TGFβ overcomes FGF-induced transinhibition of EGFR in lens cells to enable fibrotic secondary cataract

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ABSTRACT **To cause vision-disrupting fibrotic secondary cataract (PCO), lens epithelial cells that survive cataract surgery must migrate to the posterior of the lens capsule and differentiate into myofibroblasts. During this process, the cells become exposed to the FGF that diffuses out of the vitreous body. In normal development, such relatively high levels of FGF induce lens epithelial cells to differentiate into lens fiber cells. It has been a mystery as to how lens cells could instead undergo a mutually exclusive cell fate, namely epithelial to myofibroblast transition, in the FGF-rich environment of the posterior capsule. We and others have reported that the ability of TGFβ to induce lens cell fibrosis requires the activity of endogenous ErbBs. We show here that lens fiber-promoting levels of FGF induce desensitization of ErbB1 (EGFR) that involves its phosphorylation on threonine 669 mediated by both ERK and p38 activity. Transinhibition of ErbB1 by FGF is overcome by a time-dependent increase in ErbB1 levels induced by TGFβ, the activation of which is increased after cataract surgery. Our studies provide a rationale for why TGFβ upregulates ErbB1 in lens cells and further support the receptor as a therapeutic target for PCO.**

SIGNIFICANCE STATEMENT

- It has been a mystery how lens epithelial cells differentiate into myofibroblasts in the FGF-rich, antifibrotic environment of the posterior lens capsule to cause secondary cataract, the most common vision disrupting complication of cataract surgery.
- Using a serum-free, primary lens epithelial cell culture system, the authors showed that FGF induced pT669-mediated desensitization of ErbB1 (EGFR) that was overcome by the previously reported increase in ErbB1 levels induced by TGFβ.
- This work demonstrates how TGFβ modulates ErbB signaling to induce epithelial to mesenchymal transition in the presence of otherwise lens fiber-eliciting levels of FGF and raises new strategies to prevent secondary cataract.

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Abbreviations used: αSMA, α–smooth muscle actin; DCDML, dissociated cell-derived monolayer; EMT, epithelial–mesenchymal transition; EMyT, epithelial–myofibroblast transition; ERK, extracellular signal–regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FN, fibronectin; PCO, posterior capsule opacification; pdFN, plasma-derived fibronectin; TGFβ, transforming growth factor β; TGFβR, transforming growth factor β receptor; TPA, tetradecanoylphorbol acetate; VBCM, vitreous body–conditioned medium

INTRODUCTION

Fibroproliferative disorders are a major cause of human morbidity and mortality, with relatively few effective therapies (Henderson *et al.*, 2020). Although the majority of fibrotic cells in most organs are believed to be derived from nonepithelial precursors, epithelial to myofibroblast transition (EMyT; Masszi *et al.*, 2010) is thought to significantly contribute to fibrosis in some organs (Di Gregorio *et al.*, 2020; Lee and Nelson, 2012; Liu *et al.*, 2022). A prime example of the latter is the lens, which lacks a resident population of fibroblastic or endothelial cells. The vertebrate lens consists of a monolayer of epithelial cells on the anterior half of the organ and the highly elongated, crystallin-rich lens fiber cells that differentiate from them, all encased in the acellular lens capsule (Danysh and Duncan, 2009; Cvekl and Ashery-Padan, 2014). Due to its lack of blood vessels, the lens is exquisitely dependent on growth factors produced by the lens cells themselves or present in their immediate environment. The anterior face of the lens capsule is bathed by the aqueous humor, whereas the posterior face is opposed to the vitreous body. Studies in several vertebrate species have definitively shown that the relatively high levels of FGF that diffuse out of the vitreous body induce lens epithelial cells to differentiate into lens fiber cells (McAvoy and Chamberlain,1989; Le and Musil, 2001a; Schulz *et al.*, 1993). Transgenic manipulation of FGFR function has established that FGF signaling is essential for confining fiber cell differentiation to the posterior of the lens capsule, as is required for normal lens development (Chow *et al.*, 1995; Stolen *et al.*, 1997; Lovicu and Overbeek, 1998; Robinson *et al.*, 1995a,b; Robinson, 2006; Zhao *et al.*, 2008).

When wounded, lens epithelial cells can instead differentiate into myofibroblasts. EMyT is associated with the most common and costly complication of cataract surgery, namely posterior capsule opacification (PCO; also known as secondary cataract; Apple *et al.*, 2000; Saika, 2004; Awasthi *et al.*, 2009; Wormstone *et al.*, 2009; Fișuș and Findl, 2020). PCO arises from the inability to remove all lens epithelial cells from the anterior lens capsule during surgery. Postoperatively, such residual lens epithelial cells can take on one of two abnormal fates. Cells that were located near interface of the anterior and posterior halves of the intact organ (at the so-called lens equator) tend to remain in place and become lens fiber-like cells, forming a structure referred to as Soemmering's ring. Other remaining equatorial and more anterior lens epithelial cells can migrate to the posterior half of the lens capsule and differentiate into myofibroblasts. When such highly contractile, αSMA-rich cells accumulate at the posterior pole of the lens capsule, they cause the fibrotic form of PCO by depositing large amounts of opaque extracellular matrix and by wrinkling the lens capsule. Light-scattering lens fiber-like cells are also found on the posterior capsule. Both cell types interfere with transmission of light to the retina, thereby compromising eyesight. It has been estimated that up to 20% of adults develop clinically deleterious PCO after conventional cataract surgery, making PCO the second most common cause of nonretinal vision loss in this population (Shihan *et al.*, 2019; Fișuș and Findl, 2020; Ursell *et al.*, 2020; Donachie *et al.*, 2023). Rates of PCO are much higher in children (Medsinge and Nischal, 2015), and after implantation of certain types of artificial lenses (Schriefl *et al.*, 2015).

Despite over 50 y of study (Green and McDonnell, 1962), the cellular mechanisms responsible for fibrotic PCO are not fully understood. The growth factor that has been most closely linked to PCO is TGFβ. TGFβ signaling increases in lens epithelial cells shortly after cataract surgery, presumably as part of a wound-healing response (Meacock *et al.*, 2000; Saika *et al.*, 2002). Adding active TGFβ to primary lens epithelial cells from human (Wormstone *et al.*, 2002; Tiwari *et al.*, 2016), rodent (Srinivasan *et al.*, 1998; Robertson *et al.*, 2007; West-Mays *et al.*, 2010), or chick (Boswell *et al.*, 2017; or culturing them under conditions that induce the activation of endogenous TGFβ; VanSlyke *et al.*, 2018) has been demonstrated to elicit EMyT (Saika, 2004; Wormstone and Eldrid, 2016). More recently, it has been shown that inhibitors of the ErbB family of receptor tyrosine kinases, including the human therapeutic lapatinib (Tykerb), can inhibit TGFβ-induced myofibroblast differentiation in chick (VanSlyke *et al.*, 2023) and rodent (Shu *et al.*, 2019) models of fibrotic PCO. ErbB kinase blockers have also been reported to reduce PCO in human lens capsular bags (Maidment *et al.*, 2004; Wertheimer *et al.*, 2015, 2018). We have recently shown that lens epithelial cells express not only ErbB1 (EGFR; EGFR1), but also the two other active kinases in the ErbB family (ErbB2 and ErbB4; VanSlyke *et al.*, 2023). In our chick-derived primary lens epithelial cell culture system, TGFβ upregulates both total and cell surface expression of ErbB1 but reduces the levels of ErbB2 and ErbB4, leading to an increase in ErbBs with the functional properties of ErbB1/ErbB1 homodimers (VanSlyke *et al.*, 2023). The significance of this remodeling of ErbB signaling for PCO, if any, has heretofore been unknown.

Lens epithelial cells are not present on the central posterior capsule in the intact lens. Their appearance in this location after cataract surgery must therefore be due to migration from more anterior regions, consistent with prevailing concepts of the etiology of PCO in which lens epithelial cells can undergo EMyT during or after their relocation to the posterior pole (Marcantonio and Vrensen, 1999; Awasthi *et al.*, 2009; Wormstone *et al.*, 2009; Marcantonio *et al.*, 2000; Kurosaka *et al.*, 2021; McDonald, 2021; Zhang *et al.*, 2021). This migration exposes lens epithelial cells to the high levels of FGF that diffuse out of vitreous body. As reviewed above, studies in several vertebrate species have firmly established that this concentration of FGF induces lens epithelial cells to differentiate into lens fiber cells and is essential for fiber formation during normal lens development (McAvoy and Chamberlain,1989; Schulz *et al.*, 1993; Chow *et al.*, 1995; Stolen *et al.*, 1997; Lovicu and Overbeek, 1998; Le and Musil, 2001a; Robinson *et al.*, 1995a,b; Zhao *et al.*, 2008; Robinson, 2006). In none of the aforementioned studies, nor in any others to our knowledge, has it been demonstrated that FGF can induce lens epithelial cells to undergo EMyT. The question thus arises as to how epithelial cells could differentiate into myofibroblasts at the FGF-rich posterior pole of the lens capsule. Resolving this conundrum is important not only for our fundamental understanding of PCO, but also for devising strategies to mitigate the substantial health and economic burdens of this disorder throughout the world.

It has been reported that primary rat lens central epithelial explants can undergo epithelial-to-mesenchymal transition if treated with TGFβ for 24 h before culture with otherwise lens fiber-promoting levels of FGF in the absence of TGFβ (Mansfield *et al.*, 2004). The mechanism of this effect was not, however, determined. In rat lens explants, TGFβ causes the complete apoptotic loss of lens cells within 5 d that is prevented by addition of FGF (Schulz *et al.*, 1996; Mansfield *et al.*, 2004). It is therefore difficult to use this system to separate the pro-survival effects of FGF from any other possible role in lens cell fate. Unlike in rodent lens explants, TGFβ does not kill lens epithelial cells in several other models of PCO, including human lens capsular bags (Wormstone *et al.*, 2002) and primary cultures of chick lens epithelial cells (DCDMLs; Boswell *et al.*, 2017). Here, we use our DCDML system to address the interactions between TGFβ, FGF, and ErbB signaling and their role in lens cell fibrosis. Our studies reveal a previously undescribed transinhibition of ErbB function by FGF at the level the latter is present in vitreous humor. This inhibition is overcome by the TGFβ-induced upregulation of ErbB1 we have previously

