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Regulation of Lens Gap Junctions by Transforming Growth Factor Beta

Bruce A. Boswell, Judy K. VanSlyke, and Linda S. Musil

Department of Biochemistry and Molecular Biology, Oregon Health & Science University, Portland, OR 97239

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Gap junction–mediated intercellular communication (GJIC) is essential for the proper function of many organs, including the lens. GJIC in lens epithelial cells is increased by FGF in a concentration-dependent process that has been linked to the intralenticular gradient of GJIC required for lens transparency. Unlike FGF, elevated levels of TGF-β are associated with lens dysfunction. We show that TGF–β1 or -2 up-regulates dye coupling in serum-free primary cultures of chick lens epithelial cells (dissociated cell-derived monolayer cultures [DCDMLs]) via a mechanism distinct from that utilized by other growth factors. Remarkably, the ability of TGF-β and of FGF to up-regulate GJIC is abolished if DCDMLs are simultaneously exposed to both factors despite undiminished cell–cell contact. This reduction in dye coupling is attributable to an inhibition of gap junction assembly. Connexin 45.6, 43, and 56–containing gap junctions are restored, and intercellular dye coupling is increased, if the activity of p38 kinase is blocked. Our data reveal a new type of cross-talk between the FGF and TGF-β pathways, as well as a novel role for TGF-β and p38 kinase in the regulation of GJIC. They also provide an explanation for how pathologically increased TGF-β signaling could contribute to cataract formation.

INTRODUCTION

Gap junctions are clusters of intercellular plasma membrane channels that act as conduits for the direct cell-to-cell movement of low-molecular-weight (≤1 kDa) solutes in virtually all multicellular animal tissues. By mediating the regulated intercellular diffusion of substances such as second messengers, ions, and nutritional metabolites, gap junctions serve to relay signals between, and maintain metabolic homeostasis within, adjacent cells (Harris, 2001; Goodenough and Paul, 2009). In vertebrates, gap junctions are composed of members of a closely related family of four-transmembrane–containing proteins known as connexins. Mutations in connexins are responsible for at least 10 human diseases, underscoring the essential role of these proteins in tissue development and homeostasis (Alldredge, 2008).

One organ in which the importance of gap junctions has been well established is the lens. The vertebrate lens consists of a monolayer of epithelial cells on its anterior surface and the highly elongated fiber cells that differentiate from them at the lens equator, which is the region at the border of the anterior and posterior faces of the organ (Piatigorsky, 1981; see Figure 10A for a schematic of lens structure). In all species examined, gap junction–mediated intercellular communication (GJIC) is higher at the equator of the lens that at either pole (Baldo and Mathias, 1992; Mathias *et al.*, 1997). This pole-to-equator gradient in GJIC is believed to direct the overall pattern of current and solute flow in the organ, allowing the lens to stay in metabolic and ionic homeostasis (and thus transparent) in the absence of intralenticular blood vessels (Mathias *et al.*, 1997; Donaldson *et al.*, 2001). Others have suggested that the high level of gap junctional coupling at the lens equator is required to facilitate the uptake of certain substances (e.g., cysteine) into the organ (Sweeney *et al.*, 2003). Three functionally distinct and noninterchangeable connexins are expressed in the lens. The epithelial cells contain connexin43 (Cx43), a species that is also found in a wide variety of other organs throughout the body (Musil *et al.*, 1990). Gap junctions in differentiating and mature fiber cells are composed of connexin50 (Cx50; White *et al.*, 1992) and connexin46 (Cx46; Paul *et al.*, 1991), the chick orthologues of which are, respectively, Cx45.6 (Jiang *et al.*, 1994) and Cx56 (Rup *et al.*, 1993). Mice in which the genes for Cx50 (White *et al.*, 1998) or Cx46 (Gong *et al.*, 1997) have been deleted have defects in lens clarity. Mutations in Cx46 or Cx50 that compromise their ability to mediate intercellular coupling lead to a variety of cataract phenotypes in humans (Hejtmancik, 2008) and other vertebrate species (Gong *et al.*, 2007).

We use primary cultures of embryonic chick lens epithelial cells, termed DCDMLs, to study the establishment and regulation of lens GJIC (Le and Musil, 1998; Le and Musil, 2001b; Boswell *et al.*, 2008, 2009). Unlike central epithelial explants, these serum-free cultures contain cells from both the equatorial and central regions of the lens epithelium. For as yet unknown reasons, DCDMLs are much more efficient than rodent-derived systems in forming gap junctions with the morphological and functional hallmarks of those found between newly differentiated fiber cells in vivo (FitzGerald and Goodenough, 1986; Menko and Boettiger, 1987). Using DCDMLs, we have shown that gap junction–mediated intercellular dye coupling is greatly increased by fibroblast growth factor (FGF) at levels at which it diffuses out of intact

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Address correspondence to: Linda S. Musil (musill@ohsu.edu).

Abbreviations used: ASC, anterior subcapsular cataracts; BMP, bone morphogenetic protein; Cx, connexin; DCDML, dissociated cell-derived monolayer culture; EMT, epithelial-to-mesenchymal transition; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GJIC, gap junction–mediated intercellular communication; TGF-β, transforming growth factor beta; VBCM, vitreous body–conditioned medium.

vitreous bodies and would have access to cells at the lens equator (Le and Musil, 2001a,b). The concentrations of FGF in the aqueous humor, to which cells in the more anteriorly located central regions of the lens epithelium are exposed, are too low to affect GJIC. These and additional experiments led to a model in which FGF is responsible for the observed pole-to-equator gradient of GJIC in the lens. Conditions that disrupt this asymmetry would be predicted to cause an imbalance in lens homeostasis and could lead to cataract.

In contrast to FGF, transforming growth factor beta (TGF-β) is an ocular growth factor long associated with lens pathology. Infection of mouse anterior lens epithelial cells in vivo with a TGF-β1–expressing adenovirus leads to cataracts indistinguishable in structure and composition from human and animal anterior subcapsular cataracts (ASC) caused by ocular trauma, surgery, or disease (e.g., atopic dermatitis, retinitis pigmentosa; Robertson *et al.*, 2007). The levels of TGF-β signaling in lens central and equatorial epithelial cells are enhanced in both human and animal ASC, and blocking TGF-β stimulation in animal models reduced the formation of injury-induced ASC (Saika *et al.*, 2001; Saika *et al.*, 2004a,b; Ishida *et al.*, 2005; Shirai *et al.*, 2006). A single injection of TGF-β into the vitreous body of rats causes a loss of lens transparency, and it has been suggested that intraocular administration of TGF-β could contribute to the very rapid progression of cataracts in human patients undergoing treatment to heal macular holes (Thompson *et al.*, 1995; Hales *et al.*, 1999; Robertson *et al.*, 2007). Moreover, several conditions associated with an increase in the levels of active TGF-β in the lens environment (e.g., diabetes, glaucoma, atypical retinoblastoma) have been linked to higher rates of cataract formation in humans (Harding *et al.*, 1993; Klein *et al.*, 1998; Ochiai and Ochiai, 2002; Kase *et al.*, 2008), as has a decrease in an anti-TGF-β defense system, estrogen (Klein *et al.*, 1994; Benitez del Castillo *et al.*, 1997; Hales *et al.*, 1997; Freeman *et al.*, 2001). Because of the role of TGF-β in lens pathology and the importance of gap junctions to normal lens function, we examined if TGF-β affects lens cell GJIC in either the absence or presence of physiologically relevant concentrations of FGF. Our studies reveal a new role for TGF-β and of p38 kinase in the regulation of gap junction formation and function. Cross-talk between the FGF- and TGF-β–signaling pathways could contribute to cataractogenesis.

