Connexin 46 Overexpression in Lens Epithelial Cells Causes Fiber Cell Differentiation

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Abstract

Purpose: Overexpression of Cx46 may down regulate the expression of Cx43 and may up regulate the expression of crystallins and/or aquaporin O in cells in culture. This could cause terminal differentiation of the epithelial cell line, implying that connexin proteins are involved in the differentiation process. The purpose of this study is to determine the effects of overexpression of Cx46 in a lens epithelial cell line expressing Cx43 and Cx50 on growth and differentiation properties.
Methods: Cx46 was transfected into 90% confluent N/N 1003A lens epithelial cells, and stably transfected cells were selected in G418-containing DMEM growth media. Cell localization of each connexin protein was determined +/- phorbal-12 myristate 13 acetate (TPA) using immunofluorescence and confocal microscopy. We also determined whether overexpression of Cx46 results in the down regulation of Cx50 or Cx43 by utilizing Western blots and PCR. Through these same methods, we were able to determine whether or not the overexpression of Cx46 causes the up regulation of gamma crystallin or aquaporin-0 synthesis, both of which are markers of terminal differentiation in these cells. Finally, through the use of a caspase-3 detection system, we ascertained if the differentiation results in the induction of apoptosis in the cell line.
Results: N/N 1003A cells overexpressing Cx46 were found to express levels of the protein gamma crystallin, a protein involved in the differentiation of cells. A shape change in the cells themselves from a typical N/N lens epithelial cell to the shape of a lens fiber cell was noted as well. It appears that overexpressing Cx46 in the N/N 1003A cell line will cause crystallin proteins to be expressed and differentiation to occur.
Conclusion: Cx46 may have functions in addition to gap junction activity. Results suggest that Cx46 overexpression may cause lens epithelial cells to differentiate into fiber cells.

Methods

Cell Morphology
Transfected cells were cultured in DMEM (low glucose) +G418 . Confluent cells were photographed under a Nikon inverted microscope scale bar, µm
Western Blotting
Transfected cells were cultured in DMEM (low glucose) +G418. Confluent cells were scraped from sides of flasks and rinsed twice in PBS to remove any remaining growth media. The PBS was removed by centrifugation, and the cells were resuspended in 1% cell lysis buffer + protease inhibitors after which it was sonicated for 20 seconds. Protein compositions of whole cell lysate was resolved in SDS electrophoresis and visualized by Western blot analysis.
Endogenous Cx46 Plaques
N/N cells were grown in the presence or absence of 300 nM TPA for 20 minutes on glass slides in six well plates. Cells were then fixed in 2.5% paraformaldehyde, treated overnight with Goat Anti-Cx46 primary antibody, then treated with AlexaFluor 568 in 3% BSA. Data was recorded using a Nikon confocal microscope.
Sucrose Gradient Centrifugation
Whole lenses were extracted with cell-lysis buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.1% protease inhibitor cocktail, 5 mM NaF, and 2 mM PMSF, and 1% Triton X-100 and incubated on ice for 30 minutes. Whole-cell lysates were mixed with an equal volume of 80% sucrose in Mes-NaCl buffer containing 25 mM Mes (pH 6.5), and 150 mM NaCl, 0.1% protease inhibitor cocktail, 5 mM NaF, and 2 mM PMSF and laid at the bottoms of 12-mL ultracentrifuge tubes. The samples were overlaid with 8 mL of a 5% and 35% continuous sucrose gradient in Mes-NaCl buffer and centrifuged at 245,000*g* for 22 hours at 4°C in a swinging bucket rotor (SW41 Ti; Beckman, Fullerton, CA). Fractions (1 mL each, 12 fractions total) were collected from the top of each gradient. Protein samples were precipitated with 10% trichloroacetic acid (TCA), separated in 10% SDS-PAGE and immunovisualized by Western blot analysis. Fractions 3 to 6 were pooled and used for the experiments. For the coimmunoprecipitation and phosphorylation assays, the fractions were sonicated in 0.1% SDS.
Immunoprecipitation Analysis
Fractions 3 to 6 from the sucrose density were immunoprecipitated with anti-Cx46, -Cx50, or -Cav-1 at 4°C for 4 hours, as described. The immunoprecipitate–agarose bead complexes were resolved by SDS-PAGE and visualized by Western blot with antisera to Cx46, Cx50, Cav-1, or PKC.
Phosphorylation of Cx46 and Cx50 on Ser and Thr
Fractions 3 to 6 from the gradient were immunoprecipitated with anti-Cx46 or Cx50 at 4°C for 4 hours as described. The immunoprecipitate–agarose bead complexes were resolved by SDS-PAGE and visualized by Western blot analysis with antisera to pS, pT, Cx46, or Cx50.

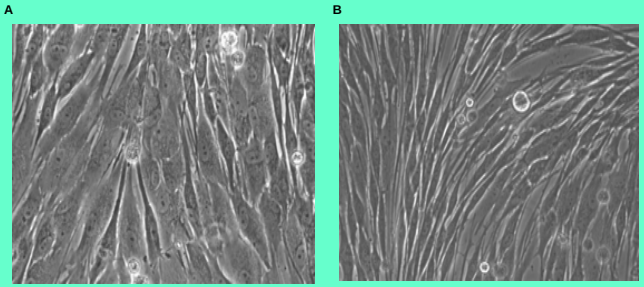


Fig. 1 Cell Morphology in N/N1003A cells overexpressing GFP and GFP-Cx46. Transfected cells were grown in DMEM + G418, and the morphology of the cells was recorded daily using a Nikon inverted microscope. **A:** Cells overexpressing GFP. **B:** Cells overexpressing GFP-Connexin 46. These pictures were taken using a stable transfected cell line.