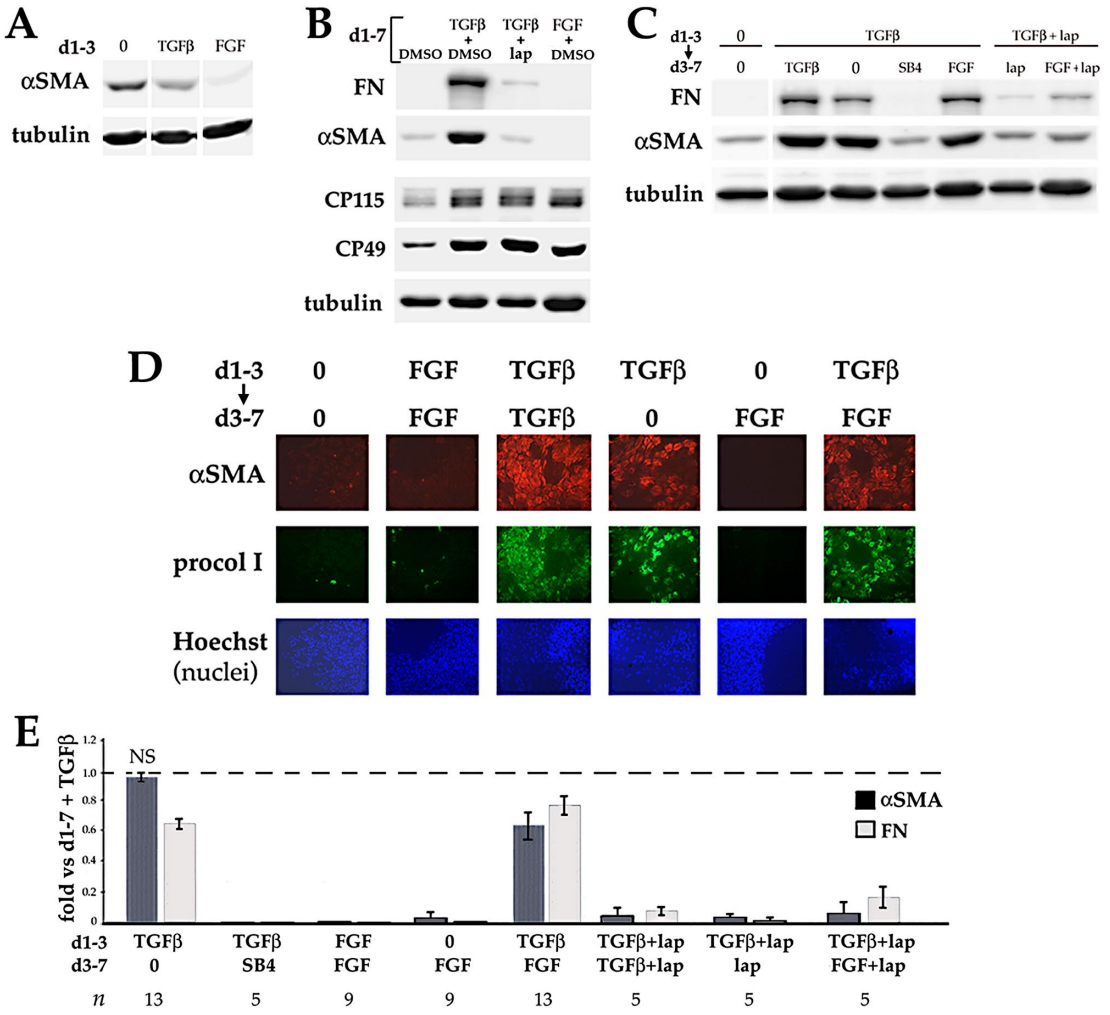


FIGURE 1: EMyT of lens epithelial cells cultured in the presence of TGFβ and/or FGF. (A) DCDML primary lens cells were cultured from d 1 – d 3 (d1–3) in the presence or absence of 4 ng/ml TGFβ1 or 10 ng/ml FGF2 before Western blot analysis of whole cell lysates for the EMyT marker α smooth muscle actin (αSMA). (B) DCDMLs were cultured from d 1 – d 7 in the continuous presence or absence of TGFβ, FGF, or TGFβ with the ErbB inhibitor lapatinib before analysis of whole cell lysates for αSMA and the EMT/EMyT marker fibronectin (FN), or for the lens fiber cell differentiation markers CP115 and CP49. (C) DCDMLs were cultured with TGFβ or FGF in the presence of absence of the TGFβR inhibitor SB-431542 or the ErbB inhibitor lapatinib as indicated, before analysis of whole cell lysates for αSMA and FN. $n = 5$. (D) DCDMLs cultured as indicated were fixed and immunostained for αSMA and the EMT/EMyT marker procollagen. Nuclei were counterstained with Hoechst 33342. (E) Quantification of the effects of the indicated treatments on expression of αSMA and FN as assessed by Western blot, expressed as fold relative to DCDMLs continuously cultured in exogenous TGFβ from d 1–7. p values were calculated compared with DCDMLs cultured with TGFβ from d 1–7; NS, $p = 0.233$; for all other values, $p \leq 0.03$. Images in (A) and (C) were montaged from single blots.

reported (VanSlyke *et al.*, 2023). Together, this work demonstrates for the first time how TGFβ can modulate ErbB signaling to induce EMyT in the presence of otherwise lens fiber-eliciting levels of FGF. In addition to their significance for understanding and treating fibrotic PCO, our findings could also be applicable to other systems in which growth factors that dictate different cell fates coexist (e.g., the developing axial skeleton [Williams *et al.*, 2019] and ovarian follicle [Lobb, 2009]) and to nonlenticular fibroproliferative disease.

RESULTS

FGF does not prevent TGFβ from inducing ErbB-dependent EMyT in DCDMLs

In the human eye, lens epithelial cells are exposed to the aqueous humor in the anterior chamber. All experiments were conducted with DCDMLs cultured in 96-well plates in 200 µl/well of M199 medium to mimic the volume and low protein content of this environment. By convention (VanSlyke *et al.*, 2024), the day the cells are prepared and plated is designated as day 0 of culture (d0). We routinely use fibronectin (FN) and α smooth muscle actin (αSMA) as markers of EMyT, and expression of the lens fiber-specific beaded filament proteins CP115 and CP49 as indicators of lens fiber cell differentiation (Boswell *et al.*, 2017).

As we have previously reported (Boswell *et al.*, 2010), a 48-h treatment with TGFβ (i.e., from d 1–3 of culture) is too short to induce EMyT in DCDMLs. At this time, the amount of αSMA detectable by Western blot does not exceed the basal levels typical of untreated lens epithelial cells (Garcia *et al.*, 2006; Figure 1A). After 6 d of culture, TGFβ upregulates EMyT in a large fraction of cells in subconfluent DCDMLs without inducing apoptosis (Figure 1, B and D); a separate population of epithelial cells undergoes lens fiber cell

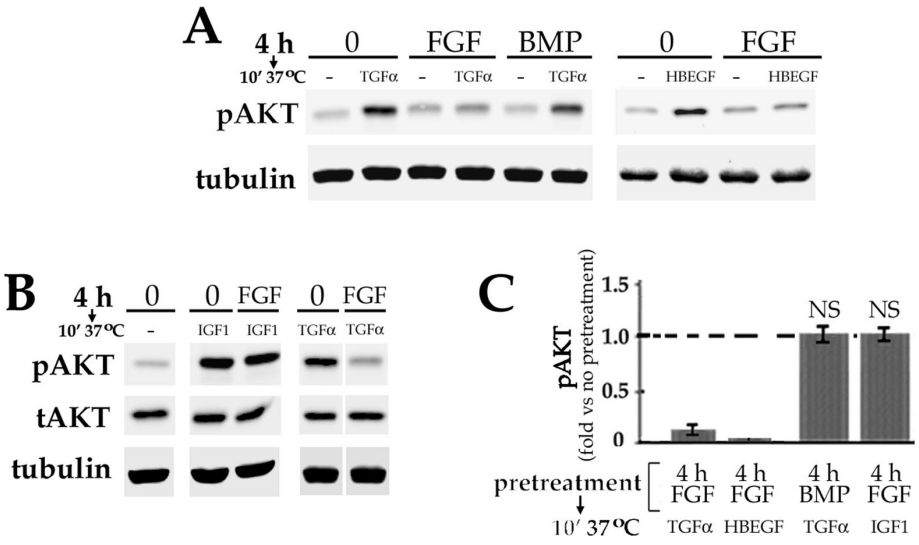


FIGURE 2: FGF abrogates ErbB1 downstream signaling. DCDMLs were exposed to 10 ng/ml FGF or BMP4 for 4 h at 37°C as indicated. Cells were then incubated for 10 min at 37°C with either no additions, TGFα, HB-EGF, or IGF1. Whole cell lysates were analyzed by Western blot for activated (pS497) and total forms of AKT. (C) Results graphed relative to the level of pAKT achieved when DCDMLs cultured in unsupplemented medium were incubated for 10 min with either TGFα, HB-EGF, or IGF1. *p* values were calculated compared with DCDMLs without pretreatment; NS, $p \geq 0.432$; rest, $p \leq 0.002$. $n = 4$. Images in (A) and (B) were montaged from single blots.

differentiation (Boswell *et al.*, 2017). In contrast, FGF at levels that mimic those present in vitreous humor (7–10 ng/ml FGF2) stimulates only the expression of markers of lens fiber differentiation (Figure 1B; Lovicu and McAvoy 1989; Schulz *et al.*, 1993; Le and Musil, 2001a). Notably, treatment with FGF for 2 (Figure 1A) or 6 (Figure 1B) d reduces the level of αSMA to marginally detectable levels, consistent with the previously established antifibrotic role of the growth factor (Dolivo *et al.*, 2017; Dolivo 2022).

Cataract surgery induces the activation of latent TGFβ in the aqueous humor as part of a wounding response (Saika, 2004). As the wound heals, this source of active TGFβ would be lost to lens epithelial cells. To examine if transient exposure to exogenous, active TGFβ can support EMyT, we treated DCDMLs with recombinant TGFβ from d 1–3 and then cultured them for the remaining 4 d in its absence (TGFβ → 0 protocol; see Supplemental Figure S1A for a diagrammatic description of growth factor protocols used in this study). On d 7, these cultures expressed high levels of markers of EMyT (Figure 1, C and D). This upregulation was blocked if SB-431542, an inhibitor of TGFβ receptors, was added to the TGFβ-free medium (Figure 1C). Short-term exposure to exogenous active TGFβ therefore appeared to trigger the production and/or activation of endogenous TGFβ to elicit subsequent EMyT in DCDMLs, as has been previously reported in human lens capsular bags (Wormstone *et al.*, 2006) and rodent lens central epithelial explants (Shu *et al.*, 2017).

After cataract surgery, lens epithelial cells are first exposed to wounding-induced TGFβ, and then to high levels of FGF only after their migration to the posterior half of the lens capsule. To model this scenario, DCDMLs were incubated with exogenous TGFβ from d 1 to d 3 of culture, and then with FGF from d 3 to d 7. Cells subjected to this TGFβ → FGF protocol continued to upregulate αSMA and FN (Figure 1C). Immunostaining confirmed that these cells expressed procollagen I and assembled αSMA into stress fibers (Figure 1D), indicating that they had undergone bona fide differentiation into myofibroblasts. Myofibroblasts persisted if DCDMLs subjected to the TGFβ → FGF protocol were cultured with FGF until d 9 to match the period of time that FGF-only cultures were exposed to the growth factor (unpublished data).

We have reported that the ability of TGFβ to induce EMyT in DCDMLs is abrogated by the highly specific ErbB inhibitor lapatinib (VanSlyke *et al.*, 2023; Figure 1B). Lapatinib also inhibited EMyT in DCDMLs subjected to either the TGFβ → 0 or TGFβ → FGF protocol, demonstrating continued dependence on ErbB signaling (Figure 1C).

In avian (Le and Musil, 2001b) as well as in mammalian (Iyengar *et al.*, 2007) lens epithelial cells, sustained activation of ERK by FGF is required for lens fiber cell formation. An obvious mechanism by which TGFβ could elicit myofibroblast differentiation (instead of lens fiber cell formation) in the presence of fiber-inducing levels of FGF would be if culturing cells in TGFβ compromised the stimulation of ERK by FGF. This, however, is not the case, in that a 2-d exposure of DCDMLs to TGFβ (a period too brief to induce differentiation into myofibroblasts; Figure 1A) did not significantly affect the ability of subsequently added 10 ng/ml FGF2 to activate ERK after an 8-h treatment. Extending the time of exposure to TGFβ from 2 d to 6 d and/or decreasing the FGF treatment from 8 h to 1.5 h also did not alter the level of pERK attained (Supplemental Figure 2S).

If TGFβ does not compromise FGFR signaling, then how does it overcome the antifibrotic effects of FGF? Based on our findings that in DCDMLs: (1) ErbB inhibitors reduce TGFβ-induced EMyT in either the absence or presence of FGF (Figure 1); (2) TGFβ increases the total and cell surface levels of ErbB1, but reduces ErbB2 and ErbB4 (VanSlyke *et al.*, 2023); (3) decreased expression of ErbB2 and ErbB4 is not required for TGFβ to induce αSMA (VanSlyke *et al.*, 2023), and the fact that ErbB1 plays a profibrotic role in some nonlenticular cell types (Liu *et al.*, 2012), we considered the possibility that TGFβ upregulation of ErbB1 could be involved in this process. We first show that FGF desensitizes ErbB1 signaling in DCDMLs in the absence of TGFβ. We then demonstrate TGFβ renders ErbB1 refractory to inhibition by FGF to restore ErbB1 function, thereby enabling (ErbB1-dependent) myofibroblast differentiation.

Effect of FGF on ErbB1 signaling and activation in the absence of TGFβ

TGFα (unrelated to TGFβ) is a direct ligand of ErbB1 but not of other ErbBs, and can stimulate ErbB1/ErbB2 and ErbB1/ErbB4 heterodimers as well as ErbB1 homodimers (Singh *et al.*, 2016). As we have previously reported (VanSlyke *et al.*, 2023), addition of TGFα to DCDMLs rapidly induces phosphorylation of the canonical ErbB downstream effector AKT on Ser473. We found that a 4-h treatment of DCDMLs with lens fiber differentiation-inducing levels of FGF reduced the ability of subsequently added TGFα to activate AKT by ~88% (Figure 2A). Similar results were obtained when TGFα was substituted with the ErbB1 and ErbB4 ligand HB-EGF, demonstrating that the effect is not confined to a single ligand (mammalian EGF does not efficiently activate avian ErbB1; Lax *et al.*, 1988). In

