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Kynurenine inhibits fibroblast growth factor 2-mediated expression of crystallins and MIP26 in lens epithelial cells

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Short title: Kynurenine and FGF2 signaling

Abbreviations

IDO, indoleamine 2,3-dioxygenase; NFK, N-formyl kynurenine; KYN, kynurenine; 3OHKYN, 3OH-kynurenine; hemTg, hemizygous transgenic; MT, 1-methyl-D,L-tryptophan; mLEC, mouse lens epithelial cells, Wt, wild type; FGF2, fibroblast growth factor-2; MAPK, mitogen activated protein kinase; ERK, extracellular-regulated kinase; MIP, major intrinsic protein; FGFR, fibroblast growth factor receptor

Abstract

Fibroblast growth factor-2 (FGF2)-mediated signaling plays an important role in fiber cell differentiation in eye lens. We had previously shown that kynurenine (KYN) produced from the overexpression of indoleamine 2,3-dioxygenase (IDO) causes defects in the differentiation of fiber cells, induces fiber cell apoptosis and cataract formation in the mouse lens, and leads to cell cycle arrest in cultured mouse lens epithelial cells (mLEC). In this study, we demonstrate that exogenous KYN reduces FGF2-mediated expression of α -, β - and γ -crystallin and MIP26 in mLEC. We show that endogenously produced KYN in mLEC of IDO transgenic animals causes similar defects in FGF2-induced protein expression and that a competitive inhibitor of IDO prevents such defects. Our data also show that KYN inhibits FGF2-induced Akt and ERK1/2 phosphorylation in mLEC, which are required for crystallin and MIP26 expression in the lens. KYN does not inhibit FGF2 binding to cells but inhibit phosphorylation of FGFR1 in mLEC. Together our data suggest that KYN might inhibit FGF2-mediated fiber cell differentiation by preventing expression of crystallins and MIP26. Our studies provide a novel mechanism by which KYN can exert deleterious effects in cells.

Keywords: Lens epithelial cell, kynurenine, FGF-2, FGFR1, crystallin, MIP26

1. Introduction

Kynurenines are present in the human lens and are perceived to be UV light filters that protect the retina from photodamage [1]. They are produced from L-tryptophan in the kynurenine pathway. This pathway is initiated by indoleamine 2,3-dioxygenase (IDO), catalyzing the oxidation of L-tryptophan to *N*-formyl kynurenine (NFK). This is eventually converted into nicotinamide adenine dinucleotide (NAD), through intermediates such as kynurenine (KYN) and 3-OH kynurenine (3OHKYN) [2].

Kynurenines are unstable under physiological conditions; they undergo spontaneous deamination to form reactive α,β -unsaturated ketones [3] that react with nucleophilic amino acids in proteins, and glutathione [4]. Several products of kynurenines have been detected in the human lens [4-8]. Using highly specific monoclonal antibodies, our laboratory provided further evidence for kynurenines modifications in aging and cataractous human lenses [9, 10]. In addition to direct modification of proteins, kynurenines indirectly modify proteins through production of reactive oxygen species [11]. Together these observations suggest that kynurenines could play an important role in lens aging and cataract formation.

To further test the role of KYN in lens protein modification, in a recent study we developed a transgenic (Tg) mouse line that overexpressed human IDO (hIDO) in the lens [12]. Lenses of homozygous Tg animals had undifferentiated fiber cells and KYN-modified proteins and furthermore exhibited dense nuclear cataract. In a subsequent study, we showed that lens epithelial cells (LEC) from hemizygous Tg mice showed KYN-mediated G2/M cell cycle arrest [13].

FGF2 plays an important role in LEC proliferation and differentiation [14, 15]. At low concentrations, FGF2 induces LEC proliferation, while at higher concentrations it induces

differentiation into fiber cells [16]. Studies on transgenic mice have shown that overexpression of FGF in the lens leads to disruption of normal development of the lens [17, 18]. FGF has several receptors (FGFR1, R2 and R3). Overexpression of either a truncated, signaling-defective FGF receptor (FGFR1) [19-21] or secreted FGFR3 [22] results in impaired lens fiber cell differentiation. Furthermore, conditional deletion of FGFR adversely affects lens morphogenesis [23].

FGF2 signaling is required for crystallin expression during LEC differentiation [24]. This signaling appears to be mediated by PI3 kinase, as a selective inhibitor of PI3 kinase blocks FGF2-mediated expression of crystallins in cultured lens epithelial cell explants [25]. FGF2/ERK1/2-mediated signaling is also necessary for the expression of major intrinsic protein (MIP26), also known as aquaporin 0, the major membrane protein in the lens [26]. Thus, it is clear that FGF signaling plays an important role in LEC differentiation.

Our previous finding that overexpression of IDO led to cell cycle arrest in LEC and moreover that KYN caused protein damage and poor differentiation of LEC, suggests that KYN might affect fiber cell differentiation. The present study was designed to investigate the effect of KYN on FGF2 induced crystallin and MIP26 expression in LEC. We provide evidence that both exogenous and endogenous KYN block FGF2-induced crystallin and MIP26 expression in LEC and that the blockade occurs via reduction in Akt and ERK1/2 phosphorylation.

2. Materials and methods

2.1. Materials

Recombinant murine fibroblast growth factor (FGF2) was obtained from PeproTech, NJ. Rabbit polyclonal antibodies specific to α A- and α B-crystallin were from Stressgen, MI. Antibodies to β - and γ - crystallin, FGF2, MIP26, FGFR1, NF- κ B (p65) were from Santa Cruz Biotechnology, CA. Antibodies to total ERK 1/2 and phospho-ERK1/2 (pERK1/2, Thr 202/Tyr 204), Akt, phospho-Akt (pAkt, Ser 473), phospho-FGFR1 (pFGFR1, Tyr 653/654) were from Cell Signaling Technology, MA. Mouse monoclonal antibody to GAPDH was from Millipore, MA. Eagles Minimum Essential Medium (MEM) was from Sigma Aldrich, MO.

2.2. Cell culture and treatment

Our studies conformed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research, and they were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Mouse lens epithelial cells (mLEC) were isolated from the lens capsule-epithelial explants of 2-3 month-old Wt and IDO hemTg mice by the method previously described [13]. Cells at passages 4 to 6 were transferred to MEM that had low fetal calf serum (1%) (LSM) and had 2 mM L-glutamine, 50 μ g/mL gentamycin. LSM was essential in order to reduce the direct reaction of KYN with serum protein and to facilitate its effects on mLEC, and to reduce effects of mitogens present in fetal calf serum. Additionally, a recent study showed that LEC in LSM resemble early differentiating fiber cells [27].

Cells at 50-60% confluence were treated with FGF2 for 5 days in LSM (without replacing the medium). In some cases, Wt LEC were treated with FGF2 and 50 μ M KYN. In other cases, hemTg LEC were treated with FGF2 and 20 μ M 1-methyl-D,L-tryptophan (MT). KYN was dissolved in 25 μ l DMSO and further diluted in 10 ml LSM to obtain the final concentration.

2.3. Cell fractionation

To determine nuclear translocation of NF- κ B (p65), subcellular fractionation was performed using the Qproteome Cell Compartment kit (Qiagen, CA) according to manufactures' instructions.

2.4. Western blotting

Trypsinized cells were rinsed thoroughly in ice cold PBS and proteins were extracted in a lysate solution (10 mM EDTA-0.02% Triton X-100, pH 10) with phosphatase inhibitor cocktail. Cell lysates corresponding to 75 μ g protein were resolved on SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane (NCM). To determine nuclear translocation of NF- κ B (p65), nuclear protein fractions corresponding to 75 μ g protein were resolved on SDS-PAGE and blotted to NCM.

