. Several studies, including those on neuronal cells, have shown that KYNs have proapoptotic effects.[21,22] Such effects may be responsible for the enhanced apoptosis in kidney tubular epithelial cells that overexpress IDO.[23] Despite these findings, the molecular mechanisms by which KYNs affect cells are poorly understood. To begin to answer this question, in a recent study (Mailankot et al., manuscript submitted) we developed a transgenic (Tg) mouse model that expresses human IDO (hIDO) in the lens. The lenses from homozygous (homTg) animals had high IDO activity and had the following characteristics that were absent in lenses from wild-type animals: The lenses (1) exhibited dense nuclear cataracts within 3 months after birth and had a significantly smaller diameter, (2) had high KYN and KYN-modified protein content, and (3) had severely decreased fiber cell differentiation, and undifferentiated fiber cells at the lens nucleus contained KYN-modified proteins and were apoptotic. Lenses in the hemizygous (hemTg) animals were similar to those in wild-type mice even though they had IDO activity; the activity, however, was approximately four times lower than that in homTg animals. From the observations in homTg animals, we concluded that overexpression of IDO leads to cytotoxic effects through formation of KYNs in the lens. To test this possibility, in this study we investigated the effects of IDO overexpression in cultured mouse lens epithelial cells (mLECs).

## Materials and Methods

### Animals and Cell Culture

mLECs were isolated from C57BL/6 animals. HomTg mice were generated by using a transgene construct with hIDO inserted between the *Eco*RI sites of minigene having a chimeric promoter that consisted of a chick $\delta$1-crystallin enhancer and the $\alpha$A-crystallin promoter (Mailnakot et al., manuscript submitted). HemTg animals were generated by breeding homTg mice with C57BL/6 Wt. mLECs were isolated and cultured from capsule-epithelial explants of 2- to 3-month-old Wt and Tg mice, as previously described.[24] Cells were grown on 35-mm

plates in Eagle's minimum essential medium (MEM; Sigma-Aldrich, St. Louis, MO) containing 50 $\mu$g/mL gentamicin and 20% fetal bovine serum. Cells at passages 4 to 6 were used for the entire study. The studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

### Treatment of mLECs

For KYN treatment, Wt mLECs were weaned into serum-free medium containing 50 $\mu$M KYN and were cultured for 3 days. In some experiments, the cells were cultured in the presence of 20 $\mu$M 1-methyl-$_{DL}$-tryptophan (MT) for 3 days to block IDO activity. Cells grown in serum-free medium alone served as control cultures.

### Measurement of IDO Activity and KYN Content

The cells were trypsinized, homogenized on ice in 200 $\mu$L PBS (pH 7.4), and centrifuged (14,000*g*, 4°C, 15 minutes). Supernatants containing the soluble proteins were added to a reaction mixture containing 50 mM sodium phosphate buffer (pH 6.5), 20 mM ascorbic acid sodium salt (Sigma-Aldrich), 200 $\mu$g/mL bovine pancreas catalase (Sigma-Aldrich), 10 $\mu$M methylene blue and 400 $\mu$M $_{L}$-tryptophan (Sigma-Aldrich). The reaction was performed at 37° C for 1 hour and was stopped by adding 40 $\mu$L 30% (wt/vol) trichloroacetic acid. The samples were incubated at 65°C for 15 minutes to convert NFK to KYN and then centrifuged (14,000*g*, 4°C, 15 minutes). Control cultures were prepared in the same way, except that the protein extract was incubated with 20 $\mu$M MT. KYN content was estimated by reversed-phase HPLC (RP-HPLC) with standards (0.2-5.0 nanomoles of $_{D,L}$-KYN [Sigma-Aldrich]) and ammonium acetate (10 mM) as solvent A and 10% methanol in ammonium acetate as solvent B. The percentage of solvent B in the gradient was 0% (10 minutes), 0% to 100% (10 minutes) and 100% to 0% (15 minutes). The flow rate was 0.8 mL/min. The column eluate was monitored for absorbance at 360 nm. Enzyme activity was expressed as nanomoles of KYN formed per milligram protein per minute. Protein was quantified by an assay kit with BSA used as the standard (Bio-Rad, Hercules, CA).

To estimate the amount of KYN formed, we counted the cells and then homogenized them in 100 $\mu$L 100% ethanol. The homogenate was kept at -20°C for 1 hour and then centrifuged (14,000*g*, 4°C, 15 minutes). The supernatant was removed and kept at -20°C while the pellet was re-extracted with 80% ethanol (100 $\mu$L). The homogenate was kept at -20°C for 1 hour and then centrifuged as before, and the supernatants were combined and dried in a concentrator (Speed-Vac; Savant-Thermo Scientific; Waltham, MA). Samples and standards (0.2-0.5 nanomoles of $_{D,L}$-KYN) were analyzed by RP-HPLC. Sodium acetate (20 mM)/acetic acid buffer (pH 4.5) was used as solvent A and 20% methanol as solvent B. The percentage of solvent B in the gradient was 0% (30 minutes), 0% to 50% (2 minutes), 50% to 100% (8 minutes) and 100% to 0% (6 minutes). The flow rate was 0.6 mL/min. Results are expressed as nanomoles per million cells. NFK and 3OHKYN were analyzed in the same runs with the use of authentic NFK and 3OHKYN standards.

To estimate KYN content in the culture medium, lyophilized medium (100 mg) was extracted with ethanol, concentrated (Speed-Vac; Savant Thermo Scientific), and processed as just described. To estimate tryptophan in the culture medium, 24 $\mu$L of medium was brought up to 200 $\mu$L with solvent A. Samples and standards were analyzed by RP-HPLC as described for KYN estimation. Results were expressed as nanomoles per milliliter medium.

### Estimation of KYN Modification in Proteins by ELISA

The estimation was performed as described previously.[16] Briefly, microplate wells were coated with cell lysate in 0.05 M carbonate buffer (pH 9.7) at a concentration of 1 $\mu$g and

blocked with 5% nonfat dry milk (NFDM). The wells were then incubated with a mouse anti-KYN mAb (1:1000 diluted in PBS) followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1:15,000 in PBST; Promega, Madison, WI). The enzyme activity was assessed by adding 100 $\mu$L 3,3′,5,5′-tetramethylbenzidine, and the reaction was stopped with 50 $\mu$L 2 N $H_2SO_4$. The samples were read at 450 nm.

