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The MAPK$^{ERK-1,2}$ pathway integrates distinct and antagonistic signals from TGFα and FGF7 in morphogenesis of mouse mammary epithelium

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Abstract

Transforming growth factor-α (TGFα) and fibroblast growth factor-7 (FGF7) exhibit distinct expression patterns in the mammary gland. Both factors signal through mitogen-activated kinase/extracellular regulated kinase-1,2 (MAPK^{ERK1,2}); however, their unique and/or combined contributions to mammary morphogenesis have not been examined. In ex vivo mammary explants, we show that a sustained activation of MAPK^{ERK1,2} for 1 h, induced by TGFα, was necessary and sufficient to initiate branching morphogenesis, whereas a transient activation (15 min) of MAPK^{ERK1,2}, induced by FGF7, led to growth without branching. Unlike TGFα, FGF7 promoted sustained proliferation as well as ectopic localization of, and increase in, keratin-6 expressing cells. The response of the explants to FGF10 was similar to that to FGF7. Simultaneous stimulation by FGF7 and TGFα indicated that the FGF7-induced MAPK^{ERK1,2} signaling and associated phenotypes were dominant: FGF7 may prevent branching by suppression of two necessary TGFα-induced morphogenetic effectors, matrix metalloproteinase-3 (MMP-3/stromelysin-1), and fibronectin. Our findings indicate that expression of morphogenetic effectors, proliferation, and cell-type decisions during mammary organoid morphogenesis are intimately dependent on the duration of activation of MAPK^{ERK1,2} activation.

Keywords: MAPK^{ERK1,2}; Kinetics; Mammary; Branching; Morphogenesis; TGFα; FGF7

Introduction

Branch formation in the mammary gland results from an intricate interplay between cell intrinsic and extrinsic factors. These extracellular factors can be grouped broadly into, but not limited to, hormones, growth factors, proteases, and the extracellular matrix (ECM), each of which have been demonstrated to be required for normal mammary development (Fata et al., 2004; Hovey et al., 2002; Parmar and Cunha, 2004; Schedin et al., 2004; Sternlicht, 2006).

Members of the epidermal and fibroblast growth factor receptor (EGFR, FGFR) families are required for normal mammary development (Mailleux et al., 2002; Sternlicht, 2006), and a number of growth factors have been shown to have functional roles including EGFR and FGFR ligands (Luetteke et al., 1999; Mailleux et al., 2002; Sternlicht et al., 2005). A major unresolved question is how these signals are temporally integrated to generate the diverse range of morphogenetic behaviours observed during mammary development and differentiation.

The mitogen-activated protein kinase (MAPK) pathway includes a three kinase cassette of Rous sarcoma associated factor (Raf), mitogen-activated protein/ERK kinase (MEK), and extracellular regulated kinase (ERK), producing a sequential activation pathway responsive to a diverse array of extracellular stimuli (Roux and Blenis, 2004). A fundamental question of signal transduction is how the canonical components of the MAPK pathway, Raf→MEK → ERK, integrate signals from multiple stimuli into distinct cellular outcomes (Marshall, 1995; Schlessinger, 2004). For instance, the rat pheochromocytoma PC12 cell line proliferates in response to EGF but differentiates in response to nerve growth factor (NGF), although both stimuli elicit activation of the Raf → MEK → ERK cassette (Qui and

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Green, 1992; Traverse et al., 1992). Efforts into resolving this question have focused on the duration of the signal through ERK-1 and ERK-2 ($MAPK^{ERK1,2}$) as a key factor determining the fate of a single cell or groups of cells grown on tissue culture plastic. Intense $MAPK^{ERK1,2}$ phosphorylation is evident by whole-mount immunohistochemistry in the course of the development of certain organs during mouse embryogenesis, including the branchial arches, limbs, and lung buds (Corson et al., 2003). Prolonged activation of $MAPK^{ERK1,2}$, in response to hepatocyte growth factor, is associated with immortalized kidney cells undergoing tubulogenesis in collagen type-1 gels (Karihaloo et al., 2004; Maroun et al., 1999). Signalling through the $MAPK^{ERK1,2}$ pathway is necessary for the development of branched organs such as the lung (Kling et al., 2002), kidney (Hida et al., 2002), and salivary gland (Kashimata et al., 2000), but whether the duration of $MAPK^{ERK1,2}$ phosphorylation is indeed a developmentally relevant regulator of organogenesis has not been addressed.

To examine the interplay between growth factor signals, $MAPK^{ERK1,2}$ activation, and mammary branching morphogenesis, we utilized an organotypic 3D mammary primary culture that models aspects of branching morphogenesis in vivo and is responsive to growth factors produced in the mammary gland. In this assay, the mammary epithelium is separated from most of the stroma but retains epithelial/myoepithelial spatial organization, allowing us a framework to evaluate the precise role of signalling modules in a controlled and reproducible environment. We show that individually as well as in combination, TGFα and FGF7 elicit distinct kinetics of $MAPK^{ERK1,2}$ activation that in turn predicts both cellular and morphogenetic outcomes.