For detection of crystallins, MIP26, total ERK1/2 and pERK1/2, NF- κ B (p65), pFGFR1 and GAPDH, NCM was blocked with 5% nonfat dry milk (NFDM) in PBS with 0.2% Tween-20 (PBST) for 2h at room temperature (RT), washed with PBST and subsequently incubated with antibodies specific to either α A-crystallin or α B-crystallin, β -crystallin (all 1: 1,000 diluted), γ -crystallin (1:10,000 diluted), GAPDH (1:10,000 diluted), MIP26, total ERK1/2, pERK1/2, NF- κ B (p65) and pFGFR1 (1:1,000 diluted) for 1 h at RT. All primary antibodies were diluted in 5%

NFDM/PBST. The membrane was subsequently washed with PBST, 3 times 5 min each and incubated with appropriate HRP conjugated secondary antibodies diluted in PBST for 1 h at RT. After washing, blots were developed with SuperSignal West Pico Chemiluminescence Kit (Pierce, IL).

For the detection of total Akt and pAkt, NCM was blocked with 5% NFDM in Tris buffered saline with 0.2% Tween-20 (TBST) for 1h at RT, washed with TBST and subsequently incubated overnight with antibodies specific to total Akt and pAkt (1:1,000 diluted) at 4°C. Primary antibodies were diluted in TBST with 5% BSA. The membrane was subsequently washed three times with TBST and incubated with goat anti-rabbit HRP conjugated IgG (1:2,000 diluted in TBST with 5% NFDM). Membrane was washed with TBST three times, 5 min each and developed as described above.

Blot for crystallin and MIP26 expression was stripped and re-probed for GAPDH (loading control). In the case of pERK1/2 and pAkt, blots were re-probed for total ERK1/2 and total Akt, respectively.

2. 5. *ELISA for crystallin expression*

ELISA was performed as described previously [13] with modifications. Briefly, microplate wells were coated with cell lysates in 0.05M carbonate buffer (pH 9.6) at a protein concentration of 1 µg/well and then blocked with 5% NFDM/PBST. The wells were incubated with antibodies to one of the following: α A-, α B-, β - and γ -crystallin (1:50 diluted in PBS) followed by incubation with HRP-conjugated goat anti-rabbit IgG (1:10,000 diluted in PBS). The enzyme activity was assessed by adding 3,3',5,5'-tetramethylbenzidine and was read at 450 nm. Results were expressed as measures of relative absorbance. For negative controls, in some cases wells

were incubated with only the secondary antibody and in others wells were coated with BSA instead of cell lysate.

2. 6. Immunocytochemistry

Cells were cultured in 24 well plates and staining was performed as previously described [13] with slight modifications. Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 80% methanol. After blocking with 3% NFDM/1% BSA, wells were incubated with antibodies to one of the following: FGF2, total ERK1/2, pERK1/2, NF- κ B (p65) and MIP26 (all 1:100 dilution) for 1 h at RT. The appropriate anti-IgG conjugated to Oregon Green (1:400 diluted) was used as the secondary antibody. For NF- κ B (p65) immunostaining donkey anti-goat antibody conjugated to Texas Red (1:400 diluted) was used as the secondary antibody.

For pAkt staining, after fixation and permeabilization steps, wells were blocked with 5% goat serum in PBS/Triton-100 for 1h, at RT and subsequently incubated overnight at 4°C with anti-pAkt (1:50 diluted in PBS/Triton-100). After washing with PBS three times for 5 min each, wells were incubated with Oregon Green goat anti-rabbit IgG (1: 400 diluted in PBS/Triton) for 2 h at RT.

In all cases, after secondary antibody incubation wells were washed with PBST for 3 times 5 min and permanently mounted with DAPI/Vectashield. Cells were viewed with an Olympus System (Model BX60) fluorescence microscope, and images were acquired with an attached digital camera (Diagnostic Instruments, Inc., Spot RT Slider) connected to a Macintosh computer using Spot RT Slider software, version 3.5.5. For negative control cells were stained with only secondary antibodies.

2. 7. Binding of FGF2 to cells

Cells in MEM medium (200 cells per well) were plated in a 96 well plate. After overnight incubation, wells were replaced with LSM (with or without FGF2, kynurenine or MT) and incubated for 12 h. Subsequently wells were washed with PBS 3 times (5 min each) and cells were fixed as described above. After blocking with 3% NFDM/1% BSA in PBS, wells were incubated with anti-FGF2 (1: 1,000 diluted in blocking solution) for 1 h at RT. After washing with PBS 3 times (5 min each), wells were incubated with HRP-conjugated goat anti-rabbit IgG (1:10,000 diluted in blocking solution) for 1 h. Wells were washed three times as before, incubated with the substrate for 20 min and then treated with 20 μ l of 2N H₂SO₄ and read at 450 nm. For negative controls, wells were incubated with only the secondary antibody. The number of cells per well was determined by DAPI (nuclear) staining.

2. 8. Statistics

Data are expressed as mean \pm SD. Statistical significance among groups was analyzed by ANOVA followed by the Fisher's least significant difference test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. FGF2 induces crystallin expression in mLEC

Many previous studies have used capsule epithelial explants of the lens to investigate the role of FGF2 in fiber cell differentiation [28-30], however we used mLEC instead. We first needed to establish that capsule-free mLEC would synthesize crystallins in response to FGF2 treatment.

For this experiment, Wt mLEC were incubated with 10-100 ng/ml of FGF2. Crystallin expression was assessed by ELISA (Fig. 1). While control experiments (no FGF2) showed very little crystallin expression, the addition of FGF2 dramatically increased expression of all crystallins (α A-, α B-, β - and γ -crystallin). As cells exposed to 100 ng/ml of FGF2 exhibited high levels of crystallin expression, we decided to use this concentration in all subsequent experiments.

3.2. KYN blocks FGF2-induced crystallin expression

To study the effect of exogenous KYN, Wt mLEC were cultured with FGF2 in the presence or absence of KYN. Similar to Fig. 1, addition of 100 ng/ml of FGF2 drastically increased all crystallin expression (Fig. 2A). However, in the presence of KYN, FGF2 failed to elicit a response; the crystallin levels were similar to those in untreated cells. Thus, KYN appears to have blocked FGF2-induced expression of all major crystallins. Furthermore, we performed immunoblot and scanning densitometric analyses of crystallins and found that FGF2 increases expression of all crystallins (Fig. 2B). However, in cells treated with FGF2 + KYN, crystallin expression was comparable to that of control. It is possible that the antibodies for crystallins did not react with KYN-modified crystallins and this contributed to the decreased protein levels in KYN treated cells. To verify this possibility, we performed western blots for all crystallins with Wt mLEC proteins reacted with KYN (0.5nmoles per 100 μ g protein) for 48 hrs. This is based on our previous observation that KYN concentration in KYN treated Wt mLEC is ~ 0.12 nmoles/50 μ g protein/ 10^6 cells [13]. Obviously, the KYN concentration used in the present study is higher than in our previous study. Even with this higher KYN concentration, when higher KYN-modification on protein is expected, the antibody reactions were similar between KYN-reacted and unreacted samples (Fig. 2C). These results suggest that exogenous KYN blocked FGF2-induced expression

of crystallins and the reduced crystallin levels are not due to the failure of the antibodies to react with KYN-modified crystallins.

The above data suggests that exogenous KYN entered cells and blocked expression of crystallins. To further verify whether KYN formed in cells could block crystallin expression, we used IDO overexpressing hemTg mLEC. In some cases we treated these cells with 20 μ M MT to block IDO activity. This concentration of MT was used based on our previous study that showed a complete inhibition of IDO in IDO hemTg mLEC with 20 μ M MT [13]. FGF2 failed to evoke crystallin-induction in IDO overexpressing cells. The crystallin levels were comparable to those in control cells (no FGF2) (Fig. 3A). However, co-treatment of cells with FGF2 and MT caused significantly higher expression of all crystallins. Western blot experiments and densitometric analyses corroborated the ELISA results. The protein bands corresponding to crystallins were denser in FGF2 + MT treated cells than in either controls or FGF2 alone treated cells (Fig. 3B). Taken together, these results strongly suggest that endogenous KYN (produced by IDO activity) can block FGF2-induced expression of all crystallins.