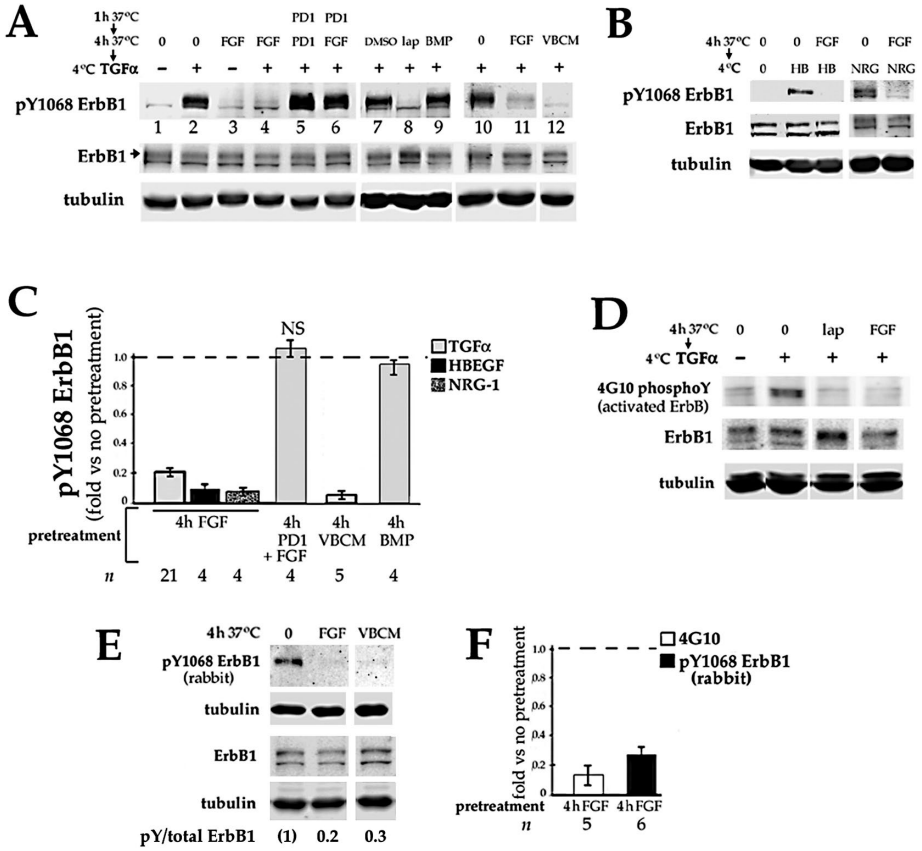


FIGURE 3: Lens fiber-inducing levels of FGF block activation of ErbB1. (A–F) DCDMLs were treated for 4 h at 37°C with 10 ng/ml FGF, 10 ng/ml BMP4, VBCM, lapatinib, or the FGFR kinase inhibitor PD173074. Where indicated, the cells were preincubated for 1 h with PD173074. (A and B) The cultures were then chilled to 4°C and incubated on ice for 15 min in either the absence or presence of the ErbB ligands TGFα, HBEGF (HB), or NRG1 before Western blot analysis of whole cell lysates with mouse anti-pY1068 ErbB1 and total (pan) ErbB1 antibodies. The arrow denotes the position of ErbB1; as previously reported (VanSlyke *et al.*, 2023), the lower band detected in some experiments with the total ErbB1 antibody is a nonspecific species. (C) Results of (A and B) graphed relative to the level of pY1068 ErbB1 attained when otherwise untreated DCDMLs were incubated at 4°C with the indicated ErbB ligand. (D) The cultures were then incubated on ice in either the absence or presence of TGFα before Western blot analysis of whole cell lysates with the pan phosphotyrosine 4G10 antibody. (E) Cultures were lysed Immediately after the indicated treatments. Half of each lysate was probed with the CST #2234 rabbit anti-pY1068 ErbB1 antibody to detect the activation of ErbB1 by endogenous ligands, and the other half of the same sample was probed with the rabbit total (pan) ErbB antibody. (F) Results of (D and E) graphed relative to the level of either 4G10 or CST #2234 immunoreactivity obtained when DCDMLs were incubated for 4 h in the absence of FGF. For (C and F), *p* values were calculated compared with the condition indicated on the y-axis of the corresponding graph; NS = $p \geq 0.374$; for all other values, $p \leq 0.08$. Images were montaged from single blots.

phosphorylation but not for downstream signaling events or for transactivation by non-ErbB receptors (Longva *et al.*, 2002; Tong *et al.*, 2014). Western blots were probed with a mouse antibody specific to the Y1068 phosphorylated form of ErbB1 (CST #2236; validated in Supplemental Figure S3); this species is a major product of ErbB1 autophosphorylation and plays an essential role in downstream activation of AKT as well as of ERK (Sebastian *et al.*, 2006; Roskoski, 2014). As expected, addition of TGFα at 4°C to otherwise untreated DCDMLs induced a large increase in pY1068 ErbB1 immunoreactivity (Figure 3A, lane 1 vs. 2) that was abolished if the cells had been preincubated with lapatinib (lane 8). Incubation of DCDMLs for 4 h with 7–10 ng/ml FGF greatly reduced their ability to increase pY1068 ErbB1 levels in response to a subsequent 4°C exposure to TGFα (lane 4). This effect was abolished by pretreatment with the FGFR kinase blocker PD173074, demonstrating a requirement for FGFR activity (lane 6). ErbB1 was also rendered unable to respond to TGFα if DCDMLs were incubated for 4 h with vitreous body conditioned medium (VBCM), the in vivo source of lens fiber cell-differentiating levels of FGF (Le and Musil, 2001a and b; lane 12). In contrast, a 4-h incubation with lens fiber-differentiating concentrations of an FGF-unrelated growth factor, BMP4, did not appreciably alter ErbB1 activation by TGFα (lane 9). As shown in Figure 3B, pretreatment with FGF also blocked activation of ErbB1 by HB-EGF (a direct ligand of ErbB1 and ErbB4) and NRG1 (which does not bind to ErbB1, and can only activate it as part of an ErbB heterodimer).

Stimulation by ligand causes ErB1 to become autophosphorylated on at least five different tyrosine residues (Biscardi *et al.*, 1999). To simultaneously assess all of these modifications, we used the pan antiphosphotyrosine antibody 4G10 (Samarakoon *et al.*, 2013; VanSlyke *et al.*, 2023; Figure 3D). As was observed with the pY1068 ErbB1-specific antibody, addition of TGFα at 4°C to otherwise untreated DCDMLs induced a large increase in 4G10 signal that was abolished if the cells had been preincubated with lapatinib. FGF pretreatment greatly reduced anti-4G10 immunoreactivity induced by a 4°C exposure to TGFα. This suggests that the inhibitory effect of FGF is not confined to phosphorylation of Y1068 of ErbB1 and is instead generalized to include other sites of autoactivation.

We have reported that a rabbit anti-pY1068 ErbB1 antibody (CST #2234) more sensitive than that used in the above experiments is able to readily detect the activity of ErbB1 in whole cell lysates of untreated DCDMLs, indicative of ongoing basal stimulation with endogenous ErbB ligands (VanSlyke *et al.*, 2023, 2024). A 4-h treatment of DCDMLs with 10 ng/ml FGF or VBCM reduced contrast, a 4-h incubation with another lens fiber-inducing factor, BMP4 (Boswell and Musil 2015), did not abrogate activation of AKT by TGFα.

Cells pretreated with 10 ng/ml FGF were still capable of upregulating pAKT levels in response to an ErbB-independent stimulus (i.e., IGF1; Figure 2B), suggesting that FGF may be acting at the level of ErbB1 itself. This was confirmed by experiments demonstrating that FGF prevents ErbB1 from being activated by exogenous ligand (protocol summarized in Supplemental Figure S1B). DCDMLs were incubated with or without FGF for 4 h at 37°C, after which the cells were chilled and treated with TGFα at 4°C, a temperature permissive for ligand-induced ErbB dimerization and auto-

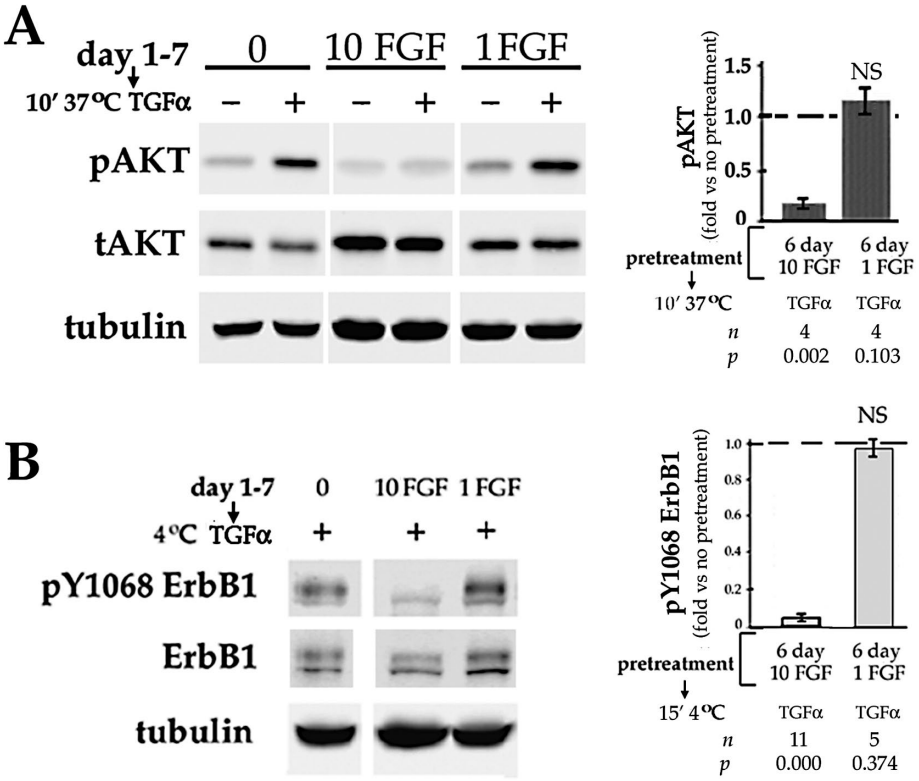


FIGURE 4: Desensitization of ErbB1 by FGF is long-lasting, and concentration-dependent. DCDMLs were cultured for 6 d with no additions, 10 ng/ml FGF2, or 1 ng/ml FGF2. (A) Cells were then treated for 10 min at 37°C with or without TGFα. Whole cell lysates were analyzed by Western blot for activated (pS497) and total forms of AKT. Results graphed relative to the level of pAKT achieved when DCDMLs cultured in unsupplemented medium were incubated for 10 min at 37°C with TGFα. (B) Cells were then chilled to 4°C and incubated on ice for 15 min in either the absence or presence of TGFα before Western blot analysis of whole cell lysates with mouse anti-pY1068 ErbB1 and total (pan) ErbB1 antibodies. Results graphed relative to the level of pY1068 ErbB1 attained when otherwise untreated DCDMLs were incubated for 15 min at 4°C with TGFα. For (A and B), *p* values were calculated compared with the condition indicated on the y-axis of the corresponding graph. Images were montaged from single blots.

CST #2234 immunoreactivity, indicating that FGF desensitized ErbB1 to endogenous as well as to exogenously added ErbB ligands (Figure 3E).

A physiologically important question is whether the desensitization of ErbB1 evoked by high levels of FGF is transient or chronic. Figure 4 shows that activation of both AKT and ErbB1 in response to TGFα remained potently blocked if cells were cultured with 10 ng/ml FGF for 6 d. In contrast, concentrations of FGF too low to induce sustained activation of ERK or efficient lens fiber formation (0.5–1 ng/ml FGF) had no such inhibitory effect.

Mechanism of inhibition of ErbB1 by FGF

One means by which FGF could inhibit ErbB1 autophosphorylation and downstream signaling would be by causing receptor degradation. A 4-h exposure to 10 ng/ml FGF did not, however, significantly reduce the level of ErbB1 in total cell lysates (Supplemental Figure S4A). Similar results were obtained with the potential ErbB1 heterodimerization partners ErbB2 and ErbB4. Another possible explanation for our findings would be if FGF induced the intracellular sequestration of ErbB1. Cell surface biotinylation revealed, however, no loss of ErbB1 from the plasma membrane after a 4-h exposure to FGF. Similar findings were obtained for ErbB2 and ErbB4 (Supplemental Figure S4B).

Several mechanisms have been reported to lead to the inability of cell surface ErbB1 to respond to ligand, including increased expression of cholesterol (Pike and Casey, 2002), certain integrins (Hang *et al.*, 2015), and ErbB1-inducible feedback inhibitors such as RALT/MIG-6 (Anastasi *et al.*, 2007). Partial loss of ErbB1 responsiveness to TGFα was detectable after 1 h of treatment with 10 ng/ml FGF (63% inhibition ± 0.06; $n = 3$; $p = 0.009$; unpublished data). In other cell types, the best characterized posttranslational means to block cell surface ErbB1 signaling is phosphorylation of ErbB1 on cytoplasmic threonine 669 (Li *et al.*, 2008; Adak *et al.*, 2011; Kluba *et al.*, 2015). The kinase most often associated with phosphorylation of T669 is ERK (Li *et al.*, 2008; Nishimura *et al.*, 2009). We considered the possibility that >1 ng/ml FGF might, by virtue of its ability to induce sustained activation of ERK in lens cells (Le and Musil, 2001b; Iyengar *et al.*, 2007), be inducing transinactivation of ErbB1 by chronically stimulating inhibitory T669 phosphorylation. Consistent with this hypothesis, we found that a 4-h treatment with 7–10 ng/ml FGF or VBCM enhanced anti-pT669 ErbB1 immunoreactivity in whole cell lysates (Figure 5A; specificity of this antibody for phosphorylated ErbB1 is demonstrated in Supplemental Figure S3). In contrast, BMP4, which does not activate ERK in lens cells (Boswell *et al.*, 2008a,b), had no effect. Elevated levels of pT669 ErbB1 were also detectable after a 24-h treatment with 10 ng/ml FGF2, but not with lower concentrations (0.5–1 ng/ml) which upregulate pERK only transiently (Le and Musil, 2001b; Iyengar *et al.*, 2007; VanSlyke *et al.*, 2024; Figure 5B). Phosphorylation of T669 in response to FGF was partially inhibited by pretreatment of cells with the specific MEK inhibitor UO126 (Bain *et al.*, 2007), confirming the involvement of ERK (Figure 5C). In contrast, pretreatment with lapatinib had no effect on this process, demonstrating that ErbB activity was not required.