### Immunocytochemistry

The cells were cultured in chambered slides 1 day before the experiments. After the cells were washed twice with PBS, they were fixed with 4% paraformaldehyde in PBS at -20°C for 15 minutes, followed by washing twice with PBS and permeabilizing with 0.1% Triton X-100 in PBS at -20°C for 5 minutes. After the slides were then washed five times with PBS to remove the detergent, they were blocked with 3% NFDM/1% BSA in PBS for 30 minutes at room temperature (RT) and washed twice for 5 minutes with PBS. The slides were incubated with the primary antibody in 0.1% BSA/PBS for 1 hour at RT and washed twice for 5 minutes with PBS. Mouse anti-$\alpha$A/$\alpha$B-crystallin mAb (1:100 dilution; Stressgen), mouse anti IDO mAb (1:40 strength; Chemicon), and mouse anti KYN-mAb (1:50 dilution) were used as primary antibodies. The slides were incubated with secondary antibody (anti-mouse IgG) conjugated with Texas red (1:200 dilution; Invitrogen-Molecular Probes, Eugene, OR). The secondary antibody was diluted in 0.1% BSA/PBS and applied to the slides for 1 hour at RT. After washing twice with PBS for 5 minutes, the slides were incubated with phalloidin (Invitrogen-Molecular Probes) for 30 minutes, washed twice with PBS and then incubated with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen-Molecular Probes) for 1 minute. The slides were washed twice for 5 minutes with PBS, permanently mounted, and viewed with a fluorescence microscope (Model BX60; Olympus, Lake Success, NY), and images were acquired with an attached digital camera (Spot RT Slider; Diagnostic Instruments, Inc., connected to a Macintosh computer using Spot RT Slider software, version 3.5.5). Secondary antibody contribution to immune reaction was verified by staining without the primary antibody.

### Assessment of Protein Expression

To assess the protein expression in mLECs, water soluble proteins (10 $\mu$g) were subjected to SDS-PAGE on 15% gel followed by staining (Bio-Safe staining solution; Bio-Rad) and destaining in water.

For Western blot analysis, cell lysates corresponding to 40 $\mu$g protein were subjected to SDS-PAGE on a 15% Tris-HCl gel, electrophoretically transferred onto a nitrocellulose membrane, blocked with 5% nonfat dry milk, and incubated with IDO mAb (1:500 dilution; Chemicon, Temecula, CA) and anti GAPDH antibody (1:300 dilution; Millipore, Billerica, MA[b]). The membrane was subsequently incubated with goat anti-mouse HRP-conjugated antibody (1:5000 dilution; Promega, Madison, WI[b]) and developed with a kit (SuperSignal West Pico Chemiluminescence Kit; Pierce, Rockford, IL).

### Assessment of Cell Proliferation

The cell proliferation was performed by adding reagent (CellTiter 96 Aqueous One Solution; Promega) directly to the wells as per the manufacturer's instructions. Briefly, 48 hours before the assay, mLECs (400 cells/well) were dispensed into 96-well plates in triplicate and cultured. The reagent (20 $\mu$L) was added into each well and incubated for 4 hours at 37°C in a humidified, 5% $CO_2$ atmosphere. Bioreduction of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] by cells to a colored formazan compound was determined spectrophotometrically at 490 nm. The number of viable cells per well was calculated from a standard curve obtained with 0 to 1000 Wt mLECs/well.

### Determination of Apoptosis

Cells grown in 12-well plates were analyzed for apoptosis (In Situ Cell Death Detection Kit; Roche Diagnostics, Indianapolis, IN), per the manufacturer's instructions. The cells were counterstained with DAPI to identify the nuclei. As a negative control, the cells were incubated without terminal transferase.

### Flow Cytometry

Two million cells were collected by trypsinization and washed twice with PBS. After fixing in 2 mL 90% methanol in PBS for 1 hour at -20°C, the cells were washed twice, and resuspended in 250 $\mu$L PBS. Then, 5 $\mu$L RNase stock solution (RNase-2 mg/mL, EDTA-10 mM, and sodium azide-0.1%) was added and incubated at 37°C for 30 minutes. The cells were chilled at 4°C for 10 minutes and incubated with 250 $\mu$L propidium iodide (PI) stock solution (PI, 100 $\mu$g/mL; Triton X-100, 0.1%; sodium azide, 0.1%) at 4°C for 60 minutes. They were then analyzed by flow cytometry (EPICS-XL; Beckman Coulter, Fullerton, CA). At least 20,000 events per sample were evaluated. All histograms were analyzed with flow cytometry software (EXPO32; Beckman Coulter) to determine the percentage of cells in the $G_1$, S, and $G_2$/M stages of the cell cycle.

### Identification of KYN-Modified Proteins

Protein from mLEC lysate (200 $\mu$g) was incubated with mouse anti-KYN mAb[16] (2 $\mu$g) for 1 hour at RT followed by addition of protein G-Sepharose (GE Healthcare, Piscataway, NJ) and incubation for 1 hour at RT with shaking. To check whether nonspecific binding occurred, proteins were incubated with protein G-Sepharose without the antibody. The sample mixture was centrifuged, and the pellet was washed five times for 5 minutes each with PBS. The gel pellet was dissolved in SDS-PAGE sample buffer, and proteins were subjected to electrophoresis on a 15% gel. The proteins were stained (Bio-Safe; Bio-Rad) and destained in water. The protein bands were cut out of the gel, minced, and subjected to in-gel digestion with trypsin. The resultant peptides were analyzed on a linear ion trap mass spectrometer (LTQ; Thermo Fisher Scientific, coupled with an Ettan MDLC system; GE Healthcare). The spectra were acquired by data-dependent methods: one full scan (m/z of 300-2000) was performed followed by MS/MS on the five most abundant precursor ions at 30% normalized collision energy. The dynamic exclusion criteria were set as follows: repeat count, 1; repeat duration, 45 seconds; and exclusion duration, 180 seconds. The obtained data were submitted to a protein search engine (Mascot; Matrix Science, Boston, MA) by searching the Swiss-Prot (sprot 50.3) mouse database (http://www.expasy.org; provided in the public domain by Swiss Institute of Bioinformatics, Geneva, Switzerland).

### Statistics

The results were analyzed by using one-way analysis of variance (ANOVA), followed by the Fisher protected least significant difference test (Statview 5.0 software; SAS Institute, Inc., Cary, NC). The level of significance was set at $P < 0.05$.

## Results

### Isolation and Characterization of mLECs

Initially, we tried to isolate and culture mLECs from homTg animals. But despite repeated efforts, the isolated cells failed to proliferate and died within 7 to 10 days of isolation, possibly because of the cytotoxic effect of the high KYN. We then successfully isolated mLECs from hemTg animals. Wt and hemTg cells showed immunoreactivity for $\alpha$A/$\alpha$B-crystallin, confirming that they are mLECs (Fig. 1). Incubating cells with only the secondary antibody showed no immunoreactivity, confirming that the staining was specific for $\alpha$-crystallins.

### IDO Expression

IDO activity in mLECs was measured by estimating the amount of KYN formed during incubation of mLEC proteins with 1 mM L-tryptophan. In cells from hemTg animals, the rate of KYN formation was estimated to be 0.15 ± 0.02 nanomoles/mg protein/min, whereas mLECs from Wt animals showed no detectable activity. Based on the KYN measured, we estimated that 2.0% of added D,L-tryptophan was converted into KYN in 1 hour. To confirm that the observed KYN formation was due to the IDO activity, we incubated cell lysates either at 65° C for 15 minutes or with the IDO inhibitor, 20 $\mu$M MT (37°C, 60 minutes). Both treatments abolished the enzyme activity. Western blot using anti-IDO mAb with GAPDH as the loading control detected IDO in hemTg mLECs but not in Wt mLECs (Fig. 2A). Immunocytochemical studies showed IDO throughout the cytoplasm in hemTg mLECs (Fig. 2B). The Wt mLECs showed no immunoreactivity. These findings further confirmed overexpression of IDO in mLECs of hemTg animals.