MATERIALS AND METHODS

Materials

Recombinant bovine FGF-2, human TGF-β1, human TGF-β2, mouse noggin/Fc chimera, and human bone morphogenic protein 4 (BMP4) were from R&D Systems (Minneapolis, MN). The following antibodies were used: for Cx43, no. C8093 (Sigma-Aldrich, St. Louis, MO); for Cx56, a polyclonal rabbit antibody (Jiang *et al.*, 1994) kindly provided by Dr. J. Jiang (University of Texas Health Science Center); for Cx45.6, a polyclonal rabbit anti-peptide antibody (no. 505; Boswell *et al.*, 2009); for N-cadherin, a polyclonal pan-cadherin antibody (no. C3678; Sigma-Aldrich); for vinculin, no. V9131 (Sigma-Aldrich); for NCAM, H-94 (no. sc8305; Santa Cruz Biotechnology, Santa Cruz, CA), 5E, and anti-NCAM Fab fragments (Keane *et al.*, 1988); for ZO-1, R40.76. provided by Dr. Daniel Goodenough (Harvard Medical School); for Cx32, 7C6.C7, a kind gift of Dr. E. Hertzberg (Albert Einstein College of Medicine); for LCAM, antibody 7D6 (Developmental Studies Hybridoma Bank, University of Iowa); for FLAG, M2 (Sigma-Aldrich); for green fluorescent protein (GFP), JL-8 (Clontech, Mountainview, CA); for α-smooth muscle actin, 1A4 (Dako, Carpinteria, CA), and for phospho-Smad3, ab51451 from Abcam (Cambridge, MA). Anti-phospho-p44/42 MAP kinase (E10), anti-phospho-p38 (no. 9211), anti-phospho-Ser133 CREB (no. 9191), anti-phospho-AKT (no. 9275), and anti-phospho-Smad1 (no. 9511) were all purchased from Cell Signaling Technology (Danvers, MA). UO126 (used at 15 μM), SB431542 (3 μM), PD173074 (100 nM), and SB203580 (20 μM) were from Calbiochem (La Jolla, CA).

Cell Culture and Treatments

Cultures were prepared from E10 chick lenses and plated at 1.6×10^5 cells/well onto laminin-coated 96-well tissue culture plates as previously described by Le and Musil (1998). Cells were cultured in M199 medium plus BOTS (2.5 mg/ml bovine serum albumin, 25 mg/ml ovotransferrin, 30 nM selenium), penicillin G, and streptomycin (M199/BOTS), with or without additives at 37°C in a 5% CO_2 incubator. Where indicated, DCDMLs were incubated with UO126, PD173074, SB431542, or SB203580 for 45 min at 37°C before addition of growth factors.

Plasmids and Transient Transfection of Lens Cells

One day after plating, DCDML cultures were transfected in M199 medium without BOTS or antibiotics using Lipofectamine Plus (Invitrogen, Carlsbad, CA) as described in our previous studies (Le and Musil, 2001b; Boswell *et al.*, 2009). A cDNA-encoding human Cx50 (gift of Dr. T. White, SUNY Stony Brook) was tagged with the FLAG epitope before subcloning into pcDNA3. The constructs for Cx43-GFP, E208K Cx32, and LCAM were kind gifts of Drs. S. Taffet (SUNY Syracuse), R. Bruzzone (HKU-Pasteur Research Center), and W. Gallin (University of Alberta, Edmonton).

Cell Surface Biotinylation

DCDMLs were biotinylated at 4°C with sulfo-NHS-SS-biotin. After the reaction was quenched and the cells lysed in SDS, biotinylated proteins were precipitated with streptavidin-agarose and analyzed by Western blot (VanSlyke and Musil, 2005).

Immunoblot Analysis

For analysis of whole cell lysates, DCDML cultures were solubilized directly in SDS-PAGE sample buffer and boiled (Le and Musil, 2001b). For analysis of endogenously expressed connexins, total membrane fractions were prepared as described by VanSlyke and Musil (2002) and boiled in SDS-PAGE sample buffer. Equal amounts of protein were transferred to polyvinylidene fluoride membranes, and immunoreactive proteins were detected as previously described (Boswell *et al.*, 2008). All results shown are representative of three or more independent experiments.

Immunofluorescence Microscopy

DCDMLs grown on glass coverslips were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) and processed for immunocytochemical detection of connexins and other proteins as previously described (Le and Musil, 1998, 2001b). Images were captured at 630× magnification using a Leica DM LD photomicrography system (Deerfield, IL) and Scion Image 1.60 software (Frederick, MD).

Scrape-Loading and Dye Transfer Assay

DCDML cultures were assessed for gap junction–mediated intercellular coupling as previously described (Le and Musil, 2001b; Boswell *et al.*, 2008, 2009). In brief, the culture medium from a 3-d-old, confluent monolayer of lens cells was removed and saved. The cells were rinsed three times with Hanks' balanced salt solution containing 1% bovine serum albumin (HBC), after which a 27-gauge needle was used to create two longitudinal scratches through the cell monolayer in the presence of a solution of Dulbecco's PBS containing one or more of the following: 0.75% rhodamine dextran (M_r = 10 kDa; Invitrogen), 1% Lucifer yellow (Sigma-Aldrich), or 0.25% Alexa594 (Invitrogen). After 1 min (for Lucifer yellow plus rhodamine dextran) or 2 min (for Alexa594 plus Lucifer yellow), the culture was rinsed three times with HBC and then incubated for an additional 8 min (for Lucifer Yellow plus rhodamine dextran) or 20 min (for Alexa594 plus Lucifer yellow) in the saved culture medium. The culture was then rinsed three times with PBS and fixed. Dyes were visualized by fluorescence microscopy. The distance that Lucifer yellow spreads into the monolayer is directly proportional to the number of coupled cells and was quantitated as previously described (Le and Musil, 2001b; Boswell *et al.*, 2008). The scrape-loading dye transfer assay allows simultaneous monitoring of dye coupling in a large population of cells; its suitability for the assessment of gap junction–mediated intercellular communication has been well documented (el-Fouly *et al.*, 1987; Venance *et al.*, 1995; Opsahl and Rivedal, 2000).

RESULTS

Effect of TGF-β on Gap Junction–mediated Dye Coupling in Lens Cells in the Absence of FGF

We use the well-characterized scrape load/dye transfer assay (el-Fouly *et al.*, 1987; Venance *et al.*, 1995; Opsahl and Rivedal, 2000) to measure GJIC in DCDMLs and other adherent cell types (Le and Musil, 2001b; Boswell *et al.*, 2008, 2009). A 27-g needle was used to make a vertical scratch

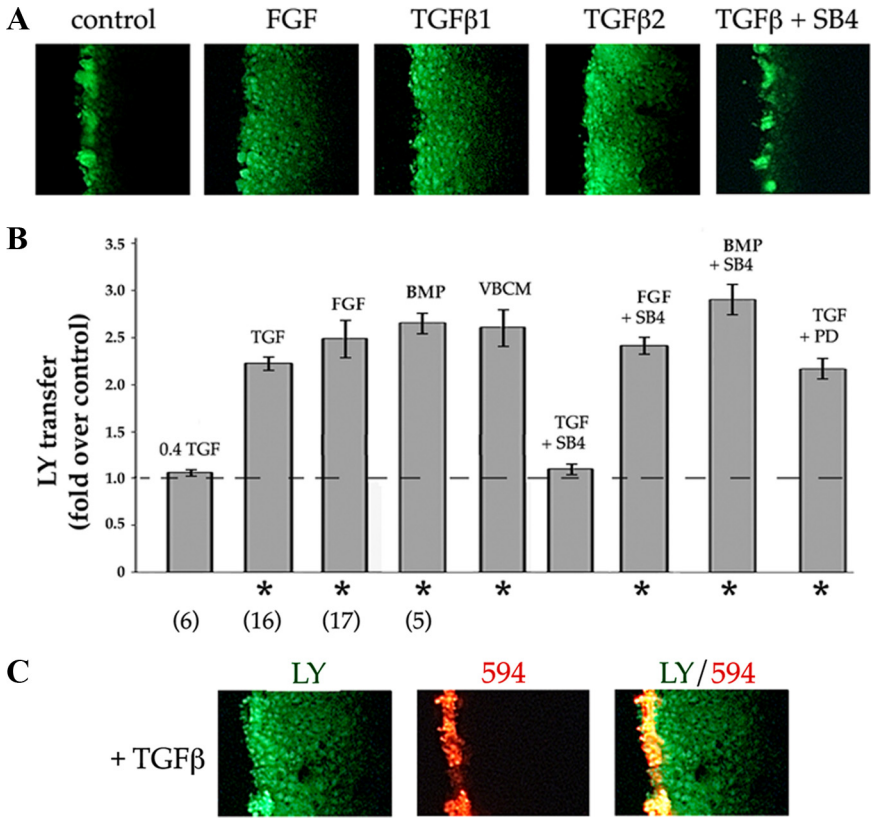

Figure 1. TGF-β increases the GJIC intercellular transfer of Lucifer yellow, but not of a Cx43-selective dye, in DCDMLs. TGF-β (at 4 ng/ml), FGF-2, BMP4 (both at 15 ng/ml), or VBCM were added to DCDMLs on day 1 of culture, with or without inhibitor (SB4, SB431542; PD, PD173074) as indicated. On day 3, scrape-load GJIC assays were conducted with a mix of Lucifer yellow (LY; permeable to Cx43, Cx45.6, and Cx56 channels) and either rhodamine dextran (impermeable to all gap junctional channels; A and B) or Alexa594 (permeable to Cx43 gap junctional channels; C) as described in *Materials and Methods*. In all cases, the M_r = 10 kDa rhodamine dextran remained confined to the cells at the wound edge into which dye had been directly introduced during the scrape-loading process (not shown). Each panel in A and C depicts a portion of the right half of the scrape/load wound. The distance of LY transfer from the wound edge into the control (untreated) monolayer is 50 μm. In B, the fold increase in the distance of transfer of LY into the cell monolayer was quantitated relative to untreated control cells within the same experiment. 0.4 TGF, 0.4 ng/ml TGF-β1. Unless indicated in parenthesis, n = 3. Values significantly different from control ($p < 0.015$) as assessed by the two-tailed paired Student's *t* test are indicated by the asterisks.

