Intratracheal Keratinocyte Growth Factor Enhances Surfactant Protein B Expression in Mechanically Ventilated Preterm Pigs

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ABSTRACT

Background: Bronchopulmonary dysplasia is a devastating disease of the premature newborn with high morbidity and mortality. Surfactant deficient preterm lungs are susceptible to ventilator induced lung injury, thereby developing bronchopulmonary dysplasia. Despite surfactant therapy and newer ventilation strategies, associated morbidity and mortality remains unchanged. Enhancing surfactant production and reducing ventilator induced lung injury in premature infants are critical. Recombinant keratinocyte growth factor previously been studied to treat adult respiratory distress syndrome. We hypothesized that administering recombinant human keratinocyte growth factor when initiating mechanical ventilation would help stimulate type II cell proliferation and surfactant production. Recombinant human keratinocyte growth factor may also help mitigate ventilator induced lung injury hereby reducing epithelial to mesenchymal transition, a possible precursor to later development of bronchopulmonary dysplasia.

Methods: To test our hypothesis, we delivered preterm pigs via cesarean section on day 102. We performed intubation and ventilation for 24 hr. using intermittent positive pressure ventilation. After ventilation began, pigs randomly received intratracheal recombinant human keratinocyte growth factor (20 μg/kg; n=6) or sham treatment (0.5 ml 0.9% saline; n= 6). We recorded physiology data and arterial blood gases during ventilation. After 24 hr. pigs were extubated and received oxygen via nasal cannulation 12 hr. before euthanasia to collect lungs for histopathology and immunohistochemistry. Immunohistochemistry staining was graded and analyzed for surfactant protein B and epithelial to mesenchymal transition markers. Data were analyzed using t-test and Fisher's exact test. Continuous variables analyzed using ANOVA.

Results: Compared with control pigs, recombinant human keratinocyte growth factor pretreated pigs had improved ventilation with higher tidal volumes and required less oxygen (FiO2) during mechanical ventilation for similar peak pressures demonstrating improved lung compliance. Recombinant human keratinocyte growth factor pretreated pig lungs showed increased surfactant protein B expression ($p< 0.05$) and significantly reduced TGF-β ($p< 0.05$), a prominent marker for epithelial to mesenchymal transition.

Conclusions: Intratracheal recombinant human keratinocyte growth factor administered at initiation of mechanical ventilation enhances surfactant production, reduce lung injury by mitigation of the changes by epithelial mesenchymal transition, thereby improving outcomes. Thus, recombinant human keratinocyte growth factor may represent a potential therapeutic strategy to prevent bronchopulmonary dysplasia.

BACKGROUND

Exposure of the immature lungs of extremely preterm infants to hyperoxia contributes to the pathophysiologic changes of bronchopulmonary dysplasia (BPD) (1). These immature lungs undergo histopathological changes from ventilator-induced lung injury (VILI) caused by the shear stress of intermittent positive-pressure ventilation. This combination of hyperoxia and mechanical shear triggers inflammation, alters production of pulmonary growth factors and surfactant, and initiates VILI (2), (3). Despite advances in mechanical ventilation, morbidity associated with BPD remains unchanged among the growing numbers of infants surviving birth before 25 weeks of gestation. The cause of this structural remodeling of the premature newborn lung remains unknown. However, exposure of premature infants to hyperoxia and mechanical ventilation causes lung injury and initiates BPD (1). The evolution of “Old BPD” to “New

BPD" represents stunted lung development (4), (5). Therefore, interventions influencing lung growth, reducing VILI and accelerating recovery when VILI occurs will reduce BPD.

Alveolar type I (AT1) and type II (AT2) cells comprise the lung alveolar epithelium. An intact epithelial barrier is essential for gas exchange. Surfactant production, active ion transport, and normal maintenance of the alveolar epithelium require AT2 cells. Surfactants reduce surface tension, improving compliance and gas exchange. Thus, surfactants can reduce early mortality due to respiratory failure (6). AT2 cell proliferation plays a key role in alveolar epithelium repair after acute lung injury. Following injury, AT2 cells replicate to produce additional AT2 cells or transdifferentiate into AT1 cells (7). Alternatively, AT2 cells differentiate into fibroblasts through epithelial to mesenchymal transition (EMT), leading to fibrosis. In neonatal rats exposed to hyperoxia, EMT precedes BPD (8). Fibroblasts and endothelial cells produce keratinocyte growth factor (KGF), which affects epithelial cells by binding epithelial-restricted FGFR2. Exogenous KGF has potentially useful roles in the management of patients with acute respiratory distress syndrome (ARDS) (9). Moreover, there is an inverse relationship between KGF concentrations in tracheal aspirates from preterm infants and the incidence and severity of BPD (10).

