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Deletion of Gremlin1 increases cell proliferation and migration responses in mouse embryonic fibroblasts

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Gremlin1 (Grem1) is an antagonist of bone morphogenetic proteins (BMPs) that plays a critical role in embryonic and postnatal development. Grem1 has been implicated as both a promotor and an inhibitor of cell proliferation driven by BMP-4 and other mitogens in a diverse range of cell types. Recent data showed that Grem1 can trigger angiogenesis via vascular endothelial growth factor receptor (VEGFR2) binding, highlighting that the precise modalities of Grem1 signalling require further elucidation.

In an attempt to enhance our understanding of the role of Grem1 in cell proliferation, mouse embryonic fibroblasts lacking grem1 (grem1−/−) were generated. Grem1−/− cells showed elevated levels of proliferation in vitro compared to wild-type and grem1+/−, with accelerated scratch wound repair but no obvious changes in cell cycle profile. Modest increases in BMP-4-stimulated Smad1/5/8 phosphorylation were detected in grem1−/− cells, with concomitant modest changes in Smad-dependent gene expression. Surprisingly, levels of ERK phosphorylation were reduced in grem1−/− cells compared to wild-type. These data suggest Gremlin1 is an inhibitor of embryonic fibroblast proliferation in vitro. Furthermore, the signalling pathways causing increased cell proliferation in the absence of Grem1 may involve other pathways distinct from canonical Smad and non-canonical ERK signalling.

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1. Introduction

Bone morphogenetic proteins (BMPs) are secreted, glycosylated members of the TGFβ1 superfamily that regulate embryonic and postnatal development [1]. The defining role for BMPs is in limb development and the induction of cartilage and endochondral bone [12]. Canonical BMP signalling involves binding of cognate BMPs to type I/II receptors which transphosphorylate and trigger phosphorylation of receptor Smads 1/5/8 (R-Smads) which then dimerise with Smad4 via docking at the R-Smad C-terminus [3]. Oligomeric Smad complexes then translocate to the nucleus and regulate transcription of BMP-responsive genes via interaction with transcription factors and transcriptional co-activators and co-repressors [3]. Data from the Masagué group has shown that R-Smads can regulate transcription independently of Smad4 via binding to factors such as transcriptional intermediary factor-γ (TIFγ) during haematopoiesis [4].

BMP action is tightly regulated, with control at the level of gene methylation, miRNA targeting, BMP proteolysis, pseudoreceptor

BAMBI [BMP and activin membrane-bound inhibitor] and inhibitory Smad proteins such as Smad6/7 [1,5]. An additional layer of regulation of BMP occurs via the action of a group of secreted, extracellular antagonists which bind to BMPs in the extracellular matrix and prevent their receptor engagement [1]. Proteins such as Gremlin (Grem1), Noggin, chordin and crosinless-2 inhibit BMP–BMP receptor binding, in a highly temporospatially regulated manner [1–3]. The function of many of these BMP antagonists extends beyond the developmental antagonism of BMP action. Many groups have reported that Gremlin1 is upregulated in fibrotic conditions such as diabetic nephropathy and pulmonary hypertension [6–8]. Elevated levels of Gremlin1 in fibrotic conditions of the kidney correlate well with disease severity [8–9].

Studies in knockout mice elegantly demonstrate the exquisite balance of BMP and Gremlin1 expression required for normal development. Mice lacking Gremlin1 do not develop kidneys [10,11], likely due to an inappropriately strong drive from BMP-4 during development [11]. Deletion of one allele of BMP-4 is sufficient to rescue kidney development in grem1−/− mice, highlighting the signalling interplay between these two proteins [12]. Gremlin1 can also bind to precursor BMP-4 intracellularly, preventing its secretion and thus inhibiting its action at the BMP receptor [13]. Apart from its role in development, Gremlin1 displays activities that may be independent of BMP action. Gremlin1 is a proangiogenic factor expressed by endothelial cells
and is highly expressed in human lung tumour vasculature [14]. This proangiogenic action of Grem1 is mediated via binding of the VEGFR2 (vascular endothelial growth factor receptor 2) in endothelial cells [15].

Several groups have highlighted the role of Grem1 in cell proliferation and growth. Grem1 promotes the proliferation and migration of vascular smooth muscle cells, potentially contributing to arterial damage as a result of balloon injury [16,17]. Grem1 is also

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**Fig. 1.** Generation of mouse embryo fibroblasts (MEFs). MEFs were generated from E13.5 embryos of grem1 +/− crosses and genomic DNA isolated as described in [22]. A. PCR reactions to detect the wild-type grem1 allele or recombinant LacZ allele were performed to identify wild-type, grem1 +/− or grem1 −/− cells. B. Phase contrast images of cells grown in normal culture conditions at 40× magnification.