### KYN and KYN-Modified Proteins

Using RP-HPLC we determined that the KYN concentration in hemTg mLECs was 0.11 ± 0.01 nanomoles/$10^6$ cells. KYN was not detected in Wt mLECs. The formation of KYN led to modification of cellular proteins, as detected by ELISA (0.34 ± 0.01 OD vs. 0.02 ± 0.01, hemTg versus Wt, respectively). No immunoreactivity was present when the primary antibody was preincubated with KYN-modified RNase A, confirming that the immunoreactivity in the ELISA resulted from KYN-modified proteins.

Immunocytochemistry was performed to localize KYN-modified proteins. KYN-modified proteins were present throughout the cytoplasm (Fig. 3). The immunoreaction was markedly reduced when the antibody was preincubated with KYN-modified RNase A. Wt mLECs were negative for immunostaining. Together, these data suggest that overexpression of hIDO results in high KYN and KYN modification of proteins in mLECs.

### KYN and Tryptophan Content of Culture Medium

KYN was detected in hemTg mLEC culture medium (0.47 ± 0.07 nanomoles of KYN per milliliter) but was absent in Wt mLEC culture medium (Fig. 4A). To determine whether mLECs from hemTg mice used tryptophan from the culture medium at a higher rate than cells from Wt mice, we measured tryptophan in the media by RP-HPLC. The tryptophan content was reduced by 0.5 nanomoles/mL (~2%) culture media in hemTg mLECs when compared to Wt (29 ± 1.92 vs. 28.5 ± 1.9 nanomoles/mL; Wt versus hemTg, respectively; Fig. 4B) This difference correlated with measured intra- and extracellular (in the medium) KYN in hemTg mLE. At 100% confluence, the average number of cells per 100-mm plate in 10 mL medium was 2 × $10^6$. The amount of KYN formed from 2 × $10^6$ hemTg mLECs was ~4.8 nanomoles. Thus, even after growth of cells to confluence, the culture medium had sufficient tryptophan.

### Effect of Exogenous KYN

To study the effect of KYN, Wt mLECs were cultured with 10 to 1000 $\mu$M KYN for 3 days. When the extracellular KYN concentration was low, the intracellular KYN concentration increased to 50 $\mu$M (Fig. 5A). However, increasing the extracellular KYN concentration beyond 50 $\mu$M did not further increase intracellular KYN. These data suggest that KYN uptake by mLECs is regulated. Similar to the findings in hemTg mLECs shown in Figure 3, immunostaining of Wt mLECs incubated with KYN showed KYN modifications in the cytoplasm, but more at the perinuclear region (Fig. 5B).

### Effect of 1-Methyl $_{D,L}$-tryptophan (MT) Treatment

To further confirm that the effects seen in mLECs from hemTg were due to IDO-mediated KYN formation, we incubated mLECs from hemTg with an inhibitor of IDO, MT. At 3 days of treatment, 20 $\mu$M MT completely inhibited IDO. As a result, neither KYN nor significant KYN modifications were detected (data not shown).

### Cell Proliferation, Apoptosis, and Cell Cycle Analysis

An MTS assay was used to determine the cell proliferation rate. After 48 hours of culturing, the number of viable cells increased by twofold in Wt mLECs (~800 cell/well from the initial ~400 cells/well), but the increase was only 1.3-fold in hemTg mLECs ($P < 0.0001$; Fig. 6A). Similar to hemTg mLECs, KYN-treated Wt mLECs also showed only ~1.3-fold increase in viable cells. Of interest, treatment with MT enhanced cell proliferation in hemTg mLECs, the number of viable cells increased by ~2-fold, similar to Wt mLECs.

To determine whether apoptosis leads to reduction in viable cells, we performed TUNEL staining in culturing cells for 3 days. The number of TUNEL-positive cells did not significantly differ between hemTg and Wt mLECs (data not shown). No significant apoptosis was found in KYN-treated Wt mLECs also. In addition, FACS analysis of TUNEL-positive cells, in which we included both adherent and floating cells, did not show a difference in either untreated or KYN-treated cells (data not shown). These results suggest that the reduction in the number of viable cells in hemTg mLECs did not result from enhanced apoptosis.

Cell cycle analysis was performed with flow cytometry. HemTg mLECs showed a markedly increased R3 fraction, suggesting a delay in $G_2$/M, or both phases (Fig. 6B). The percentage of hemTg mLECs in the $G_2$/M phase was ~1.7-fold greater compared with Wt mLECs. In both cases, the number of cells in sub-$G_1$ phase was not significant, supporting the idea that increased cell death did not contribute to reduced cell proliferation. Compared with untreated Wt cells, the number of KYN-treated Wt cells in the R3 fraction increased by twofold but did not increase in the sub-$G_1$ phase. These results suggest that exogenous KYN brings about changes in Wt mLECs similar to those in hemTg mLECs and imply that intracellular KYN results in delayed entrance into the $G_2$/M, or both phases. MT-treated hemTg mLECs showed a nearly 50% reduction in cells at $G_2$/M when compared with cells without such treatment. Although delayed entrance into the $G_2$ and or M phase is the simplest description of these results, it is also possible that there are other cell cycle effects (e.g., $G_1$ delay). We noted a marked increase in cell size in KYN-treated $G_1$ cells (Fig. 7), suggesting prolonged growth in this phase. Taken together, these data strongly support the idea that IDO-mediated KYN formation in hemTg mLECs produces significant cell cycle delays that lead to reduced cell proliferation.

### KYN-Modified Proteins

Because our evidence did not point to tryptophan deficiency, we postulated that the observed cell cycle delay might be due to KYN modification of key proteins. SDS-PAGE of cell lysates from hemTg and Wt mLECs is shown in Figure 7A. The hemTg mLEC lysates had a few high molecular weight proteins that were absent in Wt. These are possibly KYN-mediated cross-linked proteins. Immunoprecipitation of these cell lysate proteins revealed two distinct protein bands in the hemTg samples that were not present in Wt lysates (Fig. 7B). Incubation of hemTg lysate without the primary antibody or incubation without lysate showed no bands from cellular proteins, except the bands corresponding to heavy chain and light chain of antibody. The two proteins in hemTg were trypsin digested and analyzed by LC/MS. The mass spectrometry data are shown in Table 1. The two proteins were identified as myosin II and $\alpha/\gamma$-actin.

