The functional effects and mechanisms by which fibroblast growth factor 2 (FGF2) controls bovine mammary epithelial cells: Implications for the development and functionality of the bovine mammary gland¹

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ABSTRACT: Fibroblast growth factor (FGF) signaling plays essential roles in tissue development and homeostasis. Accumulating evidence reveals that fibroblast growth factor 2 (FGF2) regulates ductal elongation, which requires cell proliferation and epithelial expansion in the mammary gland. However, the function and mechanisms by which FGF2 controls functionality of epithelial cells is less well defined. Here, we demonstrate the functional effects of FGF2 on bovine mammary epithelial (MAC-T) cells and the intracellular signaling mechanisms for these FGF2-induced actions. The current results show that treatment of MAC-T cells with a recombinant FGF2 induced cell proliferation and cell-cycle progression with increased expression of proliferating cell nuclear antigen and cyclin D1. Moreover, FGF2 increased phosphorylation of serine/threonine protein kinase (protein kinase B [AKT]), extracellular signal-regulated kinases 1 and 2 (ERK1/2), Jun N-terminal kinase (JNK), 70 kDa ribosomal S6 kinase (P70S6K),

90 kDa ribosomal S6 kinase (P90S6K), ribosomal protein S6 (S6), and cyclin D1 proteins. These FGF2-induced activations of signaling pathway proteins were inhibited by blocking AKT, ERK1/2, or JNK phosphorylation. The effect of FGF2 to stimulate MAC-T cell proliferation was mediated by activation of FGF receptors (FGFR) and AKT, ERK1/2, and JNK mitogen-activated protein kinase pathways in response to FGF2 stimulation. Furthermore, expression and activation of endoplasmic reticulum (ER) stress-related factors and ER stress-induced MAC-T cell death was reduced by FGF2. Together, these results suggest that the FGF2–FGFR–intracellular signaling cascades may contribute to maintaining and/or increasing numbers of mammary epithelial cells by inducing proliferation of mammary epithelial cells and by protecting cells from ER stress responses. Therefore, this study provides evidence that FGF2 signaling is a positive factor for mammary gland remodeling and for increasing persistency of milk production.

Key words: cell proliferation, endoplasmic reticulum stress, fibroblast growth factor 2, intracellular signaling, mammary epithelial cells, mammary gland

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INTRODUCTION

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Milk is the primary source of nutrition for survival, growth, and proper development of mammalian neonates; therefore, adequate lactation is a tremendously important factor for successful reproduction and highly efficient offspring production in mammalian species (Akers, 2002; Kim and Wu, 2009). The mammary gland undergoes repeated cycles of

structural growth, development, differentiation, and regression (Accorsi et al., 2002; Akers, 2002; Seeth et al., 2015). This cycle of events in mammary development is closely intertwined with the reproductive cycle, which is controlled by a complex of hormones and other factors. Among them, various growth factors play essential roles in many mammary actions. For example, several lines of evidence indicate that GH stimulates mammary growth through increasing the stromal production of IGF-I, which is a potent mitogen for mammary epithelial cells (MEC; Bauman and Vernon, 1993; Arendt and Kuperwasser, 2015). Besides IGF-I, a number of other growth factors are considered positive or negative factors for the mammary development. These growth factors include epidermal growth factor (EGF), hepatocyte growth factor (HGF), transforming growth factors, and members of the fibroblast growth factor (FGF) family (Bauman and Vernon, 1993; Akers, 2002).

The growth factor of interest in this study is fibroblast growth factor 2 (FGF2), a potent epithelial cell mitogen. Fibroblast growth factor signaling is closely associated with both breast cancer and mammary gland development. Previous studies have demonstrated that the deletion of FGF receptors (FGFR1 and FGFR2) leads to defective mammary branching morphogenesis (Chodosh et al., 2000; Lu et al., 2008; Pond et al., 2013). A recent study with mice revealed that FGF2 controls ductal elongation, which depends on cell proliferation and epithelial expansion during mammary gland development (Zhang et al., 2014). Bovine FGF2 is expressed by various cells in bovine mammary glands such as ductal epithelial cells, myoepithelial cells, and alveolar cells during mammary development and lactogenesis (Plath et al., 1998). Cell- and stage-dependent expression and distinct localization as well as potent mitogenic effects of FGF2 on the mammary gland can provide insights into the requirement of functional FGF2–FGF receptor (FGFR) signaling for the local regulation of growth and function of the bovine mammary gland. So here, we point to the requirement for functional FGF2-mediated signaling acting in the mammary epithelium to promote epithelial cell growth and cell turnover in the mammary gland of cows.

MATERIALS AND METHODS

Cell Culture

Bovine MEC (MAC-T cells) were a gift from Dr. Hong Gu Lee (Konkuk University, Seoul, Republic of Korea). The MAC-T cell line was developed by immortalizing primary bovine mammary alveolar cells by stable transfection with a replication-defective retrovirus (simian vacuolating virus 40) large T-antigen

as described by Huynh et al. (1991). The MAC-T cells displayed a cobblestone shape when grown on plastic substratum. They formed a single monolayer at confluence. All analyses in the present study were performed on MAC-T cells between passages 25 and 30. Briefly, monolayer MAC-T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum to 80% confluence in 100-mm tissue culture dishes. For assays, in vitro cultured MAC-T cells were serum-starved for 24 h prior to treatment and then incubated in the presence of recombinant FGF2 and/or inhibitors.

