High Glucose Restraint of Acetylcholine-Induced Keratinocyte Epithelial-Mesenchymal Transition Is Mitigated by p38 Inhibition

Mark Wei Yi Tan1,2,7, Wei Ren Tan3,7, Ze Qing Kong1, Jun Hong Toh1, Wei Kiat Jonathan Wee1, Erica Mei Ling Teo2,3, Hong Sheng Cheng1, Xiaomeng Wang3,4,5,6 and Nguan Soon Tan1,3

Non-neuronal acetylcholine (Ach) plays important roles in various aspects of cell biology and homeostasis outside the neural system. Keratinocytes (KCs) have a functional cholinergic mechanism, suggesting that they respond to Ach. However, the physiological role and mechanism by which Ach modulates wound KC behavior in both nondiabetic and diabetic conditions are unexplored. We found an enrichment in neurotransmitter-related pathways in microdissected-migrating nondiabetic and diabetic KCs. We showed that Ach upregulated TGFβRII through Src-extracellular signal–regulated kinase 1/2 pathway to potentiate TGFβ1-mediated epithelial–mesenchymal transition in normoglycemic condition. Unexpectedly, KCs were nonresponsive to the elevated endogenous Ach in a hyperglycemic environment. We further showed that the activation of p38 MAPK in high glucose condition interferes with Src-extracellular signal–regulated kinase 1/2 signaling, resulting in Ach resistance that could be rescued by inhibiting p38 MAPK. A better understanding of the cholinergic physiology in diabetic KCs could improve wound management and care. The finding suggests that mitigating the inhibitory effect of diabetic wound microenvironment has a direct clinical implication on the efficacy and safety of various wound healing agents to improve chronic diabetic wounds.

INTRODUCTION

Many patients with diabetes who sustain trauma are often unaware of the injury because of peripheral neuropathy and therefore do not receive timely, appropriate treatment. It tremendously increases the risk of developing foot ulcers and can lead to amputation, after which the patients are subjected to a 5-year survival rate of ~40% (Singh et al., 2014). In addition to the high medical and socioeconomic costs, it has a profound effect on the QOL that patients with diabetes incur from disability owing to foot ulcers or poorly healing wounds (Sen et al., 2009). Although these clinical realities have been appreciated for decades, our understanding and ability to treat diabetic wounds remain unacceptably deficient.

Poor wound healing and peripheral neuropathy are two debilitating complications of diabetes that are often studied independently. Numerous studies suggested that the alleviation of one tends to improve the other (Cameron and Cotter, 1997; Schratzberger et al., 2000). It was documented that all phases of wound healing are impaired in denervated tissue, and these mechanisms are different from those related to the underlying disease of diabetes (Barker et al., 2006). Acetylcholine (Ach) is a well-established neurotransmitter in cholinergic neurotransmission. However, there is little emphasis on its non-neuronal functions, which involve the activation of cholinergic signaling in non-neuronal cells. Keratinocytes (KCs), the major cell type involved in wound re-epithelialization, have a functional cholinergic system that is suggestive of their responsiveness to Ach (Hana et al., 2007; Kurzen et al., 2007). However, the precise role and mechanism by which Ach modulates KC behavior during re-epithelialization remain obscure. Importantly, the effect of Ach on diabetic wounds is also unclear.

Wound re-epithelialization is a cooperative multifactorial process, dominated by KC migration, proliferation, and differentiation that are essential for successful wound closure. The re-epithelialization process is impaired in poorly healing wounds (Rousselle et al., 2019). It has been speculated that the initiation of re-epithelialization is similar to the epithelial–mesenchymal transition (EMT) process observed during...
Figure 1. Impaired KC EMT in diabetic wounds. (a) H&E images and immunostaining of E-cadherin and SNAI1 in the epithelial tongue of nondiabetic and diabetic wounds at days 1–3 after injury. Hoechst 33342 was used for nuclear counterstain. Bars = 100 μm. (b) Percentage of EMT cells based on the loss of E-cadherin staining (left panel) in wound tip (≤100 μm) and wound surface area occupied by these cells (right panel) in nondiabetic and diabetic wounds at days 1–3 after injury. (c) Top 50 enriched pathways of laser-captured KCs undergoing EMT. Ach- and neuronal-related pathways are highlighted in red and blue, respectively. Heatmaps show the expression of directionality. n = 9 mice per group. Data in b were expressed as means ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05 (Mann–Whitney test). Ach, acetylcholine; EMT, epithelial–mesenchymal transition; KC, keratinocyte.
Figure 2. Ach accelerates EMT in nondiabetic but not in diabetic wounds. (a) H&E images and immunostaining of E-cadherin and SNAI1 in the epithelial tongue of nondiabetic and diabetic wounds at days 1–3 after injury with and without Ach treatment. Hoechst 33342 was used for nuclear counterstain. Bars = 100 μm. (b) Percentage of EMT cells based on the loss of E-cadherin staining (left panel) in wound tip (≤100 μm) and wound surface area occupied by these cells (right panel) of vehicle- and Ach-treated wounds at days 1–3 after injury. n = 9 mice for each group. Data in b were expressed as means ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05 (Mann–Whitney U test). Ach, acetylcholine; EMT, epithelial–mesenchymal transition; n.s., not significant.
embryogenesis and cancer metastasis (Haensel and Dai, 2018; Yan et al., 2010). However, studying this process in wound healing has been technically challenging because only a small number of KCs at the epithelial tongue undergo EMT even for normal healing wounds. Thus, molecular insights of EMT in KCs during re-epithelialization and how EMT affects diabetic KCs remain unknown.

In this study, we reveal a non-neuronal role of Ach and decipher the mechanism by which it stimulates EMT. We further show that a hyperglycemic environment dampens cellular responses to Ach, that is, Ach resistance. The inhibition of the suppressive effects of high glucose (HG) enhances cellular responses to various wound healing agents.

RESULTS
Enrichment of neurotransmitter-related genes in KCs undergoing EMT
To understand whether the complications of delayed wound closure in diabetes can be attributed to the differences in the EMT of KCs, we examined nondiabetic and diabetic mice wounds at days 1–3 after injury. Mice with diabetes showed increased fasting plasma glucose levels and impaired glucose tolerance (Supplementary Figure S1a). Shifts in EMT expressions during wound closure were observed as early as on day 2 after wounding in the mice without diabetes, as indicated by reduced E-cadherin and increased SNAI1 expression in wound edge (Figure 1a and b). In contrast to nondiabetic wounds, such EMT shifts during wound closure were attenuated in diabetic wounds (Figure 1a and b), suggesting that impaired EMT of KCs was associated with poor diabetic wound healing.