3.3. KYN does not affect FGF2 binding to cell surface receptors

The binding of the FGF2 to its cell surface receptor, which exhibits receptor tyrosine kinase (RTK) activity is the first process in its signal transduction. To determine if KYN affects FGF2 binding to its receptors, and thereby blocks FGF2-mediated signaling required for crystallin expression, we performed immunostaining for FGF2. In Wt mLEC treated with FGF2, we observed immunoreactivity throughout cells (Fig. 4A). This was unaltered in cells simultaneously incubated with FGF2 and KYN. In hemTg mLEC (Fig. 4B) similar immunoreactivity was detected in cells exposed to FGF2 with or without MT. To further verify the effect of KYN on FGF2

binding, we performed ELISA using whole cells. FGF2 levels were nearly 4-fold higher in FGF2 treated cells than in control cells (Fig. 4C) and KYN or MT had no effect on FGF2 levels (Fig 4C and D). Cells incubated with the secondary antibody alone did not show immunoreactivity either (data not shown). These results confirmed that neither the exogenous nor the endogenous KYN alters FGF2 binding to cells.

3.4. *MIP26 expression is inhibited by KYN*

FGF2 is known to induce MIP26 expression [26, 31]. A recent study showed that 100 ng/ml FGF2 induced MIP26 expression in rat lens epithelial explants [26]. This is the same concentration of FGF2 we used in our experiments. We tested the effect of KYN on MIP26 expression in mLEC by western blotting and immunocytochemistry. As can be seen in Fig. 5A, MIP26 was detected in all cells. In the case of Wt mLEC a prominent band corresponding to MIP26 protein was detected in FGF2 treated cells. KYN dramatically reduced the effect of FGF2 on MIP26 expression. Densitometric analysis showed a 7-fold increase in MIP26 content in FGF2 treated cells and a content equal to control cells in FGF2 + KYN treated cells. In the case of hemTg mLEC, FGF2 mildly induced the expression of MIP26, and when added with MT, the expression was significantly increased (Fig. 5B).

Immunocytochemistry was performed to confirm the above findings. We found higher expression of MIP26 in FGF2 treated Wt mLEC (Fig. 5C). The immunoreactivity was relatively low in the presence of FGF2 + KYN. The MIP26 immunoreactivity was elevated in FGF2 treated hemTg mLEC (Fig. 5D) and it was further elevated when MT was included along with FGF2 (Fig. 5D). These results show that FGF2 induced MIP26 expression is blocked by KYN, and inhibition

of IDO by MT reverses the effect of KYN and suggests that KYN is inhibitory for FGF2 induced MIP26 expression in mLEC.

3.5. Akt activation is blocked by KYN

The P13K/Akt pathway has been implicated in the FGF2-induced expression of lens crystallins [25]. To better understand the mechanisms behind the effect of KYN on crystallin expression, we examined the phosphorylation status of Akt in Wt mLEC treated with FGF2 in the presence or absence of KYN. Western blot results showed higher levels of pAkt in cells treated with FGF2 (~8-fold higher over control), but this effect was absent in cells treated with FGF2 + KYN (Fig. 6A). In the case of hemTg mLEC, FGF2 failed to induce a response similar to Wt mLEC; pAkt levels were relatively low (Fig. 6B). However, when cells were incubated with FGF2 + MT, pAkt levels were drastically increased. The total Akt level in both cases remained similar to the levels in untreated cells.

Consistent with western blot findings, immunoreactivity to pAkt was much higher in FGF2 treated Wt mLEC (Fig. 6C) and FGF2+MT treated hemTg mLEC (Fig. 6D). Together these data imply that KYN blocks FGF2-induced Akt activation.

3.6. FGF2-induced ERK1/2 phosphorylation is inhibited in the presence of KYN

ERK1/2 activation has been identified as essential for FGF2-induced MIP26 expression [26]. To determine if the low MIP26 expression in the presence of KYN is due to KYN's effect on ERK1/2 phosphorylation, we investigated phosphorylation profile of ERK1/2 in FGF2 treated mLEC. Fig. 7A and 7B show total and phosphorylated ERKs in Wt and IDO hemTg mLEC, respectively. The two isoforms, p44 (ERK1) and p42 (ERK2) are both phosphorylated by FGF2 in

Wt cells and KYN inhibited such phosphorylation. In IDO hemTg mLEC, phosphorylation of both is induced by FGF2, however the induction was more robust in MT treated cells.

We then performed immunocytochemistry to confirm western blot results. Immunoreactivity to total ERK1/2 was detected both in Wt and hem Tg mLEC treated with or without FGF2 (Fig. 7C and D). However, immunoreactivity to pERK1/2 was greater in FGF2-treated Wt mLEC (Fig. 7E) and in FGF2 + MT treated hemTg mLEC (Fig. 7F) compared to untreated Wt mLEC and FGF2 treated IDO hemTg mLEC, respectively. These data indicate that the inhibitory effect of KYN on MIP26 is due to the inhibition of upstream ERK1/2 phosphorylation.

3.7. KYN inhibits FGF2 –mediated NF- κ B activation

Further to confirm that KYN adversely affects FGF2 signaling, we studied its effect on NF- κ B activation, which is known to be activated by FGF2 in lens epithelial cells [32]. Results showed that KYN inhibited FGF2-mediated NF- κ B (p65) nuclear translocation (activation) in Wt mLEC (Fig. 8A). In the case of hemTg mLEC, NF- κ B (p65) nuclear translocation was greater in cells treated with FGF2 + MT than in cells without treatment (Fig. 8B). NF- κ B (p65) was not detected in the nuclear fraction of control samples (no KYN). Consistent with the western blot findings, immunoreactivity for NF- κ B (p65) in the nucleus was greater in FGF2 treated Wt mLEC (Fig. 8C) than controls (no KYN). FGF2 + MT treated hemTg mLEC showed higher immunoreaction in the nucleus than cells without treatment (Fig. 8D). Together these data imply that KYN blocks FGF2-induced NF- κ B activation.

3.8. KYN blocks phosphorylation of FGFR1

Following FGF ligand binding and dimerization, FGFRs get phosphorylated at tyrosine residues [33]. FGFR1 can be phosphorylated at seven tyrosine residues in the cytoplasmic tail. Of these, tyrosine 653 and 654 are important for signaling [34]. To test if KYN interferes with FGFR1 phosphorylation, we performed western blotting for pFGFR1 (Tyr653/654). A prominent band corresponding to pFGFR1 was detected in FGF2 treated Wt mLEC (Fig. 9). KYN blocked phosphorylation of FGFR1. In the case of hemTg mLEC, a more prominent band was detected in FGF2 + MT treated cells than in FGF2 alone treated cells. Together these results suggest that KYN blocks its phosphorylation.

4. Discussion

The role of FGF2 in normal lens development and transparency is well established [35, 36]. Molecular and genetic approaches have recently identified a series of signaling molecules that participate in these processes. However, whether low molecular weight compounds present in the lens affect these pathways remains unknown. In the present study, for the first time, we provide evidence for KYN-mediated blockade of FGF2 signaling pathway.

In the human lens, kynurenines absorb UV light penetrating the cornea (300-400nm) [5, 37-39]. By absorbing UV light, they could protect the retina from photodamage. However, recent reports suggest a potential role for KYN in cataract formation. Our recent studies with transgenic mice provide evidence for deleterious effects of KYN in the lens [12, 13]. In those studies, we found that IDO overexpression in the lens led to poorly differentiated fiber cells and cell cycle arrest in lens epithelial cells [12, 13]. Furthermore, we found high levels of KYN in those

epithelial cells. Based on the results, we concluded that KYN, produced through IDO was causing protein modification and cell cycle arrest.

The present study extended these findings in order to further investigate the effect of KYN on LEC. The major findings in the present study are: 1) FGF2 induces expression of all major crystallins and MIP26 in mLEC. 2) KYN blocks FGF2-induced expression of crystallins and MIP26. 3) KYN reduces FGF2 induced phosphorylation of FGFR1, Akt and ERK1/2. 4) Inhibition of IDO reverses KYN-mediated blockade of FGF2 signaling.