Proliferation Assay

Proliferation assays were conducted using a Cell Proliferation ELISA, BrdU kit (catalog number 11647229001; Roche Applied Science, Indianapolis, IN) according to the manufacturer's recommendations. Briefly, the MAC-T cells (3×10^3 cells/well) were seeded in a 96-well plate and then incubated for 24 h in serum-free DMEM. Cells were then treated with various concentrations of FGF2 recombinant in a final volume of 100 μ L/well. After 48 h of incubation, 10 μ M bromodeoxyuridine was added to the cell culture and the cells were incubated for an additional 2 h at 37°C.

Cell Cycle Analysis

Cells (2×10^5 cells/well) were seeded in a 6-well plate and then incubated for 24 h in serum-free DMEM. Cells were then treated with FGF2 recombinant in a dose-dependent manner for 48 h. After treatment with trypsin/EDTA solutions, the cells were centrifuged (at $1,250 \times g$ for 3 min at room temperature), washed twice with cold 0.1% BSA in PBS, and fixed in 70% ethanol at 4°C for 24 h. The MAC-T cells were then centrifuged (at $500 \times g$ for 5 min at room temperature) and the supernatant was discarded. Pellets were washed twice with 0.1% BSA in PBS and stained with propidium iodide (BD Biosciences, Franklin Lakes, NJ) in 100 μ g/mL ribonuclease A (Sigma-Aldrich Corp., St. Louis, MO) for 30 min in the dark. Fluorescence intensity was analyzed using a flow cytometer (BD Biosciences).

Immunofluorescence Analysis

The effects of FGF2 on the expression of proliferating cell nuclear antigen (PCNA) and cyclin D1 were determined by immunofluorescence microscopy. The MAC-T cells (3×10^4 cells/300 μ L) were seeded on confocal dishes (catalog number 100350; SPL Life Sciences, Pocheon, Republic of Korea). They were then incubated for 24 h in serum-free DMEM. For detection

of PCNA and cyclin D1 protein, the serum-starved cells were treated with 20 ng/mL of recombinant FGF2 for 24 h. The cells were then fixed using methanol and then probed with a mouse anti-human monoclonal PCNA and rabbit anti-human polyclonal cyclin D1 at a final dilution of 1:100. Negative controls for background staining included substitution of the primary antibody with purified nonimmune mouse IgG or rabbit IgG. Cells were then incubated with a goat anti-mouse IgG Alexa 488 (catalog number A11017; Invitrogen Corp., Carlsbad, CA) or a goat anti-rabbit IgG Alexa 488 (catalog number A-11008; Invitrogen Corp.) at a 1:200 dilution for 1 h at room temperature. Afterward, the MAC-T cells were washed using 0.1% BSA in PBS and overlaid with 4',6-diamidino-2-phenylindole. For each primary antibody, images were captured by a LSM710 confocal microscope (Carl Zeiss, Inc., Thornwood, NY).

Western Blot Analyses

For collecting protein from cells after treatment, cells were lysed in lysis buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100 (Sigma-Aldrich Corp.), 5 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 0.1% SDS, and a mixture of protease inhibitors. The lysed cells were centrifuged (centrifuged at $20,000 \times g$ for 20 min at 4°C) and then supernatants were collected. Concentrations of protein in cell lysates were determined using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) with BSA as the standard. Proteins were denatured, separated using SDS-PAGE, and transferred to nitrocellulose. Blots were developed using enhanced chemiluminescence detection (SuperSignal West Pico; Pierce Biotechnology, Inc., Rockford, IL). They were quantified by measuring the intensity of light emitted from correctly sized bands under UV light using a ChemiDoc EQ system and Quantity One software (Bio-Rad Laboratories, Inc.). Immunoreactive phosphorylated and total proteins were detected using goat anti-rabbit polyclonal antibodies (catalog number 474-1506; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) or goat anti-mouse polyclonal antibodies (catalog number 474-1806; Kirkegaard & Perry Laboratories, Inc.) at a 1:1,000 dilution. As a loading control, total proteins or α -tubulin (**TUBA**) were used to normalize results from the detection of target proteins. Multiple exposures of each western blot were performed to ensure linearity of chemiluminescent signals.

Reagents

Human FGF2 recombinant protein (catalog number 233-FB/CF) was purchased from R&D Systems, Inc. (Minneapolis, MN). Tunicamycin (catalog number