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**Fig. 2.** Deletion of grem1 does not alter the cell cycle profile. MEFs from wild-type (open bars), grem1 +/− (filled bars) or grem1 −/− (shaded bars) were grown in complete medium (non-starved), serum-free medium overnight (starved) and then re-exposed to medium containing 10% FBS (FBS) for 60 min as described in Methods. The cells were prepared and stained with propidium iodide for flow cytometry. Excitation was achieved with a solid state laser (excitation wavelength 488 nm) (n=3 per genotype for each condition, in duplicate). A. Representative histogram plot of non-starved MEFs from wild-type embryos. B–D. The percentage of cells at each phase of the cell cycle was calculated and plotted. E. The percentage of cells with proliferative activity (S + G2/M) in each condition was compared for each genotype. Data were plotted as mean +/- SEM. Statistical significance was determined using one-way ANOVA with post-hoc Tukey–Kramer multiple comparison test.
expressed by tumour stromal cells and can promote basal cell carcinoma cell proliferation, possibly via BMP inhibition [18]. In contrast, Grem1 inhibited proliferation of myogenic progenitors via BMP-4 inhibition [19]. Delivery of Grem1 siRNA also inhibited renal cell proliferation and apoptosis in kidney in vitro [20]. Grem1 levels have also been found to be lower in certain tumour cell lines leading some to suggest that it may act as a tumour suppressor [21].

In this report, using mouse embryonic fibroblasts, we demonstrate that cells lacking Grem1 had higher rates of proliferation and migration in vitro, with no obvious change in cell cycle profile. Levels of pSmad1/5/8 were modestly increased in grem1−/− cells compared to wild-type, with no major changes in Smad-dependent gene expression detected. Levels of phospho-ERK were lower in grem1−/− cells compared to wild-type, suggesting that alternative mechanisms mediating the increase in cell proliferation are at play in grem1−/− cells.

2. Methods

2.1. Isolation of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were isolated at E13.5 from grem1+/− crosses. The liver and inner organs were removed and the body was placed in an eppendorf with 500 μL of trypsin, minced with scissors and incubated at 37 °C for 30 min while shaking at full speed. For each embryo a 10 cm petri dish was coated with 2 mL gelatin (0.1% w/v) and left to set for approximately 30 min. Ten mL of media (Dulbecco’s modified Eagle medium supplemented with l-glutamine (2 nM), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FBS) was added to each plate. The trypsinised embryo was added to the plate, mixed and incubated at 37 °C in a humidiﬁed chamber of 95% air and 5% CO2 for 24 h. Major debris was removed, cells were trypsinised and passaged as normal. Genotyping was carried out as previously described [22].

2.2. Cell treatments

Starvation of cells was achieved by washing cells in 1 × PBS and culturing overnight in DMEM supplemented with l-glutamine (2 nM), penicillin (100 U/mL) and streptomycin (100 μg/mL) but without FBS. Cells were then incubated for 60 min in DMEM supplemented with l-glutamine (2 nM), penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% FBS ("FBS") or 25 ng/mL BMP-4. Normal growing cells were used as a "non-starved" control (NS).

2.3. Scratch wound assay

MEFs were seeded in duplicate on 12 well plates, 2500 cells per well, and allowed to reach 80–90% confluence. For each separate

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Fig. 3. Deletion of grem1 results in increased cell number in vitro. Mouse embryo fibroblasts from wild-type, grem1+/− and grem1−/− embryos were exposed to (A) normal culture medium or (B) serum-free medium for 6, 12 or 24 h. At each time-point, an MTT assay was performed as described in Methods. Absorbance readings were taken at 570–650 nm, and results plotted (n=3–4 different cell lines for each genotype, passage 4–10). Each reading was performed in triplicate. Data were plotted as mean+/-SEM. Statistical significance was determined using one-way ANOVA with post-hoc Tukey–Kramer multiple comparison test, *p<0.05, **p<0.01, or Student’s unpaired t-test (p=0.09).
The percentage wound closure was determined in three conditions: non-starved, starved and FBS-stimulated conditions. Using a P200 pipette tip, a scratch was drawn across the base of the wells, perpendicular to the longest border of the plate. Equal pressure was applied with each scratch to ensure similar size wounds were created. The cells were washed to remove debris and starved plates covered with DMEM alone or DMEM plus 10% FBS. Images of wounds were captured using a phase contrast microscope (Nikon TMS-F microscope with JVC Colour Video Camera KY-F55B). Plates were placed in a humidified incubator at 37 °C for 12 h. At time 0 h, 3 random wound images were selected for each well. The wound size was measured at 6 points for each image and the average calculated. The resulting 3 wound sizes for the individual well were then averaged to calculate a representative wound size for that well. From the calculated wound size at time 0 and 12 h, the percentage wound closure was determined and analysed.

