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Bone morphogenetic protein-4 strongly potentiates growth factor-induced proliferation of mammary epithelial cells

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ABSTRACT

Bone morphogenetic proteins (BMPs) are multifunctional cytokines that elicit pleiotropic effects on biological processes such as cell proliferation, cell differentiation and tissue morphogenesis. With respect to cell proliferation, BMPs can exert either mitogenic or anti-mitogenic activities, depending on the target cells and their context. Here, we report that in low-density cultures of immortalized mammary epithelial cells, BMP-4 did not stimulate cell proliferation by itself. However, when added in combination with suboptimal concentrations of fibroblast growth factor (FGF)-2, FGF-7, FGF-10, epidermal growth factor (EGF) or hepatocyte growth factor (HGF), BMP-4 potently enhanced growth factor-induced cell proliferation. These results reveal a hitherto unsuspected interplay between BMP-4 and growth factors in the regulation of mammary epithelial cell proliferation. We suggest that the ability of BMP-4 to potentiate the mitogenic activity of multiple growth factors may contribute to mammary gland ductal morphogenesis as well as to breast cancer progression.

Bone morphogenetic proteins (BMPs) constitute a major subgroup within the transforming growth factor-beta superfamily of signaling molecules. Although BMPs were originally identified by their ability to induce ectopic bone formation, it has become evident that these cytokines have broader functions and regulate biological processes as diverse as cell proliferation, differentiation and cell fate determination [1,2]. With respect to proliferation, BMPs are known to either stimulate [2] or inhibit [3,4] cell growth, depending on the target cells and their microenvironmental context.

It is now well established that the nature of the response elicited by a specific cytokine is contextual, i.e., depends on the presence of other signaling cues in the pericellular environment of the responding cell. Thus, BMPs have been reported to interact synergistically or antagonistically with other cytokines, and particularly with members of the FGF family [5–8]. Here, we report that BMP-4 does not significantly stimulate the proliferation of murine mammary epithelial cells on its own, but potently enhances the mitogenic activity of suboptimal concentrations of FGF-2, FGF-7, FGF-10, EGF HGF.

Materials and methods

Reagents. Recombinant human BMP-2, BMP-4 and BMP-7 and recombinant human FGF-10 was purchased from R&D Systems Ltd. (Minneapolis, MN). Recombinant human FGF-2 and HGF were gifts from P. Sarmientos (Farmitalia Carlo Erba, Milan, Italy) and the late R. Schwall (Genentech, San Francisco, CA), respectively. Recombinant human FGF-7 (also known as keratinocyte growth factor) and EGF were obtained from PeproTech (London, UK).

Cells. J3B1A cells [9], a subclone of the murine EpH4 mammary epithelial cell line [10,11], were grown in Dulbecco's modified Eagle's medium (DMEM) with 1000 mg/L glucose and Glutamax™ (Cat. 21855-025, Invitrogen-GIBCO, Bern, Switzerland), supplemented with 10% heat-inactivated donor calf serum (DCS, Invitrogen-GIBCO). 2A4 cells [12], a subclone of the murine 31EG4 mammary epithelial cell line [13], were grown in a 1:1 mixture of DMEM and F12 medium with Glutamax™ (DMEM/F12) (Cat. No. 31331-028, Invitrogen-GIBCO, Bern, Switzerland) supplemented with 5% fetal calf serum (FCS, Invitrogen-GIBCO).