T7765) was purchased from Sigma-Aldrich Corp. The following were purchased from Cell Signaling Technology, Inc. (Beverly, MA): antibodies against phosphorylated (**p-**) protein kinase B (**AKT**; Ser⁴⁷³, catalog number 4060), extracellular signal-regulated kinases 1 and 2 (**ERK1/2**; Thr²⁰²/Tyr²⁰⁴, catalog number 9101), Jun N-terminal kinase (**JNK**; Thr¹⁸³/Tyr¹⁸⁵, catalog number 4668), 70 kDa ribosomal S6 kinase (**P70S6K**; Thr⁴²¹/Ser⁴²⁴, catalog number 9204), 90 kDa ribosomal S6 kinase (**P90S6K**; Thr⁵⁷³, catalog number 9346), ribosomal protein S6 (**S6**; Ser^{235/236}, catalog number 2211), cyclin D1 (catalog number 3300), and eukaryotic translation initiator factor 2 α (**eIF2 α** ; Ser⁵¹, catalog number 3398) and total AKT (catalog number 9272), ERK1/2 (catalog number 4695), JNK (catalog number 9252), P70S6K (catalog number 9202), P90S6K (catalog number 9335), S6 (catalog number 2217), cyclin D1 (catalog number 2922), eIF2 α (catalog number 5324), and inositol-requiring enzyme 1 α (**IRE1 α** ; catalog number 3294). The following were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): antibodies against phosphorylated protein kinase RNA-like ER kinase (**PERK**; Thr⁹⁸¹, catalog number sc-32577), total PERK (catalog number sc-13073), activating transcription factor 6 α (**ATF6 α** ; catalog number sc-166659), glucose-regulated protein 78 (**GRP78**; catalog number sc-13968), and growth arrest and DNA damage-inducible gene 153 (**GADD153**; catalog number sc-7351). Cell Signaling Technology, Inc., supplied the inhibitor for phosphoinositide 3-kinase (**PI3K**)/AKT (wortmannin, catalog number 9951). Enzo Life Sciences, Inc. (Farmingdale, NY) supplied the inhibitors for ERK1/2 (U0126, catalog number EI282) and JNK (SP600125, catalog number EI305). Selleck Chemicals (Houston, TX) supplied the FGFR inhibitor (BJG398, catalog number S2183).

Ribonucleic Acid Isolation and cDNA Synthesis

Total cellular RNA was isolated from in vitro cultured MAC-T cells using a Trizol reagent (Invitrogen Corp.) and purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The quantity and quality of total RNA were determined by spectrometry and denaturing agarose gel electrophoresis, respectively. Complementary DNA was synthesized from cellular RNA using AccuPower RT PreMix (Bioneer Corp., Daejeon, Republic of Korea) and random hexamer (Invitrogen Corp.) and oligo (dT) primers.

Semiquantitative Reverse Transcription-PCR Analysis

Specific primers for bovine *FGFR1* (forward: 5'-CAG CTG CCA AGA CAG TGA AG-3'; reverse:

5'-ATC TTA CTC CCG TTC ACC TC-3'), *FGFR2* (forward: 5'-TCG CAT TGG AGG CTA TAA GG-3'; reverse: 5'-TCC GTC ACA TTG AAC AGA GC-3'), *FGFR3* (forward: 5'-CAC CGA CAA GGA GCT AGA GG-3'; reverse: 5'-CAG GAT GAA GAG GAG GAA GC-3'), and *FGFR4* (forward: 5'-CTT GAA TGG GCA CGT TTA CC-3'; reverse: 5'-ACA CCT TGC AGA GCA GTT CC-3') were designed from sequences in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) using Primer 3 (version 4.0.0; <http://bioinfo.ut.ee/primer3-0.4.0/>). All primers were synthesized by Bioneer Inc. The expression of target mRNA in MAC-T cells was investigated using semiquantitative reverse transcription-PCR. After PCR, equal amounts of reaction product were analyzed using a 1% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under UV light using a Gel Doc XR+ system with Image Lab software (Bio-Rad Laboratories, Inc.).

Statistical Analyses

All quantitative data were subjected to a least squares ANOVA using the GLM procedures of the Statistical Analysis System (SAS Inst. Inc., Cary, NC). Western blot data were corrected for differences in sample loading using total protein or TUBA data as a covariate. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A *P*-value less than or equal to 0.05 was considered significant. Data are presented as least squares means with SE.

RESULTS

Proliferation-Stimulatory Effect of FGF2 on Bovine Mammary Epithelial (MAC-T) Cells

As illustrated in Fig. 1A, FGF2 stimulated proliferation of in vitro cultured MAC-T cells in a dose-dependent manner (0, 1, 5, 10, 20, 50, 100, and 150 ng/mL). Treatment of recombinant FGF2 significantly increased MAC-T cell proliferation by 133.5 ($P < 0.05$), 146.8 ($P < 0.01$), 157.6 ($P < 0.01$), 148.3 ($P < 0.05$), 139.2 ($P < 0.01$), and 139.4% ($P < 0.05$) at a dose of 5, 10, 20, 50, 100, and 150 ng/mL, respectively, compared with nontreated (0 ng/mL) MAC-T cells. In support of these results, we next investigated whether expression of PCNA proteins (a proliferative regulator for DNA replication) was altered in MAC-T cells in response to FGF2 stimulation (Fig. 1B). An immunofluorescence analysis revealed that PCNA proteins were abundant in nuclei of MAC-T cells treated with 20 ng/mL FGF2,

but they were nearly undetectable in nontreated control MAC-T cells. Relative fluorescence signal intensity of immunoreactive PCNA proteins was 799.7-fold ($P < 0.01$) greater in FGF2-treated cells than in control cells.