## Discussion

Although studies have demonstrated that IDO has antiproliferative effects,[5,6,9,10] the processes involved are not well defined. In the human lens, the downstream products of IDO (i.e., KYNs), can serve as UV filters. However, recent studies on KYN modifications of lens proteins point to IDO's role in cataractogenesis.[15,16,25] Our previous study (manuscript submitted) in transgenic mice overexpressing hIDO in the lens showed that KYN presence and KYN modification of proteins can harm the lens: enhanced formation of KYN in IDO overexpression results in fiber cell apoptosis, poor fiber cell differentiation, and cataract development. However, due to experimental design limitations in that study, we were only able to demonstrate deleterious effects of KYNs on fiber cells, but not on epithelial cells. Thus, the present study was undertaken to address the effect of KYN on epithelial cells. The major findings of the present work were that (1) IDO overexpression in mLECs resulted in accumulation of KYN and KYN-modified proteins in cells, (2) KYN and KYN modification of proteins correlated with cell accumulation in the $G_2$/M or in both phases of the cell cycle, (3) the effects of IDO overexpression on cell cycle were preventable by inhibiting IDO with a selective inhibitor, and (4) myosin II and $\alpha/\gamma$-actin were present as KYN-modified proteins in hemTg mLECs.

The formation of KYN in IDO-overexpressing cells was not unexpected. Therefore, the effects on the cell cycle could be directly due to KYN. We ruled out effects from other downstream metabolites of the KYN pathway, NFK and 3OKKYN, as these were not detected in hemTg mLECs. KYN formation and KYN modification in both the cytosolic and nuclear components lends credence to the possibility that KYN directly affected the cell cycle. Of interest, the cell cycle delay was not associated with apoptosis, and IDO expression has been shown to reduce T-cell proliferation without apoptosis.[26] It is possible that KYN may not induce apoptosis in epithelial cells, unlike fiber cells. This is supported by the fact that epithelial cell apoptosis was not evident in the lenses of homTg animals that had significantly more KYN than those from hemTg (Mailankot et al., manuscript submitted). These observations suggest that epithelial cells may have robust defense mechanism(s) to deal with excess KYN.

In addition to resistance to internally formed KYN, mLECs may also be resistant to exogenous KYN. We observed that adding up to 1 mM KYN to the medium did not induce cell death. In response to external KYN, intracellular KYN was regulated, as a gradual increase in KYN concentration from 50 $\mu$M to 1 mM did not produce a corresponding increase in intracellular KYN. The KYN concentration remained constant after it reached ~0.12 nanomoles per $10^6$ cells. This suggests that KYN entry into cells is regulated. Thus, lens epithelial cells may be protected from both external and internal KYN, albeit at the expense of cell cycle perturbations that result in decreased proliferation and eventually, improper fiber cell differentiation.

The methyl derivative of tryptophan MT is a pharmacologic competitive inhibitor of IDO and has been used in various studies to inhibit tryptophan depletion by IDO.[9,27-30] Inhibition of cell cycle arrest by MT confirmed that the arrest at $G_2$, M, or both in hemTg mLECs is due to enhanced IDO activity. Enhanced intracellular KYN formation as a result of increased IDO activity implies that L-tryptophan, an essential amino acid, the substrate for IDO, is not a limiting factor. To compensate for the depletion of intracellular tryptophan, cells may increase their tryptophan uptake. Our data that L-trypotphan concentration is reduced in cultures of mLECs from hemTg support this possibility. Because the culture medium had sufficient tryptophan, it is unlikely that L-tryptophan depletion by increased IDO activity resulted in cell cycle delay or arrest in $G_1$ in hemTg mLECs as observed in other experiments.

Normal lens development and growth depends on the tight spatial and temporal regulation of lens epthelial cell proliferation and differentiation. The proliferative potential of lens epithelial

cells decreases with age.[31,32] We observed an increase in KYN-modified proteins in mLECs. It is possible that KYN-modified proteins are removed from cells through the ubiquitin-proteasome pathway. If the function of this system is compromised during age-associated cataract formation, as reported previously,[33] KYN-modified proteins may accumulate. If the modified proteins are involved directly or indirectly in cell cycle regulation, such accumulation would lead to decreased proliferation of lens epithelial cells. The observation that the proliferative ability of lens epithelial cells decreases with age[34,35] supports this idea.

Although the effect of KYN on $G_2$/M arrest was apparent, less obvious is how KYN or KYN modification interferes with the cell cycle. The cell cycle is regulated by cyclin-dependent kinases.[36] The regulation of cyclin-dependent kinase inhibitors during differentiation of lens epithelial cells suggests that these proteins control cell cycle exit.[37] Whether KYN affected these cyclin-dependent kinases and their inhibitors by direct chemical modification or indirectly through modification of downstream signaling molecules remains to be investigated.

Our finding that $\alpha/\gamma$-actin and myosin II are KYN-modified proteins in hemTg mLEC lysate provides a molecular basis for impairments in cell cycle and proliferation. Modification of these two proteins could adversely affect cytokinesis in cells, as this process requires both actin and myosin (together with other structural and regulatory proteins) to form a contractile ring at the equatorial cortex and induce the furrowing that eventually results in cell division into two daughter cells.[38,39] Thus, KYN modification of these proteins may have inhibited cytokinesis and consequently cell proliferation.

Whether effects on cell cycle and cell proliferation contribute to cataract formation in humans is uncertain, but indirect evidence supports the view. In TGF-$\beta$-induced anterior subcapsular cataracts in mice, LEC proliferation is deregulated.[40] Enhanced calcium levels in the lens and aqueous humor, which is considered to cause lens opacification, cause decreased proliferation of human LEC[41]; $H_2O_2$, which causes cataract in organ cultured lenses also reduces lens epithelial cell proliferation. These observations strongly suggest that decreased proliferation of epithelial cells is associated with cataract formation. If a similar mechanism is induced by KYN modification of proteins, it might contribute to cataract formation in humans.

Human lenses have relatively low levels of IDO activity. One study reported IDO activity equivalent to 0.85 ± 0.49 nanomoles of KYN formed per hour per lens in 26- to 80-year-old human lenses.[11] If the average wet weight of an adult human lens is assumed to be ~200 mg and the protein content to be 60%, the reported IDO activity would translate to ~0.12 to 0.2 picomoles of KYN formed per minute per milligram protein. The hemTg animals we developed had IDO activity equivalent to 0.15 ± 0.02 nanomoles/min/mg protein of KYN formed in the lens. Therefore, the IDO activity in hemTg animal lenses is ~750 to 1200 times higher than human lenses. Such high activity of IDO may not occur even in senile cataractous human lenses. However, in conditions in which interferon-$\gamma$, an inducer of IDO expression, is elevated in the anterior eye (e.g., in uveitis[42]), IDO activity could increase and lead to KYN modification and $G_2$/M arrest in lens epithelial cells. It has been suggested that interferon- $\gamma$ production in LECs may be involved in cataract development.[43] It will be of interest to determine the effect of interferon-$\gamma$ on IDO activity in LECs.

In summary, our study demonstrates that enhanced IDO activity results in KYN-mediated cell cycle arrest in lens epithelial cells. We believe that this antiproliferative effect could contribute to the cataract formation in IDO-overexpressing homozygous transgenic animals that we have previously observed. In the human lens, KYN formation and KYN modifications could therefore play a role in age-related cataract formation.

## Acknowledgements

Supported by National Eye Institute (NEI) Grants R01EY-016219 and R01EY-09912, the Carl F. Asseff, MD, Professorship (RHN), NEI Grant P30EY-11373 (Visual Sciences Research Center of CWRU), Research to Prevent Blindness, the Ohio Lions Eye Research Foundation, and National Cancer Institute Grants R01CA-073413 (JWJ) and P30CA-043703 (Case Comprehensive Cancer Center Core Facilities).