through the center of a confluent monolayer of cells in the presence of one or more fluorescent dyes. Dyes incapable of penetrating gap junctions remained confined to the wounded cells bordering the scratch, whereas gap junction-permeable compounds such as Lucifer yellow were transferred to adjacent, unperturbed cells via open gap junctional channels. We found that culturing DCDMLs for 48 h in the presence of 4–40 ng/ml TGF-β1, but not 0.4 ng/ml TGF-β1, increased the intercellular spread of Lucifer yellow to an extent slightly less than that obtained with maximally effective concentrations of factors previously shown to enhance dye coupling in lens cells, namely 15 ng/ml FGF2, 15 ng/ml BMP4 (or BMP2 or BMP7; not shown), or culture medium conditioned by intact vitreous bodies (VBCM [vitreous body–conditioned medium], which contains both FGF and BMP; Le and Musil, 2001b; Boswell *et al.*, 2008; Figure 1, A and B). Up-regulation of Lucifer yellow dye coupling by TGF-β was blocked by SB431542, a highly selective small molecule inhibitor of the TGF-β-specific ALK5 receptor (Inman *et al.*, 2002), but not by the FGFR inhibitor PD173074. Similar results were obtained with recombinant TGF-β2. SB431542 had no effect on dye transfer in response to FGF or BMP. Notably, not all growth factors capable of inducing a response in DCDMLs increase GJIC. For example, neither IGF-1 or insulin has any detectable effect on dye coupling despite robust activation of downstream signaling pathways (e.g., ERK and AKT; Le and Musil, 2001b; Le and Musil, 2001a).

There are several reports of TGF-β increasing cell–cell coupling mediated by Cx43 in nonlenticular cell types (Gibson *et al.*, 1994; Chiba *et al.*, 1994; Kabir *et al.*, 2005; Rama *et al.*, 2006; Tacheau *et al.*, 2008; Hills *et al.*, 2009). To determine if lens cells behave similarly, we took advantage of the finding by Dong *et al.* (2006) that gap junction channels composed of Cx43, but not of Cx45.6, are permeable to the dye Alexa594. In the absence of exogenously added growth factors, little to no intercellular transfer of Alexa594 was detected in DCDMLs under conditions in which the dye readily passes between CHO cells that utilize Cx43 as their sole connexin (Boswell *et al.*, 2009). DCDMLs remained impermeable to Alexa594 after a 48-h treatment with 4 ng/ml TGF-β, indicating that the observed increase in Lucifer yellow coupling cannot be mediated by homomeric Cx43 gap junctional channels (Figure 1C). To our knowledge, this is the first example of up-regulation by TGF-β of the function of a nonCx43 connexin in any cell type.

As is the case with all other previously described physiologically relevant GJIC-enhancing growth factors (i.e., FGF, BMP, and VBCM), a >12-h exposure to TGF-β was required for significant up-regulation of Lucifer yellow transfer in DCDMLs. In contrast to the aforementioned factors (Boswell *et al.*, 2008), however, up-regulation of GJIC by TGF-β was not blocked if the cells were cocultured in the presence of noggin, a highly specific protein antagonist of BMP 2, 4, and 7 binding to BMP receptors (Figure 2A). Thus enhancement of GJIC by TGF-β, unlike that by FGF, is not dependent on signaling initiated by the BMPs (4 and 7) that are endogenously produced by lens cells. Up-regulation of GJIC by TGF-β is also distinguished from up-regulation by FGF in that it was not inhibited when activation of ERK kinase was blocked using UO126, a potent, nontoxic, and highly specific inhibitor of the kinases (MEK1/2) immediately upstream of ERK in the MAP kinase cascade (Favata *et al.*, 1998; Le and Musil, 2001b).

One aspect in which TGF-β differs from all other GJIC-enhancing factors examined is that it can induce epithelial cells in the lens to undergo an epithelial-to-mesenchymal transition (EMT) into myofibroblast-like cells, as it does in

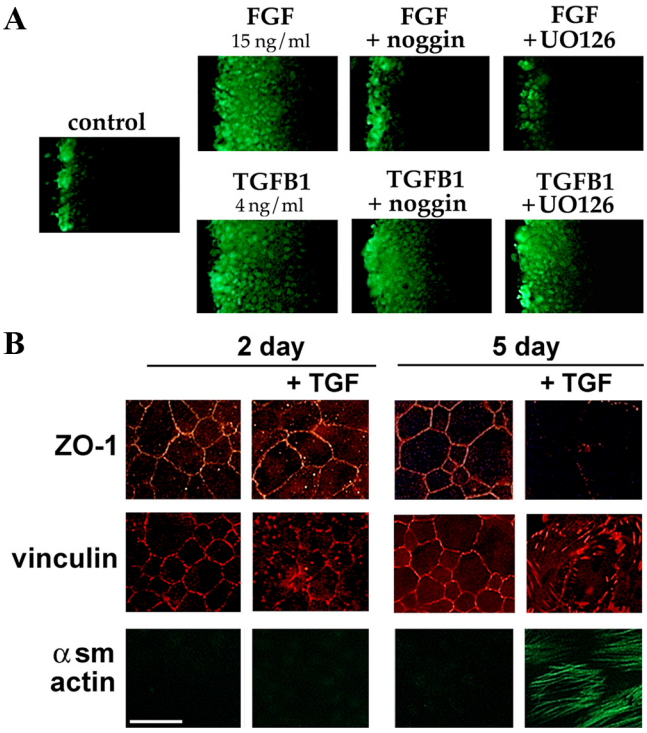

Figure 2. TGF-β up-regulates GJIC in lens cells by a novel pathway that does not require BMP or ERK signaling and is independent of TGF-β–induced EMT. (A) DCDMLs were cultured for 48 h with 15 ng/ml FGF-2 or 4 ng/ml TGF-β1 in either the absence or continuous presence of the BMP2/4/7 pathway blocker noggin or the ERK pathway inhibitor UO126. Cells were assessed for GJIC as in Figure 1A; only the Lucifer yellow images are shown. Representative of 3–5 independent experiments. Fold increase Lucifer yellow transfer over control was 2.5 ± 0.3 for TGF-β + noggin and 2.3 ± 0.1 for TGF-β + U0126. (B) DCDMLs were cultured for 2 or 5 d with 4 ng/ml TGF-β1 before immunocytochemical analysis of ZO-1, vinculin, and α-smooth muscle actin. Note that EMT-associated changes in all three proteins are detectable only at the latter time point. Scale bar, 20 μm.

many other organs (Wormstone *et al.*, 2002; Lovicu *et al.*, 2004a,b). Accepted markers for EMT in lens cells include decreased staining of ZO-1 at cell–cell interfaces and a dramatic increase in the formation of α-smooth muscle actin–positive stress fibers (Stump *et al.*, 2006). As expected, a 5-d exposure to 4 ng/ml TGF caused some of the cells in DCDMLs to display these hallmarks of EMT, in addition to redistribution of vinculin from cell–cell contacts into focal adhesions (Figure 2B). None of these markers of EMT were detectable after an only 2-d exposure to TGF-β, a time at which GJIC was already maximally elevated. EMT and up-regulation of GJIC are also separable by their concentration dependence: although TGF-β1 at 0.4 ng/ml markedly increased the expression of EMT markers (not shown), this amount was not sufficient to enhance Lucifer yellow dye coupling (Figure 1B). We conclude that up-regulation of GJIC by TGF-β is not a downstream consequence of completion of TGF-β-induced EMT.