Regulatory Effect of FGF2 on Cell Cycle Progression of MAC-T Cells

We next analyzed the dose-dependent (0, 1, 5, 10, and 20 ng/mL) effects of FGF2 on MAC-T cell cycle progression. Compared with nontreated MAC-T cells (0 ng/mL), the MAC-T cell population in the S and G₂/M phases increased in MAC-T cells treated with FGF2 in a dose-dependent manner (Fig. 2A and 2B). Meanwhile, the cell population in the G₀/G₁ phases decreased as FGF2 treatment dose increased. In support of these results, the FGF2-induced change in the amount of cyclin D1 proteins in MAC-T cells was determined by immunofluorescence analysis (Fig. 2C). Note that the cyclin D1 protein is a regulator for cell-cycle progression. Abundant levels of cyclin D1 proteins were mainly detected in the nuclei of MAC-T cells treated with 20 ng/mL FGF2 but not in nontreated cells. An immunofluorescence analysis revealed that in response to FGF2 treatment, the relative fluorescence signal intensity of immunoreactive cyclin D1 proteins increased 387.7-fold in MAC-T cells ($P < 0.01$).

FGF2-induced activation of Phosphoinositide 3-Kinase/Protein Kinase B and Mitogen-Activated Protein Kinase Signaling Pathways in MAC-T Cells

FGF2 treatment of in vitro cultured MAC-T cells induced proliferation and cell-cycle progression of the cells. The MAC-T cells expressed *FGFR2* mRNA (see Supplementary Fig. S1; see the online version of the article at <http://journalofanimalscience.org>). We therefore hypothesized that FGF2-induced effects are mediated through FGFR-conjugated intracellular signaling cascades in the cells. To investigate the signaling pathways associated with the FGF2-FGFR system stimulating MAC-T cell proliferation, we investigated changes in the phosphorylated status of signaling proteins in MAC-T cells in response to increasing doses of FGF2 stimulation (Fig. 3). We administered increasing doses of FGF2 (0, 1, 5, 10, and 20 ng/mL). Increasing the FGF2-treatment doses likewise gradually increased the phosphorylation of AKT, P70S6K, S6, ERK1/2, JNK, P90S6K, and cyclin D1 proteins. Compared with control cells (0 ng/mL), MAC-T cells treated with 20 ng/mL FGF2 saw levels of phosphorylated status AKT, P70S6K, S6, ERK1/2, JNK, and P90S6K reach a maximum increase of 6.4- ($P < 0.05$), 6.4- ($P < 0.001$), 22.6- ($P < 0.01$), 1.9- ($P < 0.01$), 2.3- ($P < 0.01$), and

Figure 1. Fibroblast growth factor 2 (FGF2) stimulates MAC-T cell proliferation. [A] Dose-dependent (0, 1, 5, 10, 20, 50, 100, and 150 ng/mL) effects of FGF2 on MAC-T cell proliferation was analyzed using a bromodeoxyuridine cell proliferation assay. FGF2-induced cell proliferation rates are presented as relative percentage changes relative to nontreated control cells (100%). [B] Abundance and localization of proliferating cell nuclear antigen (PCNA) proteins in FGF2-treated (20 ng/mL) or nontreated MAC-T cells were analyzed using immunofluorescence analyses. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue; the second horizontal panels). The scale bar represents 40 μ m (the first and third vertical panels) and 20 μ m (the second and fourth vertical panels). The relative intensity of immunoreactive PCNA proteins is presented in a graph. Pooled data from 3 independent experiments performed in triplicate are shown as means $\pm$ SEM. The asterisks indicate significant differences compared with nontreated MAC-T cells (* P < 0.05; ** P < 0.01).

7.6-fold (P < 0.01), respectively. Phosphorylation of cyclin D1 reached a maximum increase of 5.3-fold in response to 10 ng/mL FGF (P < 0.01). Although the increased level of p-cyclin D1 was slightly reduced at 20 ng/mL FGF2 treatment, it was still significantly higher (4.9-fold; P < 0.01) than the basal level (0 ng/mL).

Reciprocal Crosstalk between Phosphoinositide 3-Kinase/Protein Kinase Band Mitogen-Activated Protein Kinase Signaling Proteins Activated by FGF2 Stimulation

To further determine activation sequences of FGF2-induced signaling molecules and interrelations among

them, MAC-T cells were preincubated with pharmacological inhibitors prior to the 20 ng/mL FGF2 treatment. These inhibitors were PI3K (wortmannin, 1 μ M), ERK1/2 (U0126, 20 μ M), and JNK (SP600125, 20 μ M; Fig. 4). Pretreating MAC-T cells with PI3K inhibitor (LY294002) significantly blocked the inducible effect of FGF2 on AKT phosphorylation. The FGF2-induced AKT activation was still maintained in presence of the ERK1/2 or JNK inhibitor. Moreover, the level of FGF2-induced p-P70S6K, p-S6, and p-90S6K was returned to approximately basal levels by PI3K, ERK1/2, or JNK pathway blockage. The FGF2-induced ERK1/2 activation was completely inhibited in MAC-T cells pretreated with either PI3K inhibitor or ERK1/2 inhibitor,