We used 50 μ M KYN to study effects of exogenous KYN. In our previous study we measured KYN concentration in mLEC incubated with 50 μ M KYN and found that the levels within cells are ~ 0.12 nmoles/ 10^6 cells and are comparable to those in IDO hemizygous lens epithelial cells (~ 0.11 nmoles/ 10^6 cells) [12]. Thus, the KYN concentration used in the present study was similar to concentrations present in LEC of IDO transgenic animals. Because neither KYN incubated mLEC nor lens epithelial cells of IDO transgenic animals showed NFK or 3OH-KYN, but they did show high levels of KYN [12], we believe that the effects seen the present study are primarily due to KYN. Moreover, at the concentration used in the present study (50 μ M) we did not detect cell death in mLEC during the 5-day incubation period. This is consistent with our previous finding [13]. Furthermore, our previous study had shown that KYN concentrations within mLEC remained unchanged when we increased the KYN concentration from 50 to 200 μ M, suggesting that mLEC have the ability to regulate intracellular concentrations of KYN. The highest intracellular concentration of KYN achieved in that study was 0.12 nmoles/ 10^6 cells with 50 μ M KYN. The absence of cell death at this concentration of KYN suggests that mLEC may either have a robust mechanism to cope with KYN or that KYN does not cause apoptosis at this concentration.

Binding of FGF2 to RTKs activates the intrinsic tyrosine kinase resulting in tyrosine phosphorylation, further activating the PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways [40]. It has been reported that in differentiating LEC, ERK1/2-JNK activation is necessary for MIP26 expression [26] and Akt activation is necessary for β - and γ -crystallin expression [25]. In agreement with previous studies, we found that lens epithelial cells express α -crystallins and FGF2 induces its expression [41, 42]. Nevertheless, our finding that KYN blocks FGF2-induced ERK1/2 and Akt phosphorylation provides a mechanistic explanation for the reduced crystallin and MIP26 expression by KYN. We also showed that KYN blocks FGF2 induced NF- κ B activation, which provides additional support to our hypothesis that KYN blocks FGF2 signaling in mLEC.

Because our results suggested that FGF2 signaling is impaired by KYN, we determined whether FGF2 binding to mLEC was altered by KYN. Although we found no evidence for alteration in FGF2 binding to cell surface, we found a reduction in FGFR1 phosphorylation. Further studies are required to understand the mechanisms by which KYN reduces FGFR1 phosphorylation.

FGF2-mediated signaling occurs through its high affinity cell surface receptors that contains four major domains that are rich in cysteine [43]: the extracellular domain responsible for ligand binding, the tyrosine kinase domain that transmits biological signal intracellularly, the transmembrane domain that connects the extracellular and intracellular domains and the regulatory domain with many autophosphorylation sites. It is possible that KYN reacts with cysteines in these domains and alters their function. This notion is supported by several observations. KYN is intrinsically unstable and undergoes deamination to form a reactive α,β -unsaturated ketone [8, 44], which rapidly reacts with nucleophilic amino acids, such as, cysteine in proteins. It was found that

the rate constant for the addition of KYN derived oxo acid to cysteine is 36 ± 4 per liter $\text{mol}^{-1}\text{s}^{-1}$, which is several orders of magnitude higher than the rate with other amino acids. The rate constant for the addition reaction to L-GSH is only 2.1 ± 0.2 per liter $\text{mol}^{-1}\text{s}^{-1}$ [45]. This implies that cysteine residues in proteins are most susceptible for modification by KYN even in presence of GSH.

In addition to their receptors, FGFs bind to heparan sulfate in proteoglycan of cell membrane and extracellular matrix through a heparin sulfate binding domain [46, 47]. It has been proposed that heparin sulfate facilitates binding of FGF2 to FGFR. The anterior capsule of the lens contains heparin sulfate [48]. Considering that we used isolated LEC in the present study rather than lens epithelial explants that contain the lens capsule, we do not believe that the defective FGF2 signaling occurred due to an effect of KYN on heparin sulfate. Nevertheless, additional work is required to rule out such an effect in lens in vivo.

In cultured human lens epithelial cells (HLE-B3 cells) we have measured IDO activity and found it be ~ 0.1 nmoles of KYN/h/mg protein and we found that this activity to increase by 200-fold with pathological concentrations of interferon- γ (Mailankot and Nagaraj, unpublished observation). Interferon- γ (IFN- γ) is the most abundant cytokine in the aqueous humor in infectious and non-infectious uveitis [49]. IFN- γ is a known stimulator of IDO synthesis [1]. Since IDO is present mostly in the epithelium of the lens [50], we can speculate that in inflammatory conditions of the eye, KYN concentrations in the lens could be profoundly higher than normal concentrations of 2-40 μM [51]. Under such circumstances, elevated KYN could impair FGF2 signaling in LEC and consequently affect lens transparency (Fig. 9).

We believe that our findings are not only relevant for lens epithelial cell biology but also for neurological diseases. For example, in Alzheimer's disease, levels of kynurenine are elevated in the brain. The activation of IDO in microglial cells of the brain is believed to be the cause of

higher production of kynurenines [52, 53]. Furthermore, FGF2 is increased in the brains of Alzheimer's patients [54]. Thus, FGF2-mediated signaling may be impaired and could contribute to pathology in Alzheimer's disease. Furthermore, high levels of kynurenines have been detected in Huntington's disease as well [55]. It will be important to determine if FGF2-mediated signaling pathways are similarly affected in this disease. In summary, our study shows for the first time that an intracellular metabolite of the kynurenine pathway could specifically downregulate a signaling pathway that is important for the synthesis of major proteins of an organ.

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Figure Legends

Figure 1: FGF2 induces expression of crystallins in mLEC. Wt mLEC were treated with FGF2 (0-100 ng/ml) for 5 days. ELISA was performed with cell lysate proteins for α A-, α B-, β - and γ -crystallin expression. Results are expressed as mean \pm SD from three independent experiments.

Figure 2: Exogenous KYN inhibits crystallin expression in mLEC. **A.** ELISA was performed using 1 μ g proteins from Wt mLEC that were treated with FGF2 (100 ng/ml) and kynurenine (KYN, 50 μ M). Results are expressed as mean \pm SD from three independent experiments. **B.** Cell lysate proteins (75 μ g) were subjected to western blotting. The blot was initially probed for crystallins and subsequently striped and reprobed for GAPDH (loading control). The band intensities were quantified by scanning densitometry and expressed relative to GAPDH (bottom panels). **C.** Cell lysate protein (100 μ g) reacted with or without KYN (0.5 nmoles) was subjected to western blotting. The blot was initially probed for crystallins and subsequently striped and reprobed for GAPDH (loading control). Data are mean \pm SD from three independent experiments. * p <0.001 in comparison to controls.

Figure 3: Endogenous KYN inhibits crystallin expression in mLEC. **A.** ELISA was performed using 1 μ g proteins from IDO hemTg mLEC that were treated with FGF2 (100 ng/ml) and MT (20 μ M). Results are expressed as mean \pm SD from three independent experiments. **B.** Western blotting was performed as described in figure. 2. Data are mean \pm SD from three independent experiments. * p <0.001 in comparison to controls.

Figure 4: KYN does not inhibit FGF2 binding to cell surface receptors. Wt (**A and C**) and IDO hemTg mLEC (**B and D**) cultured in presence of FGF2 (100 ng/ml) and with or without kynurenine (KYN, 50 μ M) and MT (20 μ M). **A and B.**, immunocytochemistry for FGF2. The immunoreactivity for FGF2 was detected using Oregon green goat anti-rabbit secondary antibody. Cells were counterstained with a nuclear stain, DAPI (blue). Figures are representative of three independent experiments. **C and D.** FGF2 binding was analyzed by an ELISA using a rabbit anti-FGF2 polyclonal antibody and goat anti-rabbit IgG HRP conjugate. The number of cells per well was determined by DAPI staining. The y-axis represents OD/cell and was calculated by dividing total OD by the cell number. Data are expressed as mean \pm SD from three independent experiments. Neither exogenous nor endogenous KYN affect FGF2 binding to cell surface. * $p < 0.05$ in comparison to controls.