Cell proliferation assay. Cells harvested from confluent cultures were resuspended in chemically defined serum-free medium consisting of DMEM/F12 supplemented with ITS+ Premix (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin and 5.35 μg/ml linoleic acid; BD Biosciences, Franklin Lakes, NJ). For qualitative evaluation of cell proliferation, cells were seeded into 16-mm wells of a Falcon multiwell plate (BD Biosciences) at a density ranging from 1×10^4 to 4×10^4 cells/well. Twenty-four hours later, the cultures were either left untreated or treated with the indicated agents. Media and treatments were renewed every 2–3 days. After 6 or 7 days of treatment, the cultures were fixed and stained with crystal

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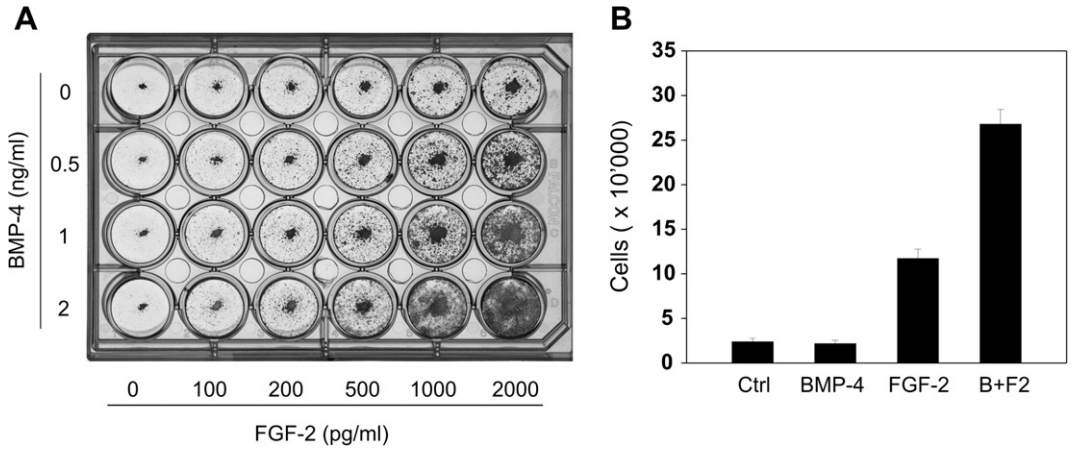

Fig. 1. BMP-4 potentiates the mitogenic activity of FGF-2 in cultures of J3B1A mammary epithelial cells. (A) J3B1A cells were seeded at a density of 1.5×10^4 cells/well in defined medium, co-treated with increasing concentrations of BMP-4 and FGF-2, and stained with crystal violet after 6 days. (B) J3B1A cells were seeded at 2×10^4 cells/well in defined medium. After 24 h, the cultures were left untreated ("Ctrl"), treated with either BMP-4 (1 ng/ml) or FGF-2 (0.5 ng/ml) alone, or co-treated with both BMP-4 and FGF-2 ("B+F2"). Cells in triplicate wells were counted after 7 days of treatment. $p < 0.0005$ for values of FGF-2-treated cultures compared with both untreated cultures and cultures treated with BMP-4. $p < 0.0005$ for values of cultures co-treated with FGF-2 and BMP-4 compared with cultures treated with FGF-2 alone.

violet. For determination of cell number, cells were seeded into 22-mm wells of a Falcon multiwell plate at 2×10^4 cells/well (for J3B1A cells) or 5×10^4 cells/well (for 2A4 cells). Twenty-four hours later, the cultures were either left untreated or treated with the indicated agents. Cells in triplicate wells were harvested by trypsinisation after 7 days of treatment and counted with a hemocytometer. Data represent the mean of at least 3 independent experiments ± SEM per condition. Mean values were compared using Student's unpaired *t*-test.

Western blot analysis. For Western blot experiments, subconfluent 2A4 cells were starved for 24 h in the absence of serum and in the presence of ITS before they were stimulated with BMP-4 (2 ng/ml), FGF-2 (1 ng/ml), FGF-7 (1 ng/ml), EGF (20 pg/ml), HGF (500 pg/ml) or with combinations of BMP-4 with either one of the other ligands for 24 h (Fig. 4A) or for 15 min, 1, 4, 16 and 48 h when compared with unstimulated controls (Fig. 4B). Whole cell lysates were prepared using ice-cold RIPA lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.5% deoxycholic acid, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% protease inhibitor cocktail for mammalian tissues (Sigma, St. Louis, MO), 0.1% SDS, 1% Triton X-100). Protein matched samples were subjected to SDS–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride microporous membrane (Millipore, Bedford, MA). Immunoblotting was performed as described previously [14]. The following primary antibodies were applied: phospho-MEK1/2, phospho-ERK1/2, phospho-p38 MAPK (all from Cell Signaling Technology, Beverly, MA), ERK2, p38 (all from Santa Cruz Biotechnology, Santa Cruz, CA).