2.4. MTT assay

The assay was performed as per protocol in the Cell Proliferation Kit (Roche). MEFs were seeded in triplicate on a 96-well plate at 5000 cells per well. Cells were allowed to reach approximately 70% confluence. For each separate MEF line the assay was performed under three conditions: non-starved, starved and FBS stimulated conditions at 6, 12 and 24 h. Non-starved cells remained in DMEM plus supplements while the other sets were incubated overnight in DMEM alone. Time 0 h was taken as when the stimulated groups received supplemented DMEM media. Plates were placed in a humidified incubator at 37 °C. At each specific time point, cells were incubated with 10 μL of the yellow MTT solution for 4 h. The solubilized formazan product was quantified using a Molecular Devices Spectramax M2 plate reader at 570 and 650 nm and the difference calculated. Triplicate results for each MEF line under different conditions at each time point were calculated and results analysed.

2.5. Cell cycle analysis

MEFs were plated in 6 cm plates and reached 70–80% confluence before the experiment was started. Each MEF line was plated in duplicate and studied under three conditions: non-starved, starved and FBS stimulated. After starving overnight, media in stimulated plates were changed to DMEM with supplements for 24 h. On day 1, culture media were removed from cells and they were washed with PBS. 500 μL trypsin–EDTA was added and cells incubated at 37 °C for 5 min. 600 μL of culture media was added to neutralise trypsin and cells were transferred to an eppendorf and spun at 2000 rpm for 5 min. Cells were resuspended in 500 μL PBS and spun again. Cells were completely resuspended in 500 μL of cold 70% ethanol and fixed at 4 °C overnight. 500 μL of PBS was added and cells spun down at 2000 rpm for 5 min. Cells were resuspended in 500 μL of PBS containing 100 μg/mL RNAse A and 50 μg/mL propidium iodide (PI) and incubated at 37 °C for 30 min. Samples were transferred to ice and kept in the dark until analysed using a Cyan ADP cytometer (Dako, Stockport, UK). Excitation was achieved with a solid state laser (excitation wavelength 488 nm).

2.6. Quantitative PCR

RNA was isolated using Trizol® (Invitrogen) and reverse transcription reactions were carried out as described [22]. Real-time quantitative PCR was carried out on an ABI Prism 7700 sequence detection system (Applied Biosystems) using the TaqMan Universal PCR Master Mix, VIC-labelled 18s rRNA as an endogenous control and FAM labelled TaqMan probe sets provided by Applied Biosystems (Id1: Mm00775963_g1; PAI-1: Mm00435860_m1). Thermal cycling for PCR was performed for 2 min at 50 °C, 10 min for 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Analysis was carried out using the ΔΔ comparative threshold (Ct) method [23,24].

2.7. Protein extraction from cells and tissues

Cells were harvested on ice in RIPA (radioimmunoprecipitation assay) buffer containing 50 mM Tris–HCl, pH 7.4, 1% (v/v) Nonidet P-40, 0.25% (v/v) sodium deoxycholate, 150 mM NaCl and 1 mM EDTA, supplemented with 1× protease inhibitor cocktail (Sigma), 2 μM microcystin (Alexis Biochemical), 10 μM Benazmidine, 1 mM NaF, 40 mM D-glycerophosphate, 1 mM sodium orthovanadate and fresh 1 mM phenylmethylsulfonyl fluoride. Cells were incubated on ice for 20 min and vortexed every 5 min. Cells were then spun at 20,000 × g at 4 °C for 20 min to remove cell debris. Samples were separated on SDS-PAGE according to the method of Laemmli [25]. Following transfer of protein samples to the PVDF, the membranes were incubated in 1× TBS-Tween (TBS-T) supplemented with 3% (w/v) milk for 60 min at RT. Membranes were then incubated with a primary antibody diluted in 3% (w/v) milk in TBS-T overnight at 4 °C (pSmad1/5/8 1:1000 Cell Signalling #9511); phospho-ERK 1:2000 (Cell Signalling #4377); total ERK 1:2000 (Cell Signalling #4695); and β-actin 1:50,000 (Sigma, A5316). Following three washes with TBS-T, membranes were incubated with the appropriate HRP-conjugated secondary antibody (Cell Signalling) in 3% milk (w/v) for 1 h at RT. Membranes were washed with TBS-T 3×10 min rinses and chemiluminescent bands were activated in a 1:1 mix of Western Blotting Luminol Reagents (Santa Cruz Biotechnology) and visualised with X-ray film.