Figure 5: KYN inhibits FGF2-induced expression of MIP26. Wt mLEC and IDO hemTg mLEC were treated with or without FGF2 (100 ng/ml). In addition to FGF2, Wt mLEC were treated with kynurenine (KYN, 50 μ M) and IDO hemTg mLEC were treated with MT (20 μ M) for 5 days. Cell lysates (75 μ g protein in each case) from Wt (**A**) and IDO hemTg mLEC (**B**) were subjected to western blotting using an anti-MIP26 antibody. The membrane was reprobed for GAPDH (loading control). Images are representative of two independent experiments. Band intensities of MIP26 relative to GAPDH are average of two independent experiments (middle panels). Immunocytochemistry for MIP26 was performed with rabbit anti-FGF2 polyclonal antibody and Oregon Green goat anti rabbit IgG in Wt mLEC (**C**) and IDO hemTg mLEC (**D**).

Cells were counter-stained with a nuclear stain, DAPI (blue). MIP26 expression was inhibited by KYN in Wt mLEC and was restored by MT treatment in IDO hemTg mLEC.

Figure 6: KYN inhibits FGF2-induced Akt phosphorylation. Cells were treated as described in figure 5. Cell lysates (75 µg protein each) from Wt (**A**) and IDO hemTg mLEC (**B**) were subjected to western blotting. The blot was initially probed for pAkt and subsequently reprobed for total Akt. Images are representative of two independent experiments. Band intensities of pAkt relative to Akt are average of two independent experiments are shown in the middle panels. Wt (**C**) and IDO hemTg mLEC (**D**) were fixed, permeabilized and subjected to immunocytochemistry using a rabbit anti pAkt polyclonal antibody and Oregon Green goat anti rabbit IgG. Cells were counter-stained with a nuclear stain, DAPI (blue). FGF2-induced Akt phosphorylation is inhibited by KYN in Wt mLEC and was restored by MT treatment in IDO hemTg mLEC. Images are representative of two independent experiments.

Figure 7: KYN inhibits FGF2-induced ERK1/2 activation. Cells were treated as described in figure 5. Cell lysate corresponding to 75µg protein from Wt (**A**) and IDO hemTg mLEC (**B**) was subjected to western blotting. The blot was initially probed for total ERK1/2 and later for pERK1/2. Images are representative of two independent experiments. Band intensities of pERK1 and pERK2 relative to ERK1 and ERK2, respectively and are average of two independent experiments (middle panels). mLEC from Wt (**C** and **E**) and IDO hemTg (**D** and **F**) were fixed, permeabilized and subjected to immunocytochemistry with an anti- ERK1/2 polyclonal antibody (**C** and **D**) or an anti-pERK1/2 polyclonal antibody (**E** and **F**). Oregon Green goat anti rabbit IgG

was used as the secondary antibody. Cells were counter stained with a nuclear stain, DAPI (blue). Images are representative of two independent experiments.

Figure 8: KYN inhibits FGF2 –mediated NF- κ B nuclear translocation. Cells were treated as described in figure 5. Nuclear protein fractions (75 μ g protein each case) from Wt (**A**) and IDO hemTg mLEC (**B**) were subjected to western blotting and probed for NF- κ B (p65). Images are representative of two independent experiments. After treatment mLEC from Wt (**C**) and IDO hemTg (**D**) were permeabilized and subjected to immunocytochemistry with an anti- NF- κ B (p65) polyclonal antibody. Texas Red donkey anti-goat IgG was used as the secondary antibody. Cells were counter-stained with a nuclear stain, DAPI (blue). Images are representative of two independent experiments.

Figure 9: KYN inhibits phosphorylation of FGFR1. Cells were treated as described in figure 5. Cell lysate corresponding to 75 μ g protein from Wt and IDO hemTg mLEC was subjected to western blotting. The blot was initially probed for pFGFR1 and subsequently for GAPDH. Images are representative of two independent experiments. Band intensities of pFGFR1 relative to GAPDH are average of two independent experiments are shown in the bottom panel.

Figure 10: Conceptual view on the mechanism of KYN-mediated inhibition of protein synthesis in mLEC. Both exogenous and endogenous KYN inhibit FGF2-induced FGFR1 phosphorylation, which results in decreased activation of Akt and ERK1/2 and consequently reduced expression of crystallins and MIP26.