Results and discussion

The present analysis of the interplay between BMPs and mitogenic growth factors in the regulation of mammary epithelial cell proliferation was prompted by observations we made in the course of previous studies. Using an in vitro assay of epithelial morphogenesis, we recently reported that BMP-4 suppresses the ability of J3B1A cells to form cystic structures in collagen gels and induces them to invade the surrounding matrix [15]. Subsequent experiments showed that simultaneous addition of FGF-2 markedly enhanced BMP-4-induced invasive growth in this culture system (R. Montesano, unpublished observations). To begin elucidating the mechanisms responsible for the observed cooperation between BMP-4 and FGF-2, we set out to assess the effect of combined treatment with these two cytokines on J3B1A cell proliferation in monolayer culture.

Qualitative checkerboard analysis of the effect of co-treatment with BMP-4 and FGF-2 revealed a potentiation of the mitogenic effect of FGF-2 by BMP-4 (Fig. 1A). To quantitate these observations, cell number was determined after 7 days of treatment with BMP-4 alone (1 ng/ml), FGF-2 alone (0.5 ng/ml) or both agents added together. The data obtained confirmed that BMP-4 does not stimulate J3B1A cell proliferation on its own, but potently enhances the mitogenic activity of FGF-2 (Fig. 1B).

To ascertain that the cooperative activity of BMP-4 and FGF-2 in cell growth stimulation was not unique to the J3B1A cell line, we next performed proliferation assays using a different mammary cell line, 2A4 cells [12]. Both qualitative checkerboard analysis (Fig. 2A) and cell counting experiments (Fig. 2B) demonstrated that BMP-4 does not stimulate 2A4 cell proliferation on its own, but robustly potentiates the mitogenic activity of suboptimal concentrations (1 ng/ml) of FGF-2. Fig. 2C illustrates the appearance of cell cultures prior to cell harvesting and counting. In untreated cultures, as well as in cultures incubated with either BMP-4 alone (2 ng/ml) or FGF-2 alone (1 ng/ml), the cells have formed small colonies. In contrast, following concomitant treatment with both BMP-4 and FGF-2, 2A4 cells have attained full confluence.

The foregoing findings raised the questions of whether BMP-4 was able to potentiate the mitogenic activity of additional growth factors besides FGF-2, and whether other members of the BMP family shared this property. To address these issues, 2A4 cells were treated with BMP-2, BMP-4 or BMP-7, together with suboptimal concentrations of growth factors known to stimulate the proliferation of mammary epithelium, including FGF-7 (also known as keratinocyte growth factor) [16,17], FGF-10 [18,19], EGF [20] and HGF [21,22]. Qualitative assessment of cell number by crystal violet staining after 7 days of treatment showed that BMP-4 increased the mitogenic activity of all growth factors tested. In contrast, neither BMP-2 nor BMP-7 enhanced growth factor-induced proliferation of 2A4 cells when added at the same concentration as BMP-4 (Fig. 3A), albeit they did so at much higher concentrations (50–100 ng/ml; not shown). The ability of BMP-4 to increase the mitogenic activity of FGF-7, EGF and HGF was confirmed by determination of cell numbers after 7 days of treatment (Fig. 3B–D).