Materials and methods

Isolation of primary mammary organoids

The 4th inguinal mammary glands were removed from 10 to 14 weeks old virgin Balb/c mice and minced with two parallel razor blades (approved by the Animal Welfare and Research Committee (AWRC) at Lawrence Berkeley Labs; animal protocol #0522). At this age the expansion of the ductal tree within the fat pad is complete and no terminal end buds exist. Minced tissue (4–8 glands) was gently shaken for 30 min at 37 °C in a 50-ml collagenase/trypsin mixture (0.2% trypsin, 0.2% collagenase type IV, 5% fetal calf serum, 5 μg/ml Insulin, 50 μg/ml gentamycin, in 50 ml of DMEM/F12). The collagenase solution was discarded after centrifugation at 1000 rpm and the pellet was re-suspended in 10 ml DMEM/F12. The suspension was pelleted again at 1000 rpm for 10 min, re-suspended in 4 ml of DMEM/F12+40 μl of DNase (2 U/μl), and incubated for 5 min at ambient temperature with occasional shaking. The DNase solution was removed after centrifugation at 1000 rpm for 10 min. The DNase solution was discarded and the epithelial pieces were separated from the single cells through differential centrifugation. The pellet was re-suspended in 10 ml of DMEM/F12 and pulsed to 1500 rpm. The supernatant was then removed and the pellet was re-suspended in 10 ml DMEM/F12. Differential centrifugation was performed at least 4 times. The final pellet was re-suspended in the desired amount of medium or Matrigel (Growth Factor Reduced Matrigel, BD Biosciences, San Jose, CA).

Morphogenesis assay

Morphogenesis assays were performed in 96 well culture plates. The culture had two layers, an underlay of 50 μl of Matrigel (Growth Factor Reduced Matrigel, BD Biosciences) and an overlying layer of organoids suspended in Matrigel. The underlay was allowed to set for 30 min at 37 °C, and then a 50-μl suspension of organoids in Matrigel (~100–200 organoids/100 μl of Matrigel) was added to the well followed by an incubation of 30 min at 37 °C. All wells were then treated with 150 μl of basal media (DMEF/F12 with 1% insulin, transferrin, selenium, and 1% penicillin/streptomycin) for 24 h. After 24 h the basal media was replenished (untreated samples) or growth factors TGFα (9 nM; Sigma, Saint Louis, MO), FGF7 (9 nM; Sigma, Saint Louis, MO), or FGF10 (9 nM; Biosource, Camarillo, CA) were added to basal media individually or in combinations. Every other day all samples were replenished with basal media alone. To determine the morphogenic response we counted all organoids within each well (~100–200 organoids/well) having 3 or more branches and divided this number by the total number of organoids per well (Simian et al., 2001). For inhibition of $MAPK^{ERK1,2}$ signaling, PD98059 (Calbiochem, San Diego, CA) was added to growth factor-supplemented medium at a concentration of 40 μM. Inhibition of MMP-3 was accomplished with 30 μM of an MMP-3-specific peptide-based inhibitor containing the sequence Ac-Arg-Cys-Gly-Val-Pro-Asp-NH_2 (Calbiochem). The broad based metalloproteinase inhibitor, GM6001, was added to media at 40 μM. Inhibition of fibronectin adhesion was accomplished with blocking antibodies against α5β1-integrin (Chemicon, Temecula, CA) at a concentration of 2 μg/ml.

RT-PCR

Total RNA was isolated with QIAGEN RNeasy Mini Kit (Valencia, CA). For cDNA synthesis, 20 ng of total cellular RNA was used to synthesize cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). MMP-3 was amplified with GTTCCTGATGTTGGTGGCTT and AGCCTTGGCTGAGTGGTAGA primers. Primers that detect fibronectin were TACCAAGGTCAATCCACACCCC and CAGATGGCAAAAGAAAGCAGAGG. As a control for total RNA, RT-PCR for GAPDH was performed with primers TGAAGGTCGGTGTGAACGGATTTGGC and CATGTAGGCCATGAGGTCCACCAC. The amplified fragments were resolved on 1% agarose gels and products were detected with a FluorChem 8900 analysis system (Alpha Innotech, San Leandro, CA). Real-time PCR was performed using LightCycler System (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Fast Start DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN) was used for PCR reaction. PCR data were analyzed with LightCycler Software ver.3 (Roche Diagnostics, Indianapolis, IN). Relative signals between fibronectin or MMP3 and 18s rRNA were quantified.

Protein isolation

For protein isolation, media were removed from the well, and Matrigel+organoids were washed once with cold 1× PBS containing NaF (1 mM) and Na_3VO_4 (1.25 mM). Matrigel and organoids were then treated with a 3D lysis solution consisting of 90% NP40 lysis mix (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP40) plus 10% 10× SDS loading buffer (83.3 μl 12 M acetate [70% glacial acetic acid (17.4 M) and 30% water], 3.67 ml water, 1.25 μl 2-mercaptoethanol, 0.01 g bromophenol blue, 5.0 ml glycerol, 1 g SDS). Samples were pipetted up and down and stored at −20 °C. The 3D lysis solution was heated to 65 °C just prior to using.

Immuno-detection of $MAPK^{ERK1,2}$ phosphorylation

For immunoblot analysis, protein samples were heated at 65 °C for 5 min then placed on ice for 3–5 min. Samples were centrifuged at 13000 rpm for 5 min and the soluble portion was resolved on a pre-cast 10% Tris–glycine polyacrylamide gel (InVitrogen, Carlsbad, CA) using the NOVEX system (InVitrogen, Carlsbad, CA). Resolved proteins were transferred to Hybond nitrocellulose (Amersham, Piscataway, NJ) followed by blocking in 1× TBS, 0.1% Tween-20 with 5% w/v non-fat dry milk for 1 h at ambient temperature. Membranes were incubated overnight at 4 °C in 5% BSA, 1× TBS, 0.1% Tween-20 containing rabbit anti-human polyclonal antibodies that recognize either phosphorylated $MAPK^{ERK1,2}$ (Thr202/Tyr204, #9101, Cell Signaling Tech., Beverly, MA), phosphorylated MEK1 (Ser 298, #9128, Cell Signaling Tech., Beverly, MA), total $MAPK^{ERK1,2}$ (#9102, Cell Signaling Tech., Beverly, MA),