To inhibit responsiveness to ligand, the pT669-modified form of ErbB1 must be present on the plasma membrane. We demonstrated that this was the case using cell surface biotinylation of DCDMLs treated with 7–10 ng/ml FGF2 for 4 h (Figure 5E). As in whole cell lysates, phosphorylation of cell surface ErbB1 on T669 in response to FGF was partially reduced by preincubation with UO126. Note that UO126 did not elevate the level of ErbB1 on the plasma membrane of cells treated with FGF, indicating a bona fide decrease in the fraction of cell surface ErbB1 phosphorylated on T669 (Figure 5F).

If ERK-dependent phosphorylation of Thr669 is the sole mechanism by which FGF inactivates ErbB1, then UO126 should completely restore the ability of TGFα to induce autophosphorylation of ErbB1 on Y1068 in FGF-treated cells. To test this possibility, we treated cells with UO126, FGF, or UO126 followed by FGF and then assessed their ability to upregulate phosphorylation of Y1068 ErbB1 in response to 4°C TGFα (Figure 6A). We found that UO126 increased the ligand responsiveness of ErbB1 in the absence of

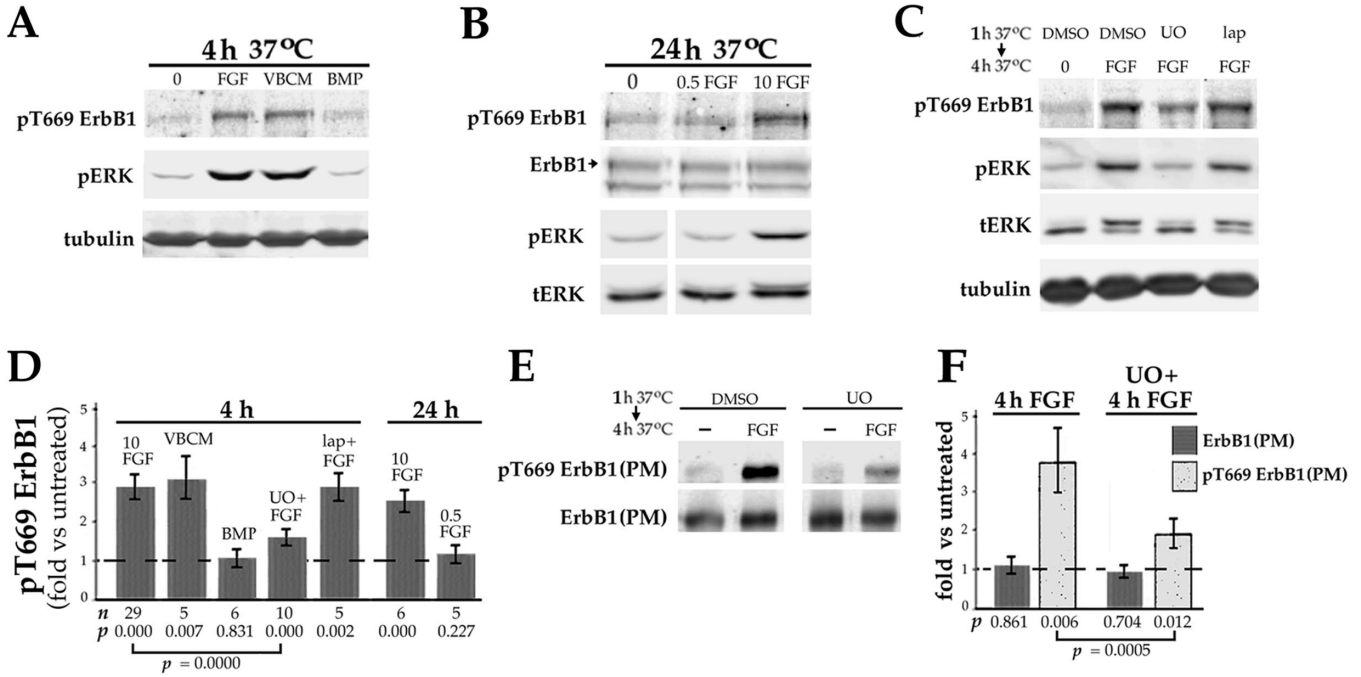


FIGURE 5: FGF stimulates phosphorylation of ErbB1 on T669 in a partially ERK-dependent process. (A and B) Upregulation of pT669 ErbB1 correlates with activation of ERK. DCDMLs were incubated at 37°C with 10 ng/ml FGF, 0.5 ng/ml FGF, VBCM, or 10 ng/ml BMP4 for the indicated period followed by Western blot analysis of whole cell lysates with antibodies against phospho T669 ErbB1, total ErbB1, phospho ERK or total ERK. Note that total levels of ErbB1 and ERK were unaffected by a 24-h treatment with FGF. (C) Phosphorylation of ErbB1 on T669 induced by FGF is partially dependent on ERK, but not on ErbB, activity. DCDMLs were preincubated at 37°C for 1 h with DMSO, the ERK activation inhibitor U0126, or lapatinib before a 4 h, 37°C treatment with or without 10 ng/ml FGF2. Western blots of whole cell lysates were probed with antibodies against phospho T669 ErbB1, phospho ERK, or total ERK. (D) Results of (A–C) graphed relative to the level of pT669 ErbB1 in untreated cells in the same experiment. (E) Regulation of pT669 ErbB1 on the cell surface. DCDMLs preincubated with DMSO or UO126 for 1 h were incubated with or without 10 ng/ml FGF2 for 4 h before cell surface biotinylation at 4°C and analysis of plasma membrane (PM) expression of pT669 ErbB1 and ErbB1. (F) Results of (E) graphed relative to the level of pT669 ErbB1 and ErbB1 on the surface of untreated cells in the same experiment. For (A-F), $n = 5$; unless indicated otherwise, p values calculated compared with the condition indicated on the y-axis of the corresponding graph. Images in (B), (C), and (E) were montaged from single blots.

exposure to FGF by approximately twofold (compare lane 3 with lane 1). Such hyper-responsiveness of ErbB1 after MEK inhibition has been observed in other cell types (Gan *et al.*, 2010). Although TGFα stimulated phosphorylation of ErbB1 on Y1068 to a greater extent in cells exposed to UO126 and then FGF (lane 4) than in cells treated with FGF alone (lane 2), the levels of pY1068 ErbB1 attained were still less than if cells had been pretreated with UO126 without FGF (compare lane 4 with lane 3). Taken together, these findings indicate that FGF desensitizes ErbB1 by a combination of ERK-dependent (UO126 inhibitable) and non-ERK (UO126 insensitive) mechanisms.

There are several reports that another member of the MAPK family, p38, can also phosphorylate ErbB1 on T669 in some cell types (Wang *et al.*, 1998; Winograd-Katz and Levitzki, 2006; Tomas *et al.*, 2015, 2017; Metz *et al.*, 2021). The fact that FGF activates p38 in DCDMLs (Boswell *et al.*, 2010; Figure 6C) combined with our finding that UO126 only partially prevents FGF from upregulating phosphorylation of T669 (Figure 5, C and E) led us to consider that this might also be the case in lens cells. Consistent with this possibility, we found that the strong p38 (but not ERK; Supplemental Figure S5B) agonist anisomycin (AM) increased the level of pT669 ErbB1 in DCDMLs in a manner unaffected by UO126 (Figure 6D). Combining UO126 and the p38 inhibitor SB-203580 almost completely prevented phosphorylation of ErbB1 on T669 in response to FGF as assessed in whole cell lysates (Figure 6A; lane 8) or after cell surface biotinylation (Figure 6E; lane 4). Moreover, pretreatment with UO126 plus SB-203580 fully restored the ability of ErbB1 to respond to ligand (4°C TGFα) as assessed using antibodies to either pY1068 ErbB1 (Figure 6A; lane 8) or to total phosphotyrosine (4G10; Supplemental Figure S6A). Similar results were obtained when SB-203580 was replaced with a mechanistically distinct p38 kinase inhibitor, BIRB 796 (Bain *et al.*, 2007; Supplemental Figure S6B). Importantly, UO126 combined with SB-203580 does not significantly increase the amount of ErbB1 on the surface of FGF-treated cells compared with untreated controls (Figure 6E; lane 4 vs. 1), indicating that the inhibitors increased the fraction of the plasma membrane pool of ErbB1 able to respond to ligand.

Taken together, these results support important roles for both ERK and p38 in the desensitization of ErbB1 by FGF. Note that SB-203580 alone (i.e., without UO126) does not reduce phosphorylation of ErbB1 on T669 in response to FGF (Figure 6A, compare lanes 6 and 2). This implies that the role of p38 in this process can be bypassed by ERK, in keeping with the ability of ERK to compensate for the loss of p38 downstream of FGF reported in other cell types (Zakrzewska *et al.*, 2019).

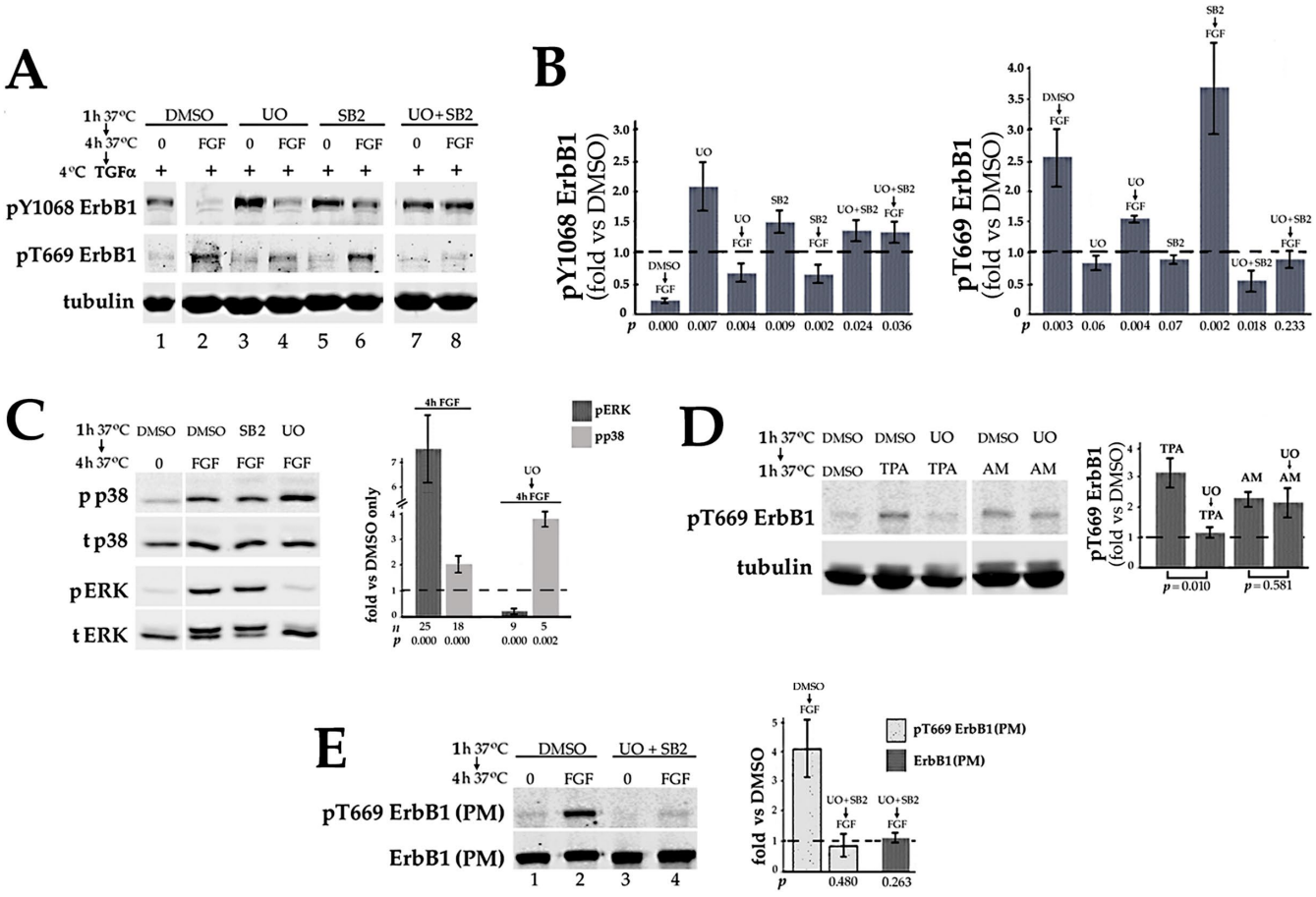


FIGURE 6: Role of p38 kinase in regulation of ErbB1 activity and T669 phosphorylation. DCDMLs were incubated for 1 h at 37°C with DMSO, UO126, and/or the p38 inhibitor SB-203580 as indicated before addition of 10 ng/ml FGF (4 h), the p38 agonist anisomycin (AM) (1 h), or the ERK agonist TPA (1 h). In (A), cells were then treated for 15 min at 4°C with TGFα. All cells were then processed for whole cell lysate (A, C, and D) or cell surface biotinylation (E) analysis of pT669 ErbB1, pY1068 ErbB1, and/or total and phosphorylated (activated) forms of p38 or ERK. (A) Desensitization, and phosphorylation on T669, of ErbB1 induced by FGF is completely reversed by combined inhibition of ERK and p38. All data shown are from a single experiment. (B) Results quantitated as fold pY1068 ErbB1 or pT669 ErbB1 obtained after the indicated incubations vs controls incubated with DMSO only before treatment with TGFα. $n = 5$. (C) FGF activates p38 in addition to pERK. Note that SB-203580 inhibits the phosphorylation of substrates by activated p38, not the activation of p38 itself; Kumar *et al.*, 1999). Results quantitated as pERK or pp38 versus untreated controls. (D) Anisomycin increases the level of pT669 ErbB1 in an ERK-independent manner. Results quantitated as fold pT669 ErbB1 obtained after the indicated treatments vs controls incubated with DMSO only. $n = 5$. (E) Blocking ERK and p38 prevents FGF from upregulating pT669 ErbB1 on the cell surface without significantly affecting the level of ErbB1 on the plasma membrane. Results quantitated as fold pT669 ErbB1 or ErbB1 detected on the cell surface vs controls incubated with DMSO only. $n = 5$. (B–E) Unless indicated otherwise, p values were calculated compared with the condition indicated on the y-axis of the corresponding graph. Images in (A), (C), and (D) were montaged from single blots. Control experiments confirmed that phosphorylation of ErbB1 on T669 was unaffected by exposure to ligand at 4°C, and that anisomycin increases the level of pp38 in an ERK-independent manner (Supplemental Figure S5).