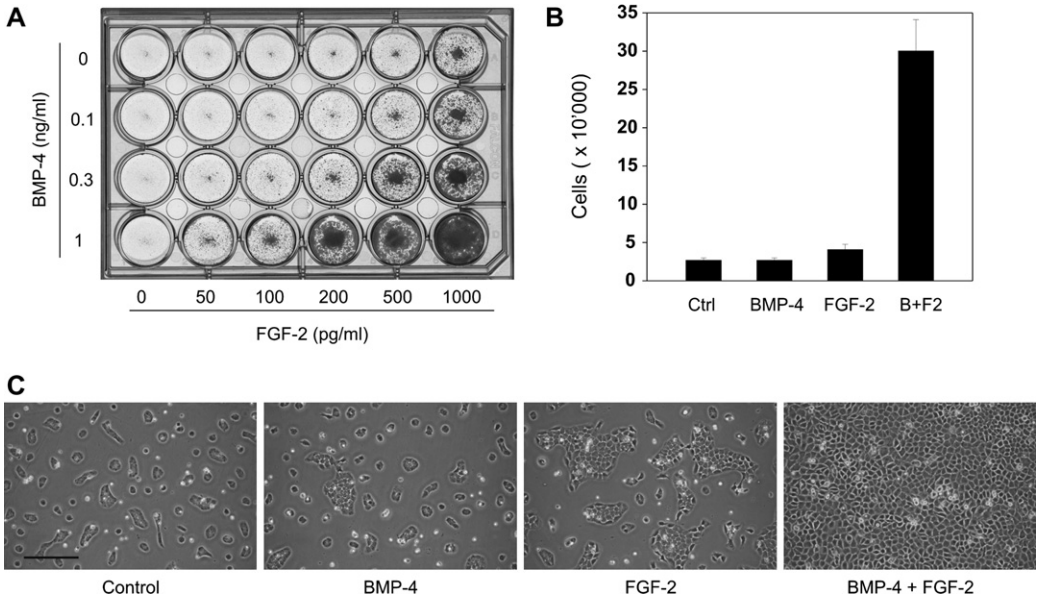

Fig. 2. BMP-4 potentiates the mitogenic activity of FGF-2 in cultures of 2A4 mammary epithelial cells. (A) 2A4 cells were seeded at a density of 2.5×10^4 cells/well in defined medium, co-treated with increasing concentrations of BMP-4 and FGF-2, and stained with crystal violet after seven days. (B) 2A4 cells were seeded at 5×10^4 cells/well in defined medium. After 24 h, the cultures were left untreated ("Ctrl"), treated with BMP-4 (2 ng/ml) or FGF-2 (1 ng/ml) alone, or co-treated with both BMP-4 and FGF-2 ("B+F2"). Cells in triplicate wells were counted after 7 days of treatment. Data represent the mean ± SEM of five independent experiments. Values of cultures treated with BMP-4 alone are not significantly different from those of control cultures. $p < 0.05$ for values of cultures treated with FGF-2 alone compared with untreated cultures. $p < 0.0005$ for values of cultures co-treated with FGF-2 and BMP-4 compared with cultures treated with either BMP-4 alone or FGF-2 alone. (C) 2A4 cells were seeded at 5×10^4 cells/well in defined medium. Seven days later, the cultures were photographed by phase contrast microscopy. In untreated cultures, as well as in cultures incubated with either BMP-4 alone (2 ng/ml) or FGF-2 alone (1 ng/ml), the cells have formed small colonies. In contrast, following concomitant treatment with both BMP-4 and FGF-2, the cells have formed a confluent monolayer. Bar, 200 μm.