To understand the mechanism of EMT during wound closure, KCs undergoing EMT from nondiabetic and diabetic wounds were microdissected for gene expression microarray (Supplementary Figure S1b). Enrichment analysis of the differentially expressed genes revealed that pathways associated with Ach, such as nicotinic Ach receptors and muscarinic Ach receptors 1–4 signaling pathways, as well as cellular pathways involved in re-epithelialization, for example, cadherin signaling, were also highly ranked. The enrichment of Ach pathways points to a non-neuronal role for Ach during wound EMT (Figure 1c).

Topical Ach accelerates EMT in nondiabetic wounds but not in diabetic wounds
To examine whether Ach can modulate EMT, nondiabetic and diabetic wounds were topically treated with Ach. As expected, vehicle-treated nondiabetic wounds were smaller than cognate diabetic wounds (Figure 1a and b). In contrast to nondiabetic wounds, such EMT shifts during wound closure were attenuated in diabetic wounds (Figure 1a and b), suggesting that impaired EMT of KCs was associated with poor diabetic wound healing.
Figure 4. Ach and TGFβ1 induce EMT in KCs under normoglycemic but not under hyperglycemic conditions. (a) Heatmap showing the upregulation (red) and downregulation (green) of EMT-related genes after exposure to Ach or TGFβ1 for 24 h in normoglycemia and hyperglycemia. (b) Immunoblots of E-cadherin, SNAI1, vimentin, and Slug after exposure to Ach or TGFβ1 in normoglycemia. β-Tubulin from the same samples serves as a loading Ctrl. (c) Cumulative distance traveled by HaCaT and Ker-CT on the basis of live-cell imaging over 24 h after exposure to either Ach (blue) or TGFβ1 (red) in normoglycemia (solid circles) and hyperglycemia (open circles). n = 15 cells per group. (d) Cell tracking of HaCaT and Ker-CT using live-cell imaging over 24 h after exposure to the indicated treatments. Displacement of each cell is indicated by the straight lines, and the radii of circles indicate the average displacement. n = 15 cells for each group.
wounds underwent EMT than those in vehicle-treated wounds, as revealed by E-cadherin and SNAI1 immunostaining (Figure 2a and b). Similarly, the total surface area of wound EMT was also greater than that of vehicle-treated nondiabetic wounds (Figure 2b). Unlike nondiabetic wounds, exogenous Ach and acetylcholinesterase (AchE) neither induced EMT nor accelerated the recovery of diabetic wounds, highlighting the insensitivity of diabetic wounds to Ach (Figure 2 and Supplementary Figure S1c).

Because the genes involved in Ach metabolism were enriched in our gene expression analysis, we hypothesized that the Ach resistance in diabetic KCs may be due to the rapid degradation of Ach in the diabetic wound microenvironment. Mean AchE, a key enzyme in the catabolism of Ach, activity was unchanged in nondiabetic and diabetic wound fluids (Figure 3a), suggesting a similar extent of Ach degradation in both wounds. Unexpectedly, a higher cumulative mean concentration of Ach was detected in the diabetic than in the nondiabetic wound fluids at 24 hours after injury (Figure 3b). Ach was elevated in diabetic wounds as early as 4 hours after injury, continued to increase over 24 hours, and began to decrease at 36–48 hours. In contrast, the Ach level in the nondiabetic wounds increased over the first 20 hours of injury, albeit at a lower level, and began to decrease at 24–48 hours (Figure 3c). To account for the Ach flux, the ratio of Ach to total choline in the wound fluid was determined in the presence of an inhibitor of AchE. A higher ratio of Ach to total choline was detected in the diabetic than in the nondiabetic wound fluids (Figure 3d and e), suggesting that the higher Ach in diabetic wound fluids was due to higher production of Ach. The high endogenous level of Ach in the diabetic wound was likely a compensatory response to the Ach resistance. Indeed, Ach and the ratio of Ach to total choline were higher in wound fluids after streptozotocin-induced diabetes (Figure 3f and g). Our observation suggests that KCs in nondiabetic wounds were responsive to Ach, whereas diabetic KCs were Ach resistant.

**Ach stimulates KCs EMT in normoglycemic conditions**

To further confirm a role for Ach in KCs EMT, live-cell imaging was used to monitor the response of immortalized TERT human KCs and HaCaT cells to either Ach, TGFβ1 (positive control), vehicle, and choline (negative control) in normoglycemia (5 mM glucose; low glucose [LG]). As expected, TGFβ1 treatment for 24 hours upregulated and downregulated the expression of mesenchymal and epithelial markers of EMT, respectively, when compared with vehicle treatment (Figure 4a and b). Although, Ach-treated cells in LG condition underwent incomplete EMT after 24 hours (Figure 4a and b), an increase in cell migration and forward cell movement were detected (Figure 4c and d and Supplementary Figure S2a). Choline-induced cells did not display any significant differences in EMT markers from the uninjured cells (Supplementary Figure S2b).

Despite a high endogenous level of Ach, KCs in the hyperglycemic milieu were refractory to Ach. The conditioned medium of KCs cultured in HG (25 mM glucose) had a higher level of Ach than the conditioned LG medium, consistent with the diabetic wound fluids (Supplementary Figure S2c). KCs in the HG medium had lower expression of muscarinic (CHRM4) and nicotinic (CHRNA9) Ach receptor than those in the LG condition, but in vitro scratch wounding enhanced their expression (Supplementary Figure S2d). Together with the higher endogenous Ach, this observation suggests that cells in the HG condition can respond to Ach. The effects by Ach and TGFβ1 on EMT markers and associated phenotypic changes were severely reduced in cells cultured in the HG medium (Figure 4a, c, and d). These observations suggest that Ach indirectly stimulates KC EMT, which can be disrupted by HG condition.