Combining FGF and TGF Reduces GJIC Up-regulated by Either Factor Alone

We first cultured DCDMLs with 4 ng/ml TGF-β in combination with a concentration of FGF (1 ng/ml) comparable to that accessible to lens central epithelial cells from aqueous humor in that it can stimulate cell division, but not fiber differentiation or GJIC (Schulz *et al.*, 1993; Le and Musil, 2001a,b). The presence of FGF at 0.3–1 ng/ml did not significantly affect the ability of 4 ng/ml TGF-β to enhance Lucifer yellow transfer (Figure 3). A very different result was obtained if DCDMLs were cocultured with FGF at the concentration at which it diffuses out of intact vitreous bodies. Although 15 ng/ml FGF and 4 ng/ml TGF-β each markedly increased Lucifer yellow transfer in DCDMLs when added separately, the combination of the two factors reduced dye movement to 0.95 ± 0.19 (n = 26) of control (untreated) levels. This inhibition required prolonged (>24 h) coincubation with FGF plus TGF-β at concentrations greater than 0.4 ng/ml. DCDMLs cultured for 2 d with 4 ng/ml TGF-β plus 15 ng/ml FGF (a combination that hereafter will be referred to as TGF + FGF) appeared as healthy and as confluent as untreated controls, and cell-to-cell spread of the Cx43-selective dye Alexa594 continued to be negligible (not shown). We have previously shown that serum, at levels that are not physiological for the avascular lens, increases Lucifer yellow transfer in DCDMLs (Le and Musil, 2001b). Fetal calf serum continued to increase dye coupling in cells cocultured with TGF-β, indicating that the inhibitory effect of TGF-β is specific for cell–cell coupling stimulated by FGF.

The Combination of TGF-β and FGF Affects GJIC at the Level of Gap Junction Assembly

As in other cell types, assembled gap junctions in DCDMLs are visualized as punctate or linear concentrations of anti-connexin staining at cell–cell interfaces. We have previously reported that FGF does not qualitatively or quantitatively affect the immunolocalization of the three connexins expressed in DCDMLs, indicating that FGF up-regulates GJIC by increasing the permeability, instead of the number, of gap junctional channels (Le and Musil, 2001b). This also appears to be the case with TGF-β, given that the staining patterns for Cx43, Cx45.6, and Cx56 in DCDMLs cultured with 4 ng/ml TGF-β were indistinguishable from those in control or FGF-treated cells (Figure 4A). Coculture of cells with 4 ng/ml TGF-β plus 1 ng/ml FGF also had no detectable effect on gap junction immunostaining (not shown). In contrast, the combination of 4 ng/ml TGF-β and 15 ng/ml FGF (TGF + FGF) led to a major decrease in gap junction immunostaining for all three connexins within 2 d (Figure 4A). Importantly, although the shape of the cells became less polygonal, they appeared to remain in close cell–cell contact. This was confirmed by the unchanged localization of the cell adhesion molecules N-cadherin (the main protein responsible for cell–cell adhesion in lens cells; Volk and Geiger, 1986; Meyer *et al.*, 1992) and NCAM (Watanabe *et al.*, 1989) at intercellular interfaces. Two proteins associated with cadherins at adherens junctions in epithelial cells, ZO-1 and vinculin, also remained at cell–cell borders (Figure 4B).

Unless clustered in gap junctions or artificially accumulated within a single organelle, connexins expressed at endogenous levels can be difficult to localize using conventional immunofluorescence microscopy. The reduction in apparent anti-Cx43, -Cx45.6, and -Cx56 immunostaining signal in DCDMLs cultured in TGF + FGF could therefore be due to either a loss of connexin protein or to a lack of assembly of connexins into gap junctions. To address this issue, we carried out quantitative Western blotting of total membrane fractions. There was no decrease in the levels of Cx43 or of Cx45.6 in TGF + FGF–treated cells relative to (untreated) controls (Figure 5A). Similar results were obtained for N-cadherin. Although culturing in TGF + FGF did reduce the levels of Cx56, this decrease was always

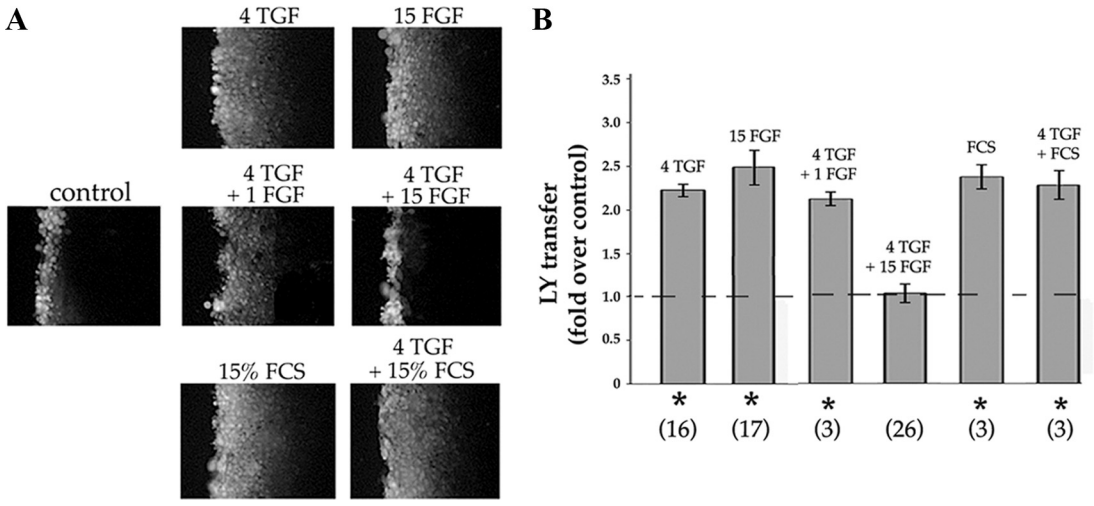

Figure 3. Reciprocal, concentration-dependent inhibition of FGF- and TGF-β–stimulated dye coupling in lens cells. DCDMLs were cultured for 48 h with FGF-2 and/or TGF-β1 at the concentrations indicated (in ng/ml). (A) Cells were assessed for gap junction–mediated intercellular transfer of Lucifer yellow as in Figure 1. (B) The fold increase in the distance of transfer of Lucifer yellow into the cell monolayer was quantitated relative to untreated control cells within the same experiment. The asterisks denote values significantly different from control ($p < 0.015$) as assessed by the two-tailed paired Student's t test. For the remaining data set, $p > 0.5$.

partial (52 ± 17%; n = 4). All three connexins migrate on SDS-PAGE as multiple bands due to posttranslational phosphorylation (Musil *et al.*, 1990; Jiang *et al.*, 1994; Shearer *et al.*, 2008; Wang and Schey, 2009). Although the intracellular site (and functional significance) of these modifications are unclear for Cx45.6 and Cx56, it is well established that Cx43 only becomes converted to slower migrating phosphospecies after its transport to the cell surface (Musil and Goodenough, 1991; Solan and Lampe, 2009). The presence of this band in TGF + FGF–treated DCDMLs (Figure 5A, asterisk) suggests that connexins continue to be delivered to the plasma membrane. Additional support for this contention is provided below (see Figure 6). The half-life of connexins in DCDMLs, as in other cell types (Fallon and Goodenough, 1981; Goodenough and Paul, 2009), appears to be quite short (Le and Musil, 2001b). Most of the connexin protein detected by Western blotting in Figure 5A is therefore <6 h old, indicative of ongoing synthesis of connexins in TGF + FGF–treated cells during the 2-d culture period.