Effect of FGF on activation of ErbB1 in the presence of TGFβ

If FGF prevents ErbB1 from responding to both exogenous and endogenous ErbB1 ligands (Figure 3), then how can TGFβ induce EMyT in the presence of FGF in a manner blocked by ErbB inhibitors (Figure 1)? One explanation would be if culturing cells in TGFβ rescued ErbB signaling from inhibition by FGF. Indeed, we found that DCDMLs cultured with exogenous TGFβ for 6 d were able to robustly phosphorylate ErbB1 on Y1068 in response to 4°C TGFα even after a 4-h incubation with either 7–10 ng/ml FGF2 or FGF-containing VBCM (Figure 7A; lanes 8 and 9). Note that TGFβ does not enhance pY1068 ErbB1 levels in the absence of added ligand (compare lanes 1 and 5), in keeping with our demonstration that TGFβ does not transactivate ErbB1 in DCDMLs (VanSlyke *et al.*, 2023). ErbB1 was also rendered resistant to desensitization by FGF on d 7 if DCDMLs were exposed to exogenous TGFβ only from d 1 to d 3 to stimulate the subsequent production and activation of endogenous TGFβ (TGFβ → 0 protocol; Figure 7B, lane 4). Similar results were obtained if HB-EGF was used as the ErbB1 ligand (unpublished data), or if antiphosphotyrosine 4G10 antibody was used as a read-out instead of mouse anti-pY1068 ErbB1 (Figure 7C). A 6-d treatment with TGFβ also made activation of ErbB1 by endogenous ligands resistant to FGF as assessed using the more sensitive CST #2234 rabbit anti-pY1068 ErbB1 antibody (Figure 7D). Importantly, a 4-h incubation with FGF did not affect the level of ErbB1 on the surface of TGFβ-cultured cells (Figure 7E), ruling out the possibility that FGF

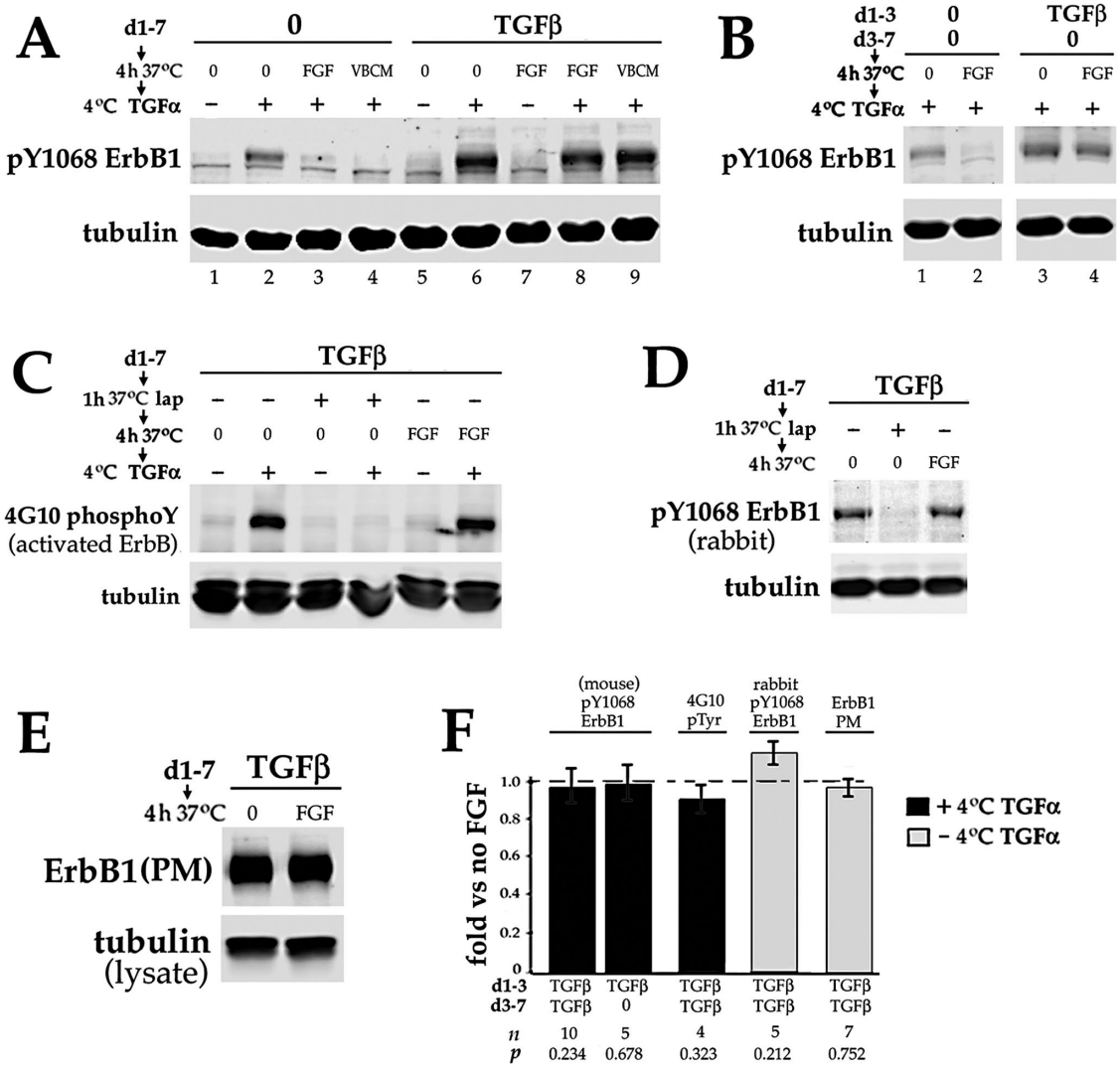


FIGURE 7: Cells cultured for 6 d in the presence of TGFβ are resistant to desensitization of ErbB1 by FGF. DCDMLs were cultured for 6 d without additions, with TGFβ, or with exogenous TGFβ for 2 d followed by 4 d with no additions. Where indicated, cells were then incubated for 1 h with lapatinib before a 4-h treatment at 37°C with no additions, 10 ng/ml FGF2, or VBCM. In (A–C), cells were then subjected to a 15-min incubation at 4°C with or without TGFα, and whole cell lysates probed with antibodies against pY1068 ErbB1 (A and B) or total phosphotyrosine (4G10) (C) to detect receptor activated by exogenous TGFα. In (D), endogenous levels of active ErbB1 were examined using the CST #2234 rabbit anti-pY1068 ErbB1 antibody. (E) Cells were subjected to cell surface biotinylation to measure the level of ErbB1on the cell surface. (F) Results of (A–E) quantitated as the value obtained after a 4-h treatment with FGF relative to the same conditions, but without exposure to FGF. *p* values were calculated compared with no FGF treatment. Images in (B) were montaged from a single blot.

recruited additional receptor to the plasma membrane under these conditions.

Resistance to FGF desensitization correlates with TGFβ-induced increased expression of ErbB1

We have reported that culturing DCDMLs with TGFβ for 6 d upregulates cell surface and total cell lysate levels of ErbB1, but decreases both pools of ErbB2 and ErbB4 (VanSlyke *et al.*, 2023). We wondered if the TGFβ-induced increase in ErbB1 might be linked to acquisition of resistance of the receptor to inactivation by FGF. Consistent with this possibility, we found that DCDMLs cultured for only 2 d with TGFβ, a treatment period too short to upregulate expression of ErbB1, were still sensitive to inhibition of ErbB1 by a 4-h treatment with FGF when measured on d 3. Increasing the time of exposure to TGFβ to 4 or 6 d conferred both increased resistance to inactivation by FGF and elevated levels of ErbB1 (Figure 8A). We have reported that plating DCDMLs on platelet-derived fibronectin instead of on laminin results in the activation of enough endogenous TGFβ to upregulate both EMyT (VanSlyke *et al.*, 2018) and expression of ErbB1 (VanSlyke *et al.*, 2023). DCDMLs grown on pdFN were also rescued from desensitization of ErbB1 by FGF in a TGFβ-dependent manner (Supplemental Figure S7).

Next, we examined how long-term exposure to FGF affects ErbB1 expression and activation. DCDMLs subjected to the TGFβ → FGF protocol (i.e., fed with TGFβ-containing medium on d 1, and then with FGF on d 3 and d 5) undergo ErbB-dependent EMyT by d 7 of culture (Figure 1C). We found that DCDMLs cultured under TGFβ → FGF conditions were capable of activating ErbB1 in