Figure 2. Fibroblast growth factor 2 (FGF2) induces MAC-T cell cycle progression. [A] Dose-dependent (0, 1, 5, 10, and 20 ng/mL) regulatory effects of FGF2 on MAC-T cell cycle progression was investigated by flow cytometric analyses. A bar chart presents the percentages of cell populations in the G₁, S, and G₂/M phases. Histograms show changes in the DNA content of MAC-T cells positive for propidium iodide. [B] Abundance and localization of cyclin D1 proteins in FGF2-treated (20 ng/mL) or nontreated MAC-T cells were analyzed using immunofluorescence analyses. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue; the second horizontal panels). The scale bar represents 40 μ m (the first and third vertical panels) and 20 μ m (the second and fourth vertical panels). The relative intensity of immunoreactive cyclin D1 proteins is presented in the form of graph. Pooled data from 3 independent experiments performed in triplicate are shown as means $\pm$ SEM. The asterisks indicate significant differences compared with nontreated MAC-T cells (** P < 0.01).

whereas the FGF2-induced JNK phosphorylation was significantly reduced by pretreatment of PI3K inhibitor as well as JNK inhibitor. In addition, inhibition of PI3K, ERK1/2, or JNK pathways led to complete blockage of the inducible effect of FGF2 to activate cyclin D1.

Fibroblast Growth Factor Receptor–Mediated Action of FGF2 to Activate Intracellular Signaling Pathways in MAC-T Cells

We further investigated the FGF2-induced activation of intracellular signaling pathways occurring

through the FGFR. For this investigation, MAC-T cells were preincubated with a pharmacological inhibitor for FGFR (BGJ398, 20 μ M) prior to 20 ng/mL FGF2 stimulation (Fig. 5). Consistent with findings shown in Fig. 3 and 4, FGF2 alone significantly increased the abundance of p-AKT, p-P70S6K, p-S6, p-ERK1/2, p-JNK, p-P90S6K, and p-cyclin D1 by approximately 7.6- (P < 0.01), 3.6- (P < 0.01), 12.9- (P < 0.01), 1.9- (P < 0.001), 2.9- (P < 0.01), 3.0- (P < 0.01), and 1.5-fold (P < 0.01), respectively, compared with the control cells (no FGF2 and BGJ398 treatment). However, these increased levels of phosphorylated signaling proteins

Figure 3. Fibroblast growth factor 2 (FGF2) activates phosphoinositide 3-kinase (PI3K), extracellular signal-regulated kinases 1 and 2 (ERK1/2), and Jun N-terminal kinase (JNK) intracellular signaling proteins. [A] Phosphorylation of protein kinase B (AKT), [B] 70 kDa ribosomal S6 kinase (P70S6K), [C] ribosomal protein S6 (S6), [D] ERK1/2, [E] JNK, [F] 90 kDa ribosomal S6 kinase (P90S6K), and [G] cyclin D1 in response to different concentrations of FGF2 (0, 1, 5, 10, and 20 ng/mL) was determined using western blot analyses. Bar graphs above blot images show the abundance of phosphorylated (p-) proteins normalized with abundance of total (t-) proteins, whose values are presented as fold changes relative to nontreated (0 ng/mL) control MAC-T cells. The graph presents means $\pm$ SEM from 3 independent experiments. The asterisks indicate significant differences compared with control cells (* $P < 0.05$; ** $P < 0.01$; † $P < 0.001$).

Figure 4. Fibroblast growth factor 2 (FGF2)-induced signaling pathways are not entirely separated; rather, they are interrelated. The abundance of phosphorylated statuses in [A] protein kinase B (AKT), [B] 70 kDa ribosomal S6 kinase (P70S6K), [C] ribosomal protein S6 (S6), [D] extracellular signal-regulated kinases 1 and 2 (ERK1/2), [E] Jun N-terminal kinase (JNK), [F] 90 kDa ribosomal S6 kinase (P90S6K), and [G] cyclin D1 were analyzed in MAC-T cells treated with FGF2 (20 ng/mL) plus wortmannin (1 μ M, phosphoinositide 3-kinase [PI3K] inhibitor), U0126 (20 μ M, ERK1/2 inhibitor) or SP600125 (20 μ M, JNK inhibitor). After preincubation of MAC-T cells with each pharmacological inhibitor for 2 h, the cells were treated with FGF2 for 15 min. The above bar graphs' blot images show an abundance of phosphorylated (p-) proteins normalized with an abundance of total (t-) proteins. The values are presented as fold changes relative to control cells (non-FGF2 and inhibitors). The graph presents means $\pm$ SEM from 3 independent experiments. The asterisks indicate significant differences between 2 groups (* $P < 0.05$; ** $P < 0.01$; † $P < 0.001$).

Figure 5. The effects of fibroblast growth factor 2 (FGF2) to activate intracellular signaling pathways are dependent on fibroblast growth factor receptors (FGFR). Western blot analyses show an abundance of phosphorylated status in protein kinase B (AKT), [B] 70 kDa ribosomal S6 kinase (P70S6K), [C] ribosomal protein S6 (S6), [D] extracellular signal-regulated kinases 1 and 2 (ERK1/2), [E] Jun N-terminal kinase (JNK), [F] 90 kDa ribosomal S6 kinase (P90S6K), and [G] cyclin D1 in MAC-T cells treated with FGF2 (20 ng/mL) alone, BGJ398 (20 μM, FGFR inhibitor) alone, or FGF2 plus BGJ398 (20 μM, FGFR inhibitor). The above bar graphs' blot images show an abundance of phosphorylated (p-) proteins normalized with an abundance of total (t-) proteins. These values are presented as fold changes relative to control cells (non-FGF2 and non-BGJ398). Each bar in the graph presents a mean ± SEM from 3 independent experiments. The asterisks indicate significant differences between 2 groups (* $P < 0.05$; ** $P < 0.01$; † $P < 0.001$).

through FGF2 stimulation were significantly reduced or completely returned to basal levels by FGFR inhibition, despite the existence of FGF2 stimulation.