KGF may exert protective effects in rat models of radiation- and bleomycin-induced lung injury. Intratracheal KGF (5 mg/kg) instillation 48 and 72 hr before exposure to 18 Gy bilateral thoracic irradiation does not significantly improve survival in rats (11). However, histology shows less pneumonitis and fibrosis in KGF pretreated rats than in control irradiated rats. In neonatal rats exposed to hyperoxia, exogenous KGF protects the lung epithelium, enhances repair, and reduces inflammation, contributing to reduced mortality (12). KGF administration

also increases surfactant production in preterm rabbit lungs (13), whereas mechanical ventilation decreases both surfactant and KGF in preterm lungs (14).

Here, we performed intratracheal administration of recombinant human KGF (rhKGF) to preterm pig lungs at initiation of 24 hr of mechanical ventilation. We hypothesized rhKGF would enhance surfactant production and ameliorate the lung injury preceding BPD development. We also predicted KGF may reduce the severity of VILI and the extent of EMT that contributes to lung fibrosis and BPD in premature newborns following VILI.

MATERIALS AND METHODS

Source of preterm pigs and initial care. The Institutional Animal Care and Use Committees of the University of Tennessee Health Science Center (location of caesarean section) and the University of Memphis (site of ventilation and critical care) approved the protocols for the harvest, care, and sampling of preterm pigs (*Sus scrofa*). Antenatal steroids were not provided, and preterm pigs were delivered via caesarean section on gestational day 102 (89% of 115-day term) from two specific pathogen-free, artificially inseminated sows obtained from a production herd with genetics derived from crossing multiple pig strains. After suctioning and clearing the airway, the pigs were placed in a 38-39°C incubator with supplemental oxygen. After spontaneous breathing was established, the pigs were placed in a warmed transport carrier with supplemental oxygen provided by masks that fit over the snout and transported to a neonatal intensive care unit developed for the care of preterm pigs (pNICU).

Instrumentation, processing, and intensive care of preterm pigs. Pigs were weighed in the pNICU. An umbilical catheter (UAC; 3.5F Argyle TM, Covidien, MA) was inserted via one of the two umbilical arteries. The UAC was advanced to the descending aorta, and the position was confirmed by radiography (Duoview high Resolution Digital Radiography System, Revo2,

Kennesaw, GA). The UAC was used to provide parenteral nutrition (PN), sample arterial blood, and administer Cefazolin (50 mg/kg/dose) as a prophylactic antibiotic. Maternal plasma (5 ml/kg) was also administered via the UAC to provide passive immunity and compensate for the absence of colostrum.

From each litter, 6 pigs (spontaneously breathing and of similar body weights) were intubated with red rubber 2.5 French endotracheal (ET) tubes (Jorgensen Laboratories, Loveland, CO) that minimize leaks during mechanical ventilation (<10%). The position of the ET tubes was confirmed using digital radiography and repositioned, if necessary. The pigs were connected to Dräger VN500 ventilators (Dräger Medical, Incorporated, Dräger, Telford, PA) with initial assist control volume guarantee (AC+VG) settings of a tidal volume of 5 cc/kg, a respiration rate of 40 breaths per min, positive-end expiratory pressure (PEEP) of 5 cm H_2O, iTime 0.35 seconds, and FiO_2 of 40%. Surfactant was not administered as it would be difficult to distinguish between endogenous and exogenous sources. The peak inspiratory pressure (PIP) and ventilation rate were adjusted based on the blood gases to maintain normal gas exchange values.