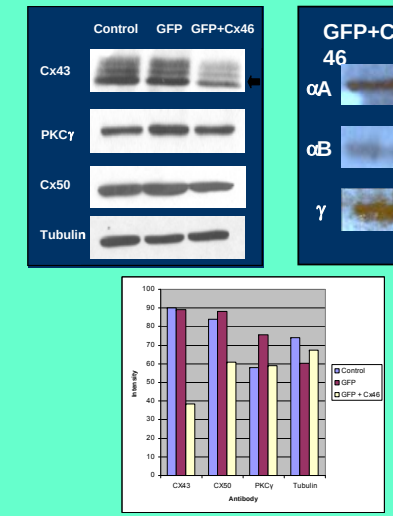
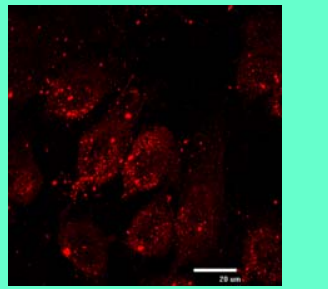
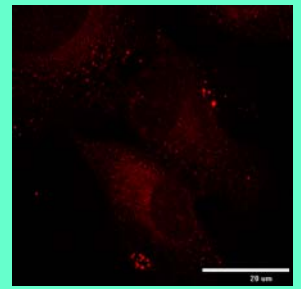


Fig 2. Downregulation of Cx43 and Presence of Crystallins in N/N Cells Overexpressing Cx46. **A:** Western blotting of N/N cells overexpressing GFP and GFP + Cx46 after being co-immunoprecipitated with Cx43, Cx50, PKCγ, and tubulin. **B:** Western blotting of N/N cells overexpressing GFP + Cx46 after co-immunoprecipiation with αA, αB, and γ crystallins. **Results:** αB Crystallin is known to always be expressed in N/N Cells. Therefore, the overexpression of Cx46 causes the expression of both αA and γ crystallin proteins. This overexpression also causes the downregulation of Cx43 in cells.





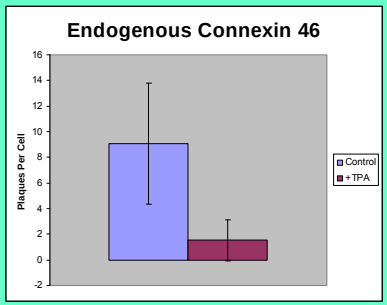


Fig. 4 Endogenous Cx46 Plaques in Presence or Absence of TPA. N/N Cells were grown in the presence or absence of TPA and reacted with Goat Anti-Cx46 antibodies. Data was gathered using a Nikon confocal microscope. Cx46 plaques 1µm or greater were counted.

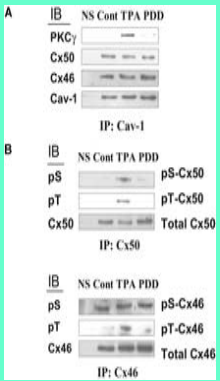


FIGURE 3. (**A**) Coimmunoprecipitation of PKC with Cx46, Cx 50, or Cav-1. Activated PKC was translocated to fractions containing Cx46 and Cx50. Lens plasma membrane fractions were immunoprecipitated with anti-Cav-1 and the complexes analyzed by Western blot with anti-Cx46 and -Cx50 or -PKC. Note that detectable levels of PKC translocation into membrane fractions occurred only in membranes from TPA-treated lenses. In contrast, treatment with TPA did not alter the distribution of Cx46 or Cx50 in the fractions. (**B**) Phosphorylation of Cx50 and Cx46 by TPA. Activated PKC phosphorylates Cx46 and Cx50. To determine connexin phosphorylation, membrane fractions of untreated and treated lenses were precipitated with anti-Cx50 and -Cx46 and then probed with antibodies against phosphoserines and phosphothreonines. Treatment with TPA induced phosphorylation of Cx50 at both serines and threonines but induced phosphorylation of Cx46 only at threonines. Treatment with the nonfunctional phorbol ester PDD had no effect. The experiments were performed in triplicate and nonspecific rabbit IgG was used as a negative control (NS). Blots loaded with antibodies against Cx46 or Cx50 served as the loading control (Zampighi et al. *IOVS*, 46, 3247-3255).

Summary

1. Overexpression of Cx 46 causes cell morphology changes.
2. Cx 46 overexpression causes a decrease in Cx 43 expression.
3. Cx46 overexpression causes an increase in αA and γ crystallin expression
4. Cx 46 does not respond to TPA as does Cx50 (different phosphorylation)
5. Cx46 does disassemble after TPA

Conclusions

Cx46 overexpression causes a decrease in Cx43 expression, and the expression of αA and γ crystallin

Support

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