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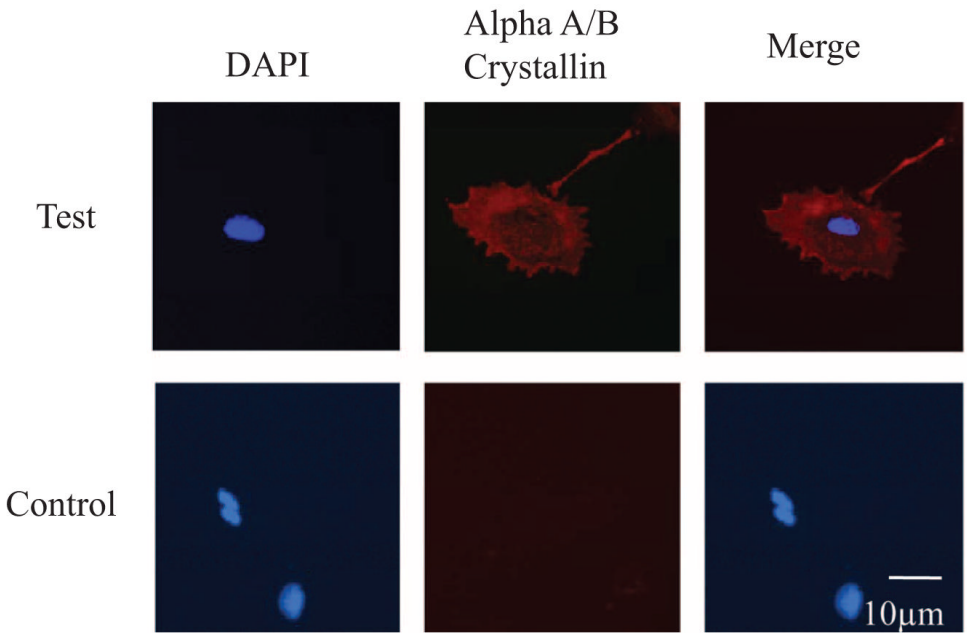



**Figure 1.**
Characterization of mLECs. Isolated Wt and hemTg mLECs were immunostained for αA/αB-crystallin (*red*) in the presence (test) and absence (control) of mouse anti αA/αB-crystallin monoclonal antibody. The cells were counterstained with DAPI (*blue*). The images are representative of three independent cultures from three mice.

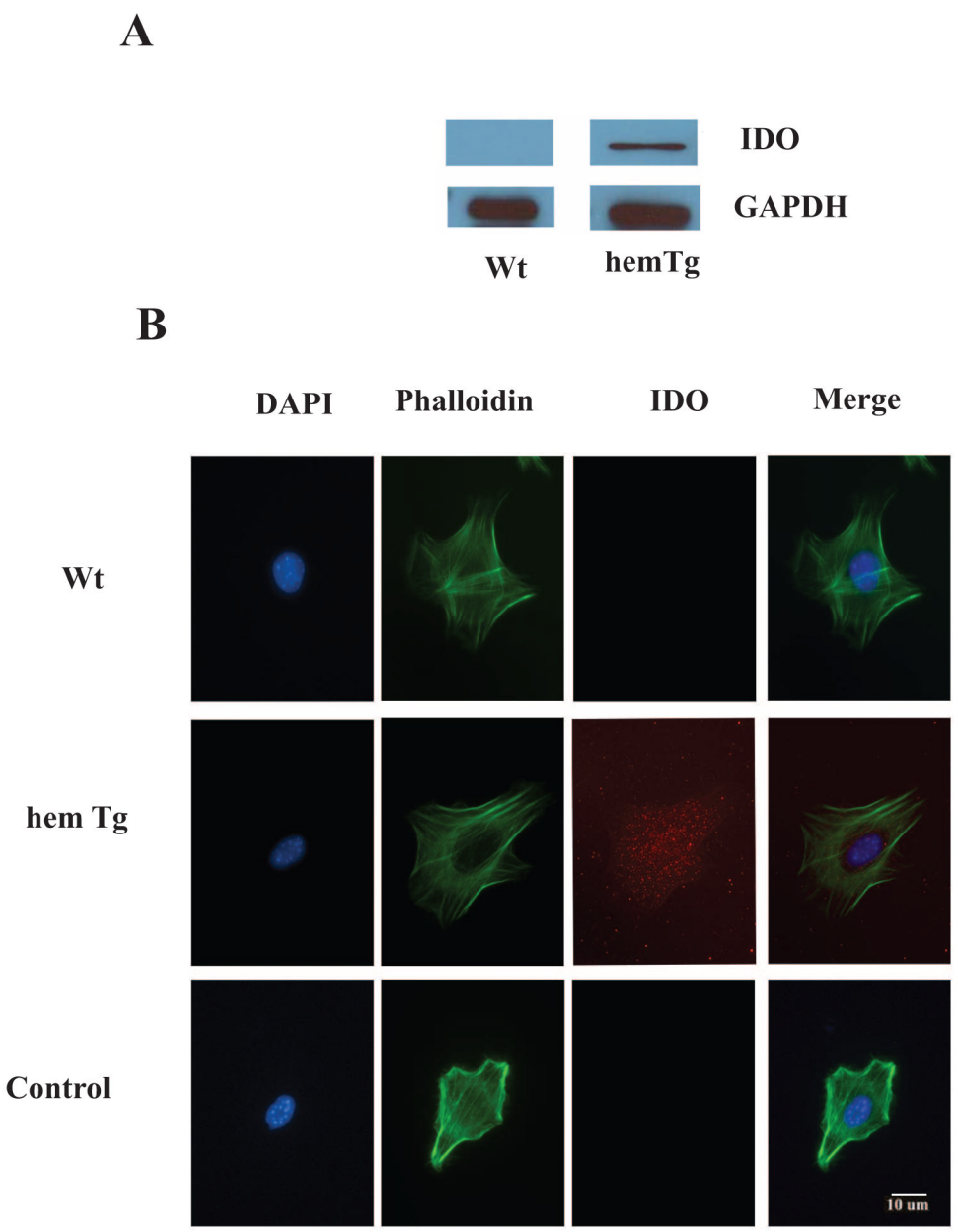



**Figure 2.**
IDO expression in Wt and hemTg mLECs. (**A**) Western blot analysis. IDO was detected by using mouse anti-IDO monoclonal antibody in hemTg mLECs but not in Wt mLECs. GAPDH was used as the loading control. (**B**) Immunocytochemistry for IDO. IDO was detected in hemTg mLECs by using the monoclonal antibody and Texas red-conjugated secondary antibody. The cells were also stained for F-actin with phalloidin (*green*) and counterstained with DAPI (*blue*). Data are representative of results in three independent experiments.