Within 1 hr after ventilation was established, the pigs were randomized to the KGF treatment (3 per litter) or sham/control group (3 per litter). rhKGF (ProSpec Protein Specialists, Ness Ziona, Israel) is produced by E. coli as a single, non-glycosylated polypeptide chain and reconstituted in normal saline. Based on the mouse homolog, this gene is required for embryonic epidermal morphogenesis, including brain development and lung morphogenesis. This gene may also be a primary factor in wound healing. A single dose of rhKGF (20 µg/kg) was diluted and mixed with normal saline to prepare a volume of 1 ml that was divided into two equal aliquots. These aliquots were administered via the ET tube to each side of the lung. rhKGF was then hand bagged (PIP: 15 cm H_2O; PEEP: 5 cm H_2O) to enhance adequate distribution throughout the

lungs. The control pigs received a similar volume of normal saline. The heart rate, oxygen saturation, and perfusion index were monitored continuously (Masimo Radical 7, Masimo, Irvine, CA). Arterial blood gas measurements (iSTAT®, Abbott, Abbott Park, IL) were performed after placement of the UAC and every 3 hr or after adjustment of ventilator settings to maintain pulse oximetry saturation of > 90-95%, pH 7.25 to 7.4, pCO_2 40 to 55 mmHg, and pO_2 > 60. Ventilators automatically recorded the mean airway pressure, PIPs, tidal volumes and oxygen requirements every 5 min. The pigs were repositioned each hour from one side to the other to avoid dependent edema. All study pigs could spontaneously breathe during the study and were not paralyzed. Pigs that became excessively active during the 24 hr of mechanical ventilation were sedated using ketamine (Bioniche Teoranta, Galway, Ireland) via the UAC. After 24 hr, the pigs were extubated, and supplemental oxygen was provided by nasal cannula for 12 hr. The pigs were then followed for survival. At the end of the 36-hr study period, all surviving pigs were euthanized (Euthasol; Virbac AH, Inc., Fort Worth, TX, 1 ml/kg; IV).

PN was provided continuously at a rate of 4 ml/kg-hr, beginning immediately after placement of the UAC. For the first 4 to 6 hr, the pigs received a low potassium (2 mmol) PN solution that provided (per L) 116 g dextrose, 60.5 g amino acids (Travasol), and 31.3 g lipid (Intralipid 30%) with electrolytes, vitamins and trace elements. Thereafter, a PN solution with normal potassium (5 mmol) was provided. Supplemental fluid was provided via the UAC as needed to maintain tissue perfusion using lactated Ringers and averaged 3-4 ml/kg-hr, with the same relative volume (by weight~ 100 ml/kg/day) administered to all pigs to avoid possible differences caused by variable fluid volumes. The volume of fluid administered was insufficient to cause significant pulmonary edema. Metabolic acidosis was corrected with a normal saline bolus (10 ml/kg) as indicated by a base deficit on arterial blood gas.

Radiography. A chest x-ray image was obtained after placement of the ET tube and UAC to confirm proper positioning and to assess initial lung volume recruitment. An additional chest x-ray was obtained at the end of the study to assess lung volume recruitment.

Necropsy. The lungs were collected from pigs that died prior to 36 h and from pigs that were euthanized after 24 h of mechanical ventilation, followed by 12 h of oxygen provided by nasal cannulation. The lungs were removed en bloc and inflated using the ET tube and a NeoPuff ™ (Fisher & Paykel Healthcare, Irvine, CA) to a PIP of 20 cm H_2O and PEEP of 5 cm H_2O pressure. The trachea was immediately clamped, and the right lower lobe was tied off, excised, and submerged in formalin for routine histology and immunohistochemistry (IHC).

Histologic analysis. Formalin-fixed tissues were processed in paraffin, embedded in paraffin and sectioned (4 μm). For routine histology, the sections were stained with hematoxylin and eosin. A pediatric pathologist (JZ) who was blinded to the study protocol semi-quantitatively graded inflammation, hemorrhage, edema, necrosis and atelectasis of each lung. Each parameter was individually scored using a Likert scale from 0 (no injury), 1 (25% injury), 2 (50% injury), 3 (75% injury), and 4 (100% injury) (15).

Immunohistochemistry. For IHC, the sections were deparaffinized, rehydrated with graded ethanol and treated with methanol and hydrogen peroxide to remove any endogenous peroxidase. The sections were treated with guanidinium hydrochloride followed by trypsin to enhance antigen detection. Then, the sections were incubated for 20 min in PBS containing 3% goat serum (Gibco, Thermo Fisher Scientific, Waltham, MA) to block nonspecific binding sites. Following manufacturer instructions, the slides were incubated overnight with primary antibodies for surfactant protein B (SP-B rabbit polyclonal, 20 μg/ml, Hycult Biotech, Wayne, PA) and transforming growth factor β1 (TGF-β1, 2 ng/ml, EMD Millipore, Billerica, MA).

