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# TGF-β1 prevents rat retinal insult induced by amyloid-β (1–42) oligomers

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ABSTRACT

To set up a retinal degenerative model in rat that mimics pathologic conditions such as age-related macular degeneration (AMD) using amyloid-β (Aβ) oligomers, and assess the effect of TGF-β1. Sprague-Dawley male rats were used. Human $A\beta_{1-42}$ oligomers were intravitreally (ITV) injected (10 μM) in the presence or in the absence of recombinant human TGF-β1 (1 ng/μl ITV injected). After 48 h, the animals were sacrificed and the eyes removed and dissected. The apoptotic markers Bax and Bcl-2 were assessed by western blot analysis in retina lysates. Gene-pathway network analysis was carried out in order to identify pathways involved in AMD. Treatment with Aβ oligomers induced a strong increase in Bax protein level (about 4-fold; $p < 0.01$) and a significant reduction in Bcl-2 protein level (about 2-fold; $p < 0.05$). Co-injection of TGF-β1 triggered a significant reduction of Bax protein induced by Aβ oligomers. Bioinformatic analysis revealed that Bcl-2 and PI3K-Akt are the most connected nodes, for genes and pathways respectively, in the enriched gene-pathway network common to AMD and Alzheimer disease (AD). Overall, these data indicate that ITV injection of $A\beta_{1-42}$ oligomers in rat induces molecular changes associated with apoptosis in rat retina, highlighting a potential pathogenetic role of Aβ oligomers in AMD. Bioinformatics analysis confirms that apoptosis pathways can take part in AMD. Furthermore, these findings suggest that human recombinant TGF-β1 can prevent retinal damage elicited by Aβ oligomers.



## 1. Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible central vision loss in elderly populations in developed countries. Two main forms of AMD exist, the dry and the wet one. Dry AMD is characterized by drusen bodies (cellular debris) that accumulate between choroid and retina; wet AMD includes abnormal growth of choroidal blood vessels leading to detachment of retina along with edema due to vascular leakage.

Drusen are extracellular deposits that accumulate under the basement membrane of the retinal pigmented epithelium (RPE) and the inner collagenous layer of the Bruch membrane (Fig. 1). An age-related accumulation of amyloid-β (Aβ) in the normal mouse retina and human retina has been recently demonstrated (Hoh Kam et al., 2010). Many protein and lipid constituents of drusen are similar to those found in deposits characteristic of other age-related degenerative disorders such as Alzheimer disease (AD). Several studies have led to the comprehension that prefibrillar soluble oligomers, rather than amyloid fibrils, might be the primary toxic agents in AD brain (Kayed et al., 2003; Kayed et al., 2004; Lambert et al., 1998). The presence of prefibrillar oligomers in drusen has been demonstrated (Luibl et al., 2006), suggesting that amyloid oligomers may be involved in drusen biogenesis and/or participate directly in local RPE toxicity (Isas et al., 2010).

Transforming-growth-factor-β1 (TGFβ1) is an anti-inflammatory cytokine with neurotrophic and neuroprotective properties (Caraci et al., 2011; ten Dijke and Hill, 2004). It has been proposed that TGF-β1-TGFβ receptor I (TβRI) axis plays a key role in the function of retinal vascular barrier, by promoting endothelial cell survival and homeostasis (Walshe et al., 2009).

Based on these grounds, we set up an *in vivo* model of AMD using Aβ oligomers to induce retinal damage and investigate the

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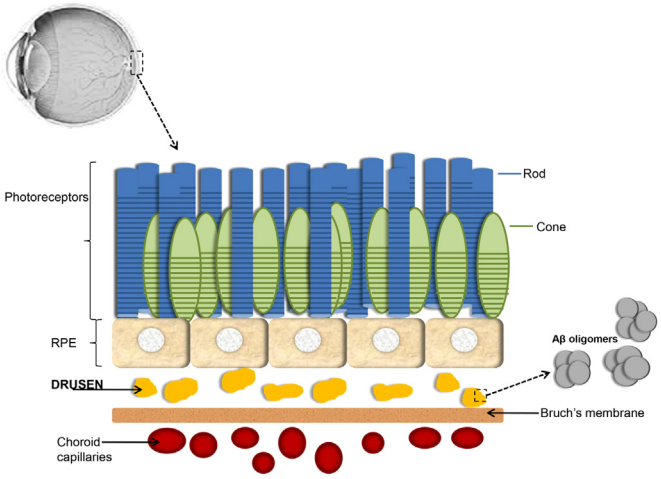


**Fig. 1.** Schematic diagram of the age-related macular degeneration.

potential protective role of TGF-β1. Furthermore, we carried out a bioinformatics analysis in order to sort out gene pathways commonly related to AMD and AD.