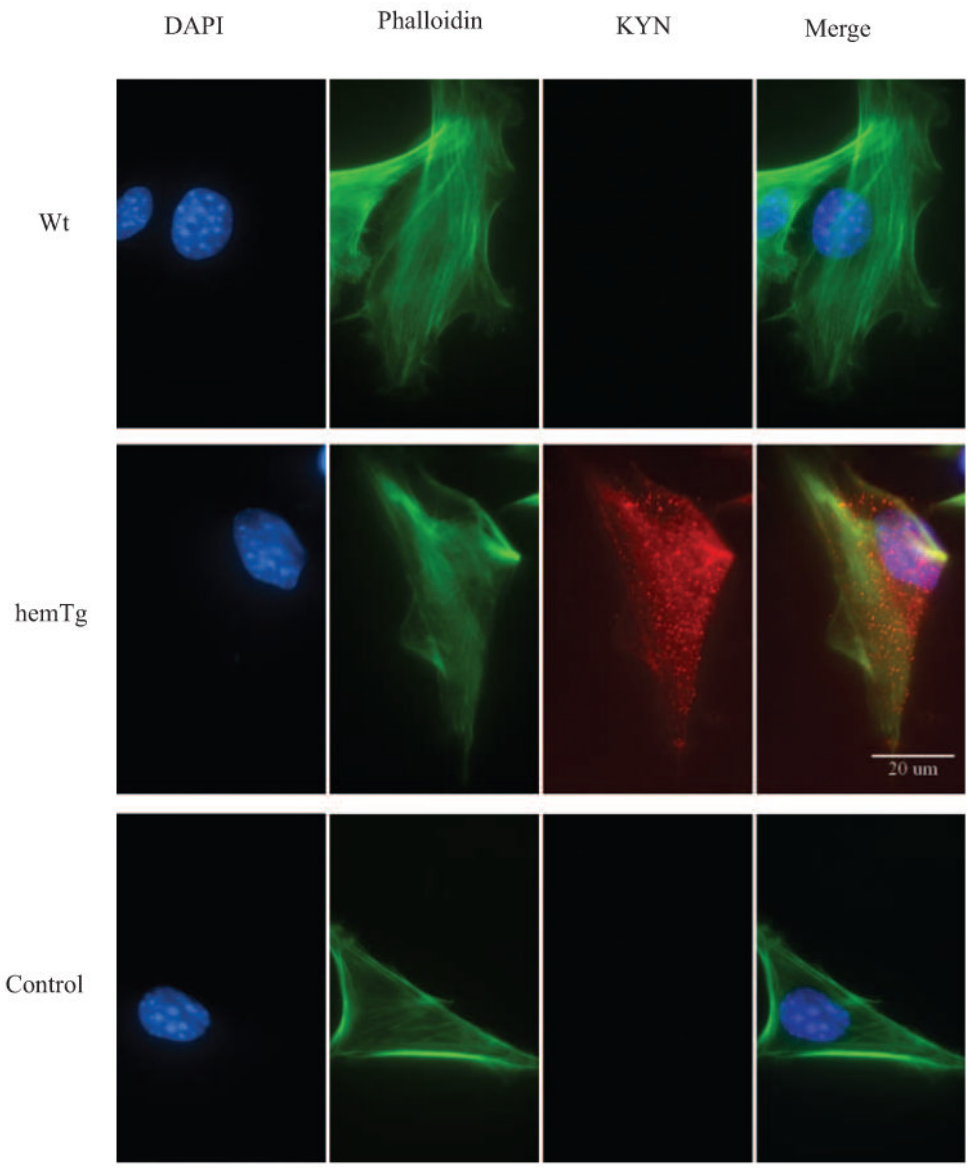



**Figure 3.**

Immunostaining for KYN-modified proteins. KYN-modified proteins were detected using a monoclonal antibody and Texas-red conjugated secondary antibody. Cells were also stained for F-actin by phalloidin (*green*) and nuclei with DAPI (*blue*). KYN-modified proteins (*red*) were detected in KYN treated mLECs but not in untreated cells. Data are representative of results in three independent experiments.

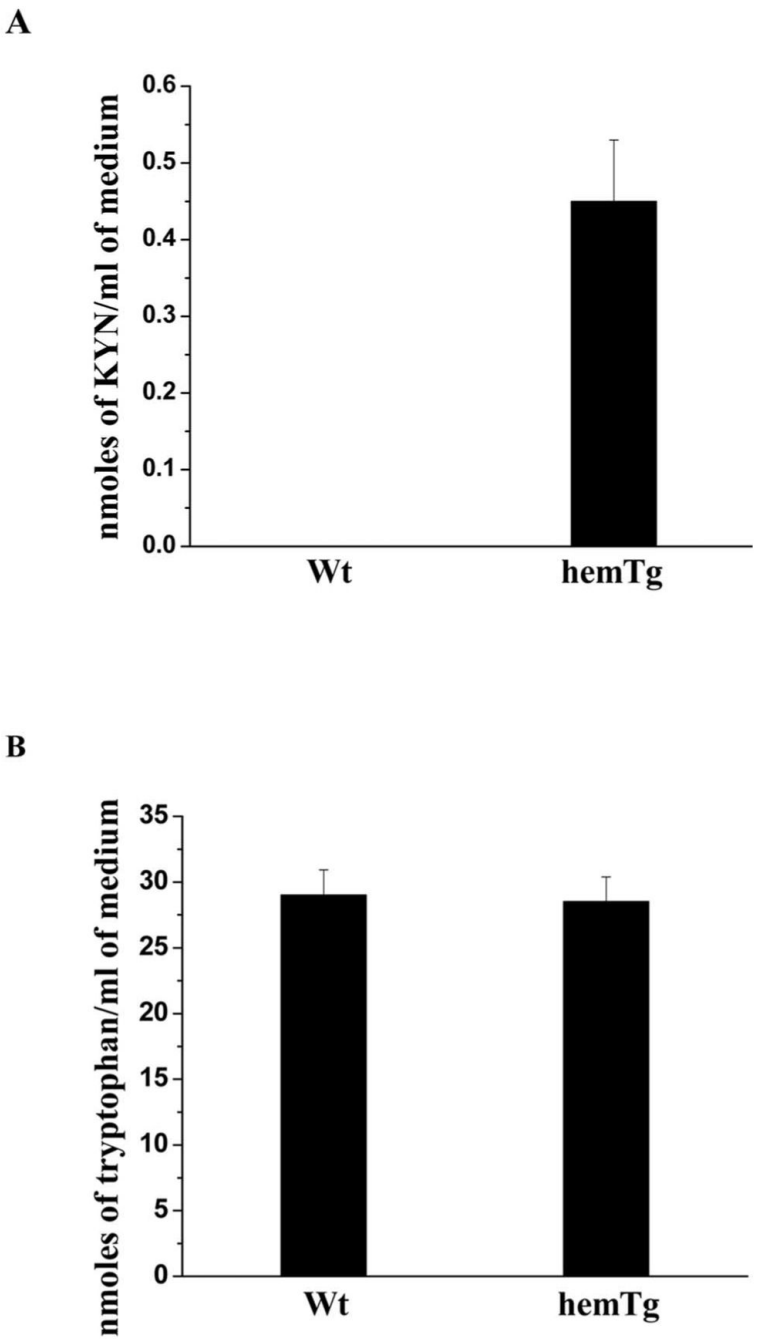



**Figure 4.**

KYN and L-tryptophan in culture medium. Wt and hemTg mLECs were cultured for 3 days, the cell-free medium was collected, lyophilized and processed for measurement for IDO activity. KYN and L-tryptophan were quantified by HPLC. (**A**) KYN in the medium. KYN was detected in the hemTg mLEC culture medium but not in that of Wt mLECs. (**B**) L-Tryptophan in the medium. L-tryptophan content decreased slightly in culture medium from hemTg mLECs but not in that of Wt mLECs. Results shown are the mean ± SD of three independent experiments.