Slides were incubated for 1 hr with primary antibodies for E cadherin (0.5 μg/ml, Novus Biologicals, Centennial, CO), Vimentin (1:300), Ki-67 (1:100) and β-catenin (1:50) (Dako North America Inc., Carpinteria, CA). After washing, secondary antibodies (anti-rabbit or anti-mouse biotinylated horseradish peroxidase) were applied for 30 min according to the primary antibody. Color was developed by 3,3'-diaminobenzidine (DAB), and the slides were counterstained with hematoxylin.

Aperio© Image Analysis Algorithm (version 9, Leica Biosystems, Wetzlar, Germany) was used to quantify IHC-stained cells. The algorithm was optimized for fetal pig lung sections stained for β-catenin, E-cadherin, Ki-67, vimentin, prosurfactant, SP-B, and TGF-β. The algorithm classified nuclei as 0, 1+, 2+, and 3+ based on staining intensity. The percentage of positively stained nuclei, average staining intensity of positive nuclei, and percentage of nuclei in each classification were exported as Excel spreadsheets. The spreadsheets were combined into a single master file for each animal.

Statistical analysis.

Categorical data were compared using unpaired Student's t-test and Fisher's exact test. Continuous variables were analyzed using ANOVA for physiologic parameters. *Post hoc* Tukey's tests were performed on continuous data. Quantitative immunohistochemistry and histology data were analyzed using a Mann-Whitney U test after testing for normality. Data are presented as the means ± SD. The selected level of significance was $p < 0.05$.

CONCLUSIONS

KGF improves aeration and reduces oxygen needs.

Preterm pigs harvested from two sows at gestational day 102 were similar in size, body weight, and gender distribution (Table 1). Physiologic parameters (heart rate, oxygen saturations

and arterial blood gases) did not differ between the control and KGF pretreatment groups during the initial 24-hr ventilation period (Table 2). PIP also remained the same for both groups (data not shown). However, tidal volumes were higher (5.8 ± 2.1 vs 5.1± 1.8, $p< 0.05$) (Fig. 1A) and oxygen (FiO_2) needs (Fig. 1B) were lower for KGF pretreated pigs than for control pigs (58.40 ± 14.32 vs 68.78 ± 22.34, $p< 0.05$). These findings demonstrate improved compliance with KGF pretreatment and decreased oxygen needs due to improved aeration and gas exchange from surfactant in the lungs.

Pigs that died following extubation developed worsening respiratory symptoms. Symptoms included chest wall retractions, poor oxygen saturation, and progressively diminishing respiratory effort, eventually leading to cardio-respiratory failure. Since the animals were not mechanically ventilated post extubation, survival was our only outcome (Table 2). All KGF pretreated pigs and 5 out of the 6 control pigs survived for 24 hr. After extubation and placement of the nasal cannula, survival to 36 hr was higher among KGF pretreated pigs than among control pigs.

KGF ameliorates surfactant loss.

Compared with control lungs (Fig. 2A), KGF pretreated lungs showed significantly less inflammatory cell infiltrate and necrosis (1.48±1.18 vs 0.83±1.04, $p< 0.05$, Fig. 2B). SP-B expression was significantly higher in KGF pretreated lungs (Fig. 2D) than in control lungs (19.4±2.76 vs 11.88±2.30, $p< 0.05$, Fig. 2C). Compared with control, KGF significantly reduced TGF-β expression (5.31±2.11 vs 2.72±0.08, $p< 0.05$, Fig. 2E-F). However, E-cadherin, vimentin, β-catenin and Ki-67 expression was similar between the control and experimental groups (data not shown).