## 2. Material and methods

### 2.1. Reagents

SB431542, a selective inhibitor of TGFβ-1 receptor, and protease inhibitors cocktail were purchased from Sigma-Aldrich (St Louis, MO). Rabbit polyclonal antibody against Bax, and rabbit monoclonal antibodies against Bcl-2, and GAPDH were purchased from Cell Signaling Technology (Danvers, MA); secondary goat anti-rabbit IRDye 680 conjugated antibody was purchased from LI-COR (Lincoln, US).

### 2.2. Amyloid-β oligomers

Human $A\beta_{1-42}$ oligomers were prepared according to the original protocol of Klein's group (Caraci et al., 2015b; Gong et al., 2003). Briefly, the $A\beta_{1-42}$ lyophilized peptide, purchased from Novus Biologicals (Littleton, USA), was dissolved in trifluoroacetic acid (TFA, 1 mg/ml) and sonicated in a water bath sonicator for 10 min. Then TFA was evaporated under a gentle stream of argon and 1 ml 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was added to the peptide. After 1 h incubation at 37 °C, the peptide solution was dried under a stream of argon, and then solubilized again by adding 2 ml of HFIP. Finally, HFIP was removed by argon streaming followed by further drying in a lyophilizer for 1 h and $A\beta_{1-42}$ resuspended in 5 mM anhydrous dimethyl sulfoxide (DMSO), before dilution to 100 μM in ice-cold PBS. Aliquots of 100 μM $A\beta_{1-42}$ were incubated for 72 h at 4 °C and then stored at −20° until use. For *in vivo* experiments human $A\beta_{1-42}$ oligomers were diluted in sterile PBS and 1 μl intravitreally (ITV) injected at the final concentration of 10 μM.

### 2.3. Animal treatment

Male Sprague-Dawley rats (250–300 g) were purchased from Harlan (Udine, Italy). The animals were fed on standard laboratory food and were allowed free access to water in an air conditioned room with a 12-h light/12-h dark cycle. The animals were randomly divided in four experimental groups: 1) control; 2) ITV injected with 1 μl Aβ at a concentration of 10 μM in PBS; 3) ITV injected with 1 μl of Aβ and TGFβ1 (1 ng/μl; the dose was selected based on previous work, (Caraci et al., 2015a); 4) ITV injected with 1 μl of Aβ, TGFβ1 and the inhibitor of TβRI (SB431542; 20 μM; the dose was selected based on previous work) (Caraci et al., 2015a). Before ITV injection, animals were anesthetized by intravenous injection of 5 mg/kg Zoletil (tiletamine HCl and zolazepam HCl, Virbac, Milano, Italy); and 1 drop in the eye of the local anesthetic Novesina (Novartis, Origgio, Italy). Animals were sacrificed 48 h after treatment, and retinas were dissected. Housing and treatments were in accordance to Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research.

### 2.4. Western blotting

Retinas from control and treated rats were homogenized and sonicated in RIPA buffer (Life Technologies, Monza, Italy) in the presence of protease inhibitor cocktail (Sigma P2714), serine/threonine phosphatase inhibitors (Sigma P0044) and tyrosine protein phosphatase inhibitors (Sigma P5726). Protein concentrations were determined by Bradford's method using bovine serum albumin as standard. Retina lysates (40 μg protein) were loaded into SDS-PAGE, blotted and probed for different target proteins. Membranes were incubated with primary antibodies against total Bcl-2 (Rabbit monoclonal, 1:1000 dilution), Bax (rabbit polyclonal, 1:1000 dilution), GAPDH (Rabbit, monoclonal 1:1000 dilution). The membranes were then incubated with secondary fluorescent antibodies (1:20,000 dilution) for 1 h at room temperature, and the immunocomplexes were detected by Odyssey imaging system (LI-COR, Lincoln, NE). All blots were controlled for equal loading by probing with GAPDH. Densitometric analysis was performed using Image J software.

### 2.5. Statistical analysis

Statistical significance between two groups was analyzed by Student's *t-test*. One-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test, was used for multiple comparisons. $P$ values $< 0.05$ were considered as statistically significant.

