Role of Early Growth Response 1 (Egr-1) in the Corneal Fibrotic Process

Megan Twite, Ching Yuan
Department of Ophthalmology and Visual Neurosciences, University of Minnesota, Minneapolis, MN, USA

Introduction

- Transforming growth factor beta (TGF-β) has been shown to be one of the most important fibrotic signaling pathways by in vitro experiments and animal models [1]. TGF-β plays a role in mediating the transformation of myofibroblasts and ECM protein deposits [1, 2]. Cellular responses to TGF-β are mediated through Smad-dependent and Smad-independent signal transduction mechanisms, refer to Figure 1.
- Egr-1 overexpression has been linked to animal models of fibrosis and human fibrotic disorders, such as idiopathic pulmonary fibrosis and scleroderma. Notably, instead of activation by post-translational modifications (such as phosphorylation), regulation of Egr-1 signaling is mediated via its biosynthesis and by interaction with regulatory molecules, such as Nab2 [1].
- Halofuginone has previously been identified to have anti-fibrotic effects in corneal fibroblasts. However, it also acts competitively with proline as an inhibitor of the t-RNA charging activity and activates the amino acid response pathway (AAR). The addition of proline has been seen to reverse the effects of halofuginone in mouse embryonic fibroblasts [3].

Methods:

- Human cornea fibroblasts were transfected by the liposome transfection method with Egr-1 transgene or short hairpin RNA to delineate the profibrotic roles of Egr-1 by either constitutively overexpressing or suppressing Egr-1 expression.
- Fibroblasts were treated with Halofuginone and TGF-β1 to depict their affects on Egr-1 expression and analyzed by Western blot.
- Human fibroblasts were also treated with halofuginone and various concentrations of the amino acid proline to explore the influence of the amino acid response (AAR) pathway on halofuginone's interaction with Egr-1.

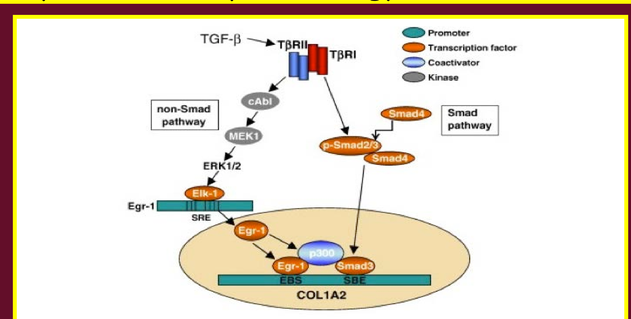

Figure 1. TGF-β signaling pathways; c-Abl stimulates egr-1 via MAP kinase. Egr-1 binds to the Egr-1 binding elements (EBS) to stimulte COL1A2 transcription. Figure adapted from Bhattacharyya et al., Matrix Biol. 2011.

Signaling pathway(s) targeted by Halofuginone

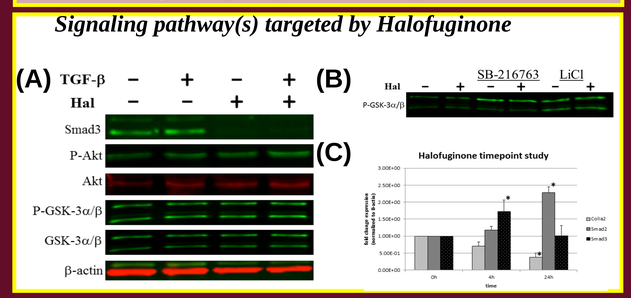

Figure 2. Human fibroblasts were first treated with Halofuginone ("**Hal**", 10 ng/ml) for 24 hours, and then TGF-β1 (2 ng/ml) for 1 hour. The cells were harvested for western blot analysis using various antibodies as shown in **(A)**. To confirm the GSK-3α/β protein bands, cells were treated with GSK-3 inhibitors (SB-216763 or LiCl) and Halofuginone, as shown in **(B)**. **(C)** qRT-PCR analysis of type I collagen, Smad2 and Smad3 expressions at 0, 4 and 24 hours (n=3, mean ± SD; *: $p < 0.05$).

Egr-1 is expressed in cultured corneal fibroblasts and the cornea

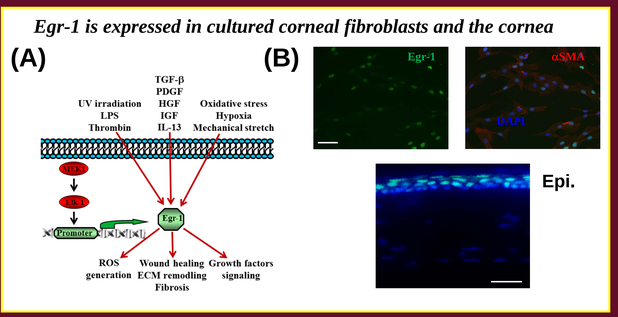

Figure 3. **(A)** Schematic description of Egr-1-mediated cellular events. **(B)** Immunostaining results revealed that Egr-1 is present in cultured corneal fibroblasts (upper panels), and corneal epithelial cells (lower panel). In contrast, keratocytes were negative for Egr-1 staining.