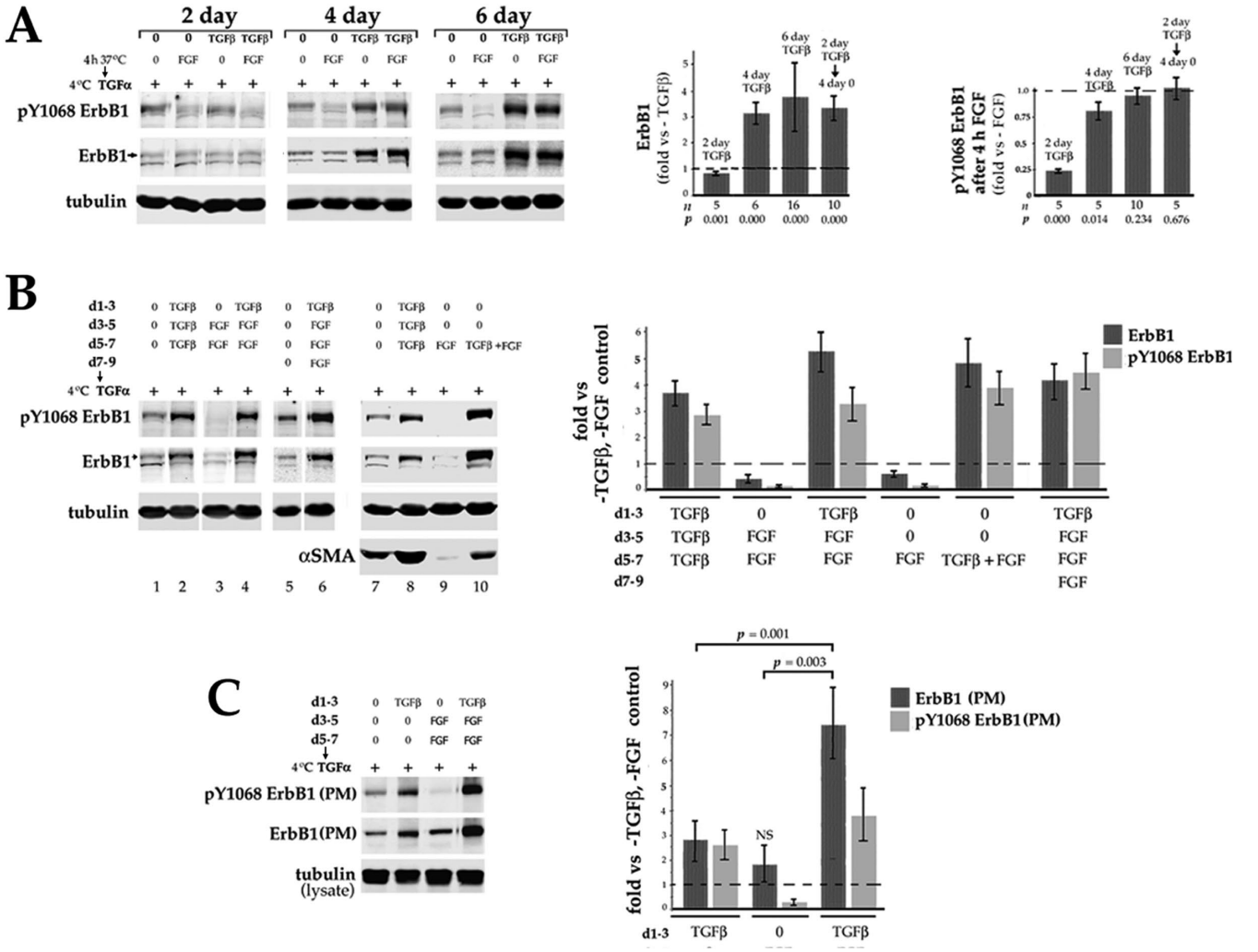


FIGURE 8: TGFβ-induced rescue of ErbB1 from desensitization by FGF correlates with increased expression of ErbB1. DCDMLs were cultured in the presence or absence of TGFβ, FGF, or SB-431542 for 2-9 d as indicated. In (A), cells were then incubated for 4 h at 37°C in the absence or presence of FGF. Cells in (A-C) were then exposed to TGFα for 15 min at 4°C to assess activation of ErbB1 by ligand. Whole cell lysates (A and B) or plasma membrane pools acquired after cell surface biotinylation (C) were then probed with antibodies against pY1068 ErbB1, total ErbB1, or αSMA. (A) Time course of TGFβ upregulation of ErbB1 expression, and of TGFβ rescue of ErbB1 ligand sensitivity from inhibition by short-term exposure to FGF. Total ErbB1 values graphed as the fold obtained in DCDMLs cultured with TGFβ for the indicated period compared with controls cultured without TGFβ. pY1068 ErbB1 values graphed as the fold obtained in DCDMLs cultured with TGFβ for the indicated period and then incubated with FGF for 4 h before addition of TGFα, compared with cells not incubated with FGF but otherwise treated identically. The graph also presents data from cells subjected to the TGFβ → 0 protocol in which cells were exposed to exogenous TGFβ from d 1 to d 3 to stimulate the subsequent production and activation of endogenous TGFβ. *p* values were calculated compared with the condition indicated on the y-axis of the corresponding graph. (B) TGFβ rescues ErbB1 expression and ligand sensitivity from inhibition by long-term exposure to FGF. Total and pY1068 ErbB1 values graphed as the fold obtained in DCDMLs cultured under the indicated conditions compared with controls cultured without TGFβ or FGF. ErbB1 remained able to be activated by ligand if DCDMLs subjected to the TGFβ → FGF protocol were cultured with FGF until d 9 to match the time that FGF-only cultures were exposed to the growth factor. For all, $n = 5$; $p \leq 0.003$ compared with no TGFβ, no FGF controls. (C) Cells subjected to the TGFβ → FGF protocol show higher levels of ErbB1 on the cell surface than cells treated with either TGFβ or FGF only. Cell surface ErbB1 and pY1068 ErbB1 values graphed as the fold obtained in DCDMLs cultured under the indicated conditions compared with controls cultured without TGFβ or FGF. For all, $n = 5$; $p \leq 0.03$ compared with no TGFβ, no FGF controls, except for NS ($p = 0.106$). Images in (A) and (B) were montaged from single blots.

response to 4°C TGFα despite the 4-d exposure to FGF (Figure 8B, compare pY1068 ErbB1 lane 4 with lane 3). Rescue of ErbB1 activity by TGFβ was correlated with increased expression of ErbB1 protein, both in whole cell lysates (Figure 8B) and on the cell surface (Figure 8C). Interestingly, cells subjected to the TGFβ → FGF protocol showed significantly higher levels of ErbB1 on the cell surface than cells treated with only either TGFβ or FGF (Figure 8C).

In the course of these experiments, we noticed that subjecting lens cells to a 48-h simultaneous treatment with FGF plus TGFβ starting on d 5 of culture increased the level of total ErbB1 (Figure 8B,

compare ErbB1 lane 10 to lane 7) and induced the resistance of ErbB1 to desensitization by FGF (compare pY1068 ErbB1 lane 10 to lane 9). These conditions did not, however, elevate the level of αSMA over untreated controls (compare lane 10 to lane 7; $p = 0.721$; $n = 6$). Although such a treatment is unlikely to be physiologically relevant, these findings demonstrate that resistance to FGF is associated with increased expression of ErbB1 instead of with acquisition of the myofibroblast phenotype.

Mechanism by which TGFβ prevents desensitization of ErbB1 by FGF

The simplest explanation for why culturing DCDMLs for >2 d in TGFβ renders ErbB1 resistant to inactivation by FGF would be if TGFβ makes the cells unable to phosphorylate ErbB1 on the inhibitory T669 site. Anti-pT669 ErbB1 immunoreactivity was, however, readily detectable in cells cultured for 6 d with TGFβ, both in whole cell lysates and by cell surface biotinylation (Figure 9A). An alternative possibility would be if ErbB1 in TGFβ-cultured cells was impervious to ERK and/or p38 activity. This was ruled out by the finding that in TGFβ–cultured cells, a 1-h incubation with the ERK activator TPA plus the p38 AM increased the level of pT669 ErbB1 in a manner reversed by pretreatment with the ERK/p38 inhibitor mix UO126 + SB-203580 (Figure 9B).

A third explanation arose from a comparison of cells cultured with TGFβ for 0, 2, or 6 d. DCDMLs cultured for only 48 h with TGFβ behaved like no TGFβ cells by having low basal levels of pT669 ErbB1 that were increased ~2.83-fold by a 4-h treatment with FGF (Figure 9C; compare lanes 4 and 3). Cells cultured with TGFβ for 6 d had ~2.63X higher levels of pT669 ErbB1 than no TGFβ cells (compare lane 7 with lane 5), nearly identical to the amount of pT669 ErbB1 observed in no TGFβ cells incubated for 4 h with FGF (compare lane 7 with lane 6). The amount of pT669 ErbB1 in 6-day TGFβ-cultured cells was not, however, further increased by exposure to FGF (compare lane 7 with lane 8). When normalized to total ErbB1 in the same sample, the ratio of pT669 ErbB1/ total ErbB1 in 6 d TGFβ-cultured cells was similar to that in untreated (i.e., no TGFβ or FGF) cells (Figure 9C table; 0.90 vs. 1). Although exposure to FGF increased the ratio of pT669 ErbB1/ total ErbB1 in no TGFβ cells from 1 to 2.86, it did not in TGFβ-cultured cells (0.90 vs. 0.94).

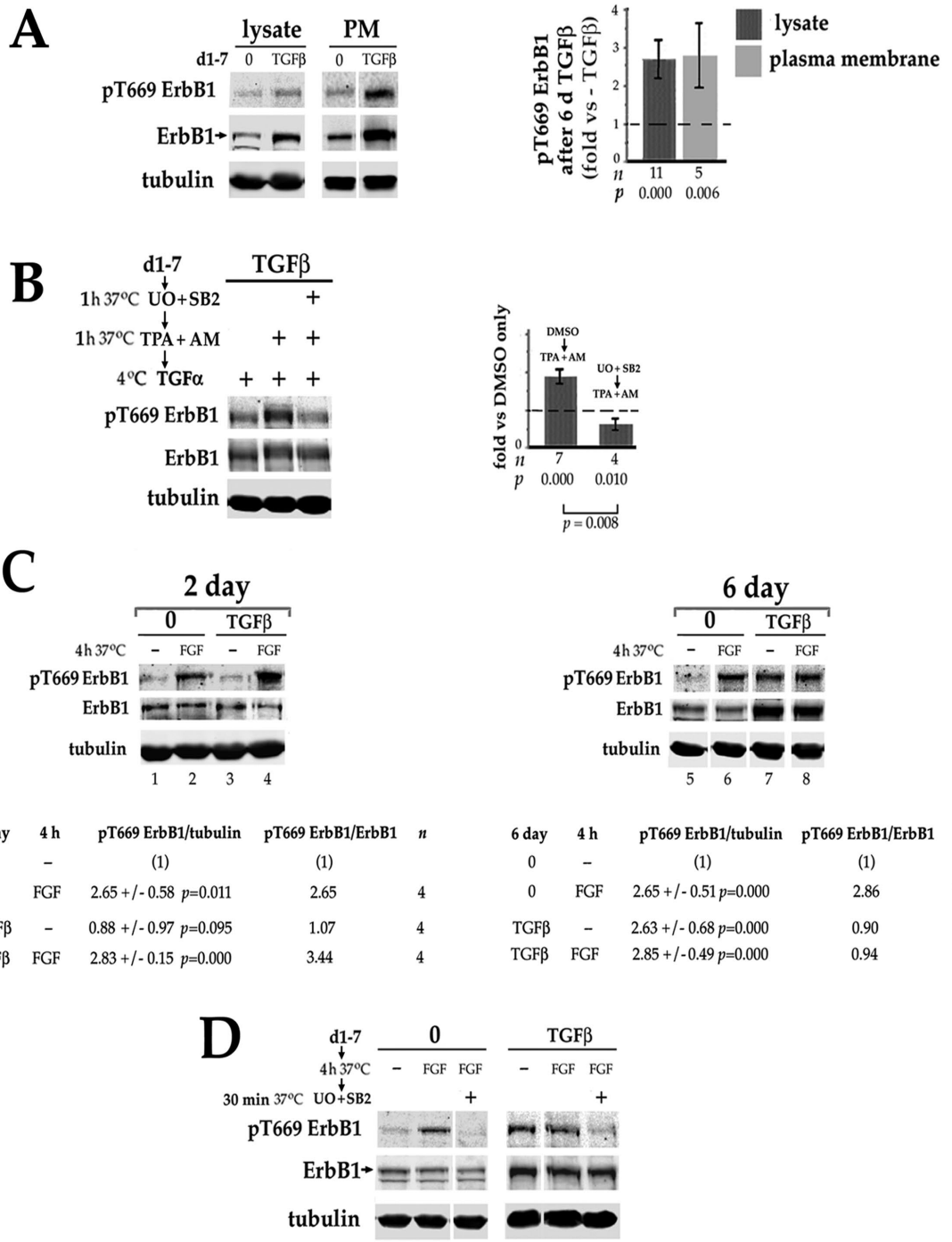
The results shown in Figures 7–9 suggest a model in which the ability of DCDMLs to accumulate ErbB1 phosphorylated on T669 in response to FGF is limited by, for example, a relative scarcity of proteins that link ERK and/or p38 to ErbB1, and/or to high levels of pT669 phosphatases. Evidence for the latter came from experiments showing that FGF-induced modification of ErbB1 on T669 in both -TGFβ and +TGFβ cells is reversed within 15–30 min under conditions that prevent further phosphorylation of ErbB1 (i.e., incubation with UO126 + SB-203580; Figure 9D). According to this model, in cells cultured without TGFβ, treatment with 7–10 ng/ml FGF induces the phosphorylation of T669 in a large enough fraction of total cellular ErbB1 that the remaining pool of non-desensitized receptor is too small to effect more than a minor increase in pY1068 ErbB1 levels in response to subsequently added ligand. Culturing the cells in TGFβ for >48 h enhances the expression of total (and cell surface) ErbB1 without increasing the capacity of the cells to accumulate the pT669 ErbB1 form. By this means, TGFβ increases the level of activatable (i.e., not phosphorylated on T669) ErbB1 in DCDMLs to an extent that results in a substantial upregulation of pY1068 ErbB1 levels in response to 4°C TGFα in either the absence or presence of FGF pretreatment.

If increased expression of ErbB1 is the main mechanism by which culturing in TGFβ renders DCDMLs resistant to blockade of ErbB1 by FGF, then forced expression of ErbB1 should phenocopy the effects of >2-d exposure to TGFβ. To test this prediction, we transfected DCDMLs with a plasmid encoding wild-type human ErbB1-GFP and cultured the cells in the absence of TGFβ. ErbB1-GFP was transiently expressed in DCDMLs at 8-40X the level of endogenous ErbB1, resulting in a robust increase in Y1068 phosphorylation after exposure to TGFα at 4°C (Figure 10A). As expected, endogenous ErbB1 in control cells transfected with a plasmid encoding an irrelevant integral membrane protein was rendered insensitive to a 4°C treatment with TGFα by a 4-h preincubation with FGF. In contrast, ErbB1 transfectants were resistant to inactivation by FGF, with levels of pY1068 ErbB1 (endogenous plus GFP-tagged exogenous receptor) induced by a 4°C treatment with TGFα approaching those obtained in cells not exposed to FGF (Figure 10A). Unlike overexpression of ErbB1, overexpression of ErbB2 to >50-fold endogenous ErbB2 levels (range 57-310X) did not render cells able to respond to TGFα after exposure to FGF. Exogenous ErbB2 also did not make TGFβ-cultured cells sensitive to FGF inhibition (Supplemental Figure S8). pT669 ErbB1 is detectable in ErbB1-GFP transfectants with or without FGF treatment, but only at levels comparable to those present in FGF-treated control transfectants despite the ~8–40X increase in total ErbB1 (Figure 10B; compare lanes 3 and 4 to lane 2). This finding is consistent with the concept that the machinery responsible for the initiation and/or maintenance of phosphorylation on T669 in response to physiological activation of ERK and p38 by FGF is limiting. A preference of this (substoichiometric) machinery for native chick ErbB1 over human ErbB1-GFP could explain why endogenous ErbB1 becomes more phosphorylated on T669 in response to FGF than its exogenous counterpart (Figure 10B, lane 4; compare the intensity of the exogenous pT669 ErbB1-GFP band labeled with an asterisk with endogenous pT669 ErbB1 indicated with an arrowhead). In keeping with this interpretation, experiments in which ErbB1-GFP was expressed to even higher levels (~960X over endogenous) to increase the competition between exogenous and endogenous ErbB1 for the T669 phosphorylation machinery showed a marked increase in the relative phosphorylation of T669 in the ErbB1-GFP species (Figure 10B, lane 6; compare the band designated with an asterisk with the species indicated with an arrowhead). As in 6 d-TGFβ-cultured control cells (Figure 9C), FGF did not elicit an increase in pT669 ErbB1 levels after exogenous overexpression of ErbB1(Figure 10B; compare lanes 5 and 6).