### 2.6. Bioinformatics analysis

Gene(s) to pathway(s) information was derived from the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database. AMD is a complex disease and gene association studies highlighted a series of genes involved in development of AMD: *HGS*, *TNF*, *RAD51B*, *CFH*, *CFB*, *C3*, *ARMS2*, *COL8A1*, *CX3CR1*, *FBLN5*. Analysis of pathways, and pathways interconnection, regulated by these genes was carried out with the web application KENeV-KEGG Enriched Network Visualizer (KENev) (Pilalis et al., 2015). However, output associated to this list of genes was not informative enough. Therefore, an enrichment information strategy was carried out. In a previous work (Romano et al., 2015) we have looked at pathways that are common to neurodegenerative diseases (glaucoma, AMD and AD), with particular focus on glaucoma. Results were obtained through bioinformatic prediction of microRNAs (miRNA) involved in such diseases. The enrichment analysis was carried out with the following steps, because one miRNA can regulate more than one gene:

- searching of miRNAs known to be deregulated in AMD (Romano et al., 2015);
- searching of miRNAs that putatively target genes, retrieved from gene association studies (Romano et al., 2015). This search was carried out through the web server microRNA.org (Betel et al., 2008). Only miRNAs commonly deregulated both in AMD and AD were analyzed;

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- searching for biochemical pathway commonly regulated by miRNAs, through DIANA-miRPath, by using the miRTarBase algorithm (Vlachos et al., 2012);
- retrieving of genes that are targets of selected miRNA, which regulate pathways known to have a known role in AMD and AD;
- building of enriched gene-pathway network interaction with KENev.

Output from KENev, a genes-pathways network, was analyzed and visualized with Cytoscape 3.2.1 (Shannon et al., 2003). Genes and pathways are represented as nodes in the network, if a gene regulates a pathway then an edge between two nodes is created.

## 3. Results

Bcl-2 and Bax are cytoplasmic proteins involved in the regulation of apoptosis. This family of apoptosis regulatory genes affects mitochondrial membrane dysfunction during programmed cell death. In the early stage, pro-apoptotic Bcl-2 family member such as Bax are translocated to mitochondrial membranes where they increase mitochondrial permeability (Mattson, 2000). Bcl-2 proteins prevent apoptotic death in cells, while Bax proteins induce the opening of mitochondrial permeability transition pores and the subsequent release of cytochrome C, which results in cell apoptosis (Garcia et al., 1992; Vander Heiden et al., 1999). We observed a significant ($p < 0.01$) reduced level of Bcl2 and a significant ($p < 0.01$) increase in the level of Bax in retina of $A\beta_{1-42}$ oligomers treated rats (Fig. 2).

As a result, intraocular injection of $A\beta_{1-42}$ oligomers significantly ($p < 0.01$) increased retinal Bax/Bcl2 ratio in Aβ-treated rats in comparison with control group. Interestingly, TGFβ1 significantly prevented ($p < 0.01$) both Bax induction and the Aβ-induced decrease of Bax/Bcl2 ratio, suggesting an anti-apoptotic effect of this neurotrophic factor in our experimental model of AMD. Furthermore, co-treatment with TGFβ1 and SB431542, a selective inhibitor of ALK5/TβRI, blunted the effect of TGFβ1 on Bax/Bcl-2 ratio confirming that the protective effect of exogenous TGFβ1 was exerted through the activation of TβRI (Fig. 2).

In order to test the hypothesis that genes related to PIK3-Akt signaling or cell cycle activation are dysregulated in AMD we built a gene-pathway network. In a first step we tried to build a gene-pathway network starting from genes retrieved from gene association studies related to AMD; however, retrieved pathways did not give any information about AMD. Therefore, considering that miRNAs can target more than one gene, and deregulated miRNAs can regulate biochemical pathways with combinatorial effects, we proceeded to identify miRNAs known to be deregulated in AMD. Additional miRNAs, commonly deregulated in AMD and AD, where predicted to regulate genes associated to AMD (Romano et al., 2015, Table 1).

These miRNAs (Table 1) can impact the following pathways: cell cycle, Toll-like, RIG-1 like, NF-kB, Apoptosis, p53 signaling, Notch signaling, PI3K-Akt, Focal Adhesion, Neurotrophins (NT), HIF-1, Adherence junctions, Wnt signaling.