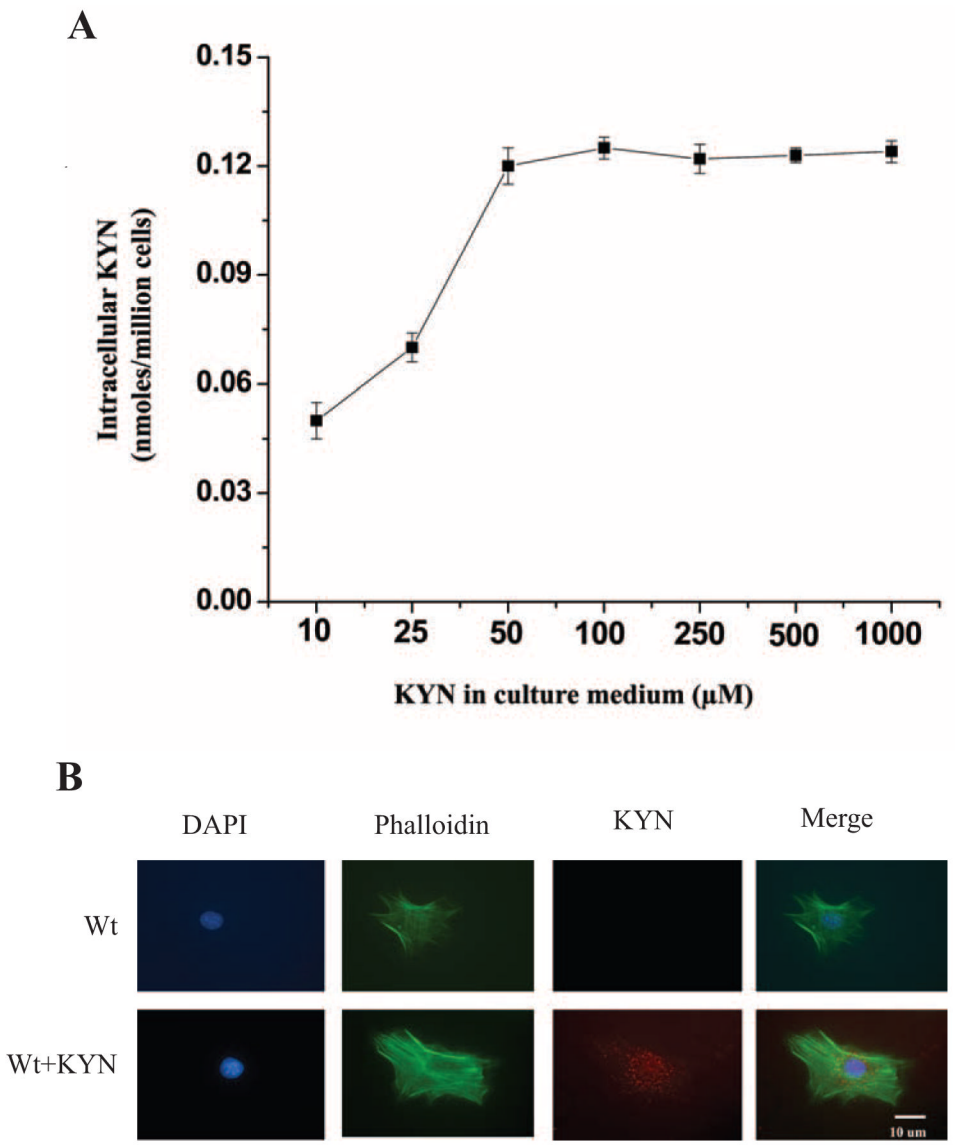
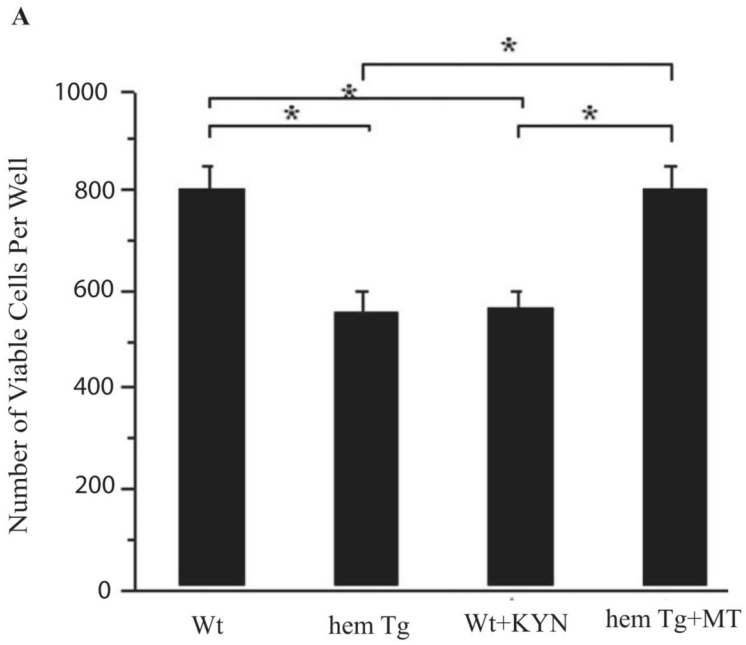
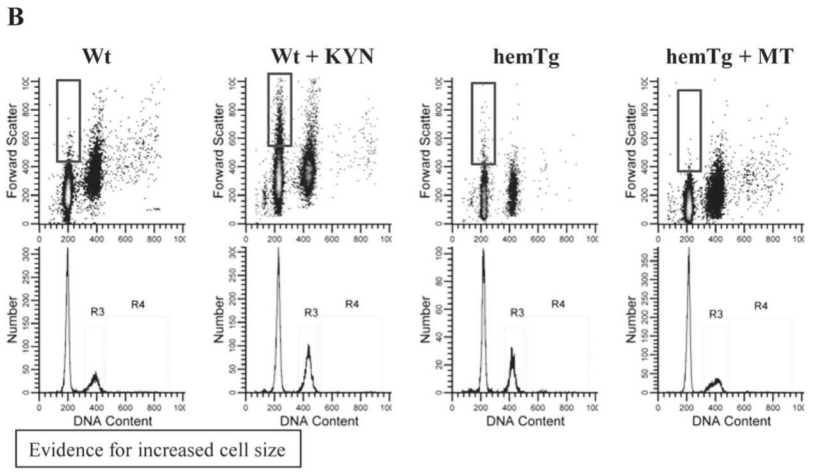



**Figure 5.**

Effect of exogenous KYN on intracellular KYN and KYN modification in mLECs. (**A**) mLECs from Wt were incubated with 0 to 1.0 mM KYN for 3 days. Cells were lysed, protein precipitated, and KYN estimated in the protein-free supernatants by HPLC. Results are the mean ± SD of results in three independent experiments. (**B**) Wt mLECs were incubated with 50 $\mu$M KYN for 3 days and immunostained for KYN-modified proteins. KYN-modified proteins (*red*) were detected in KYN-treated mLECs but not in untreated cells. Cells were also stained for F-actin by phalloidin (*green*) and nuclei with DAPI (*blue*). Images are representative of three independent experiments.