Cx43, as well as Cx45.6 and Cx56 and their mammalian orthologues (Cx50 and Cx46, respectively), are all capable of forming gap junctions in the absence of any other connexin species (Rup *et al.*, 1993; Hopperstad *et al.*, 2000; Tong *et al.*, 2004). Deletion of Cx43, Cx46, and/or Cx50 from the mouse lens does not prevent gap junction formation by the remaining connexin(s) (Gong *et al.*, 1997; White *et al.*, 1998). Because each lens connexin can function independently, the partial reduction in Cx56 levels observed in DCDMLs after exposure to TGF + FGF cannot account for the inability of (normally expressed) Cx43 and Cx45.6 to form gap junctions. A 50% reduction in Cx46 levels in Cx46 ±, Cx50 +/+, Cx43 +/+ mice proportionately reduces (but does not abol-

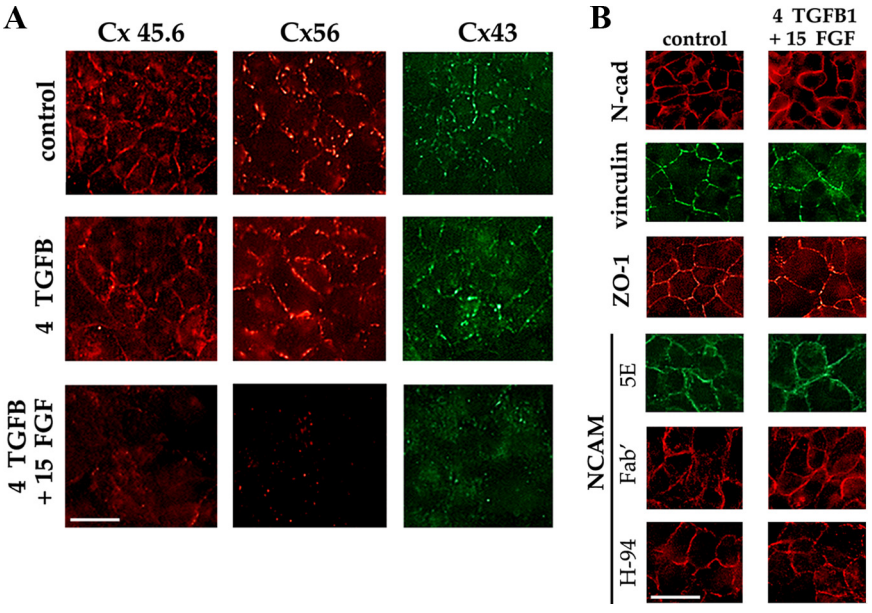

Figure 4. Loss of gap junctions, but not cell–cell contact, in TGF- + FGF-treated DCDMLs. DCDMLs were cultured in M199/BOTS medium for 2 d without additions (control), or in the continuous presence of the indicated factors. (A) Cultures were then immunostained for Cx45.6, Cx56, Cx43 and for (B) N-cadherin, vinculin, ZO-1, vinculin, or NCAM, the latter with three different antibodies. Scale bars, 20 μm.

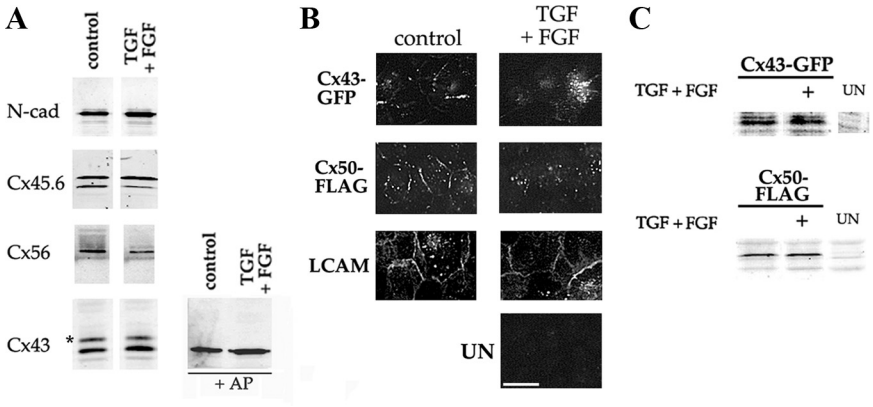

Figure 5. Loss of gap junctions in TGF-β + FGF–treated DCDMLs is not due to a lack of connexin synthesis. (A) Total membrane fractions prepared from DCDMLs cultured for 48 h in the absence or presence of 4 ng/ml TGF-β1 plus 15 ng/ml FGF were analyzed for expression of endogenous N-cadherin, Cx45.6, Cx56, or Cx43. The asterisk indicates the position of phosphorylated Cx43. In lanes marked +AP, samples were dephosphorylated with alkaline phosphatase (Musil *et al.*, 1990) before analysis. Fold expression of connexins in TGF + FGF–treated cells relative to untreated controls was for Cx45.6, 1.22 ± 0.2 (n = 4); for Cx56, 0.52 ± 0.17 (n = 4), and for Cx43, 1.6 ± 0.8 (n = 5). (B and C) One-day-old DCDML cultures were transfected with plasmids encoding Cx43-GFP, Cx50-FLAG, or the cell–cell adhesion molecule LCAM, after which they were cultured for 48 h with or without TGF + FGF. Expression of the exogenous protein was assessed by immunocytochemistry (B) and/or by Western blotting of total cell lysates (C) using antibodies directed against the epitope tag or LCAM. UN denotes anti-FLAG/anti-mouse IgG-Alexa488 immunostaining in untransfected cells (Figure 5B), and anti-GFP or anti-FLAG immunoblots from untransfected cell lysates. All lanes contained equal amounts of total protein. Scale bar, 20 μm.

ish) Cx46 immunostaining in gap junctions, and decreases gap junction–mediated intercellular coupling in differentiating fiber cells by only 25% (Gong *et al.*, 1998). It is therefore highly unlikely that the ~50% decrease in Cx56 levels in TGF + FGF–treated DCDMLs is responsible for the virtual absence of Cx56 gap junctions in these cells. Instead, it appears that TGF + FGF inhibits the accumulation of connexins in gap junctions at a step after connexin synthesis. This was confirmed by experiments in which DCDMLs were transfected with plasmids encoding lens connexins under the control of a constitutively active (and therefore growth factor-insensitive) CMV promoter (Figure 5, B and C). In untreated cells, Cx43-GFP and Cx50-FLAG were localized at cell–cell interfaces in a linear or punctate pattern, and were detectable in whole cell lysates by Western blot. Despite similar levels of protein expression, gap junctional staining for both constructs was greatly reduced in cells cultured for 48 h in TGF + FGF. LCAM is closely related to E-cadherin, a cell–cell adhesion molecule expressed in central epithelial cells in the mammalian lens (Xu *et al.*, 2002). Transiently expressed LCAM accumulated at cell–cell interfaces in both control and in TGF + FGF–treated DCDMLs, as expected given our other evidence (Figures 4B and 5A) that TGF + FGF does not compromise cell–cell adhesion.

The lack of punctate anti-connexin staining attributable to either cell surface or internalized gap junctions in TGF + FGF–treated cells (Figures 4A and 5B) is consistent with a defect in gap junction assembly. In other types of cells deficient in gap junction assembly, connexins are rapidly internalized from the plasma membrane. Some of these connexin molecules recycle back to the cell surface, whereas another fraction is degraded in lysosomes (VanSlyke and Musil, 2005). In TGF + FGF–treated cells, staining for connexins in the plasma membrane, endosomes, or lysosomes was not obvious, indicating that they do not stably accumulate in any of these compartments. Post-Golgi connexin pools were, however, readily visualized if cells were exposed to chloroquine, a lysosomotropic amine that traps connexins in enlarged endosomes and lysosomes (Musil *et al.*, 2000; VanSlyke and Musil, 2005). In contrast, a connexin mutant incapable of transport to the cell surface was excluded from these structures (Figure 6A). More direct evidence that connexins continue to be transported to the plasma membrane in TGF + FGF–treated DCDMLs was obtained using cell surface biotinylation (Figure 6B). As in all other cell types examined (Musil and Goodenough, 1991; VanSlyke and Musil, 2005), biotinylation with the membrane-impermeant biotinylating reagent sulfo-NHS-SS-biotin is confined to the plasma membrane in DCDMLs as demonstrated by the inability to biotinylate the uncleaved, intracellular precursor form of N-cadherin. The amounts of N-cadherin and Cx43 detectable on the cell surface were not reduced by a 48 h exposure of DCDMLs to TGF + FGF. In both control and treated cells, cell surface biotinylated Cx43 was recovered almost exclusively in the slower migrating form that arises from phosphorylation of Cx43 after its delivery to the plasma membrane. Cx56 could also be biotinylated from the surface of TGF + FGF–treated DCDMLs, although less was obtained than from untreated cells as was also the case when total membranes were assayed (Figure 5A). Cx45.6 (as well as its human ortholog Cx50) lacks lysine residues in its extracellular domains amenable to biotinylation with sulfo-NHS-SS-biotin, precluding its analysis with this technique. We conclude that treatment with TGF + FGF does not prevent connexins from being transported to the cell surface. More detailed studies will be required to determine if these factors change the kinetics with which connexins are internalized from, or recycled back to, the plasma membrane.