In particular hsa-miR-107 targets VEGFA, hsa-miR-146a targets NF-kB. Interestingly hsa-miR-181c, upregulated in AD, is predicted to down-regulate Bcl-2, then leading to apoptosis.

An enriched list of 104 genes (supplemental material), that are predicted to be targeted by miRNAs listed in Table 1, was created from miRNA-pathway analysis carried out with DIANA-miRPath. These genes were used for building an enriched gene-pathway network. Submission of these genes to the KENeV web application gave the result illustrated in Fig. 3.Genes–pathway interaction network was visualized with a circular layout, increasing closeness centrality was used as hierarchical parameter. High closeness

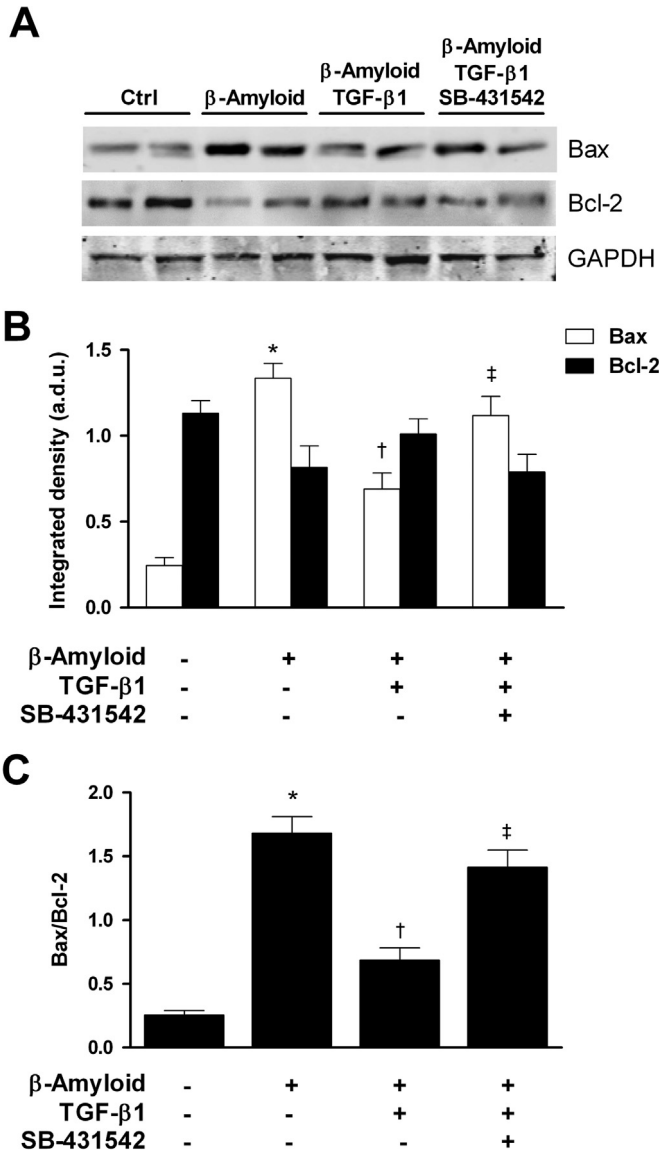


**Fig. 2.** Western blot analysis of Bax and Bcl-2 in Aβ-treated rats with or without TGF-β1 in the presence or absence of selective inhibitor of TβRI (SB431542). Representative western blotting of Bax, Bcl-2 and GAPDH are shown (panel A). The values, expressed as arbitrary densitometric units (a.d.u.), were obtained by the reading of blots with the Image J program and are means ± SD of at least three independent experiments, each performed in triplicate (panel B). Bax/Bcl-2 ratio (panel C). $^{*}p < 0.01$ *vs.* control; $^{\dagger}p < 0.01$ *vs.* Aβ-treated rats, $^{\ddagger}p < 0.01$ *vs.* Aβ +TGF-β1treated rats.

centrality nodes have the shortest distance from other nodes of the network; thus, high closeness centrality nodes are connected through few links to any other node of the network. MYC and Bcl-2 are the genes with the higher closeness centrality. The most high closeness centrality pathway is PI3K-Akt, along with "miRNA in cancer" and "pathways in cancer". This network, built from genes targeted by miRNAs listed in Table 1, supports the hypothesis that dysregulation of Bcl-2 and PI3K-Akt pathways would lead to apoptotic events occurring in both AMD and AD.