**Ach increases TGFβRII to potentiate EMT in KCs**

TGFβ1 has the broadest spectrum of effects on wound healing and is also a potent EMT stimulus. We hypothesize that Ach indirectly stimulates EMT through the TGFβ1 pathway. Thus, the effect of Ach on the expression of TGFβ1 and its receptor TGFβRII was examined. LG and HG conditions per se did not alter the surface expression of TGFβRII (Supplementary Figure S2e). An increase in TGFβRII mRNA and higher surface expression of TGFβRII, as confirmed by flow cytometry, were detected in Ach-treated LG immortalized TERT human KCs, HaCaT cells, and primary human KCs (Figure 5a and Supplementary Figures S3 to S5). Notably, cells exposed to Ach followed by TGFβ1 exhibited more robust changes in the expression of EMT markers and a significantly higher level of phosphorylated SMAD3 (p-SMAD3) than those exposed to Ach or TGFβ1 alone, indicating an enhanced TGFβ1 signaling (Figure 5b and c and Supplementary Figures S3b and c and S4b and c). In concordance, cells exposed to Ach followed by TGFβ1 showed more forward cell movement than those exposed to Ach or TGFβ1 alone (Supplementary Figure S2c). Despite the high endogenous or ectopic exogenous Ach, HG-treated KCs did not respond to Ach as depicted by the lack of increase in TGFβRII mRNA and protein (Figure 5d and Supplementary Figures S3d and S4d). Ach did not further enhance TGFβ1 signaling in these cells and showed little change in EMT marker expression profile (Figure 5e and f and Supplementary Figures S3e and f and S4e and f). To verify the effect of Ach in vivo, wound epithelial tongues of Ach- and vehicle-treated nondiabetic wounds as well as those of diabetic wounds were stained for p-SMAD3. In Ach-treated nondiabetic wounds, more p-SMAD3 staining (Figure 5e and Supplementary Figure S5). Despite the elevated endogenous Ach in diabetic wounds, p-SMAD3–positive wound KCs were only evident on day 3 after injury, indicating delayed activation of TGFβ1 signaling.
Figure 5. Ach upregulates TGFβRII through the Src-ERK1/2 pathway to sensitize KCs to TGFβ1-mediated EMT in Ker-CT. (a, d) Relative gene expression of TGFβ1 and TGFβRII and surface expression of TGFβRII in vehicle-treated (blue) and Ach-treated (red) Ker-CT in (a) low glucose and (d) high glucose conditions. (b, c, e, f) Relative fold change in (b, e) mRNA and (c, f) protein of SNAI1, Slug, vimentin, and E-cadherin levels in Ker-CT, subjected to indicated treatments, cultured in (b, c) low glucose and (e, f) high glucose conditions. Immunoblots of p-SMAD, t-SMAD, SNAI1, Slug, vimentin, and E-cadherin are shown. β-Tubulin from the same samples serves as a loading Ctrl. (g) Relative fold change of TGFβRII mRNA in Ach-treated Ker-CT after kinase inhibitor screening in low glucose.

MWY Tan et al. Acetylcholine Enhances Wound Healing

Journal of Investigative Dermatology (2021), Volume 141
The above observation raises the question of how Ach increases the expression of TGFβRII to enhance TGFβ1-mediated EMT. Ach-stimulated LG KCs were subjected to an unbiased kinase inhibitor screening to identify the signaling pathways triggered by Ach. TGFβRII mRNA was increased by Ach within 6 hours in LG KCs (Figure 5g). We reasoned that kinase inhibitors that negated the Ach-mediated upregulation of TGFβRII mRNA indicate the involvement of their kinase targets in the signaling cascades (Supplementary Figure S6a). A total of 13 kinase inhibitors attenuated the Ach-mediated increase in TGFβRII transcription (Figure 5g). Pathway analysis revealed Src and extracellular signal–regulated kinase (ERK) as key signaling nodes required for the upregulation of TGFβRII by Ach (Figure 5h). Immunoblot analysis confirmed enhanced phosphorylated Src and ERK1 on Ach treatment (Figure 5g and Supplementary Figures S3g and S4g).

In summary, Ach increases the expression of TGFβRII through Src-ERK–mediated pathway in LG KCs. These Ach-treated LG KCs have enhanced TGFβ1 signaling and EMT markers. In contrast, TGFβRII expression was not increased by Ach in HG KCs.

**HG activates p38 pathway to attenuate the cellular response to Ach**

HG KCs produced more Ach than LG KCs (Supplementary Figure S2b). Unlike LG KCs, HG KCs did not respond to Ach-induced EMT. To decipher the mechanism underlying this Ach resistance, another kinase inhibitor screening using HG KCs was performed. In this instance, kinase inhibitors that alleviate the suppressive effect of HG on Ach-mediated upregulation of TGFβRII mRNA indicate that these kinases are involved (Supplementary Figure S6b). Three kinase inhibitors resulted in the upregulation of TGFβRII by Ach in HG KCs (Figure 6a). Pathway and immunoblot analyses revealed that HG activated the p38 MAPK pathway, which interfered with Ach-Src-ERK pathway, to suppress the KC response to Ach (Figure 6a and b and Supplementary Figures S3h and S4h). We rationalize that if p38 kinase was inhibited, Ach could activate Src-ERK pathway and increase TGFβRII in HG KCs to facilitate EMT. Indeed, immunoblot analysis showed that HG KCs treated with solamargine, a p38 kinase inhibitor, have higher phosphorylated ERK1/2, phosphorylated MAPK/ERK kinase 1/2, and increased surface expression of TGFβRII (Figure 6c and d and Supplementary Figures S3j and S4i). The cotreatment of solamargine with TGFβ1 facilitated EMT as evidenced by changes in the SNAI1 and E-cadherin expressions (Figure 6e and Supplementary Figure S3k). In vivo, the topical application of solamargine on diabetic wounds accelerated wound closure compared with application of the vehicle (Supplementary Figure S6c). Thus, HG restrains KCs to Ach-induced EMT, which is mitigated by the inhibition of p38 kinase.

**DISCUSSION**

Diabetic neuropathic injuries represent a serious health care burden to patients and society. Whereas the management of chronic diabetic wounds has improved in recent years, it remains a frustrating problem for clinicians and caretakers. As the most abundant neurotransmitter, Ach is involved in almost all aspects of diabetes and its complications. A non-neuronal role of Ach in diabetic wound healing and the mechanism remain unclear.