Fig. 1

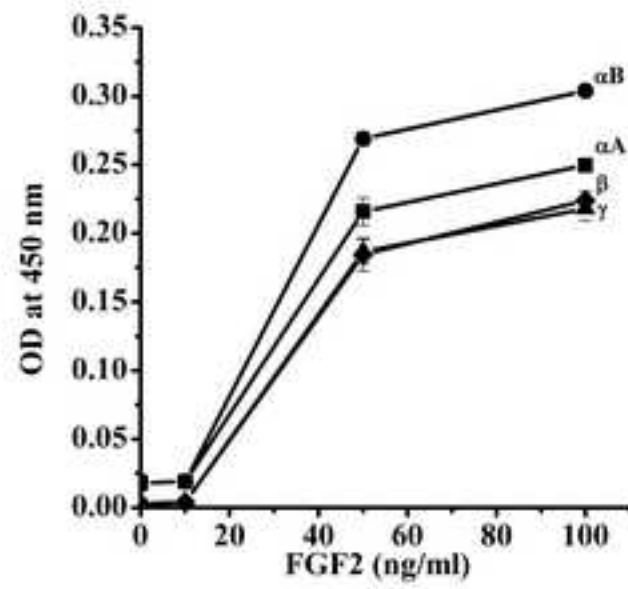


Fig. 2

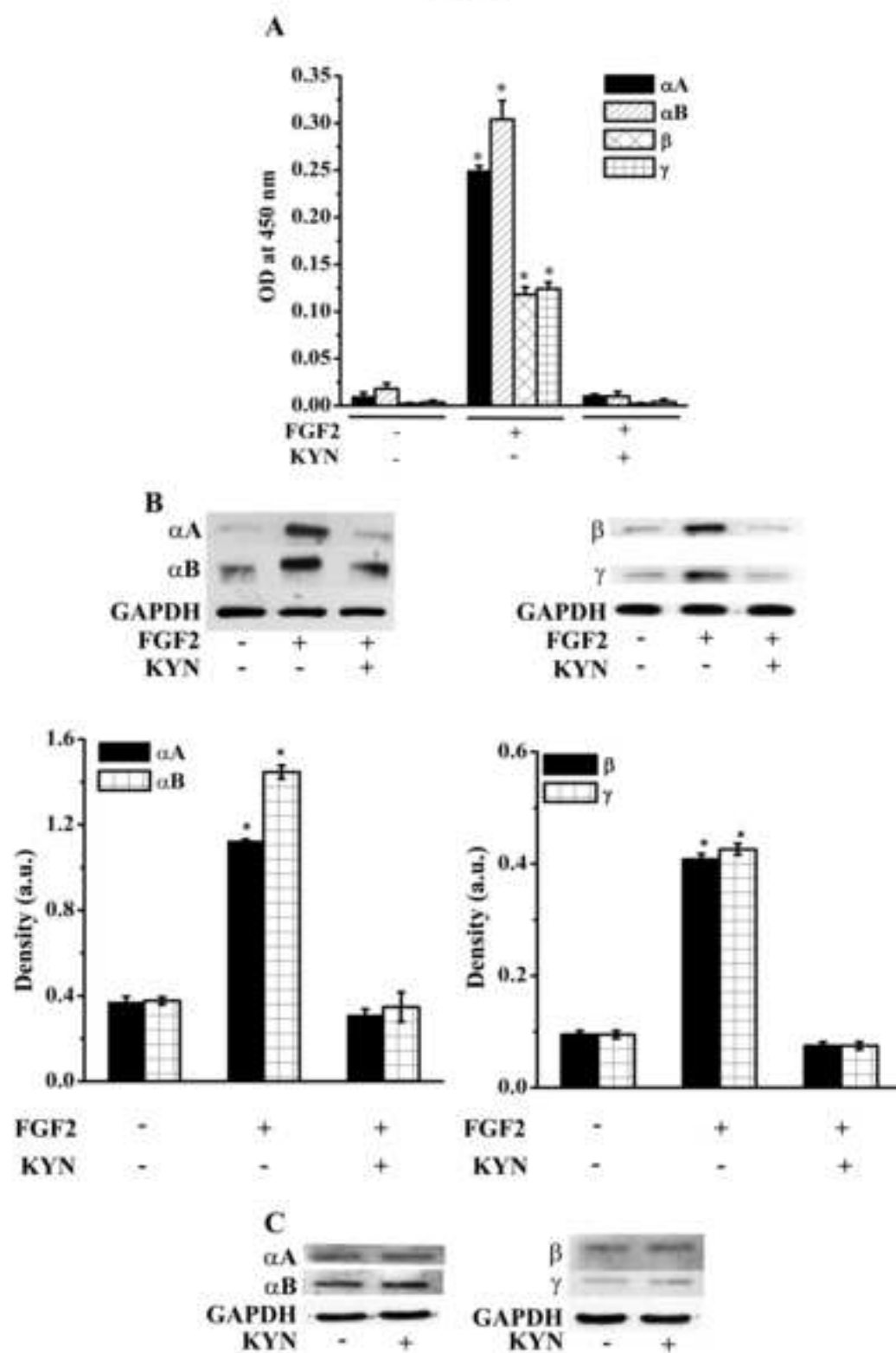


Fig. 3

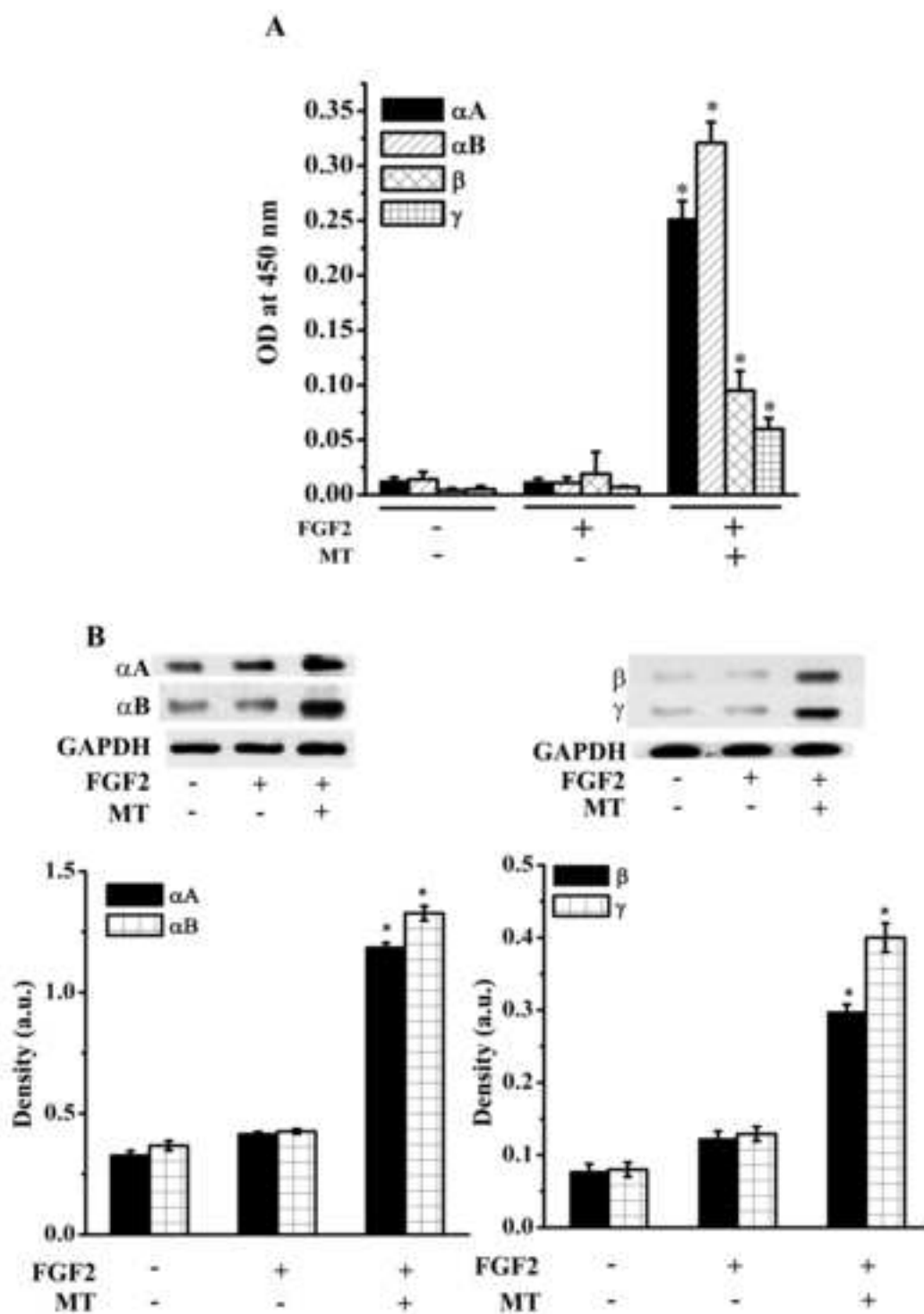