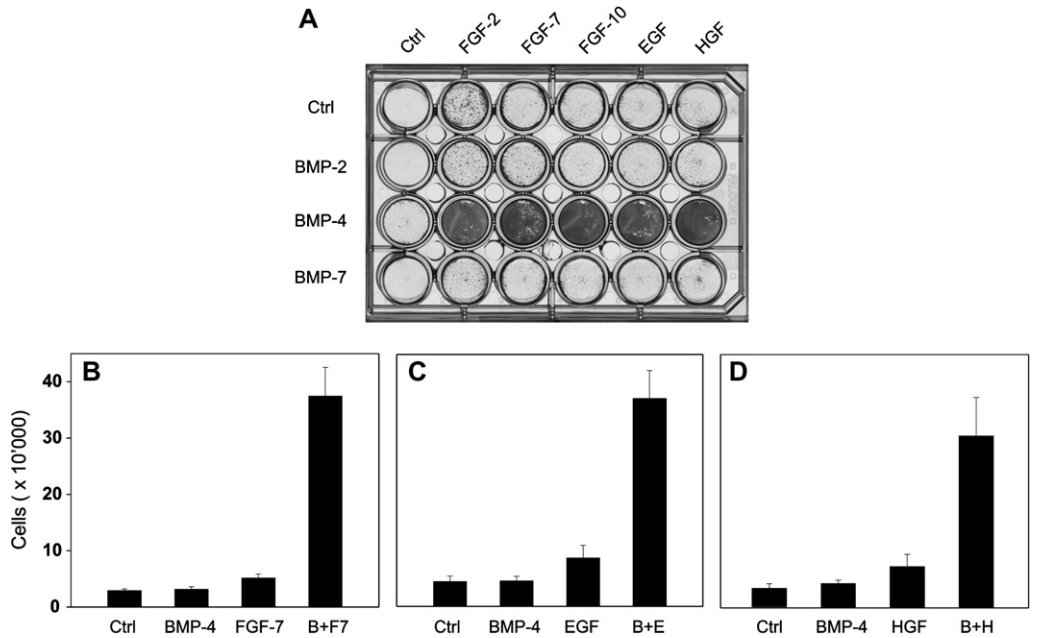

Fig. 3. BMP-4 potentiates the mitogenic activity of multiple growth factors. (A) 2A4 cells were seeded at a density of 4×10^4 cells/well in defined medium, and subsequently treated with FGF-2 (1 ng/ml), FGF-7 (1 ng/ml), FGF-10 (5 ng/ml), EGF (10 pg/ml) or HGF (500 pg/ml), alone or in combination with 0.2 ng/ml of BMP-2, BMP-4 or BMP-7. Seven days later, the cells were stained with crystal violet. (B) 2A4 cells were seeded at 5×10^4 cells/well in defined medium. After 24 h, the cultures were left untreated ("Ctrl"), treated with BMP-4 (2 ng/ml) or FGF-7 (1 ng/ml) alone, or co-treated with both BMP-4 and FGF-7 ("B+F7"). Cells in triplicate wells were counted after 7 days of treatment. Values of cultures treated with BMP-4 alone are not significantly different from those of control cultures. $p < 0.0025$ for values of cultures treated with FGF-7 alone compared with untreated cultures. $p < 0.0005$ for values of cultures co-treated with BMP-4 and FGF-7 compared with cultures treated with either BMP-4 alone or FGF-7 alone. (C) The proliferation assay was carried out as described in B, except that the cultures were treated with BMP-4 (2 ng/ml) or EGF (20 pg/ml) alone, or co-treated with both BMP-4 and EGF. Values of cultures treated with BMP-4 alone are not significantly different from those of control cultures. $p < 0.05$ for values of cultures treated with EGF alone compared with untreated cultures. $p < 0.0005$ for values of cultures co-treated with BMP-4 and EGF compared with cultures treated with either BMP-4 alone or EGF alone. (D) The proliferation assay was carried out as described in (B), except that the cultures were treated with BMP-4 (2 ng/ml) or HGF (500 pg/ml) alone, or co-treated with both BMP-4 and HGF. Values of cultures treated with BMP-4 alone are not significantly different from those of control cultures. $p < 0.05$ for values of cultures treated with HGF alone compared with untreated cultures. $p < 0.0025$ for values of cultures co-treated with BMP-4 and HGF compared with cultures treated with HGF alone. $p < 0.0005$ for values of cultures co-treated with BMP-4 and HGF compared with cultures treated with BMP-4 alone.