## 4. Discussion

Complex and multi-factorial diseases, such AMD, need appropriate experimental models for identification of new pharmacological targets and development of new neuroprotective drugs.

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**Table 1**
Human microRNAs associated to AMD and AD.

| AMD* | AMD[a] ∩ AD | GAS[b] ∩ AD |
|---|---|---|
| hsa-miR-30b | hsa-miR-9 | hsa-miR-107 |
| hsa-miR-23a | hsa-miR-146a | hsa-miR-137 |
| hsa-miR-9 | hsa-miR-21 | hsa-miR-146a |
| hsa-miR-146a | hsa-miR-34 | hsa-miR-181c |
| hsa-miR-146b | | hsa-miR-197 |
| hsa-miR-31 | | hsa-miR-21 |
| hsa-miR-21 | | hsa-miR-22 |
| hsa-miR-184 | | hsa-miR-328 |
| hsa-miR-34a | | hsa-miR-590 |

Age related macular degeneration (AMD), Alzheimer's disease (AD), gene association studies (GAS).

[a] miRNA retrieved from literature (Romano et al., 2015). hsa-miR is the tag for human miRNA.

[b] These miRNAs are common to AMD and AD, furthermore these miRNAs are predicted to target genes retrieved from GAS for AMD. In GAS ∩ AD column, miRNA were underlined in order to highlight those predicted miRNA that have been also experimentally validated (hsa-miR-146a and hsa-miR-21 are also in AMD ∩ AD column).

Besides the validity of genetic animal models for AMD, animal models of choroidal neovascularization are among the most simple and diffused models of wet AMD (Pennesi et al., 2012). Consistent with the neurodegenerative hypothesis of AMD, Liu et al. (2013) developed a model of early stages of AMD. This model was based on intravitreal injection of toxic fragments of $A\beta_{1-40}$ in Long Evans rats; Aβ treatment led to upregulation of proinflammatory cytokines (*e.g.* IL-6, TNF-α, IL-1β, and IL-18) in the RPE and neuroretina (Liu et al., 2013).

Different groups have demonstrated the neurotoxic effects of amyloid-β peptides ($A\beta_{1-40}$ and $A\beta_{1-42}$) in cortical neurons (Jen et al., 1998; Paradis et al., 1996). In particular, in human neuron primary cultures, $A\beta_{1-40}$ and $A\beta_{1-42}$ can trigger the execution phase of apoptotic death via induction of Bax protein and down-regulation of Bcl-2 (Paradis et al., 1996). Furthermore, Jen et al. (1998) showed that treatment with $A\beta_{1-40}$ and $A\beta_{1-42}$ severely decrease Bcl-2 immunoreactivity in Müller glial cells. In the present study we developed an animal model of AMD characterized by apoptotic events elicited by intravitreal injection of $A\beta_{1-42}$ oligomers. Recently, the presence of a wide spectrum of amyloid structures in drusen, from patients with AMD, has been demonstrated; in particular non-fibrillar oligomers were detected (Isas et al., 2010). In an early phase of AD, soluble Aβ monomers aggregate into soluble Aβ oligomers and insoluble Aβ plaques, both considered as toxic to neurons (Klein, 2013). Furthermore, it has been suggested that Aβ oligomers might represent the primary neurotoxic species in amyloid-related neurodegeneration in AMD. Therefore, we challenged rat retina with prefibrillar small soluble Aβ oligomers, because they are known to induce apoptotic death in neuronal cultures (Giuffrida et al., 2009), to set up a model of AMD.

Here we show, for the first time, that Aβ oligomers increase the ratio of Bax/Bcl2 in this new experimental model of AMD. We hypothesize that Aβ oligomers convert into fibrils during the 48 h of treatment, which could finally contribute to apoptotic retinal