Stimulation of MAC-T Cell Proliferation through Fibroblast Growth Factor 2–Fibroblast Growth Factor Receptors–Phosphoinositide 3-Kinase/Protein Kinase B and Mitogen-Activated Protein Kinase Pathways

Next, we investigated whether there is a close correlation between the FGF2-induced PI3K and mitogen-activated protein kinase (MAPK) pathways and examined the functional effects of FGF2 for stimulating MAC-T cell proliferation. Consistent with findings shown in Fig. 1, 20 ng/mL FGF2 alone stimulated MAC-T cell proliferation by 157.5% ($P < 0.01$) compared with the control MAC-T cells (no FGF2 and inhibitor treatment). However, compared with MAC-T cells treated with FGF2 alone, the stimulatory effect of FGF2 on MAC-T cell proliferation was completely inhibited by a blocking of the PI3K, ERK1/2, or JNK pathways (Fig. 6A). As shown in Fig. 6B, the increase of MAC-T cell proliferation in response to FGF2 stimulation was completely blocked by FGFR inhibition, despite the existence of FGF2 stimulation.

Effect of FGF2 to Reduce Endoplasmic Reticulum Stress–Mediated Cell Death in MAC-T Cells

To determine the effects of FGF2 on endoplasmic reticulum (ER) stress–mediated cell death, we analyzed changes in the level of ER stress regulatory proteins and the cell proliferation rate in response to 20 ng/mL FGF2 alone, tunicamycin (0.25 μg/mL) alone, or their combination (Fig. 7). Tunicamycin, an inducer of ER stress, significantly reduced MAC-T cell proliferation. However, the co-treatment of FGF2 with tunicamycin mitigated the tunicamycin-mediated decrease of MAC-T cell proliferation. Moreover, the MAC-T cells treated with tunicamycin showed an increased abundance of major stress sensor proteins IRE1α, and ATF6α. However, these tunicamycin-induced increases of IRE1α and ATF6α were attenuated in the presence of FGF2. Similarly, tunicamycin stimulated phosphorylation of PERK and eIF2α. Tunicamycin induced the expression of GRP78 and GADD153. However, the abundance of tunicamycin-induced p-eIF2α, GRP78, and GADD153 was significantly reduced by co-incubation of MAC-T cells with tunicamycin and FGF2.

Figure 6. The functional effect of fibroblast growth factor 2 (FGF2) to induce MAC-T cell proliferation is mediated by fibroblast growth factor receptors (FGFR) conjugated with phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT), extracellular signal-regulated kinases 1 and 2 (ERK1/2), and Jun N-terminal kinase (JNK) signaling pathways. [A] The MAC-T cells were treated with FGF2 (20 ng/mL) alone, FGF2 plus wortmannin (1 μ M, PI3K inhibitor), FGF2 plus U0126 (20 μ M, ERK1/2 inhibitor), or FGF2 plus SP600125 (20 μ M, JNK inhibitor). [B] The MAC-T cells were treated with FGF2 (20 ng/mL) alone, BGJ398 (20 μ M, FGFR inhibitor) alone, or FGF2 plus BGJ398 (20 μ M, FGFR inhibitor). The effects of PI3K, ERK1/2, JNK, or FGFR inhibition on FGF2-induced MAC-T cell proliferation were determined using a bromodeoxyuridine cell proliferation assay. Cell proliferation rates are presented as percentage changes relative to nontreated control cells (100%). Pooled data from 3 independent experiments performed in triplicate are shown as means $\pm$ SEM. The asterisks indicate significant differences between 2 groups (** P < 0.01; † P < 0.001).

Figure 7. Fibroblast growth factor 2 (FGF2) reduces endoplasmic reticulum (ER) stress-induced death of MAC-T cells. [A] The effects of tunicamycin (0.25 μ g/mL) and FGF2 alone or their combination on tunicamycin-induced MAC-T cell death was analyzed using a bromodeoxyuridine cell proliferation assay. Cell proliferation rates are presented as percentage changes relative to control cells (nontunicamycin and non-FGF2; 100%). [B–G] The changes in expression and activation of ER stress-related proteins in response to tunicamycin and FGF2 (20 ng/mL) alone or their combination were analyzed by western blot analyses. The above bar graphs' blot images show an abundance of proteins that interestingly normalized with an abundance of total (t-) proteins or α -tubulin (TUBA). These values are presented as fold changes relative to control MAC-T cells (nontunicamycin and non-FGF2). Pooled data from 3 independent experiments performed in triplicate are shown as means $\pm$ SEM. The asterisks indicate significant differences between 2 groups (* P < 0.05; ** P < 0.01; † P < 0.001). IRE1 α = inositol-requiring enzyme 1 α ; ATF6 α = activating transcription factor 6 α ; p- = phosphorylated; PERK = protein kinase RNA-like ER kinase; eIF2 α = eukaryotic translation initiator factor 2 α ; GRP78 = glucose-regulated protein 78; GADD153 = growth arrest and DNA damage-inducible gene 153.