Reciprocal Inhibition of Gap Junctions by TGF and FGF Is Mediated by p38

The p38 kinase pathway has been shown to be downstream of FGF in several types of mammalian cells, including those from lens epithelium. Although not an integral part of the canonical TGF-β signaling pathway, p38 has also been reported to be activated in response to TGF-β treatment in several cell types (Moustakas and Heldin, 2005), including a human lens epithelial cell line (Dawes *et al.*, 2009). As expected, the well-known p38 agonist anisomycin activated p38 in DCDMLs as assessed with an antibody specific for the activated (phospho) form of the kinase. We found that 15 ng/ml FGF and 4 ng/ml TGF-β (1 and 2) also increased the amount of phospho-p38 in DCDMLs, as did the combination of TGF-β plus FGF (Figure 7A).

To assess the role of p38 activity in DCDMLs, we used SB203580. SB203580 has been well characterized to inhibit the SAPK2a and 2b isoforms of p38 by ≥90% without appreciably (<30%) affecting related kinases such as ERK (extracellular signal–regulated kinase) or JNK (c-Jun N-terminal kinase), or 17 other widely expressed kinases (Davies

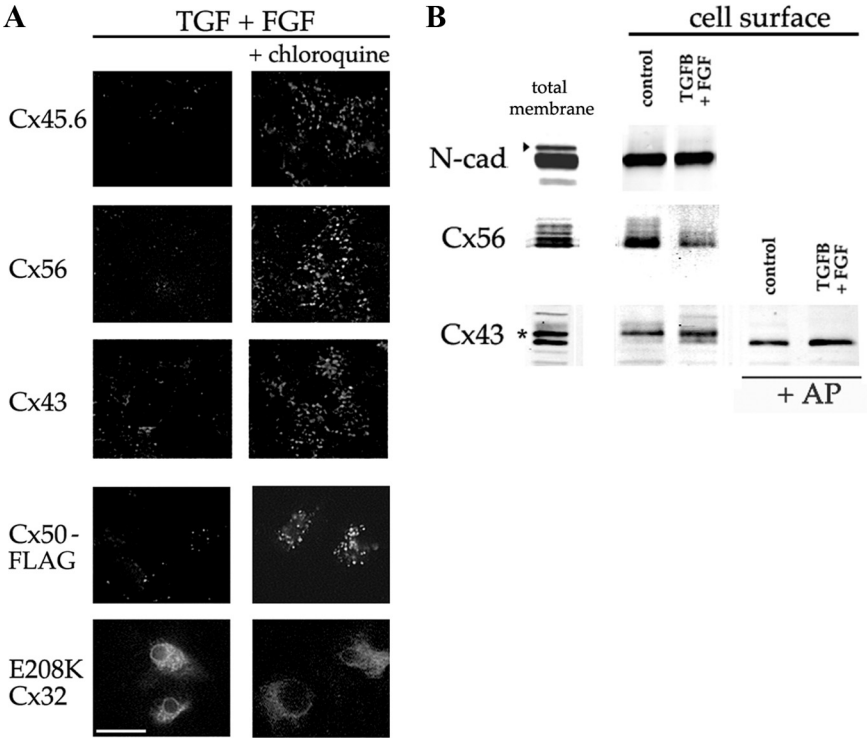

Figure 6. TGF- + FGF does not prevent transport of connexins to the cell surface. (A) DCDMLs cultured for 2 d with TGF + FGF were incubated for 7 h in either the absence or presence of 200 μM chloroquine. The lysosomotropic amine caused endogenous Cx45.6, Cx56, and Cx43, as well as exogenously expressed Cx50-FLAG, to accumulate in swollen endosomes and lysosomes. The distribution of an ER-retained connexin mutant (E208K Cx32; VanSlyke *et al.*, 2000) was unaffected by chloroquine. Scale bar, 20 μm. (B) DCDMLs were cultured for 2 d in either the absence or presence of TGF + FGF before cell surface biotinylation. After cell lysis, streptavidin-precipitated biotinylated proteins were analyzed by Western blotting for N-cadherin, Cx56, or Cx43. In lanes marked +AP, samples were dephosphorylated with alkaline phosphatase before SDS-PAGE. Lanes on left are blots of total membrane fractions. The arrowhead indicates the position of the intracellular, uncleaved proform of N-cadherin, which is not detectable by cell surface biotinylation. *, phosphorylated Cx43. Fold expression of cell surface biotinylated connexin in TGF + FGF–treated cells relative to untreated controls was 1.22, 1.15, 1.6, and 1.45 for Cx43 in four independent experiments and for Cx56, 0.24 and 0.24 in two experiments.

et al., 2000). Its suitability for inhibiting p38 in primary cell systems (including in vivo) over a multiday period has been documented in many studies (Hayashi *et al.*, 2003; Hu *et al.*, 2003; Wu and Bennett, 2005; Xu *et al.*, 2007). As expected, preincubating DCDMLs with 20 μM SB203580 for 45 min up to 6 d blocked the ability of anisomycin to stimulate phosphorylation of CREB on serine 133, a major downstream substrate of p38 (Figure 7B). Anisomycin also activates JNK, but as in other cell types (Iordanov *et al.*, 1997), JNKs do not activate CREB in DCDMLs as demonstrated by the inability of a JNK inhibitor (SP600125) to inhibit anisomycin-induced CREB phosphorylation. Importantly, a 1–2-d treatment of DCDMLs with SB203580 did not block processes that are not mediated by p38 activity, including phosphorylation of 1) ERK stimulated by FGF2, 2) AKT stimulated by IGF1, 3) CREB stimulated by the cAMP analogue 8-CPT, 4) Smad3 stimulated by TGF-β, 5) Smad1 stimulated by BMP4, and 6) p38 stimulated by FGF or TGF-β (SB203580 inhibits the phosphorylation of substrates by activated p38, not the activation of p38 itself; Kumar *et al.*, 1999; Supplemental Figure 1). SB203580 did not reduce the viability of DCDMLs, or their capacity for protein synthesis (not shown).

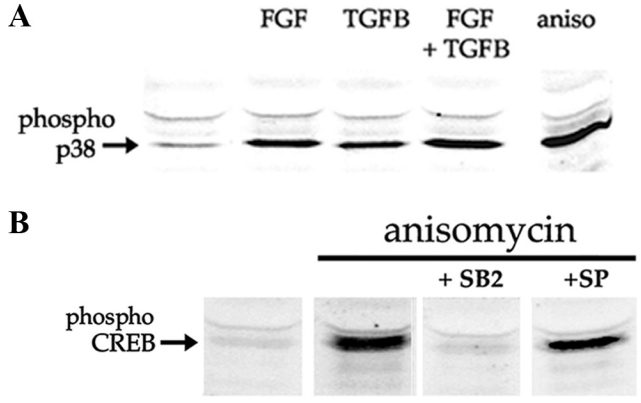

Figure 7. Activation of the p38 kinase pathway by TGF-β and/or FGF in DCDMLs, and its inhibition by SB203580. (A) DCDMLs were incubated without factors, or with 15 ng/ml FGF2, 4 ng/ml TGF-β1, 15 ng/ml FGF2 plus 4 ng/ml TGF-β1, or 3 μg/ml anisomycin for 1.5 h. (B) DCDMLs were incubated with or without 3 μg/ml anisomycin for 30 min. Where indicated, cells were pretreated with either 20 μM SB203580 (SB2) or 20 μM SP600125 (SP). (A and B) Whole cell lysates were probed with antibodies specific for the phosphorylated (activated) forms of p38 (A) or CREB (phospho-Ser133) (B).

With this demonstration that SB203580 is a potent and specific blocker of p38 in DCDMLs, we examined its effect on gap junctions. We found that inhibiting p38 with SB203580 completely reversed the effects of TGF + FGF on gap junctional immunostaining of Cx45.6, Cx56, and Cx43, provided it was added to the cells for $\geq$24 h (Figure 8). To determine whether these gap junctions were functional and to explore the potential mechanism of this effect, we conducted a series of dye transfer experiments (Figure 9). SB203580 had no effect on GJIC in cells cultured in otherwise unsupplemented medium or in DCDMLs treated with either TGF-β or FGF. SB203580 did, however, dramatically increase dye transfer in cells cultured with TGF + FGF for 24–48 h. GJIC was also enhanced in TGF + FGF–treated cells by another highly specific, mechanistically distinct inhibitor of p38, BIRB 0796 (Bain *et al.*, 2007; Supplemental Figure 2). In contrast, small-molecule inhibitors of two other pathways reported to be downstream of FGF or TGF-β in lens cells, JNK (SP600125) and PI3K/AKT (LY294002; Golestaneh *et al.*, 2004; Iyengar *et al.*, 2006; Cho *et al.*, 2007; Choi *et al.*, 2007), had no effect on GJIC in TGF + FGF–treated cells (not shown).