Interestingly, when 2A4 cells were seeded at high density (i.e., more than 5×10^4 cells per 16-mm well), BMP-4 stimulated cell proliferation even in the absence of added growth factors (data not shown). To gain insight into the mechanisms underlying the density-dependent proliferative responses to BMP-4, we treated low-density cultures of 2A4 cells with BMP-4 (1 ng/ml) in the presence or absence of 25% (v/v) serum-free conditioned medium from confluent cultures of 2A4 cells. In this experimental setting, BMP-4 did not stimulate cell proliferation when added alone, but greatly enhanced the growth-promoting effect of autologous conditioned medium (data not shown). These finding suggests that, in high-density cultures, BMP-4 enhances cell proliferation in the absence of exogenous mitogens by potentiating the activity of autocrine growth factors released by 2A4 cells.

To explore the possibility that BMP-4 increases growth factor-induced cell proliferation through the synergistic activation of mitogenic signaling pathways, we assessed the impact of BMP-4 alone, suboptimal concentrations of growth factors (FGF-2, FGF-7, HGF and EGF) alone, or a combination of BMP-4 with either one of the other ligands, on the phosphorylation of MEK1/2, ERK1/2, and p38 MAPK. After 24 h of incubation, all five ligands led to a marginal increase in the phosphorylation of both MEK1/2 and ERK1/2 in 2A4 cells when compared with unstimulated controls (Fig. 4A). When EGF (20 pg/ml) or HGF (500 pg/ml) where applied in the presence of 2 ng/ml BMP-4, a slight additive effect on MEK1/2 phosphorylation was detected (Fig. 4A). Five nanogram per milliliter EGF served as a positive control for the phosphorylation of the mitogenic MEK1/2-ERK1/2 signaling module after 24 h. ERK2 protein expression remained unaltered under all conditions tested (Fig. 4A). Moreover, short-term stimulation of 2A4 cells with 2 ng/ml BMP-4, 20 pg/ml EGF or a combination of these two ligands led to a significant induction of ERK1/2 phosphorylation after 15 min of incubation without an additive effect (not shown). In addition, 20 ng/ml EGF and 2 ng/ml BMP-4 induced a time-dependent phosphorylation of p38 MAPK (Fig. 4B). While EGF elicited transient p38 MAPK phosphorylation after 15 min of incubation, which thereafter decreased to basal levels, BMP-4-stimulated p38 MAPK phosphorylation was maximal after 1 h and remained elevated for at least 16 h (Fig. 4B). When BMP-4 was administered together with EGF, a similar time-dependent p38 MAPK phosphorylation was observed, with a reduction and a shift of maximal p38 MAPK phosphorylation levels to later time points. p38 MAPK protein expression remained unaltered under all experimental conditions tested. These findings suggest that the observed potentiation of growth factor-induced cell proliferation by BMP-4 is unlikely to be accounted for by synergistic activation of either the MEK1/2-ERK1/2 or p38 MAPK pathways. However, different members of the MAPK family of protein kinases may cooperate in supporting cell proliferation [23]. Moreover, integration of signals from MAPK pathways and other intracellular mediators may lead to distinct responses to different ligands. Further studies are required to establish whether the potentiation effect we observed is due to interactions between BMP-4-mediated intracellular signals and other mitogenic pathways activated by classical growth factors.