Taken together, these studies support the contention that increased expression of ErbB1, either induced by TGFβ or engineered by transfection, can overcome FGF-induced desensitization of the receptor conferred by phosphorylation of T669. This is accomplished by increasing the level of ErbB1 so that proportionally less of the total receptor pool is subjected to this modification. The elevation in TGFβ signaling after cataract surgery would allow continued ErbB signaling on the posterior of the lens capsule, thereby facilitating the EMyT of lens cells and the development of fibrotic PCO (summarized in Supplemental Figure S9).

DISCUSSION

Myofibroblasts and lens fiber cells are considered to be mutually exclusive cell types. How lens epithelial cells could undergo EMyT in the FGF-rich, lens fiber cell-inducing environment of the posterior capsule has been an important unanswered question in the etiology of PCO. In a previous investigation, we reported that a 6-d treatment of DCDMLs with TGFβ upregulates both total and cell surface levels of ErbB1 to an extent well beyond the amount required to respond to endogenous ligand (VanSlyke *et al.*, 2023). The potential biological significance of this excess capacity has heretofore been



2 day	4 h	pT669 ErbB1/tubulin	pT669 ErbB1/ErbB1	*n*
0	–	(1)	(1)	
0	FGF	2.65 +/- 0.58 *p*=0.011	2.65	4
TGFβ	–	0.88 +/- 0.97 *p*=0.095	1.07	4
TGFβ	FGF	2.83 +/- 0.15 *p*=0.000	3.44	4

6 day	4 h	pT669 ErbB1/tubulin	pT669 ErbB1/ErbB1	*n*
0	–	(1)	(1)	
0	FGF	2.65 +/- 0.51 *p*=0.000	2.86	18
TGFβ	–	2.63 +/- 0.68 *p*=0.000	0.90	9
TGFβ	FGF	2.85 +/- 0.49 *p*=0.000	0.94	8

FIGURE 9: Culturing DCDMLs in TGFβ for 6 d reduces the fraction of ErbB1 phosphorylated on T669 in response to FGF. (A) A 6-d treatment with TGFβ increases the level of pT669 ErbB1 detected in whole cell lysates and by cell surface biotinylation relative to no TGFβ controls. Cells were not exposed to either FGF or ErbB ligand. *p* values were calculated compared with no TGFβ cells. (B) TGFβ-cultured cells were incubated at 37°C for 1 h with DMSO or UO126 + SB-203580 before a 1-h treatment with or without TPA plus anisomycin. Cells were then incubated at 4°C for 15 min with TGFα before analysis of whole cell lysates. Results are graphed relative to the pT669 ErbB1 values obtained in cells cultured with TGFβ and treated with DMSO only. Unless indicated otherwise, *p* values were calculated compared with DMSO only cells. (C) Cells cultured for 2 d (d1–3) or for 6 d (d1–7) in either the absence or presence of TGFβ were incubated at 37°C for 4 h with or without FGF before analysis of whole cell lysates for pT669 ErbB1 and total ErbB1. The data are tabulated as the level of pT669 ErbB1 obtained after the indicated treatments, normalized either to tubulin or to the level of total ErbB1 in the same sample. *p* values were calculated compared with the corresponding no TGFβ, no FGF controls. (D) DCDMLs were incubated for 4 h with or without FGF and then chased for 30 min with UO126 plus SB-203580 or DMSO only before analysis of whole cell lysates. Blocking ERK and p38 reduced the level of pT669 ErbB1 to 0.532 ± 0.196 fold of untreated (no FGF; DMSO only) controls. $n = 6$; $p = 0.002$ compared with untreated control. Images in (A), (C), and (D) were montaged from single blots.

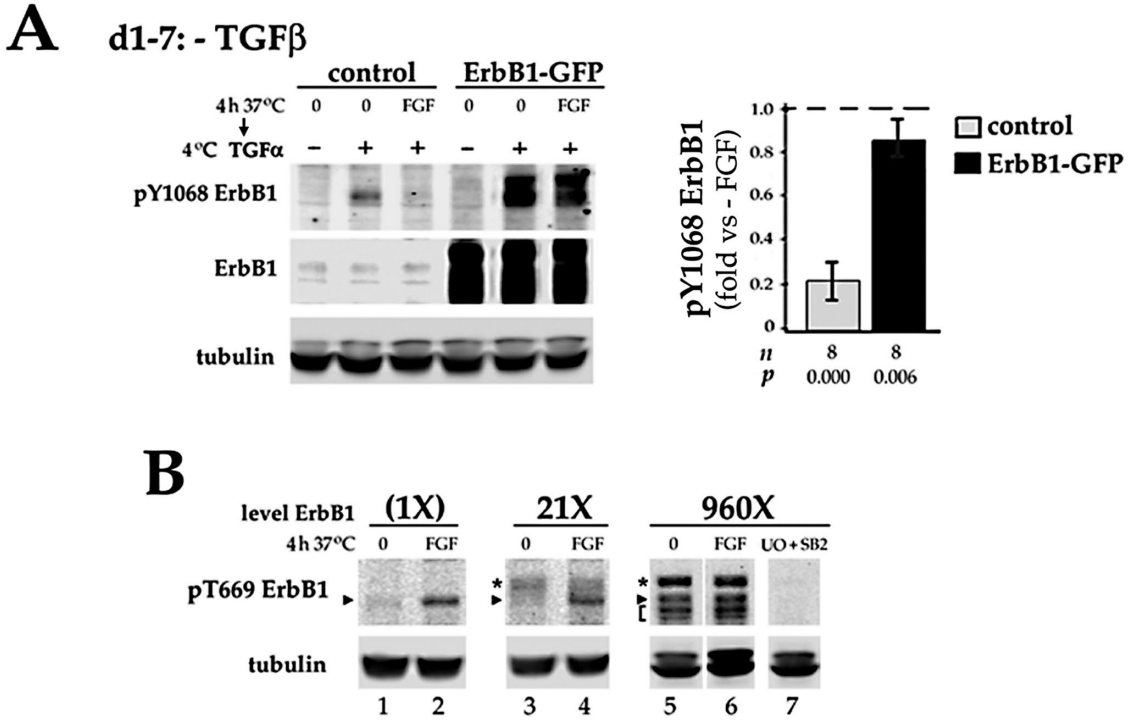


FIGURE 10: Overexpression of ErbB1 renders ErbB1 resistant to desensitization by FGF. (A): DCDMLs were transiently transfected with plasmids encoding either ErbB1-GFP or an irrelevant transmembrane protein (E208K Cx32; Boswell *et al.*, 2010). After 6 d, cells were subjected to a 4-h incubation at 37°C in the presence or absence of 7 ng/ml FGF2, followed by a 15-min incubation at 4°C with or without TGFα. Whole cell lysates were analyzed by Western blotting. pY1068 ErbB1 values graphed as the fold obtained in transfectants incubated with FGF for 4 h before addition of TGFα compared with cells not incubated with FGF but otherwise treated identically. *p* values were calculated compared with the corresponding no FGF control. (B) Effect of FGF on the level of pT669 ErbB1 in ErbB1-overexpressing cells. DCDMLs were transiently transfected with plasmids encoding either ErbB1-GFP. Where indicated, cells were then subjected to a 4-h incubation with FGF at 37°C. Whole cell lysates were analyzed using antibodies to pT669 ErbB1. The position of full-length T669 ErbB1-GFP and endogenous T669 ErbB1 are labeled with an asterisk or arrowhead, respectively. Lane 7 depicts an experiment in which phosphorylation of ErbB1 on T669 was abolished by coinhibition of ERK and p38 with a UO126 + SB-203580 mix, which confirms that the bracketed lower M_r immunoreactive bands represent fragments of pT669 ErbB1. Reblotting with antibodies against total ErbB1 (unpublished data) was used to determine the indicated level of overexpression. Typical of five experiments. Images in lanes 5–7 were montaged from a single blot.

unknown. Here, we provide an answer to this enigma, namely that the increased expression of ErbB1 induced by TGFβ serves to prevent FGF in the lens cell environment from inhibiting the ErbB signaling required for myofibroblast differentiation. Taken together, our data support the following model to explain the observed relationship between ErbB1 expression and desensitization by FGF.

1. Under unstimulated conditions (i.e., no exogenous TGFβ or FGF), a small fraction of ErbB1 is phosphorylated on T669, possibly due in part to basal levels of ERK and p38 activity. Most of the total cellular pool of ErbB1 is not modified on T669, and is therefore able to be activated (as assessed by autophosphorylation on Y1068) by ligand.
2. Exposure to exogenous FGF activates ERK and p38 (and possibly additional kinases; discussed below) that phosphorylate ErbB1 on T669 (Figure 6). This modification desensitizes the receptor, rendering most ErbB1 unable to be activated by either exogenous or endogenous ligand (Figure 3). In other systems, phosphorylation of T669 has been proposed to inhibit the ability of ErbB1 to form a functional dimer, as is required for ligand-mediated ErbB tyrosine autophosphorylation and downstream signaling (Sato *et al.*, 2013; Kluba *et al.*, 2015; Thirukkumaran *et al.*, 2020). Because we have not assessed ErbB1 dimer formation in DCDMLs, we cannot rule out the possibility that phosphorylation of T669 perturbs the function of cell surface ErbB1 by some other mechanism.
3. A >2-d incubation with TGFβ increases the level of ErbB1 by ~3.7X (Figure 8). This increase in substrate increases the rate of T669 phosphorylation under basal kinase conditions, resulting in a similar fraction of ErbB1 modified on T669 as in no TGFβ or FGF cells. Although we have not determined the mechanism by which TGFβ increases ErbB1 levels, Shu *et al.* (2019) have reported that a 24-h treatment of rat lens epithelial cell explants with TGFβ results in a ~1.6-fold increase in *Egfr* transcript as detected by RT-qPCR. It is therefore likely that TGFβ acts, at least in part, on the transcriptional and/or posttranscriptional level, but effects on ErbB1 protein stability cannot currently be ruled out.
4. Constraints on the machinery responsible for the accumulation of phosphate on T669 prevents FGF from further increasing the number of ErbB1 molecules phosphorylated on this residue in plus TGFβ cells. These constraints could include limited quantities and/or colocalization of protein(s) that link ERK and/or p38

to ErbB1, or high levels of pT669 phosphatases. Evidence for the latter in DCDMLs is presented in Figure 9D. Although the identity of the enzyme that removes phosphate from T669 is unknown (Kluba *et al.*, 2015), rapid dephosphorylation of ErbB1 has been reported and proposed to be an important means to regulate receptor downstream signaling (Kleiman *et al.*, 2011). The majority of ErbB1 in TGFβ-cultured cells therefore remains unmodified on T669. This restores ErbB1 signaling in the presence of FGF (Figure 7). Upregulation of ErbB1 levels by TGFβ can be phenocopied by exogenous overexpression of ErbB1 in no TGFβ cells (Figure 10).

5. The higher levels of ErbB1 expression and function induced by 4 d of exposure to TGFβ allows TGFβ to drive full EMyT 48 h later in the presence of FGF.

Antagonistic role of FGF in ErbB signaling

We show here for the first time that FGF blocks ErbB1 activation and signaling in lens cells. Transinhibition of cell surface pools of ErbB1 was maximal after 4 h of exposure to lens fiber-inducing levels of FGF. Rapid inhibition of ErbB1 signaling by ligands of certain other receptors including those for AT_2, HGF, prolactin, and PDGF has been noted in other systems (Bedecs *et al.*, 1997; Quijano and Sheffield, 1998; Bagowski *et al.*, 1999; Sato *et al.*, 2013). To our knowledge, however, this is the first demonstration that ErbB1 can be transinhibited by FGFR activity in nontransformed cells.

Cisplatin and UVC light have previously been reported to induce the phosphorylation of ErbB1 on T669. This has been linked to intracellular accumulation of ErbB1 (Winograd-Katz and Levitzki, 2006; Tomas *et al.*, 2015), and to its activation as assessed by increased anti-pY1068 ErbB1 immunoreactivity (Tomas *et al.*, 2015). The effects we report here for FGF differ from these genotoxic stresses in that FGF does not induce a redistribution of ErbB1 into intracellular compartments, nor does it increase the level of pY1068 ErbB1 in the presence of either exogenous or endogenous ligand.

Although our data indicate that phosphorylation of T669 is required for FGF-induced desensitization of ErbB1, they do not preclude a role for additional mechanisms. Our observation that the p38 inhibitors SB-203580 and BIRB 796 partially rescue ErbB1 responsiveness from inhibition by FGF without markedly reducing FGF-induced phosphorylation of T669 (Figure 6A; Supplemental Figure S6B) suggests that p38 might mediate one or more other inhibitory modifications of ErbB1. The best characterized candidate for such an alteration is p38-dependent phosphorylation of S1046/7 (Countaway *et al.*, 1992; Adachi *et al.*, 2009; Nishimura *et al.*, 2009; Sato *et al.*, 2013). We were, however, unable to detect this modification in DCDMLs after activation of p38 with either anisomycin or FGF, although it cannot be ruled out that the phosphospecific antibody used does not recognize the avian protein. ErbB1 has been reported to undergo other, posttranslational modifications, including phosphorylation on T654, acetylation, and SUMOylation, that are negatively correlated with its activation state (Lin *et al.*, 1986; Horita *et al.*, 2017). It is possible that p38 activity increases such modifications either directly or indirectly to reduce ErbB1 sensitivity to ligand. In principle, an essential role of T669 phosphorylation in ErbB1 desensitization could be demonstrated by showing that overexpression of a T669 ErbB1 phosphomimetic mutant in DCDMLs fails to rescue ErbB1 activity from inhibition by FGF, unlike overexpression of wild-type ErbB1. It has, however, been reported that neither substitution of E or D in position 669 confers upon ErbB1 a desensitizing effect (Adak *et al.*, 2011), indicating that such an approach would not be informative.