Culturing DCDMLs with TGF + FGF in the presence of the ALK5 TGF-β receptor inhibitor SB431542 restored GJIC to the levels obtained in cultures incubated with 15 ng/ml

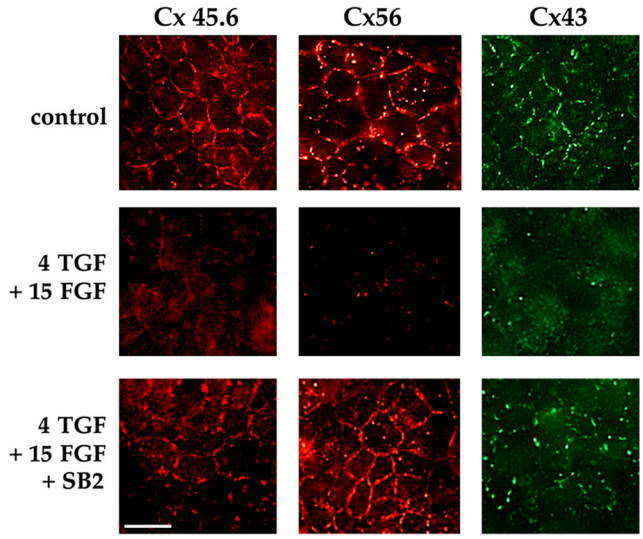

Figure 8. Inhibition of p38 restores gap junctions in TGF + FGF–treated lens cells. DCDMLs were untreated (controls), or incubated with 4 ng/ml TGF-β plus 15 ng/ml FGF2 in either the absence or presence of SB203580 (SB2). After 48 h, cultures were immunostained for Cx45.6, Cx56, and Cx43. Scale bar, 20 μm.

FGF alone. Blocking FGF receptor function with PD173074 (Boswell *et al.*, 2008) also increased GJIC in TGF + FGF–treated cells (Figure 9B). These results indicate that in order for the combination of TGF-β and FGF to inhibit gap junctions, signaling through the FGF receptor is required to block TGF-β signaling, and, at the same time, signaling through the TGF-β receptor is required to block FGF signaling. Therefore, SB203580 could be elevating GJIC in TGF + FGF–treated cells by 1) blocking TGF-β from inhibiting FGF-mediated up-regulation of GJIC, thereby allowing FGF to enhance GJIC; 2) blocking FGF from inhibiting TGF-β–mediated up-regulation of GJIC, thereby allowing TGF-β to enhance GJIC; or 3) both. To distinguish between these three possibilities, we took advantage of the finding that noggin blocks FGF, but not TGF-β, from enhancing GJIC (Figure 2A). Lucifer yellow transfer in cells cultured with TGF + FGF in the presence of noggin was much lower than in cells exposed to TGF-β alone, indicating that noggin does not prevent FGF from inhibiting TGF-β–mediated GJIC (Figure 9C). If SB203580 acted only by allowing FGF to enhance GJIC (possibility 1), then GJIC would be expected to remain low under conditions that block FGF-induced up-regulation of GJIC, namely in the presence of noggin. Instead, dye transfer in cells treated with TGF + FGF and SB203580 plus noggin was very high, ruling out this mechanism. If SB203580 acted in a converse manner, i.e., only by allowing TGF-β to enhance GJIC (possibility 2), then cells treated with TGF + FGF and SB203580 would behave like cells exposed to TGF-β alone. Instead, dye transfer is significantly greater under the former conditions (2.95 ± 0.3-fold over control, n = 8) than under the latter (2.3 ± 0.18-fold over control, n = 6). Taken together, these results make it likely that SB203580 derepresses the ability of both FGF and of TGF-β to up-regulate GJIC in the presence of the other growth factor (possibility 3; Figure 9D).

DISCUSSION

Up-Regulation of Dye Coupling by TGF-β in Lens Cells

TGF-β has previously been reported to enhance Cx43-mediated gap junctional cell–cell coupling in several nonlenticular cell types, an effect most often attributed to a TGF-β–induced increase in the number of Cx43 gap junctions. This cannot be the mechanism by which TGF-β increases dye coupling in DCDMLs because 1) TGF-β does not cause a marked increase in anti-Cx43 gap junction immunostaining (Figure 4A) and 2) homomeric Cx43 channels are not responsible for most GJIC in DCDMLs in either the absence (Boswell *et al.*, 2009) or presence (Figure 1C) of TGF-β as assessed by the lack of intracellular transfer of the Cx43-selective dye Alexa594. Instead, TGF-β appears to increase GJIC mediated by Cx45.6 and/or Cx56 at the level of channel gating, as is also the case for FGF (Le and Musil, 2001b). In some nonlens systems, only a relatively small percentage (e.g., ≤20%) of gap junction intercellular channels are open under basal conditions (Weidmann and Hodgkin, 1966; Lin and Faber, 1988; Bukauskas *et al.*, 2000). If this is also the case in lens epithelial cells, then increasing the channel open probability would be a very effective (but not the only; Le and Musil, 2001b) means to raise GJIC in DCDMLs. A rise in this parameter in response to either FGF or TGF-β would also explain why the extent of Lucifer yellow transfer in DCDMLs exposed to TGF + FGF is comparable to that in untreated control cultures despite the much higher levels of morphologically detectable gap junctions in the latter: if FGF or TGF-β increases the amount of dye that can pass through a gap junction channel by 10-fold, then even a 90% decrease in the number of such channels upon exposure of cells to the combination of TGF- + FGF would result in the same level of dye transfer as in untreated controls.

Interactions between the TGF-β and FGF Pathways in Lens Cells

An extensive literature supports the concept that the low concentrations of FGF in the aqueous chamber may contribute to the proliferation of lens epithelial cells, whereas the higher concentrations of FGF in the vitreous humor initiate epithelial-to-fiber differentiation at the lens equator (Lovicu and McAvoy, 2005; Robinson, 2006). We have reported that FGF increases GJIC in lens cells at the higher, but not at the lower, level (Le and Musil, 2001b). The current studies reveal another important concentration-dependent difference in how FGF affects lens cells: TGF-β enhances GJIC in the presence of low FGF, but blocks gap junction formation and function when combined with FGF at the levels present in vitreous humor. As in other cell types, TGF and FGF have been reported to have antagonistic effects on lens cells. For example, TGF-β has been reported in several lens-derived systems to inhibit FGF-induced epithelial cell proliferation (Wallentin *et al.*, 1998; Awasthi and Wagner, 2006). This also appears to be true in DCDMLs (Supplemental Figure 3), reducing the levels of phosphohistone H3 staining to below that in untreated controls. Unlike the block in GJIC, however, the reduction in proliferation induced by TGF + FGF was not overcome by SB203580. To our knowledge, this study is the first to describe a reciprocal, p38-dependent antagonism between FGF and TGF-β in the lens, or in any organ.

The fact that all three structurally and functionally distinct connexins endogenously expressed in lens cells (as well as exogenously expressed Cx43-GFP and Cx50-FLAG) are defective in gap junction formation in the presence of TGF + FGF makes it likely that these factors interfere with an as yet unknown process essential to gap junction assembly. We have no evidence that general cell–cell contact is compromised in TGF + FGF–treated cells. One potential possibility is that culturing DCDMLs with TGF- + FGF creates a novel