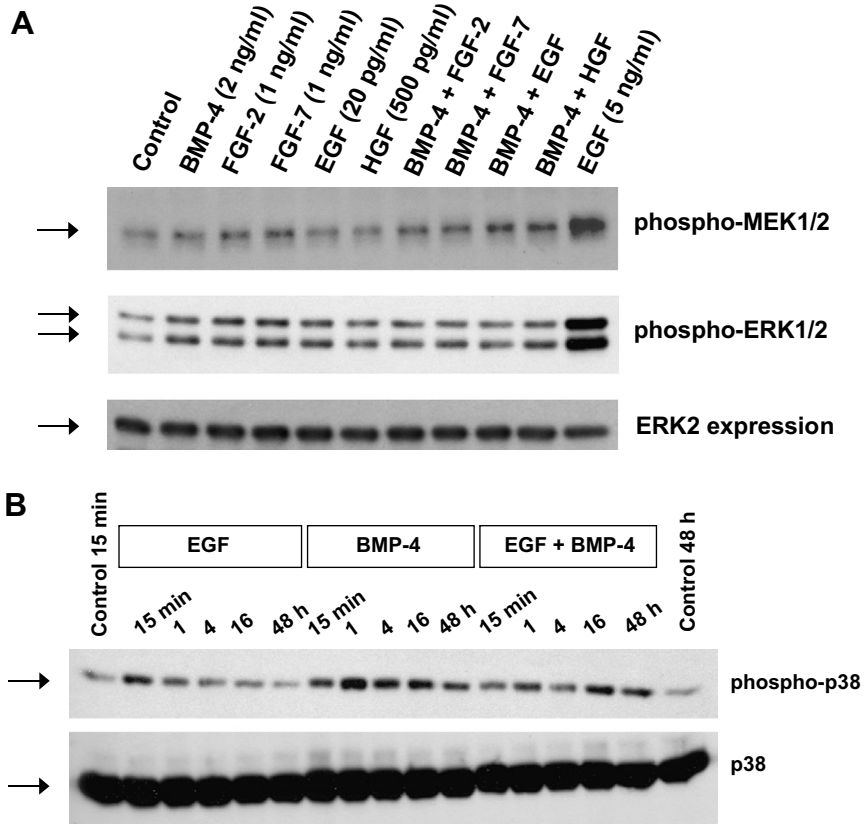

Fig. 4. Effects of BMP-4, FGF-2, FGF-7, EGF, and HGF on phosphorylation of MEK1/2-ERK1/2 and p38 MAPK. (A) Subconfluent 2A4 cells were starved for 24 h in the absence of serum and in the presence of ITS before they were stimulated with BMP-4 (2 ng/ml), FGF-2 (1 ng/ml), FGF-7 (1 ng/ml), EGF (20 pg/ml), HGF (500 pg/ml) for 24 h when compared with unstimulated controls. Protein matched samples of stimulated cells and unstimulated controls were separated on SDS 10% PAGE and analyzed by Western immunoblot for phosphorylation of MEK1/2 and ERK1/2 as well as for protein expression of ERK2. The results from one representative Western blot of $n = 3$ separate experiments are depicted. (B) Subconfluent 2A4 cells were starved for 24 h in the absence of serum and in the presence of ITS before they were stimulated with BMP-4 (2 ng/ml), EGF (20 pg/ml) or EGF + BMP-4 for 15 min, 1, 4, 16 and 48 h when compared with unstimulated controls (15 min, 48 h). Protein matched samples were analyzed by Western immunoblot for phosphorylation of p38 MAPK as well as for protein expression of p38. The results from one representative Western blot of $n = 3$ separate experiments are depicted.

What is the biological relevance of our findings? In the mammary gland, FGFs, EGF and HGF are involved in the process of epithelial duct elongation and branching [19–21,24,25]. BMP-4, which is expressed in the stroma of both embryonic and post-natal mammary gland [26], may therefore facilitate ductal morphogenesis by enhancing the mitogenic activity of FGFs, EGF and/or HGF. On the other hand, inappropriate signaling by FGFs [27], EGF [28] and HGF [29] has been implicated in breast carcinogenesis, and BMP-4 has recently been shown to be highly expressed in breast cancer [30,31]. The ability of BMP-4 to robustly potentiate growth factor activity may therefore play a significant role in the progression of breast cancer.

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