DISCUSSION

Extremely preterm pigs provide clinically relevant insights because of compatibility with chronic ex utero care and similarities to preterm infants. These similarities include lung anatomy and developmental trajectory. By contrast, neonatal rodents have sufficiently developed lungs for spontaneous breathing of atmospheric air, and fetal lambs retain a placental connection. Even with clinically relevant settings and adjustments, AC+VG can damage preterm pig lungs similar to extremely preterm infant lungs ((15), present study). Diminishing compliance and gas exchange caused by mechanical ventilation are antecedents of chronic lung disease. The hyperoxia necessary to maintain PaO_2 within a targeted range would exacerbate this damage. Here, intratracheal administration of rhKGF at the initiation of mechanical ventilation reduced immature lung damage and the ensuing morbidity and mortality. These findings may explain why higher endogenous KGF production by preterm infants less than 30 weeks of gestation at birth correlates with reduced BPD incidence and severity (10).

The lack of surfactant administration likely contributed to acute lung injury, atelectasis, and inflammatory cell infiltrate in both groups. Although mechanical ventilation can rescue extremely preterm infants who develop RDS, it further reduces surfactant protein and KGF expression (14). This effect explains why surfactant administration helps improve survival (16, 17). We found KGF augments SP-B expression in immature lungs after preterm delivery. This finding is clinically relevant and consistent with other animal models of prematurity (13) and neonatal term rodents (11, 12). Increased SP-B expression in response to KGF administration may have compensated for surfactant deficiency. Multiple findings support this potential compensatory mechanism. These findings include less severe lung injury, lower FiO_2 after 24 hr of mechanical ventilation, and improved outcomes after extubation and reliance on nasal

cannula. Thus, rhKGF administered to preterm infants who require mechanical ventilation may stimulate endogenous surfactant production, thereby accelerating successful extubation to non-invasive ventilation. In previous research, KGF pretreatment at 72 hr, not at 24 or 48 hr, considerably ameliorated HCl-induced morphologic damage in lungs. Moreover, the 72-hr KGF pretreatment markedly decreased inflammatory cells in bronchoalveolar lavage 3 and 7 days post HCl instillation (18).

Our IHC findings provide novel insights into immature lung responses to rhKGF following exposure to mechanical ventilation after preterm birth. The epithelial cell proliferation marker Ki-67 (19, 20) was similar between groups. This finding suggests increased AT2 cell proliferation alone did not increase surfactant expression in KGF pigs. In mature lungs, epithelial damage, inflammation, and oxidative stress can trigger AT2 cells to differentiate into fibroblasts. Similarly, newborn rats exposed to prolonged hyperoxia exhibit EMT, a precursor of BPD (8). EMT is a complex process that involves a large interactome including protein to protein and genetic interactions that are initiated and controlled as a response to extracellular cues. TGF-β1 which, on addition to epithelial cultures, causes the cells to undergo EMT (21). TGF-β1 is involved in several cellular functions including cell proliferation, cell differentiation and apoptosis. During EMT, epithelial cells transdifferentiate into motile mesenchymal cells while losing their epithelial characteristics (22). TGF-β_1 or endothelin-1 and oxidative stress can induce EMT in airway epithelial cells (AECs) (23). After preterm delivery, knowledge of EMT is scarce following exposure of essentially fetal lungs to damaging mechanical ventilation and hyperoxia. Hyperoxia negatively affects alveolar epithelial barrier function, rendering neonatal lungs susceptible to injury and/or BPD (24).

In this study, we measured E-cadherin expression. E-cadherin is an adherens junction protein altered by oxidants and other stressors in adult alveolar epithelial cells (25). E-cadherin mediates cell-cell adhesion and recognition. E-cadherin expression appeared lower in KGF pretreated pigs but was nonsignificant. This finding may suggest involvement of E-cadherin in EMT and fibrosis development. However, mesenchymal transition markers vimentin and β-catenin showed no changes. These proteins may be unsuitable EMT markers for preterm lungs or 36 hr was inadequate for detecting changes in expression. Alternatively, fetal lungs may have a limited response. No changes in collagen content in fetal lamb lungs after 15 days of mechanical ventilation indicate a limited response (26). Therefore, we need better explanations of how mechanical ventilation disrupts the developmental trajectory of immature lungs in extremely preterm infants.