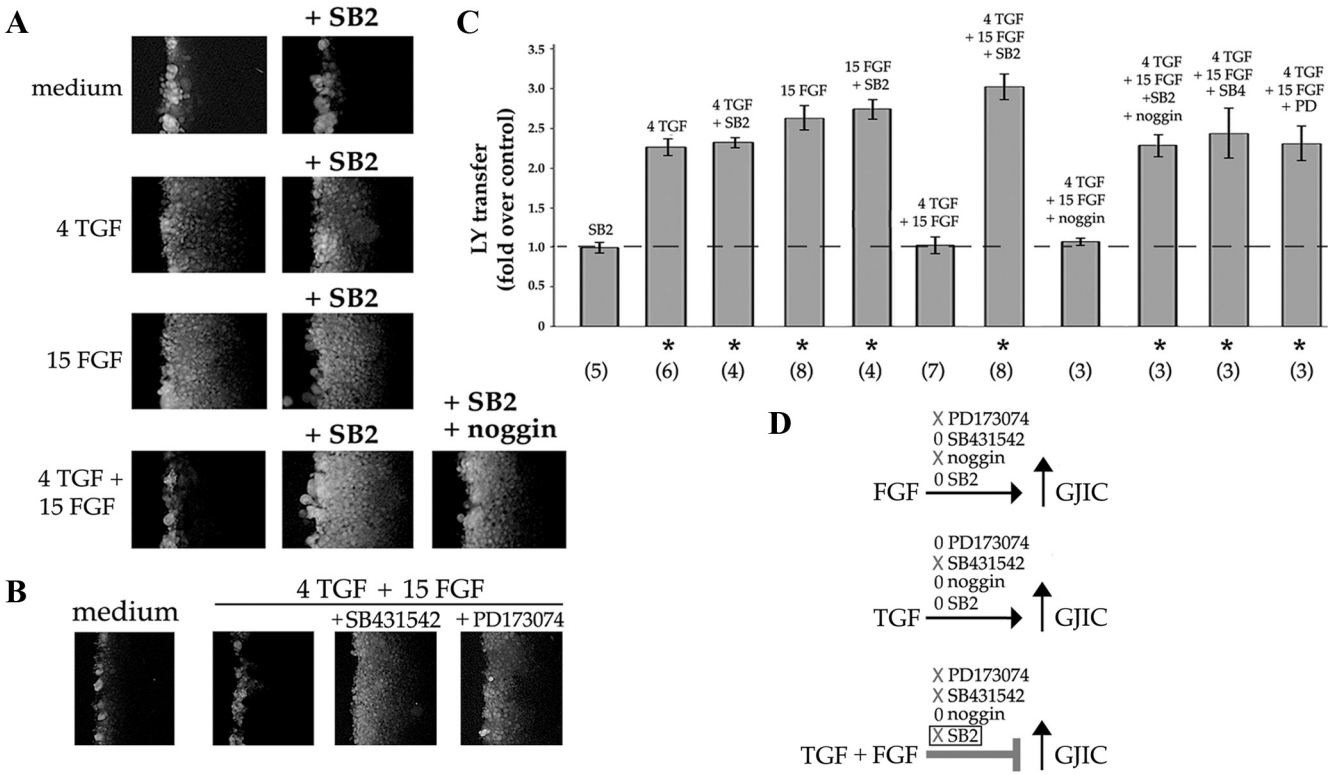

Figure 9. Up-regulation of GJIC in TGF + FGF–treated lens cells by SB203580. (A and B) DCDMLs were incubated for 48 h without growth factors or with 15 ng/ml FGF2 and/or 4 ng/ml TGF-β1. Where indicated, cells were pretreated with either the TGF receptor inhibitor SB431542 (SB4), the FGF receptor inhibitor PD173074 (PD), the p38 inhibitor SB203580 (SB2), or SB203580 plus the BMP inhibitor noggin before addition of growth factor(s). The cultures, along with medium-only controls, were then assayed for intercellular transfer of Lucifer yellow. Culturing cells for 2 d in PD173074 or SB431542 alone had no effect on dye transfer (not shown). (C) The fold increase in the distance of transfer of Lucifer yellow into the cell monolayer was quantitated relative to untreated control cells within the same experiment. N values are given in parentheses. Values significantly different from control ($p < 0.015$) as assessed by the two-tailed paired Student's *t* test are indicated by the asterisks. For the remaining data sets, $p > 0.5$. (D) Summary of the effects of growth factors and inhibitors on GJIC in DCDMLs, as assessed by Lucifer yellow transfer. See text for details.

Eph/ephrin boundary between cells that is incompatible with the formation of gap junctions, provided Eph/ephrin play a role in the function of lens fiber connexins comparable to that described for Cx43 in nonlens cells (Davy *et al.*, 2006). Establishment of such a boundary would be dependent on p38 activity and blocked by SB20358, as has been described for induction of expression of ephrin-A1 by TNF-α in endothelial cells (Cheng and Chen, 2001).

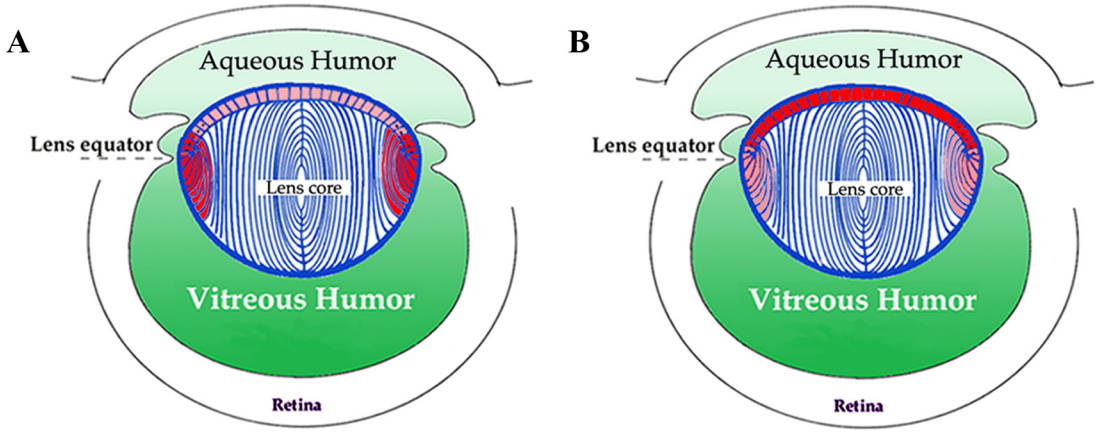

Figure 10. Model of the changes in lenticular GJIC induced by pathologically elevated levels of TGF-β. (A) In the unperturbed lens, GJIC (indicated in red) is greater in the equatorial region than at the anterior pole, a difference dependent on the higher levels of FGF in the vitreous humor than in the aqueous humor (green). (B) In response to injury or disease, the level of biologically active TGF-β is increased at the anterior of the lens, which results in the reversal of the normal pole-to-equator gradient of lenticular communication and increased cataractogenesis.

Relevance of TGF-β and Gap Junctions to Lens Transparency

After accidental or surgically induced injury, TGF-β signaling is elevated in lens central and equatorial epithelial cells (Saika *et al.*, 2001, 2004a,b; Shirai *et al.*, 2006). This is thought to be part of a wounding response and to involve increased levels of biologically active TGF-β at the anterior of the lens, including in the aqueous humor (Shirai *et al.*, 2006). The amount of active TGF-β in the aqueous humor has also been reported to be increased in other conditions that, like injury, are linked to increased cataractogenesis (e.g., glaucoma and diabetes; Ochiai and Ochiai, 2002). Unlike TGF-β, FGF is more concentrated in the vitreous humor than in aqueous humor (Wallentin *et al.*, 1998; Shirai *et al.*, 2006). Consequently, it is the population of cells at the border of the anterior and posterior chambers at the lens equator that would be simultaneously subjected to high levels of both FGF (from the vitreous humor) and TGF-β (largely from the aqueous humor) signaling under such pathological conditions. From the results presented here, we would expect that GJIC at the lens equator would be reduced. This would be predicted to disrupt the normal pole-to-equator gradient of gap junctional coupling in the organ, causing a dysregulation of ion and solute movement in the lens and possibly a loss of transparency (Figure 10). In keeping with this concept, a single injection of TGF-β into the vitreous body of rats (which would expose equatorial cells to high levels of exogenous TGF-β, in addition to endogenous FGF) caused cortical lens fibers to turn cloudy in 100% of the treated animals (Hales *et al.*, 1999). The was accompanied by swelling of the fibers, indicative of osmotic imbalance. Moreover, some animals also developed dense focal cataracts at the lens equator. Such fiber defects do not occur when isolated lenses are cultured in the presence of TGF-β alone, despite the rapid development of subcapsular cataracts at the lens anterior (Hales *et al.*, 1997, 1999). These findings are consistent with (but do not prove) the notion that TGF-β must act in combination with a factor in vitreous humor (i.e., FGF) to induce cortical cataracts in the equatorial region. Unlike those at the equator, cells in the central epithelium of the lens are exposed to the low FGF environment of the aqueous humor. A pathological increase in the concentration of TGF-β at the anterior of the lens would be predicted (Figure 1) to abnormally increase GJIC in this cell population, a perturbation that would contribute to the disruption of the normal pole-to-equator intralenticular gradient of GJIC. In light of the long-term activation of TGF-β signaling after a single, relatively brief (2 d) exposure of lens cells to elevated levels of TGF-β (Saika *et al.*, 2002; Wormstone *et al.*, 2006), even a transient increase in active TGF-β in equatorial and/or central lens epithelial cells could potentially cause a sustained perturbation of lens GJIC leading to a loss of lens homeostasis and increased cataractogenesis.

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