In our study, the transcriptomic profiles of KCs undergoing EMT revealed an enrichment of neurologically related and Ach-related pathways. This highlights a strong relationship between neuropathy and the migratory potential of KCs at the tip of the epithelial tongue during re-epithelialization. When an injury occurs, Ach in wound fluids begin to increase and plateau by 24 hours. The early onset of Ach production after wounding is functionally engaged to TGFβ1, which is a potent inducer of EMT and a major player of wound healing. In normoglycemic wounds, Ach upregulated the expression of TGFβRII in KCs through the Src-ERK pathway to promote TGFβ1-SMAD2–mediated EMT. Taken together, Ach encourages EMT in KCs in vitro and in vivo.

In contrast, hyperglycemic wound microenvironment dampens the responsiveness of KCs at the tip of diabetic wound tongues to Ach. Diabetic wound fluids have higher Ach over 24 hours after injury than nondiabetic wounds. Analogous to insulin resistance in diabetes, KCs in hyperglycemic conditions were resistant to Ach, leading to diminished TGFβRII, impaired TGFβ1-mediated signaling, and delayed EMT. Consistent with delayed EMT at the epithelial tongue of diabetic wounds, low TGFβ1 activity in diabetic wounds as a result of reduced TGFβ1 and TGFβRII expression has been reported (Al-Mulla et al., 2011; Jude et al., 2002; Kim et al., 2003). We observed fewer KCs at the tip of the diabetic wound tongue expressing p-SMAD3 than at the tip of nondiabetic wounds treated or not with Ach, once again underlining Ach resistance in diabetic KCs. Ach resistance is linked to the activation of p38 kinase pathway that interferes with Src-ERK cascade. Thus, this suppressive effect by HG must be resolved for Ach to participate in wound re-epithelialization. Indeed, the inhibition of p38 kinase by solamargine sensitized HG KCs to endogenous Ach and TGFβ1-mediated EMT. A p38 MAPK inhibitor is currently tested for neuropathic pain management (NCT00390845) and chronic inflammation, which are prevalent in patients suffering from diabetic foot ulcers. To our knowledge, no p38 MAPK inhibitors are widely explored for diabetic wound therapy. Our findings highlight the potential clinical condition.

Kinases involved in Ach-mediated upregulation of TGFβRII are highlighted in red. Immunoblots of total and phosphorylated Src and ERK1/2 vehicle- and Ach-treated Ker-CT cells are shown. (b) Connectivity network of the identified kinases (red) involved in the Ach-mediated upregulation of TGFβRII. Data were from n = 4 independent experiments with triplicates. Data in a−g were expressed as means ± SEM. Data in a, d, and g were analyzed by Mann–Whitney test. Data in b, c, e, and f were analyzed using Dunnett’s post-hoc test after one-way ANOVA. ***P < 0.001, **P < 0.01, *P < 0.05. Ach, acetylcholine; Ctrl, control; EMT, epithelial–mesenchymal transition; ERK, extracellular signal–regulated kinase; KC, keratinocyte; Ker-CT, immortalized TERT human keratinocyte; n.s., not significant; PE, phycoerythrin; p-ERK, phosphorylated extracellular signal–regulated kinase; p-SMAD, phosphorylated SMAD; p-Src, phosphorylated Src; t-SMAD, total SMAD.
Figure 6. High glucose restrains Ach-induced EMT in KCs through p38 MAPK. (a) Relative fold change of TGFβRII mRNA in Ach-treated Ker-CT after kinase inhibitor screening in high glucose condition (left panel). Kinases involved in suppressing Ach-mediated upregulation of TGFβRII are indicated in blue. Immunoblots of total and phosphorylated p38 kinase in vehicle- and Ach-treated Ker-CT cultured in low and high glucose are shown (right panel). (b) Connectivity network of the identified kinases (blue) involved in high glucose suppression of the Ach-mediated upregulation of TGFβRII. Blue lines indicate the possible mechanisms by which p38 MAPK exerts its repressive effects. (c) Immunoblots of total and p-Src, ERK1/2, and MEK1/2 in Ker-CT treated with vehicle and p38 MAPK inhibitor (solamargine) cultured in high glucose condition. (d) Relative gene expression of TGFβRII and surface expression of TGFβRII in vehicle- and p38 inhibitor–treated Ker-CT under hyperglycemia. (e) Immunoblots of p-SMAD, SMAD, SNAI1, Slug, vimentin, and E-cadherin in HG–treated Ker-CT.
significance of targeting p38 MAPK as an adjunctive treatment for this diabetic complication.

The role of Ach, AchE, and its receptors on diabetic neuropathy is still emerging. Patients with diabetic neuropathy showed significantly reduced vasodilator responses to Ach (Quattrini et al., 2007). AchE deficiency also contributes to neuromuscular junction dysfunction in type 1 diabetic neuropathy (Garcia et al., 2012). Sensory neurons can produce, release, and respond to Ach. Surprisingly, diabetic muscarinic Ach receptor type 1-deficient mice were protected from the physiological and structural indices of sensory neuropathy. The pharmacological blockade of muscarinic Ach receptor type 1 also prevented or reversed indices of peripheral neuropathy, including the depletion of sensory nerve terminals (Calcott et al., 2017; Jolivalt et al., 2020).

High blood sugar primarily affects cells that have a limited capacity to regulate their glucose intake. These cells include capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons of the peripheral nervous system and CNS. As a result, hyperglycemia leads to largely intractable complications such as retinopathy, nephropathy, hypertension, and neuropathy (Rajchgot et al., 2019). Nondiabetic KCs respond to insulin and regulate glucose uptake through glucose transporters. In contrast, diabetic KCs are resistant to Ach. Although we did not examine the effect of Ach on sensory neurons in diabetic wounds, it will be interesting to know whether neurons also exhibit Ach resistance. Clearly, future investigation on Ach resistance in various cell types in wound healing will be of great interest.