Figure 8. The schematic diagram shows the current working hypothesis of the fibroblast growth factor 2 (FGF2)–fibroblast growth factor receptor (FGFR) signaling system in bovine mammary epithelial cells. FGF2 induces MAC-T cell cycle progression and stimulates cell proliferation through various intracellular signaling cascades. The cell proliferation-stimulating effect of FGF2 depends on activating these signaling cascades. Furthermore, FGF2 alleviates endoplasmic reticulum (ER) stress–induced MAC-T cell death with changes in the expression and activation of various ER stress–related proteins. AKT = protein kinase B; ATF6α = activating transcription factor 6α; CREB = cAMP response element binding protein; ERK1/2 = extracellular signal–regulated kinases 1 and 2; FGFR2 = fibroblast growth factor receptor 2; IRE1α = inositol-requiring enzyme 1α; JNK = Jun N-terminal kinase; PCNA = proliferating cell nuclear antigen; PERK = protein kinase RNA-like ER kinase; PI3K = phosphoinositide 3-kinase; Raf1 = rapidly accelerated fibrosarcoma 1; RAS = small, monomeric GTP-binding proteins; P70S6K = 70 kDa ribosomal S6 kinase; P90S6K = 90 kDa ribosomal S6 kinase; S6 = ribosomal protein S6.

DISCUSSION

This is the first report describing the functional effects of FGF2 on MAC-T cells and intracellular signaling mechanisms for these FGF2-induced actions. Although accumulating evidence indicates that FGF signaling is closely associated with mammary gland development and that various growth factors exert their mitogenic effects on MEC, the potential role of FGF2 ligand on the development and function of the MEC remains unclear. So we seek to initiate studies on the functional and regulatory effects of FGF2–FGFR signaling on the MEC of cows, using a well-established bovine mammary epithelial (MAC-T) cell line. In this initial study, we 1) investigated functional effects of FGF2 on MAC-T cells to regulate proliferation and cell cycle progression, 2) identified the inducible effects of FGF2 on activation of signaling proteins involved in pathways regulating cell proliferation, 3) examined whether FGFR and FGF2-induced intracellular signaling pathways are involved in the FGF2-induced changes in functionality of MAC-T cells, and 4) determined the reducible effects of FGF2 on ER stress-mediated MAC-T cell death.

The mammary gland is the foundation of lactation in mammals. Understanding the molecular mechanisms involved in mammary gland development and functionality would thereby improve the efficiency of milk production and advance knowledge of lactation biology. Most of the mammary development events take place while the female is pregnant and preparing for lactation. A complex of steroid hormones usually controls this process. For example, estrogen and progesterone are known to be responsible for mammary growth by stimulating proliferation of MEC (Topper and Freeman, 1980; Capuco et al., 2001; Ormandy et al., 2003; Inman et al., 2015). Signaling via members of the FGF family is another major factor essential for mammary gland biology (Dillon et al., 2004). Different FGF ligands can cause distinct cellular responses including proliferation, growth, survival, and differentiation. However, little attention has been paid to the specific role for FGF signaling on MEC of the mammary gland.

In the present study, we used in vitro cultured MAC-T cells and then serum-starved them prior to any treatment to synchronize their cell cycle and reduce basal cellular activity. We found that treatment of serum-starved MAC-T cells with recombinant FGF2 affected cell cycle progression and induced cell proliferation. These results were accompanied by an upregulation of PCNA and cyclin D1 expression. The mammary gland in lactating mammals undergoes repeated cycles of structural and functional development and regression. Following cessation of milk production or weaning of the young, regression of the mammary gland occurs.

This regression is characterized by apoptosis or loss of the mammary alveolar epithelial cells (Accorsi et al., 2002; Seeth et al., 2015). Afterward, redevelopment and remodeling of the mammary gland occurs. Therefore, the number of functional MEC greatly affects the synthetic capacity of the mammary gland and milk yield (Capuco et al., 2003; Rezaei et al., 2016). Together, our data suggest that FGF2 is a positive factor for bovine mammary gland development and remodeling. FGF2 stimulates MEC proliferation and helps to maintain a high number of MEC. Because MEC are the functional unit of the mammary gland for milk production (Akers, 2002), a comprehensive understanding of bovine MEC and their development mechanisms may eventually contribute to improving milk production in cows.