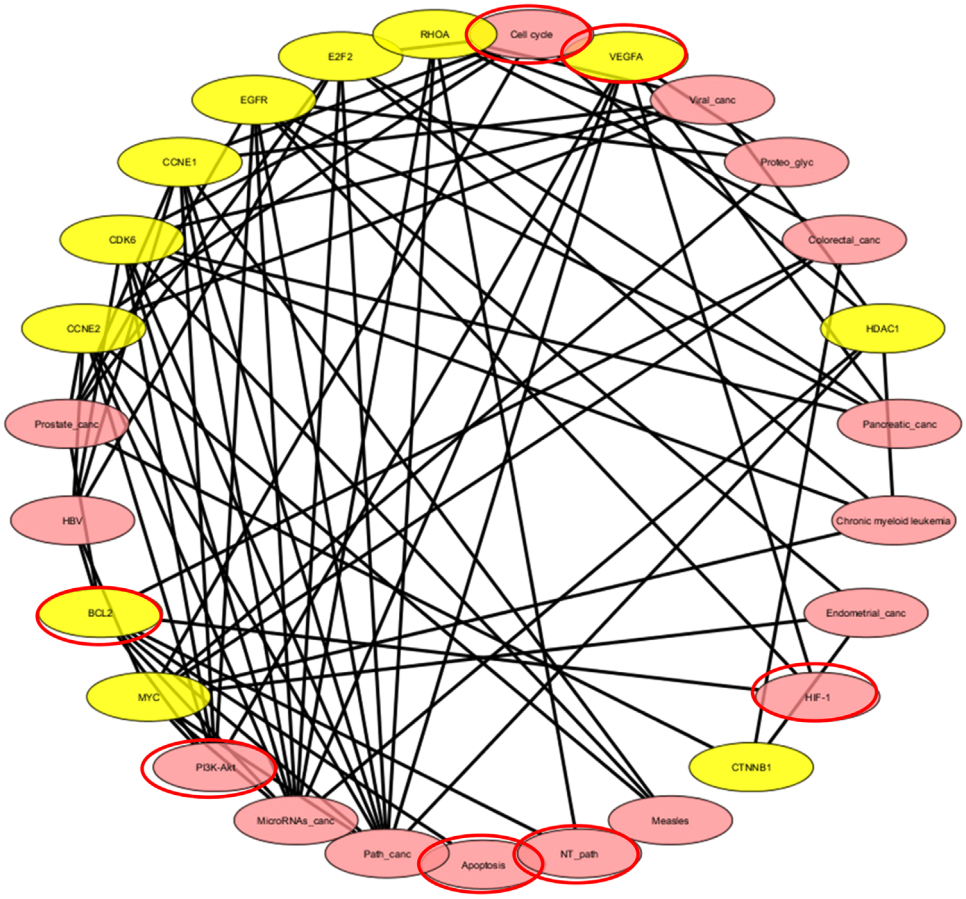


**Fig. 3.** Gene-pathway network. Genes are the yellow nodes. Pathways are the pink nodes. Genes are named with their "HUGO gene nomenclature committee" symbols. Other abbreviations: viral carcinogenesis (viral_canc), proteoglycan in cancer (proteo_glyc), colorectal cancer (colorectal_canc), pancreatic cancer (pancreatic_canc), endometrial cancer (endometrial_canc), hypoxia inducible factor HIF signaling pathway (HIF-1), neurotrophins signaling pathway (NT_pathway), pathways in cancer (path_canc), microRNA in cancer (microRNA_canc), prostate cancer (prostate_canc), hepatitis B virus (HBV). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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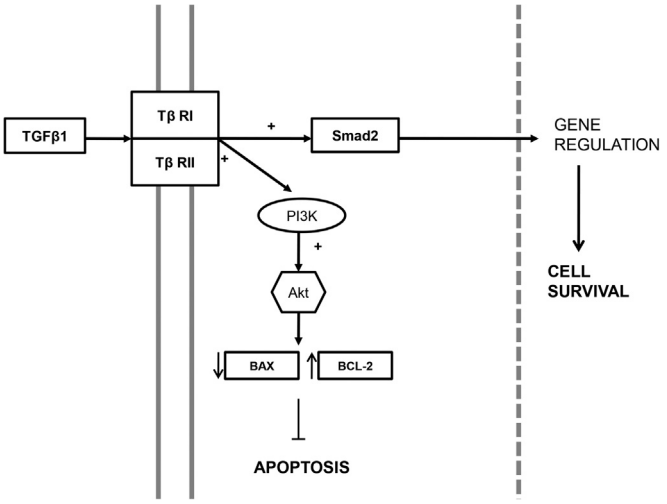


**Fig. 4.** Smad-dependent and smad-independent TGF-β1 signaling pathways.

cell death; in this context, the ratio of pro-apoptotic Bax to anti-apoptotic Bcl2 can be taken as an index of the extent of apoptosis. Furthermore, we showed that retinal damage is reversed by exogenous TGF-β1 treatment. Treatment with human recombinant TGF-β1 reversed both the induction of Bax and the reduction of retinal Bcl-2 in rats treated with $A\beta_{1-42}$ oligomers, leading to a reduction of the ratio of Bax/Bcl2, which reduces execution phase of apoptotic cell death.