Evidence for increased cell size

| | Wt | Wt + KYN | hemTg | hemTg + MT |
|---|---|---|---|---|
| R3 (2N G2/M + 4N G1) | 20.8 ± 1.8 | 33.4 ± 2.0 | 28.0 ± 1.7 | 21.4 ± 1.1 |
| R4 (4N S + G2/M) | 2.5 ± 0.8 | 0.9 ± 0.3 | 0.7 ± 0.2 | 1.94 ± 0.3 |
| 2N S | 2.3 ± 0.4 | 1.1 ± 0.1 | 1.0 ± 0.1 | 0.5 ± 0.1 |

**Figure 6.**

Cell proliferation and cell cycle analysis. (**A**) MTT assay for cell proliferation. The number of viable hemTg mLECs was lower than Wt mLECs. Treatment of Wt mLECs with KYN reduced the number of viable cells, and MT treatment of hemTg mLECs increased the number. **P* < 0.0001. Results shown are mean ± SD of three independent experiments. (**B**) Flow cytometric analysis. hemTg mLECs and KYN-treated Wt mLECs showed an increased number of $G_2$/M and 4N $G_1$ cells. The cell cycle perturbation in hemTg mLECs was normalized by MT treatment. R3, $G_2$/M + 4N $G_1$; R4, 4N S+$G_2$/M.

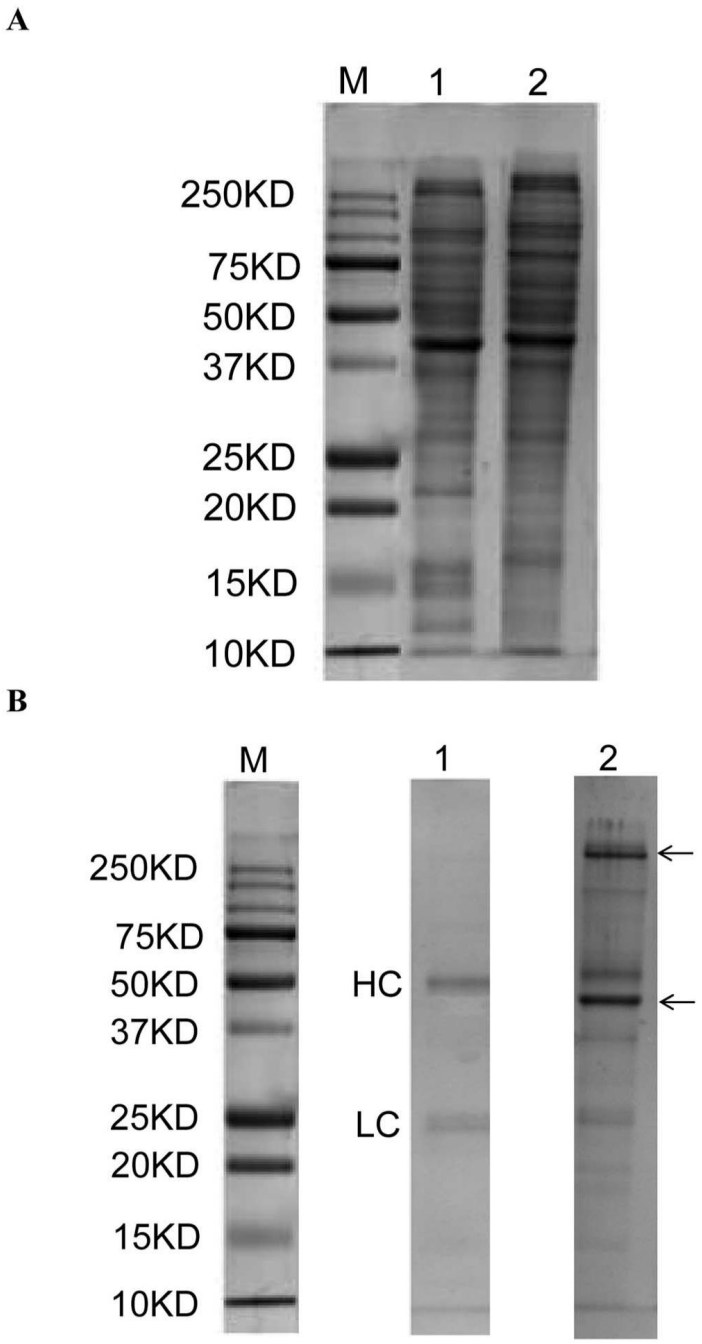



**Figure 7.**
KYN-modified proteins in mLECs. (**A**) SDS-PAGE of mLECs lysates. Cell lysate of hemTg mLECs showed high-molecular-weight proteins that were absent in Wt mLECs. (**B**) SDS-PAGE of immunoprecipitated KYN-modified proteins. Two major KYN-modified proteins (*arrows*) were detected in hemTg mLECs lysate. *Lane M*: molecular weight marker; *lane 1*:Wt mLECs; *lane 2*: hemTg mLECs. HC, IgG heavy chain; LC, IgG light chain.

**Table 1**

KYN-Antibody Binding Proteins Identified by LC/MS

| Protein ID | Protein Name | Sequence Mass | Coverage % | Mascot Score | Unique Peptide |
|---|---|---|---|---|---|
| Band 1 | | | | | |
| MYH9_MOUSE (Q8VDD5) | Myosin-9 (Myosin heavy chain, nonmuscle IIa) | 226,086 | 63 | 11,549 | 150 |
| MYH10_MOUSE (Q61879) | Myosin-10 (Myosin heavy chain, nonmuscle IIb) | 228,855 | 33 | 2,025 | 59 |
| FLNA_MOUSE (Q8BTM8) | Filamin-A ($\alpha$-filamin) | 280,887 | 28 | 1,916 | 61 |
| FLNB_MOUSE (Q80X90) | Filamin-B ($\beta$-filamin) | 277,579 | 5 | 192 | 11 |
| Band 2 | | | | | |
| ACTG_MOUSE (P63260) | Actin, cytoplasmic 2 ($\gamma$-actin) | 41,766 | 74 | 13,619 | 26 |
| ACTC_MOUSE (P68033) | Actin, $\alpha$ cardiac | 41,992 | 64 | 7,593 | 22 |
| HSP47_MOUSE (P19324) | 47-kDa heat shock protein precursor | 46,560 | 41 | 608 | 15 |
| VIME_MOUSE (P20152) | Vimentin | 53,524 | 34 | 341 | 19 |