Identifying injury biomarkers for the lungs of preterm infants and future risk of BPD is a research priority (27). TGF-β in tracheal aspirate indicates lung inflammation and is associated with fibrosis and abnormal lung development (28). Additionally, TGF-β reduces surfactant production in AT2 cells. Transitional AT2 cells and likely AT1 cells inhibit matrix and growth factor expression in fibroblasts (29). Kunzmann et al. suggest possible molecular mechanisms involving progesterone, including inhibition of TGF-β1-activated Smad signaling and TGF-β1-regulated genes involved in BPD pathogenesis, which likely attenuate the development of BPD by inhibiting TGF-β1-mediated airway remodeling (30). The correlation between lower TGF-β expression in KGF pretreated pigs with increased surfactant and improved outcomes confirms previous reports on preterm infants. Furthermore, this correlation validates the preterm pig as a relevant model for identifying other signaling molecules.

We demonstrated KGF reduces epithelial remodeling in lungs following VILI in the preterm pig model. Shyamsundar and colleagues studied bronchoalveolar lavage fluid from KGF pretreated human volunteers (31). The fluid showed increased alveolar epithelial proliferation along with increased surfactant protein D and matrix metalloproteinase 9 (MMP-9) levels. Based on these findings, active MMP-9 enhances alveolar epithelial wound repair. Our animal model of preterm ARDS supports this conclusion.

In this interventional short-term ventilation study, extremely preterm pigs received intratracheal administration of rhKGF at initiation of mechanical ventilation. This intervention increases SP-B expression and reduces VILI. Furthermore, rhKGF administration may facilitate earlier extubation to non-invasive ventilation. Based on the improved outcomes, rhKGF delivery to premature lungs may provide a therapeutic strategy to preserve the alveolar epithelium during VILI. However, we need further studies to evaluate long-term outcomes and identify additional lung injury biomarkers. These studies should also determine the responses to such interventions. This research is vital to identify new treatments for RDS in premature newborns.

ABBREVIATIONS

BPD: Bronchopulmonary dysplasia

rhKGF: Recombinant human keratinocyte growth factor

TGF -β: Tumor growth factor – β

VILI: Ventilator-induced lung injury

AT1 and AT2: Alveolar type I and type II cells

EMT: Epithelial to mesenchymal transition

pNICU: Intensive care unit developed for the care of preterm pigs

UAC: Umbilical artery catheter

RDS: Respiratory distress syndrome

ANIMAL USE APPROVAL

The Institutional Animal Care and Use Committees of the University of Tennessee Health Science Center (location of caesarean section) and the University of Memphis (site of ventilation and critical care) approved the protocols for the harvest, care, and sampling of preterm pigs (*Sus scrofa*).

AVAILABILITY OF DATA AND MATERIALS

The authors are willing to share the raw data and details of experimental materials used as per appropriate request.

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COMPETING INTERESTS

Mr. Frank Caminita is an employee of Dräeger Medical, the provider of the pediatric ventilators used for this study. None of the other authors have financial relationships to disclose.

AUTHORS CONTRIBUTIONS

RK: contributed to study design, methods, data and statistical analysis, and manuscript writing.

EA: contributed to study methods, data collection, manuscript review and editing.

FC: contributed to ventilator management, animal laboratory methods and testing, ventilator data collection and analysis, manuscript review and editing.

JZ: contributed to histopathology and IHC studies, pathology data interpretation, manuscript review and editing.

RB: contributed to study design, methods, data and statistical analysis, and manuscript writing.

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Figure Legends

Fig. 1. Physiologic parameters measured during 24 hr of mechanical ventilation. *A:* Tidal volumes (ml/kg) are significantly higher in the KGF group (n=6) than in the control group (n=6) (ANOVA, $p< 0.05$). *B:* Oxygen requirements (percentage FiO_2) are significantly lower in the KGF group than in the control group (ANOVA, $p< 0.05$).

Fig. 2. Immunohistochemistry performed on lung sections collected after 24 hr of mechanical ventilation. *A-B:* Hematoxylin and eosin (H&E)-stained sections show increased areas of atelectasis and necrosis in control lungs compared to those in KGF lungs (1.48±1.18 vs 0.83±1.04, $p< 0.05$) *C-D:* Surfactant protein B (SP-B) expression is significantly higher in KGF pretreated lung tissue than in control lung tissue (19.4±2.76 vs 11.88±2.30, $p< 0.05$). *E-F:* Transforming growth factor β (TGF-β) is lower in KGF pretreated tissue than in control tissue (2.72±0.08 vs 5.31±2.11, $p< 0.05$). Scale bars in *A-B* are 300 μm. All other scale bars are 200 μm.