The role of nicotine on wound healing remains unclear (Martin et al., 2009). Low doses of nicotine accelerate angiogenesis and wound healing in mice with and without diabetes (Jacobi et al., 2002; Morimoto et al., 2008). Other studies showed that nicotine impaired wound contraction (Mosely et al., 1978) and has deleterious effects on wound healing through increased vasoconstriction and its immunosuppressive effect (Davies and Ismail, 2016; Kalra et al., 2004). It was proposed that nicotine dosages have a bimodal impact and may act using several nicotinic Ach receptor subtypes or cell types. Thus, low versus high concentrations or acute versus chronic stimulation with nicotine can affect different cell types and outcomes (Chernavsky et al., 2004; Martin et al., 2009). Although nicotine is usually used to imitate the action of Ach, there are differences in their mechanism and metabolism. In contrast to nicotine that mainly acts through nicotinic Ach receptors, Ach can activate the nicotinic Ach receptors and muscarinic Ach receptors. It is also conceivable that HG differentially regulates the receptors’ expression in a cell-dependent manner. The availability of endogenous Ach in the wound fluid is a balance between the degradation by AchE and cellular production, whereas exogenous nicotine concentration depends on the level of Cyp2A6 (Benowitz et al., 2006). Importantly, such differences in their metabolism and action can alter their concentrations, affecting wound healing outcomes.

Recombinant PDGF-BB remains the only pharmacological biologics approved by the Food and Drug Administration for the treatment of chronic wounds. Numerous GFs and cytokines have been shown to improve diabetic wound healing; however, their clinical adoption has generally been disappointing (Okonkwo and DiPietro, 2017; Zubair and Ahmad, 2019). These therapies often use doses that far exceed their endogenous levels, which increase their risk of side effects. It is tempting to speculate that hyperglycemic condition exerts a similar suppressive effect on the action of other prohealing GFs. Thus, modulators that mitigate HG effect can be potential adjunctive therapeutic agents. This finding has wide-ranging implications on the safety and efficacy of these GFs to improve diabetic wounds.

MATERIALS AND METHODS

Cell treatments

Cell lines used in this study include immortalized TERT human KCs and primary human KCs. Cells for each respective treatment were serum-starved overnight before treatment exposure. Treatments provided included 25 mM glucose, 10 ng/ml TGFβ1 (PeproTech, Rocky Hill, NJ), 5 mM Ach chloride (Sigma-Aldrich, St. Louis, MO), 5 mM choline chloride (Sigma-Aldrich), and kinase inhibitors (Target Molecule Corporation, Boston, MA) at their respective half-maximal inhibitory concentration. For exposure to multiple agents, the agents were added at 4-hour intervals from each other.

Mice wounding

Type 2 diabetes was induced in male C57BL/6j mice aged 8 weeks as previously described (Furman, 2015). Mice fasted for 16 hours, and blood glucose levels were monitored with a glucometer. Mice with a fasting blood glucose >5 mmol/L and impaired glucose tolerance were considered diabetic. Full-thickness excisional wounds were inflicted as previously described (Tan and Wahl, 2013) on mice after being diabetic for >1 week. Wound fluid or exudate was collected as described (Goh et al., 2010). In Ach-treated wounds, 2.5 nM Ach was added to the wounds immediately after the injury. Wounds were harvested at indicated intervals and processed for immunofluorescence staining as previously described (Teo et al., 2017). In AchE-treated wounds, 10 µl of 3 U/ml of AchE (Sigma-Aldrich) in 0.25 M Tris-hydrogen chloride (pH 8.0) and buffer alone were added immediately to the inflicted treated and control wounds, respectively. For solamargine treatment, diabetic wounds were topically treated with 10 µl of 10 µM/ml solamargine or vehicle (0.1% DMSO in 0.9% saline). Wounds were imaged and harvested at indicated intervals. All mice experiments were carried out in accordance with the guidelines of the Nanyang Technological University Singapore Institutional Animal Care and Use Committee (A0250, A0322, A19043, and A19032).

subjected to the indicated treatments. (f) Cell tracking of Ker-CT cultured in high glucose using live-cell imaging over 24 hours after exposure to the indicated treatments. Displacement is indicated by straight lines, and the radii of the circles indicate the average displacement. n = 15 cells for each group. Data were from n = 3 independent experiments with triplicates. Data in a, c–e were expressed as means ± SEM. Data in a, c, and d were analyzed using Mann–Whitney test. Data in e were analyzed using Dunnett’s post-hoc test after one-way ANOVA. **P < 0.01, ***P < 0.001, *P < 0.05. Ach, acetylcholine; ERK, extracellular signal–regulated kinase; EMT, epithelial–mesenchymal transition; HG, high glucose; KC, keratinocyte; Ker-CT, immortalized TERT human keratinocyte; MEK, MAPK/ERK kinase; n.s., not significant; p-ERK, phosphorylated extracellular signal–regulated kinase; p-MEK, phosphorylated MAPK/ERK kinase; p-p38, phosphorylated p38; p-SMAD, phosphorylated SMAD; p-Src, phosphorylated Src; t-SMAD, total SMAD.
Live-cell imaging and analysis
Live-cell imaging was performed in six-well plates at 1-hour intervals over a 24 hours using JuLi Stage real-time cell history recorder (NanoEnTek Inc, Seoul, Korea) using a UPlanSApo 4X 0.16 numerical aperture objective lens (Olympus, Tokyo, Japan). Cell movement was tracked and measured using the Manual Tracking plugin on the Imager software (National Institutes of Health, Bethesda, MD). Directionalities were then made using the Chemotaxis and Migration Tool (Ibidi, Gräfelfing, Germany).

Kinase inhibitor array
Immortalized TERT human KCs were treated with different kinase inhibitors (Target Molecule Corporation) for 4 hours, after which 5 mM Ach was added, and they were further incubated for 6 hours before qPCR analysis.

Real-time qPCR
Real-time qPCR was performed as previously described (Lam et al., 2011) using the respective primers (Supplementary Table S1). Except for Figure 4 (24 hours), all samples were obtained at 6 hours after exposure to the last agent.

Far-infrared immunoblotting
Cells were lysed using ice-cold M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA). Far-infrared immunoblotting was performed as previously described (Chan et al., 2018; Chong et al., 2014). Primary and secondary antibodies used are in Supplementary Table S2.

Ach/AchE assay
Ach amount was measured using the Amplex Red Acetylcholine/ Acetylcholinesterase Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Samples were measured with a microplate reader (Infinite 200, Tecan, Crailsheim, Germany) at ex = 560 nm and em = 590 nm. Standard curve was derived using exact choline concentrations. The concentration of Ach was obtained by subtracting the concentration of free choline from total choline.