Fibroblast growth factor signaling is associated with both breast cancer and mammary gland development. The signaling induced by FGF ligands occurs through ligand binding with high affinity to one or more of their cognate receptors. These receptors are a highly conserved family of 4 single-pass membrane receptor tyrosine kinases, FGFR1 through FGFR4 (Ornitz and Itoh, 2001). The FGF–FGFR binding induces receptor dimerization, autophosphorylation, and activation via one or more of the downstream signaling sub-branches (Mohammadi et al., 1996; Eswarakumar et al., 2005; Itoh and Ornitz, 2008; Lew et al., 2009; Turner and Grose, 2010). The FGF-induced intracellular signaling cascades vary in different target cells and different FGF ligands. The present results show FGF2 induced phosphorylation of various intracellular signaling proteins. These are involved in PI3K, ERK1/2, and JNK MAPK signaling cascades in the MAC-T cells. In MEC, PI3K/AKT1 and MAPK signaling cascades are associated with cell survival pathways. These signaling cascades increase proliferation and/or reduce apoptosis in response to growth factor stimulation (Fata et al., 2007; Maroulakou et al., 2008). The suppressive effects of PI3K, ERK1/2, or JNK blockage on the FGF2-mediated activation of these signaling proteins indicate that the FGF2-induced cell signaling pathways are not completely independent of each other. Rather, they are closely interrelated.

Based on the results from the reverse transcription-PCR analysis (Supplementary Fig. S1; see the online version of the article at <http://journalofanimalscience.org>), in vitro cultured MAC-T cells expressed *FGFR2* mRNA. However, there was extremely weak or no mRNA expression of other FGFR, *FGFR1*, *FGFR3*, and *FGFR4*. Most FGF ligands bind more than one FGFR, which results in redundant effects on the target cells. Despite this, our supplemental results suggest FGFR2 is the leading receptor for FGF2 in MAC-T cell lines. Likewise, several previous reports implied the importance of FGFR2 during mammary gland development. These reports found

that a reduction in *Fgfr2* resulted in a failure to form mammary placodes and that a *Fgfr2*-null epithelium was unable to undergo ductal branch initiation and elongation (Mailleux et al., 2002; Kim et al., 2013). In humans, FGFR2 is also well known, because its excessive expression is associated with the development of human breast cancer (Hunter et al., 2007; Meyer et al., 2008).

Next, we confirmed whether the FGFR participate in the process where FGF2 transduces signals into the MAC-T cells. We found that the inducible effects of FGF2 on the activation of these signaling and downstream-target proteins were partially or completely reduced by a blockage of FGFR in the cells. This result indicates that the functional effect of FGF2 to activate intracellular signaling pathways likely occurs through FGFR2 activation. In addition, we examined the effects of FGF2 on MAC-T cell proliferation when activation of FGFR or signaling proteins (PI3K, ERK1/2, or JNK) were blocked prior to FGF2 stimulation. We found that the proliferation-stimulating effect of FGF2 was completely inhibited by their blockage. Although results of the present study do not indicate whether other receptor subtypes for FGF2 are expressed in MAC-T cells, current data highlight the regulatory functions of FGF2 on behaviors of MAC-T cells that are primarily dependent on the activation of FGFR and PI3K, ERK1/2, and JNK cell signaling pathways.

We also found FGF2 reduced ER stress-mediated MAC-T cell death. The ER is an eukaryotic cell organelle. It is closely associated with the biosynthesis of proteins and lipids and the regulation of calcium ion influxes (Anelli and Sitia, 2008; Kim et al., 2008). Endoplasmic reticulum stress occurs by extracellular or intracellular stress. This stress includes the accumulation of misfolded proteins, damage in DNA, or deprivation of nutrients, gas, or Ca (Oakes and Papa, 2015). The ER stress-activated unfolded protein response is accompanied by the activation of 3 main ER transmembrane sensors: IRE1 α , ATF6 α , and PERK. The activation is characterized by an increase in ER chaperone protein GRP78 and transcription factor GADD153 (Kaufman et al., 2002). In the present study, the treatment of MAC-T cells with tunicamycin induced the expression and activation of ER stress-related molecules. These molecules included IRE1 α , ATF6 α , PERK, eIF2 α , GRP78, and GADD153 in MAC-T cells. Also, tunicamycin treatment reduced the viability of MAC-T cells. FGF2 stimulation alleviated tunicamycin-induced ER stress and cell death. At different stages of the lactation cycle, MEC experience ER stress, especially at the onset of lactation when they are subject to the extreme metabolic stress required for increases in synthesis of fat and proteins (Invernizzi et al., 2012). Changes in the ER stress pathway are a nor-

mal aspect of the mammary tissue's adaptation to the changing physiological states. These changes are also required for functional and structural maturation of milk-secreting epithelial cells (Invernizzi et al., 2012). However, the role of FGF2 on this response was not investigated here. Taken together, the present results suggest that FGF2 would enhance development and remodeling of the mammary gland of lactating cows by both preventing ER stress-mediated apoptosis and increasing MEC survival and proliferation.

In conclusion, the findings of the current study indicate that FGF2 induces proliferation of MAC-T cells and cell-cycle progression by activating PI3K/AKT, ERK1/2, and JNK MAPK signaling cascades and that FGF2 protects MAC-T cells from ER stress responses including decreases in cell proliferation induced by tunicamycin (Fig. 8). Based on these findings, we suggest that the FGF2-FGFR signaling system is important for mammary gland development. Specifically, this system may be important to increase and/or maintain proliferation of MEC and protect MEC from ER stress responses in lactating cows. Further research is required to elucidate the precise mechanisms and actions of FGF2 signaling in the mammary gland in vivo. Furthermore, more in-depth knowledge of the FGF system in the mammary gland will improve our fundamental understanding of the physiology of lactation and optimize milk production in lactating cows and other mammals.

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