An impairment of TGF-β1 signaling pathway plays a central role in the pathogenesis of AD and it contributes to Aβ accumulation and microglia activation in animal models of AD (Chen et al., 2015; Tichauer and von Bernhardi, 2012). Accordingly, exogenous TGF-β1 is neuroprotective against Aβ toxicity both in *in vitro* and *in vivo* models of AD (Caraci et al., 2011). TGF-β1 has been shown to protect cortical neurons from Aβ-induced neurodegeneration, through activation of the neuroprotective pathway PI3K-Akt (Caraci et al., 2008). However, the role of TGF-β1 in promotion of either cell survival or apoptosis in retina is not completely understood. The TGF-β1 signaling pathway could induce either apoptosis or cell survival, most likely depending on other cell signaling pathways, such as PI3K-Akt pathway that can be activated independently of Smad-signaling (Wilkes et al., 2005).

Members of the TGF superfamily act through a receptor complex constituted by the activin-like kinase 5 (ALK5)/TGF-β type I receptor (TβRI) and TGF-β type II receptor (TβRII), strongly expressed in the CNS, particularly in hippocampus (Derynck and Zhang, 2003). TGF-β1 binding to TβRII induces the assembly of TβRI and TβRII receptors into a complex, with the subsequent transphosphorylation of TβRI by the type II receptor kinase. The subsequent activation of TβRI receptor leads to phosphorylation of selected SMAD proteins that, in turn, translocate into the nucleus and regulate the expression of different target genes involved in cell survival and proliferation (ten Dijke and Hill, 2004, Fig. 4). Besides Smad-mediated gene transcription, TGFβ activates Smad-independent pathways, including NF-kB (Konig et al., 2005), and PI3K/Akt (Bakin et al., 2000; Caraci et al., 2008). TGF-β/Smad-independent pathways play a key role in mediating different biological effects of TGF-β such as cell cycle inhibition, epithelial-to-mesenchymal trans-differentiation, immune suppression and neuroprotective effects (Caraci et al., 2008; Derynck and Zhang, 2003).

Yin et al. (2011) have reported that upregulation of Akt prevents alteration of Bcl-2 family members (including Bcl-xL, Bcl-w, Bad, and Bax) elicited by Aβ and that overexpression of Akt significantly attenuates Aβ-induced apoptosis, while simultaneous inhibition of PI3K, the immediate upstream regulator of Akt, abolishes the protective effect of Akt activation. Therefore, because TGFβ1 activates PIK3-Akt signaling (Zhu et al., 2004) which is anti-apoptotic, its protective effect could be attributed to the activation of this pathway; however, other mechanism/s of cell protection by TGFβ1 through SMAD2 cannot be ruled out (Zhu et al., 2004, Fig. 4).

We carried out a bioinformatics analysis, characterized by several enrichment information steps, in order to verify the importance of PI3K-Akt pathway in retinal cell survival. The genes-pathways network, built with the web-application KENeV, revealed that Bcl-2 and PI3K-Akt are respectively the gene and the pathway that can be dysregulated in AMD (Fig. 3). This bioinformatics analysis included also the assumption that AMD and AD share common pathogenic mechanisms. The KEGG pathway related to TGFβ signaling suggests that p-SMAD2 a TGF-β1 downstream pathway effector, would regulate apoptosis (Fig. 4). Recently, we demonstrated that p-SMAD2 is involved in the effects of TGF-β1 on long term potentiation (LTP) formation (Caraci et al., 2015a). Aβ oligomers are known to induce a severe impairment of Smad-dependent TGF-β1 signaling in AD brain (Caraci et al., 2012). Thus, we speculate that activation of the PI3K/Akt pathway by exogenous TGF-β1 in AMD might counteract the deficit of Smad-dependent TGF-β1 induced by Aβ oligomers. Further studies are needed to elucidate whether or not the PI3K/Akt pathway contributes to the neuroprotective effects of TGF-β1 in AMD, under conditions of defective activation of Smad-dependent TGF-β1 signaling similar to that occuring in AD brain.

## 5. Conclusions

In conclusion, the novel experimental model hereby proposed can recapitulate the apoptotic events induced by Aβ oligomers occurring during AMD, and represents a tool to assess the pharmacological activity of new potential candidates for AMD management such as TGF-β1.

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