Statistical analysis
Statistical differences were evaluated with either a two-tailed Mann- Whitney U test or one-way ANOVA test with Statistical Package for the Social Sciences software wherever appropriate. P-values < 0.05 indicated statistical significance.

Additional methods are provided in Supplementary Materials and Methods.

Data availability statement
Datasets related to this article can be found at Gene Expression Omnibus accession number GSE141956, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE141956, hosted at Gene Expression Omnibus.

ORCIDs
Mark Wei Yi Tan: http://orcid.org/0000-0003-3927-8600
Wei Ren Tan: http://orcid.org/0000-0002-5076-0286
Ze Qing Kong: http://orcid.org/0000-0002-2132-6152
Jun Hong Toh: http://orcid.org/0000-0003-1967-7276
Wei Kiat Jonathan Wee: http://orcid.org/0000-0002-8392-3497
Erica Mei Ling Teo: http://orcid.org/0000-0002-2748-5218
Hong Sheng Cheng: http://orcid.org/0000-0001-9745-7872
Xiaomeng Wang: http://orcid.org/0000-0002-1036-2764
Nguan Soon Tan: http://orcid.org/0000-0003-0136-7341

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
MWYT and EMLT are scholarship recipients of the Interdisciplinary Graduate School of Nanyang Technological University Institute for Health Technolog. We thank Walter Wahl for his useful discussion and input, Yun Sheng Yip and Justin Yin Hao Lee for their logistic assistance. This research is sup- ported by the Singapore Ministry of Education under its Singapore Ministry of Education Academic Research Fund Tier 1 (2014-T1-002-138-03) and Tier 2 (MOE2018-T2-1-043) to NST.

AUTHOR CONTRIBUTIONS
Conceptualization: MWYT, NST; Formal Analysis: MWYT, WRT, HSC, WX, NST; Funding Acquisition: NST; Investigation: MWYT, WRT, ZQK, JHT, WKJW, EMLT, HSC; Supervision: WX, NST; Writing - Original Draft Preparation: MWYT, WRT, WX, NST; Writing - Review and Editing: MWYT, WRT, HSC, WX, NST

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.10.026.

REFERENCES
Chan JSK, Sng MK, Teo ZQ, Chong HC, Twang JS, Tan NS. Targeting nuclear receptors in cancer-associated fibroblasts as concurrent therapy to inhibit development of chemoresistant tumors. Oncogene 2018;37:160–73.
Davies CS, Ismail A. Nicotine has deleterious effects on wound healing through increased vasoconstriction. BMJ 2016;353:i2709.
Furman BL. Strepotozocin-induced diabetic models in mice and rats. Curr Protoc Pharmacol 2015;70. 5.47.1.


SUPPLEMENTARY MATERIALS AND METHODS
Sources of cell lines and culture methods
Immortalized TERT human keratinocytes and primary human keratinocytes were obtained from ATCC (Manassas, VA) and cultured in KGM-GOLD BulletKit medium (Lonza, Basel, Switzerland). HaCaT cells were obtained from AddexBio (T0020001, San Diego, CA) and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT). Both cell cultures were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Mouse and housing conditions
Male C57BL/6J mice aged 8 weeks were used. All mice were housed in a temperature-controlled room (22±1 °C) on a 12:12 light–dark cycle with ad libitum access to standard chow diet and water.

Laser capture microdissection
Laser capture microdissection was performed on immunohistologically stained sections of wounds using the Zeiss PALM Laser Capture Microdissection system (Carl Zeiss, Oberkochen, Germany) according to manufacturer’s instructions. All the samples were collected using separate adhesive CapSure Macro LCM Caps (Life Technologies, Carlsbad, CA). A total of 50 sections per sample were accumulated within each tube. Images were taken using the Fluor ×10/0.25 and Korr LD Plan-Neofluar ×40/0.60 objectives alongside the AxioCam ICC1 color camera installed in the microscope.

Microarray gene analysis and gene ontology enrichment analysis
RNA amplification and single-stranded DNA synthesis were carried out using the GeneChip WT Pico Kit (Affymetrix, Santa Clara, CA) on laser-captured samples. Hybridization was done using GeneChip Mouse Gene 1.0 ST Array as recommended by the manufacturer (Affymetrix). Raw microarray data files (Gene Expression Omnibus accession numbers: GSE141956) were analyzed using Partek Genomic Suite (version 6.6). Mice with diabetes were contrasted against those with diabetes, and pathway predictions were carried out with PantherGO using a 1.5-fold cutoff (Mi et al., 2007; Thomas et al., 2003). Heatmaps were generated using R 4.0.0 for expressed genes in highlighted pathways.

Ingenuity pathway analysis
For the low-glucose pathway, kinase list (in red) was derived from the unbiased kinase inhibitor screen, which was then connected to two major nodes of acetylcholine and its receptor and the TGFβRI, TGFβRII kinases, and TGFβR nodes. Intermediate nodes (denoted in gray) are grown off by the curated pathway analysis algorithm. For the high-glucose pathway, the p38-MAPK node (in blue) was integrated into the low-glucose pathway.

Flow cytometry
Cells were rinsed with PBS before being treated with 10 mM of EDTA to dislodge cells from culture flasks. Cells were resuspended in 50 μl of blocking buffer (3% BSA, 2 mM of EDTA in 1× PBS) for 45 minutes before incubation with the indicated antibody for 1 hour (Supplementary Table S1) (1:50 dilution). Samples were washed and stained with secondary phycoerythrin-conjugated antibody (BioLegend, San Diego, CA) before flow cytometry analysis using BD Accur C6 Plus (BD Biosciences, Franklin Lakes, NJ). Post-FACS analysis was performed using FlowJo, version 10.0.7 (FlowJo LLC, Ashland, OR).