Studies in other systems demonstrate that ErbB1 heterodimers as well as homodimers are subject to inhibitory phosphorylation of T669 (Sato *et al.*, 2013). That ErbB1/ErbB4 heterodimers are desensitized by FGF in DCDMLs is indicated by our finding that 4-h pretreatment with FGF blocks the ability of the ErbB4, but not ErbB1, ligand NRG1 to induce the phosphorylation of ErbB1 on pY1068 (Figure 3B). We have reported that TGFβ dramatically reduces the total and cell surface levels of ErbB4, and that overexpression of ErbB4 does not impede TGFβ-induced EMyT (VanSlyke *et al.*, 2023). Combined with reports that TGFβ can upregulate at least some aspects of EMyT in a human lens-derived cell line that does not express ErbB4 (Dawes *et al.*, 2007), it is unlikely that ErbB4 plays an essential role in lens cell fibrosis. We have reported that lens epithelial cells also express ErbB2 (VanSlyke *et al.*, 2023), which cannot bind ligand but which can form functional heterodimers with other ErbBs. Treatment of DCDMLs with TGFβ reduces both cell surface and total pools of ErbB2 concomitant with the increase in ErbB1 (VanSlyke *et al.*, 2023). Our demonstration that overexpression of exogenous ErbB2 cannot rescue ErbB1 from being inactivated by FGF (Supplemental Figure S8) is inconsistent with a mechanism by which TGFβ restores sensitivity to ErbB1 ligands by reducing ErbB2 levels to decrease the number of ErbB1/ErbB2 heterodimers. Taken together, these results indicate that ErbB1 is the predominant ErbB required for TGFβ-induced EMyT.

An inverse relationship between ErbB1 and FGF signaling has previously been described in the oncology literature. For example, inhibition of a constitutively active FGFR3 mutant in RT112M bladder cancer cells upregulates ErbB1 activity (e.g., phosphorylation on Y1068), rendering the cells more sensitive to ErbB inhibitors. This antagonism involves an FGFR inhibitor-induced reduction in ErbB1 endocytosis, and indicates that FGF signaling increases the internalization of ErbB1 in this system (Herrera-Abreu *et al.*, 2013). The relationship between ErbB1 and FGFRs we describe here in DCDMLs is distinct in that FGF signaling in DCDMLs is mediated by ligand-activated wild-type FGFRs, and because FGF does not reduce the cell surface expression of ErbB1 under conditions (4-h treatment) in which FGF desensitizes ErbB1 (Figure 5). It remains possible that in some CA FGFR oncogene-driven cells, as in normal lens cells, FGF desensitizes ErbB1 to ligand by stimulating phosphorylation on T669. Inhibition of FGFR signaling would reverse this inhibitory modification, thereby increasing ErbB signaling and rendering the cells more sensitive to ErbB inhibitors. If so, then our findings in lens cells could be relevant to cancer. Given that TGFβ can stimulate the expression of ErbB1 in some transformed cells (Zhao *et al.*, 2018), it would also be interesting to examine if TGFβ can render any CA FGFR oncogene-driven cells more sensitive to ErbB inhibitors.

Therapeutic implications of enhanced upregulation of ErbB1 by TGFβ for PCO

Our data support a model in which TGFβ-mediated upregulation of ErbB1 is essential for EMyT of lens cells in the presence of the high levels of FGF found at the posterior of the lens capsule. It would therefore follow that the development of fibrotic PCO after cataract surgery could be prevented by keeping the level of ErbB1 at basal, presurgery levels instead of requiring the complete obliteration of ErbB1 expression. This strategy would provide an alternative to blocking the function of all ErbBs with small molecule pan-ErbB kinase inhibitors, some of which (gefitinib; erlotinib) have been shown to have deleterious ocular side effects in human patients (Ibrahim *et al.*, 2012; Chow *et al.*, 2013). ErbB1-specific interventions in current clinical use and in development include function-blocking anti-ErbB1 antibodies, ErbB1-selective small molecule kinase inhibitors,

ErbB1-directed siRNA, and PROTAC strategies that target ErbB1 for degradation (Zhang, 2023; Zhang *et al.*, 2023). Indeed, in vivo administration of ErbB1-silencing siRNA has been reported to reduce PCO in a rat model (Huang *et al.*, 2011). Importantly, modern IOLs are designed to provide a strong physical barrier to the movement of lens epithelial cells to the posterior of the lens capsule by 2 wk after cataract surgery (Nishi *et al.*, 2002; Buehl *et al.*, 2004; Nixon, 2004; Fișuș and Findl, 2020). An ErbB1-targetted intervention may therefore only need to prevent ErbB1 upregulation, and only during the immediate postoperative period, to lead to a decrease in the initial formation of fibrotic PCO.

A clinically intractable problem is the delayed development of PCO months to years after cataract surgery (Dewey, 2006; Awasthi *et al.*, 2009). By preventing lens cells from becoming myofibroblasts in the acute postsurgical period, reducing ErbB1 expression and/or function would also be expected to decrease the production of EMyT-associated, pro-fibrotic extracellular matrix and integrins that cause the chronic activation of TGFβ thought to contribute to late-onset PCO and other fibrotic conditions (Mamuya and Duncan, 2012; VanSlyke *et al.*, 2018). Without ongoing TGFβ stimulation, levels of ErbB1 in lens epithelial cells would remain low, and the ErbB1 that is still expressed would be desensitized by FGF in the vitreous humor. Interventions that reduce ErbB1 expression could therefore lead to a robust and durable block in fibrotic PCO. Lastly, FGF2 has been reported to have antifibrotic effects in tissues in which myofibroblasts are reportedly mainly derived from nonepithelial sources (Dolivo, 2022). Given that TGFβ (Frangogiannis, 2020) and ErbB1(Blaine *et al.*, 2009; Fuchs *et al.*, 2014; Harris, 2021; Schramm *et al.*, 2022) can promote myofibroblast formation in multiple organs, it is possible that aspects of the TGFβ/ErbB1/FGF axis we describe here for lens cells are relevant to nonlenticular fibroproliferative diseases.

MATERIALS AND METHODS

Materials

Recombinant human TGFβ1, TGFα, HB-EGF, NRG1(EGF domain; #396-HB-050), BMP4, and bovine FGF2 were from R & D Systems (Minneapolis, MN). R3IGF-1, an analogue of human IGF1, was from GroPep (Adelaide, Australia). Mouse laminin (#23017015) and bovine plasma fibronectin (#33010018) were from Invitrogen (Carlsbad, CA, USA). The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): anti-pT669 ErbB1 (#3056), antiphospho-p44/42 ERK (#9106), anti–total p44/42 ERK (#9102), antiphospho-p38 (#9211), anti-total AKT (#9272), and antiphospho (Ser473) AKT (#9275). Total cellular pools of ErbB 1, 2, and 4 were detected using antibodies specific for the cytoplasmic tail domain of each receptor: for ErbB1, SC-03 from Santa Cruz Biotechnology (Santa Cruz, CA); for ErbB2, A0485 from Agilent (Santa Clara, CA), and for ErbB4, SC-243 (Santa Cruz Biotechnology). Two antibodies specific for the tyrosine1068 autophosphorylated, activated form of ErbB1 were used, a mouse monoclonal (#2236), or, where specified, a rabbit polyclonal (#2234), both from Cell Signaling Technology. Other commercial antibodies used in this study: for total p38, sc-535 from Santa Cruz Biotechnology; for α tubulin, T5168 from Sigma-Aldrich (St Louis, MO); for αSMA, clone 1A4 from Agilent; for β actin, clone C4 (MilliporeSigma, Billerica, MA, USA); for phosphotyrosine, clone 4G10 (MilliporeSigma); for chick fibronectin, B3/D6 (from D. Fambrough, Johns Hopkins University; Developmental Studies Hybridoma Bank, University of Iowa), and for procollagen I, SP1.D8 (from H. Furthmayr, Stanford University; Developmental Studies Hybridoma Bank). Rabbit antimouse CP49 polyclonal serum (#899 or 900) was a generous gift of Paul FitzGerald, University of California, Davis, as was the rabbit anti-CP115 antiserum (#76). SB-431542 was from Sigma-Aldrich. UO126, PD173074, SB-203580, tetradecanoylphorbol acetate (TPA), and anisomycin were from Millipore Sigma. Lapatinib was purchased from LC Labs (Woburn, MA), and BIRB 796 from Selleckchem (Houston, TX). Vitreous body–conditioned medium (VBCM) was prepared from E10 embryonic chick eyes as previously described (Le and Musil, 2001a).

DCDML cell culture and treatments

DCDML cultures were prepared from E10 chick lenses as previously described (Le and Musil, 1998). During this process, cells exterior to the lens capsule are removed and mature lens fiber cells die, leaving a preparation of purified lens epithelial cells. Cells were plated at subconfluent density (0.9×10^5 cells/well) onto laminin-coated 96-well tissue culture plates. Starting on day 1 of culture, the cells were fed every two days with 200 µl M199 medium supplemented with penicillin G and streptomycin. In experiments shown in Supplemental Figure S7, DCDMLs were plated on 12.5 µg/ml bovine plasma fibronectin (pdFN) instead of on laminin (VanSlyke *et al.*, 2018). Drugs were used at the following final concentrations: 4 µM lapatinib, 3 µM SB-431542, 15 µM UO126, 0.1 µM PD173074, 20 µM SB-203580, 1 µM BIRB 796, 3 µg/ml anisomycin, and 1 µM TPA. DCDMLs were incubated at 4°C with 10 nM of the ErbB ligands TGFα, HB-EGF, and NRG-1.

Immunofluorescence microscopy

DCDMLs grown on laminin-coated glass coverslips were fixed in 2% paraformaldehyde in phosphate-buffered saline and processed as previously described (Le and Musil, 1998, 2001b). Images were captured using a Leica (Teaneck, NJ, USA) DM LD photomicrography system and Scion (Frederick, MD, USA) Image 1.60 software.

Cell surface biotinylation

DCDMLs were biotinylated on ice using freshly prepared 0.25 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Thermoscientific #21331, Thermo Fisher Scientific, Waltham, MA, USA) as previously described (VanSlyke *et al.*, 2023). After solubilization of cells in sodium dodecyl sulfate (SDS), a portion of the total cell lysate was reserved for Western blot analysis of β actin or α tubulin as a loading control. The remainder of the sample was collected on strepavidin beads to isolate biotinylated cell surface proteins.

Alkaline phosphate digestion

DCDMLs were subjected to cell surface biotinylation at 4°C, after which biotinylated proteins were bound to strepavidin beads. The beads were washed three times in AP immunoprecipitation buffer (100 mM NaCl, 20 mM Na Borate, 0.02% NaN_3, pH 8.2) plus 0.5% Triton X-100 and 0.1% SDS, followed by one wash with AP reaction buffer (150 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.0) plus 0.05% Triton X-100 and 0.1% SDS. Beads were resuspended in 200 µl of detergent-free AP reaction buffer and one-half of each sample was incubated at 37°C for 1 h, either with or without 50 U calf intestinal alkaline phosphatase (Roche, #10713023001). The beads were then washed with immunoprecipitation buffer as previously described (VanSlyke and Musil, 2000), resuspended in BME-containing SDS–PAGE sample buffer, boiled, and the eluted proteins analyzed by Western blot.

Plasmids and transient transfection

One day after plating, DCDML cultures were transfected in M199 medium without antibiotics using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) following the manufacturer's suggested

protocol. Control experiments confirmed that the efficiency of transient transfection of DCDMLs is consistently ~70% (Boswell *et al.*, 2009). pcDNA3 plasmids encoding the following ErbB species were obtained from Addgene: ErbB2-EGFP (#39321), originally from Martin Offterdinger; EGFR-GFP (#32751) from Alexander Sorkin, and ErbB4 (#29527) from Yardena Samuels.

Immunoblot analysis

Whole cell lysates were solubilized directly into SDS–PAGE sample buffer and boiled. Equal volumes of total cell lysate were transferred to polyvinylidene fluoride membranes, and the blots probed with primary antibodies. Immunoreactive proteins were detected using secondary antibodies conjugated to either IRDye800 (Rockland Immunochemicals, Pottstown, PA) or Alexa Fluor 680 (Invitrogen, Waltham, MA) and directly quantified using the LI-COR Biosciences Odyssey infrared imaging system (Lincoln, NE) and associated software. The level of each protein was normalized to the level of β actin or α tubulin in the same sample. Data are expressed as the means ± SD obtained in the number of experiments indicated. Data were analyzed for significance using the two-tailed paired Student's *t* test. Unless indicated otherwise, *p* values are calculated compared with the condition indicated on the y-axis of the corresponding graph.

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