Fig. 4

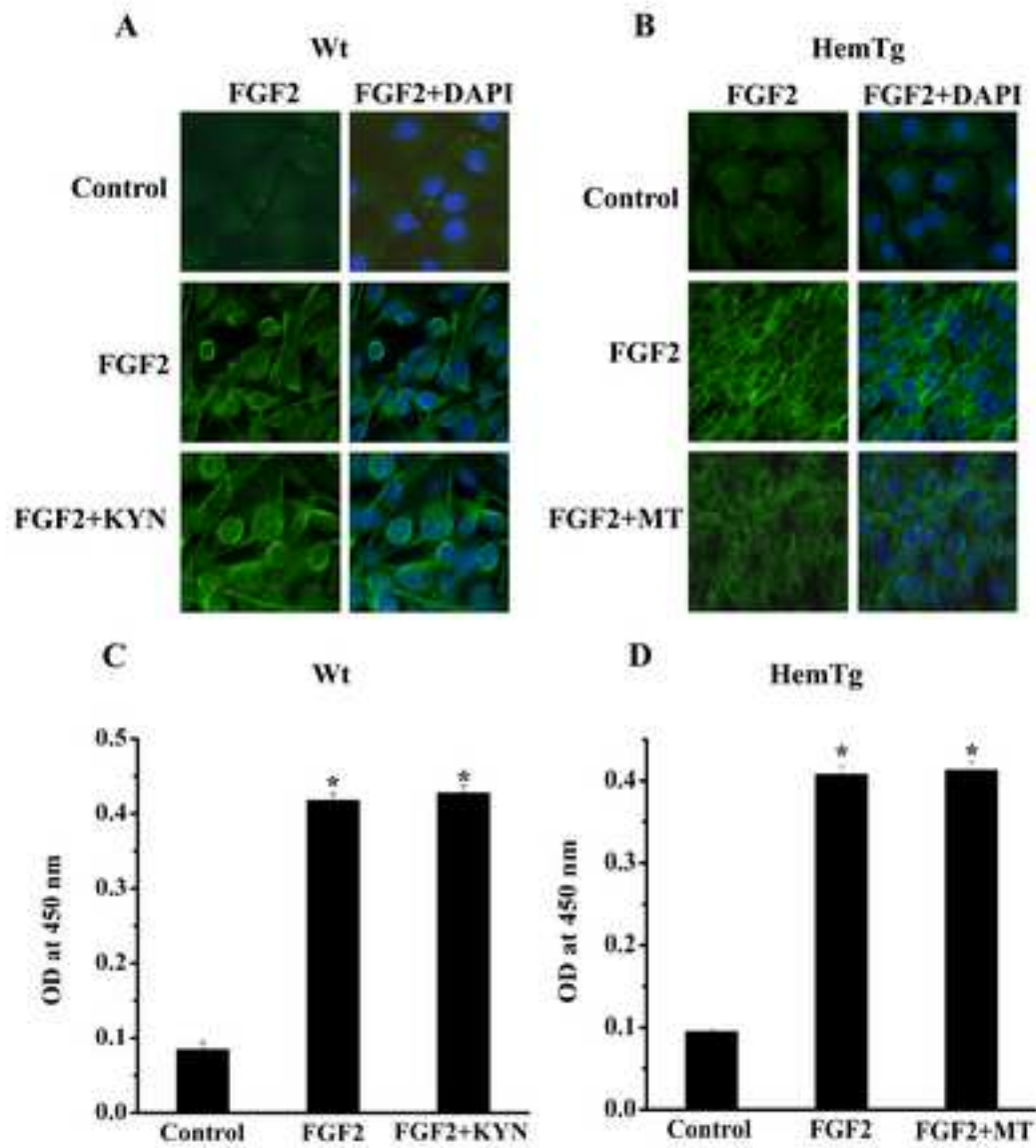


Fig. 5

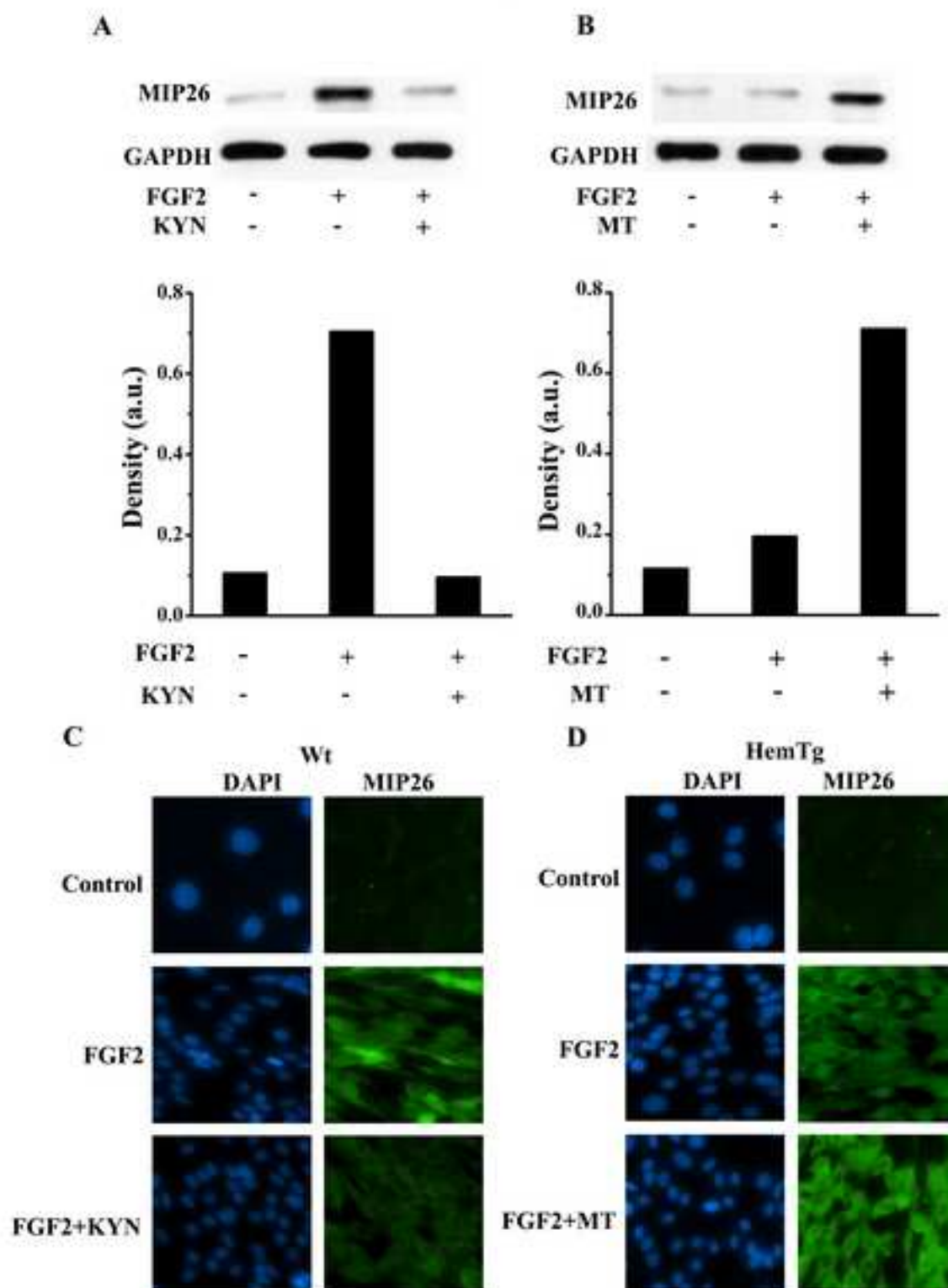


Fig. 6

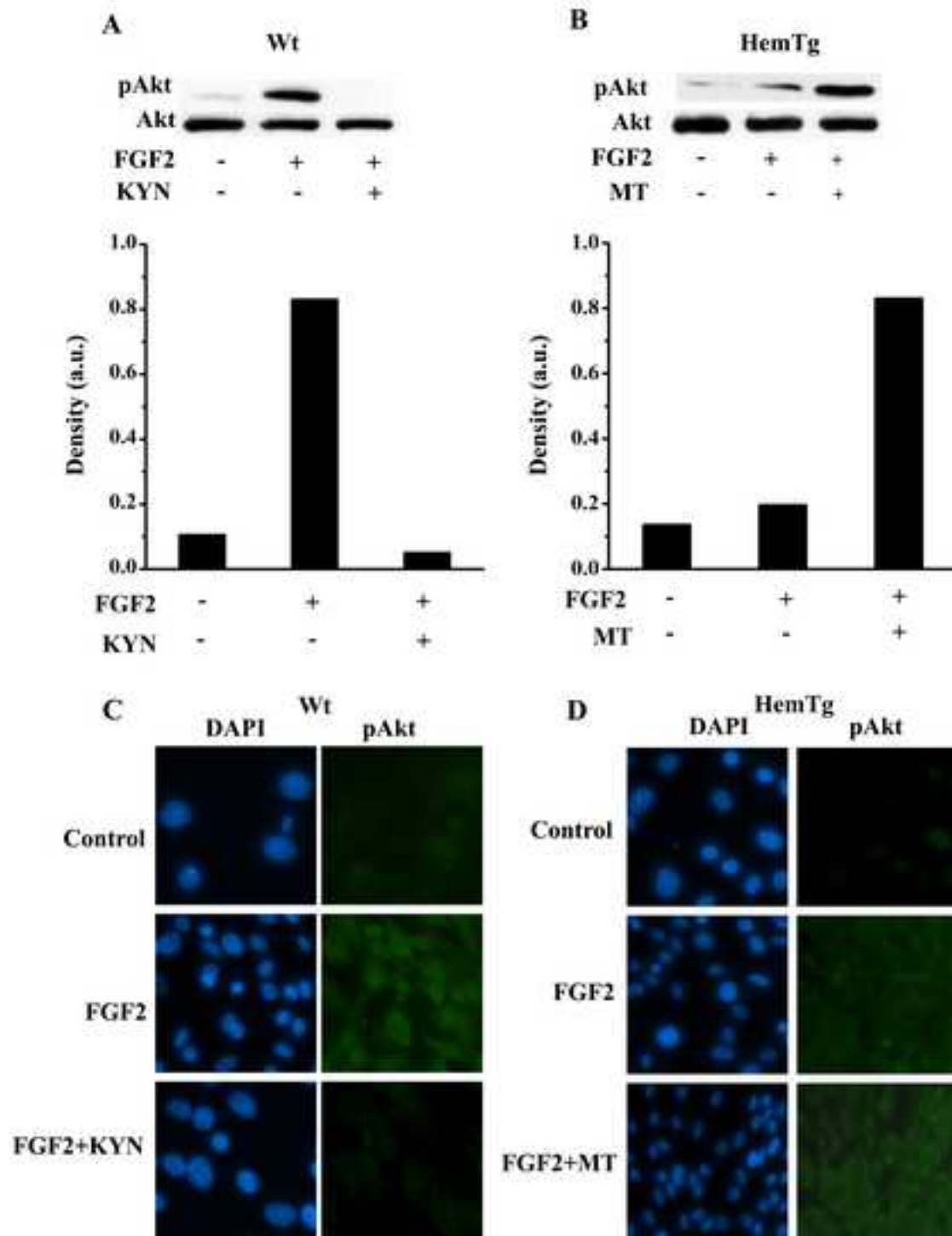


Fig. 7