SUPPLEMENTARY REFERENCES
Supplementary Figure S1. Immunohistochemically stained and macroscopic images of wounds from mice with and without diabetes. (a) IGTT curves for mice with and without diabetes. (b) Representative wound images on day 3 after injury obtained from mice with and without diabetes before and after laser capture microdissection. Immunohistochemical staining of E-cadherin identified KCs undergoing EMT. Bars = 75 μm. (c) Macroscopic images of nondiabetic and diabetic mouse wounds before (day 0) and after (day 4) topical treatment of vehicle (PBS) or Ach. The graph shows the percentages of wound closure with respect to day 0 wounds. n = 9 mice for each group. Data in a and b were expressed as means ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05 (Mann–Whitney test). Ach, acetylcholine; AchE, acetylcholinesterase; EMT, epithelial–mesenchymal transition; IGTT, intraperitoneal glucose tolerance test; KC, keratinocyte; min, minute, n.s., not significant.
**Supplementary Figure S2. Impact of choline and glucose on Ach-mediated EMT in KCs.** (a) Heatmap showing the expression changes to EMT-related genes (right panel) in HaCaT and Ker-CT after exposure to choline for 24–48 h in normoglycemic condition. Immunoblots of SNAI1 (left panel) from HaCaT and Ker-CT exposed to choline in a low glucose condition. β-Tubulin from the same samples serves as a loading Ctrl. (b) The ratio of acetylcholine to total choline in the conditioned medium from HaCaT and Ker-CT cultured in normoglycemic and hyperglycemic conditions. (c) Fluorescence intensity of surface CHRM4 and CHRNA9 expression in cells cultured in low or high glucose and after in vitro scratch wound. Unstained Ctrl was used for gating. (d) Cell tracking of HaCaT and Ker-CT cells using live-cell imaging over 24 h after exposure to the indicated conditions. Displacement is indicated by the straight lines, and the radii of the circles indicate the average displacement. n = 15 cells for each group. (e) The surface expression of TGFβRII in HaCaT and Ker-CT exposed to normoglycemic (blue) and hyperglycemic conditions (red). Data were from n = 4 independent experiments with triplicates. Data in a, b, and d were expressed as means ± SEM. **P < 0.01 (Mann–Whitney test). Ach, acetylcholine; Ctrl, control; EMT, epithelial–mesenchymal transition; h, hour; KC, keratinocyte; Ker-CT, immortalized TERT human keratinocyte; n.s., not significant; PE, phycoerythrin; TF, transcription factors.
Supplementary Figure S3. Ach upregulates TGFβRII through the Src-ERK1/2 pathway to sensitize KCs to TGFβ1-mediated EMT in HaCaT cells. (a, d) Relative gene expression of TGFβ1 and TGFβRII and surface expression of TGFβRII in vehicle-treated (blue) and Ach-treated (red) HaCaT in (a) low and (d) high glucose. (b, e) Relative fold change in (b, e) mRNA and (c, f) protein of EMT marker levels in HaCaT, subjected to the indicated treatments, cultured in (b) low and (e) high glucose. Immunobots of p-SMAD, t-SMAD, Snai1, and E-cadherin are shown. β-Tubulin from the same samples serves as a loading Ctrl. (g, h) Immunobots of (g) total and p-Src and ERK1/2 and (h) total and phosphorylated p38 kinase in vehicle- and Ach-treated HaCaT cultured in low and high glucose. (i) Immunobots of total and p-Src, ERK1/2, and MEK1/2 in vehicle-treated and p38 MAPK inhibitor (solamargine)-treated HaCaT cultured in high glucose.
Relative gene expression of TGFβRII and surface expression of TGFβRII in vehicle- and p38 inhibitor–treated HaCaT under hyperglycemic condition. (k) Immunoblots of p-SMAD, SMAD, SNAI1, and E-cadherin from HaCaT cultured in high glucose and subjected to the indicated treatments. Data were from n = 3 independent experiments with triplicates. Data in a–g were expressed as means ± SEM. Data in a, d, and g were analyzed using Mann–Whitney test. Data b, c, e, and f were analyzed using Dunnett’s post-hoc test after one-way ANOVA. ***P < 0.001, **P < 0.01, *P < 0.05. Ach, acetylcholine; Ctrl, control; EMT, epithelial–mesenchymal transition; ERK, extracellular signal–regulated kinase; KC, keratinocyte; MEK, MAPK/ERK kinase; n.s., not significant; PE, phycoerythrin; p-ERK, phosphorylated extracellular signal–regulated kinase; p-MEK, phosphorylated MAPK/ERK kinase; p-SMAD, phosphorylated SMAD; p-Src, phosphorylated Src; t-SMAD, total SMAD.
Supplementary Figure S4. Ach upregulates TGFβRII through the Src-ERK1/2 pathway to sensitizes KCs to TGFβ1-mediated EMT in primary human KCs. (a, d) Relative gene expression of TGFβ1 and TGFβRII and surface expression of TGFβRII in vehicle-treated (blue) and Ach-treated (red) primary human KCs in (a) low and (d) high glucose. (b, e) Relative fold change in (b) mRNA and (c, f) protein of EMT marker levels in primary human KCs, subjected to the indicated treatments, cultured in (b, c) low and (e, f) high glucose. Immunoblots of p-SMAD, t-SMAD, SNAI1, and E-cadherin are shown. β-Tubulin from the same samples serves as a loading Ctrl. (g, h) Immunoblots of (g) total and p-Src and ERK1/2 and (h) total and phosphorylated p38 kinase in vehicle- and Ach-treated primary human KCs cultured in low and high glucose. (i) Immunoblots of total and p-Src, ERK1/2, and MEK1/2 in vehicle-treated and p38 MAPK inhibitor (solamargine)-treated primary human KCs cultured in high glucose. (j) Relative gene expression of TGFβRII and surface expression of TGFβRII in vehicle- and p38
Supplementary Figure S5. Exogenous Ach promotes SMAD3 activation in nondiabetic but not in diabetic wounds. (a) H&E images and immunostaining of p-SMAD3 (green) in the epithelial tongue of nondiabetic and diabetic wounds on days 1–3 after injury with and without Ach treatment. Hoechst 33342 was used for nuclear counterstain. Arrows indicate epithelial wound tip. Dotted lines indicate the basal membrane. Bars = 100 μm. (b) Percentage of p-SMAD3–positive cells in the epithelial tongue (≤100 μm) of nondiabetic and diabetic wounds on days 1–3 after injury with and without Ach treatment. n = 6 mice for each group. Data in b were expressed as means ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05 (Mann–Whitney U test). Ach, acetylcholine; hf, hair follicle; n.s., not significant; p-SMAD3, phosphorylated SMAD3.