Halofuginone suppressed the expression of Egr-1

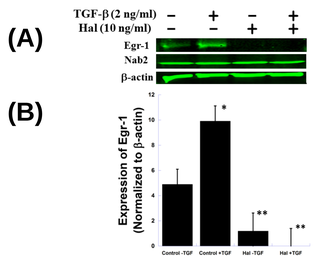

Figure 4. **(A)** Representative western blot showed reduced Egr-1 expression by Halofuginone. The results from five independent experiments were summarized in ***(B)*** (n=3, mean ± SD; * and **: $p < 0.05$).

Manipulation of Egr-1 expression in cultured fibroblasts

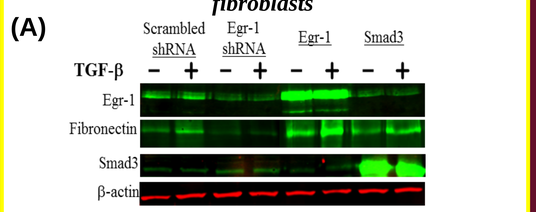

Figure 5. **(A)** RNA interference **(**"Egr-1 shRNA") and transgene overexpression ("Egr-1" and "Smad3") were used to manipulate the expression level of Egr-1 in cultured fibroblasts. Transfected cells were treated with TGF-β1 for 24 hours before harvest.

Effect of Halofuginone through AAR pathway

(A)

Protein	Percent Proline
Egr-1	12.89%
Smad 2 (isoform 1)	4.49%
Smad 2 (isoform 2)	7.78%
Smad 3 (isoform 1)	8.47%
β–Actin	6.42%

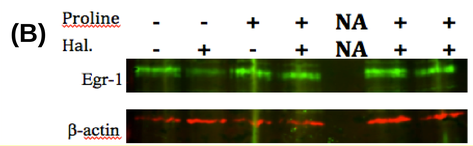

Figure 6. **(A)** Egr-1 contains a higher percentage of proline than other fibrotic signaling proteins, which affects the interaction of halofuginone and protein production level. **(B)** Fibroblasts were treated with proline at varying concentrations for 1 hour before the addition of Halofuginone ("Hal", 10 ng/ml) for 24 hours.

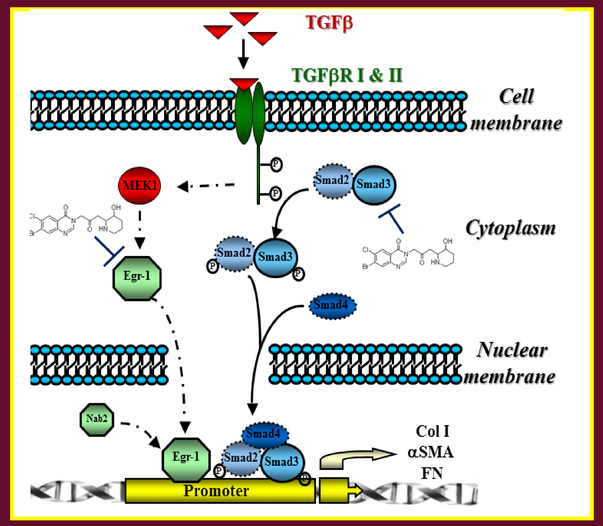

Figure 7. Proposed antifibrotic mechanism of Halofuginone. Halofuginone reduces the expression of both Smad3 and Egr-1that are essential regulators for fibrosis events in the corneal fibroblasts.

Conclusions:

- Halofuginone suppresses the Egr-1 expression in cultured human corneal fibroblasts; the addition of proline reverses the effect of halofuginone through the AAR pathway. However, the Western blots results were inconsistent and would need to be studied more in depth.
- Egr-1 is a profibrotic regulator in the corneal fibroblasts and may play roles in corneal fibrosis.

References

1. Nelson EF, Huang CW, Ewel JM, Angela AC, Yuan C. Halofuginone down-regulates Smad3 expression and inhibits the TGFbeta-induced expression of fibrotic markers in human corneal fibroblasts. Mol Vis 2012; 18: 479-487.
2. Fini ME. Keratocyte and Fibroblast Phenotypes in the Repairing Cornea. Progress in Retinal and Eye Research 1999; 18(4): 529-551.
3. Keller TL, et al. Halofuginone and other febrifugine derivatives inhibit proyl-tRNA synthetase. Nature Chem Biol 2012; 8: 311-317.