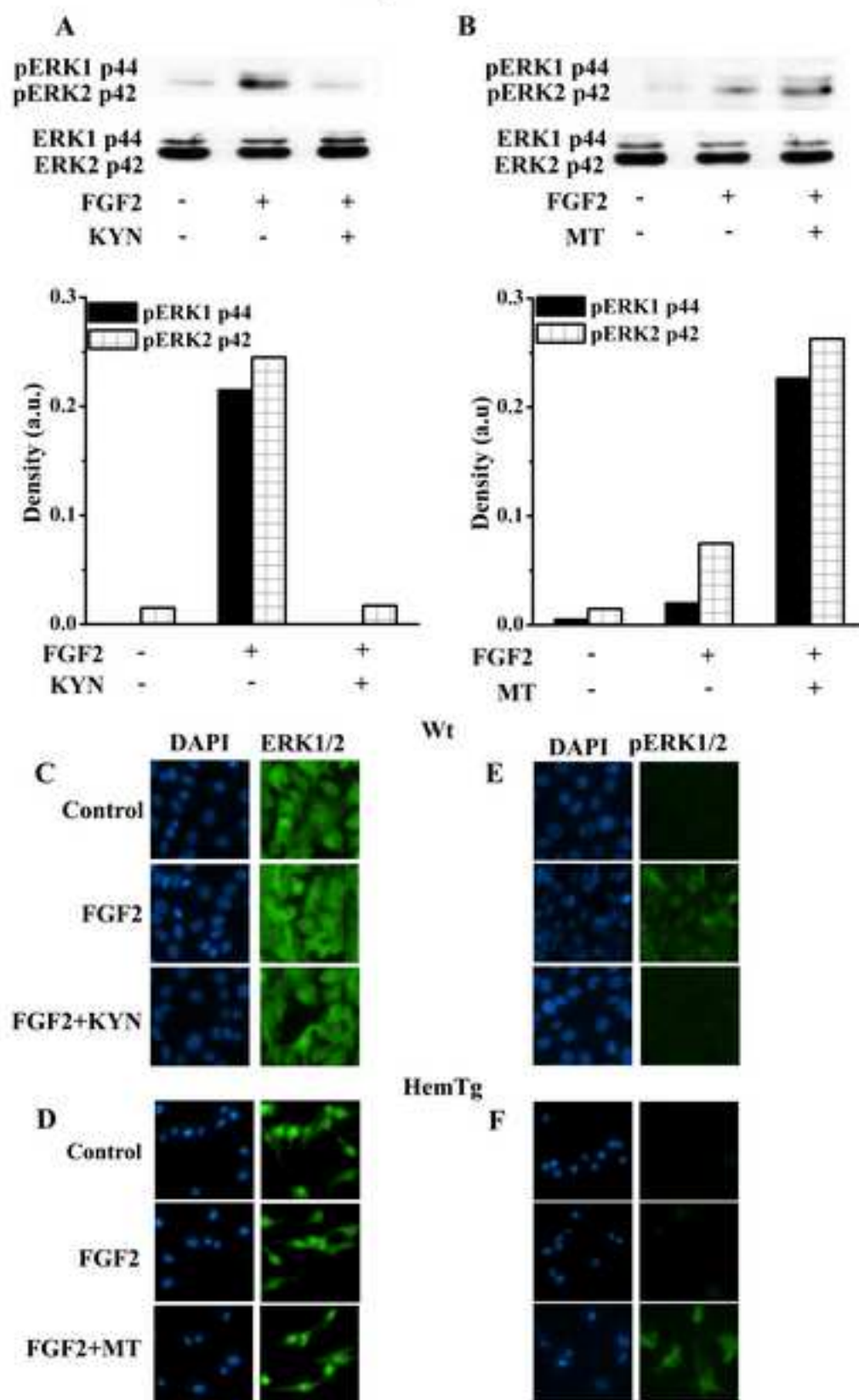


Fig. 8

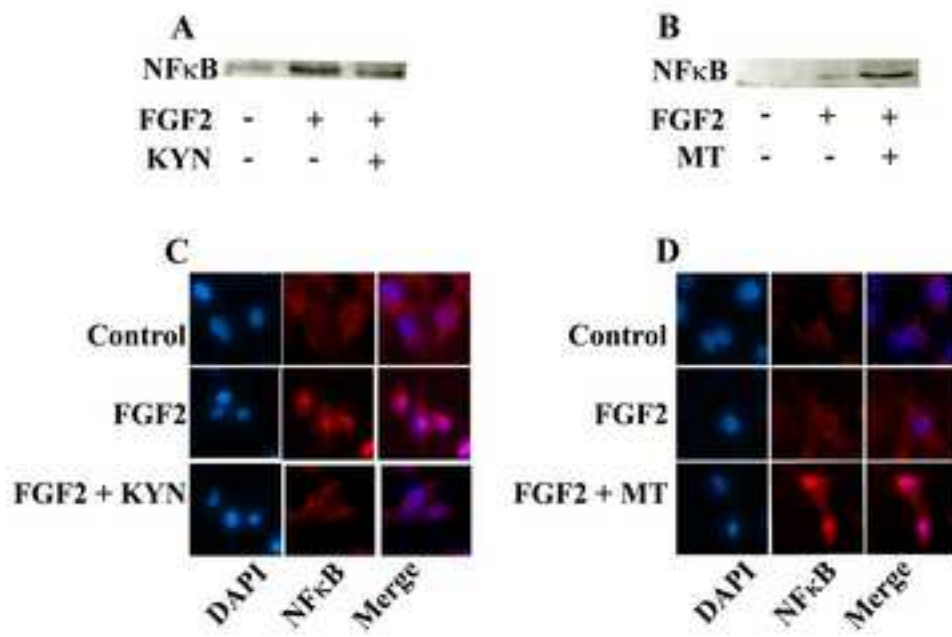


Fig. 9

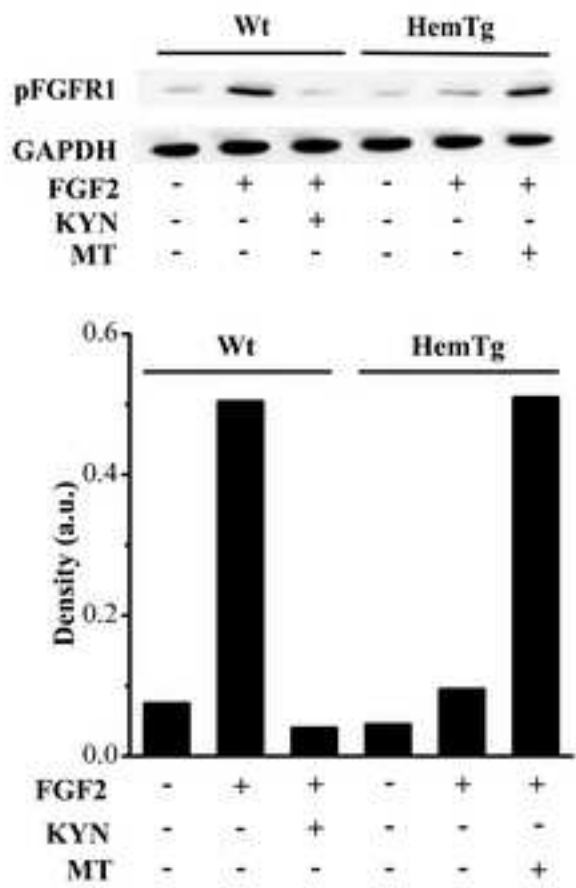


Fig. 10