MWY Tan et al. Acetylcholine Enhances Wound Healing

Journal of Investigative Dermatology (2021), Volume 141
Supplementary Figure S6. Schematic illustration of the possible outcomes from the kinase inhibitor screening. (a, b) A schematic diagram of the rationale behind the kinase inhibitor screening, which led to mapping the IPA pathways in (a) Figure 5h and (b) Figure 6b. Possible outcomes of the kinase inhibitor screening of Ker-CT cultured in (a) low and (b) high glucose conditions during Ach-mediated upregulation of TGFβRII. (c) Graph showing the percentage of diabetic wound closure when treated with solamargine or vehicle. The percentage that would closure was calculated with respect to wound area on day 0. Representative images of wounds are shown. Bars = 1 cm. n = 5 mice for each group. *P < 0.05 (Mann–Whitney U test). Ach, acetylcholine; IPA, indigenous pathway analysis; Ker-CT, immortalized TERT human keratinocyte; n.s., not significant.
### Supplementary Table S1. Primers Used in the Study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCLN forward</td>
<td>GACTTCAGGCACCTCCGTTAC</td>
</tr>
<tr>
<td>OCLN reverse</td>
<td>GCGTAGTAGTCAGTCTCGTCA</td>
</tr>
<tr>
<td>CDH1 forward</td>
<td>GCCGGAGGCTACAGCTTCAA</td>
</tr>
<tr>
<td>CDH1 reverse</td>
<td>GACCGGTGAATCTCCTAAA</td>
</tr>
<tr>
<td>ERBB3 forward</td>
<td>GACCCAGGTCTACGATGGGAA</td>
</tr>
<tr>
<td>ERBB3 reverse</td>
<td>GTGAGCTGAGTCAAGCGGAG</td>
</tr>
<tr>
<td>NCAD forward</td>
<td>AGCCAACCTTAATGAGGAGT</td>
</tr>
<tr>
<td>NCAD reverse</td>
<td>GGGAGGAGCTACACGGAG</td>
</tr>
<tr>
<td>VIM forward</td>
<td>AGTCCACTGATACCCAGGAGAC</td>
</tr>
<tr>
<td>VIM reverse</td>
<td>CTTTCAGCAGCTCGGAGGTC</td>
</tr>
<tr>
<td>TWIST1 forward</td>
<td>GTCCGGCACTCTACAGGAGAG</td>
</tr>
<tr>
<td>TWIST1 reverse</td>
<td>GCTTAGGGTCTGAATCTGCT</td>
</tr>
<tr>
<td>SNAI1 forward</td>
<td>GCCCTTCAACTGAAACTGCT</td>
</tr>
<tr>
<td>SNAI1 reverse</td>
<td>CTTCCTTGAGATCTCAGTGGTC</td>
</tr>
<tr>
<td>SNAI2 forward</td>
<td>CGAATGGCAGACACACACAGT</td>
</tr>
<tr>
<td>SNAI2 reverse</td>
<td>CTGAGGATCTGTTTGGT</td>
</tr>
<tr>
<td>18S forward</td>
<td>GTAACCCTTCAGAACCCTCAT</td>
</tr>
<tr>
<td>18S reverse</td>
<td>CCATCCAATCGGGATAGC</td>
</tr>
<tr>
<td>TGFβ1 forward</td>
<td>CAACAATTTGGTCCAGCAGT</td>
</tr>
<tr>
<td>TGFβ1 reverse</td>
<td>GCTAAGCGCGCAAACCTCAGT</td>
</tr>
<tr>
<td>TGFβR2 forward</td>
<td>CGTGGAGTCTGTCAGACGAG</td>
</tr>
<tr>
<td>TGFβR2 reverse</td>
<td>CCGCAGCTTTGGAACCCAAATGG</td>
</tr>
</tbody>
</table>

### Supplementary Table S2. Antibodies Used in the Study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Vendor</th>
<th>Catalog No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>31955</td>
</tr>
<tr>
<td>Total SMAD3</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>ab40854</td>
</tr>
<tr>
<td>p-SMAD3</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>ab52903</td>
</tr>
<tr>
<td>SNAI1</td>
<td>Rabbit</td>
<td>Abnova</td>
<td>PAB27142</td>
</tr>
<tr>
<td>TGFβR2</td>
<td>Mouse</td>
<td>Miltenyi Biotec</td>
<td>REA903</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Mouse</td>
<td>DHSB</td>
<td>E7</td>
</tr>
<tr>
<td>Total ERK1/2</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-94</td>
</tr>
<tr>
<td>p-ERK1/2</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-16982</td>
</tr>
<tr>
<td>Total Src</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>21085</td>
</tr>
<tr>
<td>p-Src</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>69435</td>
</tr>
<tr>
<td>Total P38 MAPK</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>86905</td>
</tr>
<tr>
<td>p-P38 MAPK</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>45115</td>
</tr>
<tr>
<td>Total MEK1/2</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>91225</td>
</tr>
<tr>
<td>p-MEK1/2</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>91215</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>5741</td>
</tr>
<tr>
<td>Slug</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>9585</td>
</tr>
<tr>
<td>CHRNA9</td>
<td>Rabbit</td>
<td>MyBioSource</td>
<td>9203650</td>
</tr>
<tr>
<td>CHRM4</td>
<td>Rabbit</td>
<td>Biorbyt</td>
<td>461669</td>
</tr>
<tr>
<td>IRDye 680RD anti-Mouse IgG (H + L)</td>
<td>Goat</td>
<td>LI-COR</td>
<td>LIC-925-68070</td>
</tr>
<tr>
<td>IRDye 680RD anti-Rabbit IgG (H + L)</td>
<td>Goat</td>
<td>LI-COR</td>
<td>LIC-925-68071</td>
</tr>
<tr>
<td>IRDye 800CW anti-Mouse IgG (H + L)</td>
<td>Goat</td>
<td>LI-COR</td>
<td>LIC-926-32210</td>
</tr>
<tr>
<td>IRDye 800CW anti-Rabbit IgG (H + L)</td>
<td>Goat</td>
<td>LI-COR</td>
<td>LIC-925-32211</td>
</tr>
</tbody>
</table>

Abbreviations: DHSB, Developmental Studies Hybridoma Bank; ERK, extracellular signal-regulated kinase; H, heavy chain; L, light chain; MEK, MAPK/ERK kinase; No., number; p-ERK, phosphorylated extracellular signal-regulated kinase; p-MEK, phosphorylated MAPK/ERK kinase; p-SMAD3, phosphorylated SMAD3; p-Src, phosphorylated Src.