2.8. Statistical analysis

All data were expressed as mean +/− standard error of the mean (SEM). Statistical analysis was carried out using InStat software.
package. Analyses of variance (ANOVA) with post-hoc Tukey–Kramer Multiple Comparison test or Student’s unpaired t-test were carried out to determine significant differences. p<0.05 was considered to be statistically significant.

3. Results

Mice lacking both copies of grem1 are born without kidneys and die shortly after birth due to both renal and lung insufficiencies [10]. To examine its role in cell proliferation, fibroblasts (MEFs) lacking both copies of grem1 were isolated from E13.5 mouse embryos (Fig. 1). Grem1−/− embryos were readily isolated from grem1+/- crosses, although at lower than expected Mendelian frequencies (data not shown). These fibroblasts provided a robust model in which to test proliferative responses in the total absence of Grem1, as opposed to previous experiments examining cells with reduced Grem1 expression [20,22]. We have previously shown that Grem1 expression is detected in MEFs isolated from E13.5 embryos [22]. Preliminary observations from cell culture indicated that grem1+/- and grem1−/− cells reached confluence more rapidly than wild-type cells (data not shown). Cell cycle profiling using flow cytometry showed no major differences in percentage of cells in G0/1, S and G2/M phases when serum starved or exposed between wild-type, grem1+/- and grem1−/− MEFs (Fig. 2). The growth rate of these cells was assessed using an MTT assay in normal culture conditions, after serum deprivation and in response to FBS-treatment. Both grem1+/- and grem1−/− MEFs displayed higher levels of baseline growth compared to wild-type at all three time points measured (Fig. 3A). Serum starvation reduced the growth of all three cell lines, but again, grem1−/− cells still maintained a higher growth rate compared to wild-type and grem1+/− (Fig. 3B). In response to serum starvation, grem1−/−
MEFs displayed a lower fold change in cell proliferation at all three time-points, which was significantly different to wild-type cells at 6 and 24 h (Fig. 4A). Particularly evident was the serum starvation-induced reduction in cell growth in both wild-type and grem1+/− cells between 12 and 24 h which was not evident in grem1−/− cells (Fig. 4A). Grem1−/− cells also exhibited a blunted response to FBS re-exposure after 24 h starvation, with smaller fold-change increase in cell proliferation compared to wild-type and grem1+/− cells at all three time-points (Fig. 4B). These data suggest that fibroblasts lacking grem1 display higher levels of cell proliferation and a reduced responsiveness to serum starvation/stimulation when cultured in vitro.

To extend these observations, scratch wound assays were performed to measure cell proliferation and migration [26]. The effect of serum starvation and FBS-stimulation on scratch wound repair was then assessed in wild-type, grem1+/− and grem1−/− cells (wild-type shown, Fig. 5A). In the presence of normal complete medium, wild-type MEFs displayed lower 12 h wound closure compared to wild-type and a reduced responsiveness to serum starvation/stimulation when cultured in vitro.

Upon serum re-exposure, wild-type and grem1+/− cells exhibited slightly higher cell migration than wild-type cells (Fig. 5B). These data support the hypothesis that reduction in the levels of Grem1 increases the proliferative/migratory capacity in mouse embryonic fibroblasts.

Grem1 binds to BMP proteins such as BMP2, 4 and 7 and antagonises BMP signalling to intracellular Smad1/5/8 during development and disease [1]. Changes in Smad1/5/8 phosphorylation were examined, as a potential contributory mechanism to observed changes in cell proliferation in grem1−/− MEFs. Baseline levels of Smad1/5/8 phosphorylation were not significantly different in wild-type, grem1+/− or grem1−/− MEFs (Fig. 6). Incubation of cells with serum-containing medium following starvation induced significant Smad1/5/8 phosphorylation in all three genotypes (Fig. 6). Levels of pSmad1/5/8 phosphorylation in FBS-treated grem1−/− MEFs were lower than wild-type (Fig. 6). In addition, no difference in phospho-Smad1/5/8 nuclear localization was detected between wild-type and grem1−/− cells (data not shown). Since FBS is a complex mixture of different growth factors and other agonists, the ability of recombinant BMP-4, a direct target of Grem1, to specifically induce Smad1/5/8 phosphorylation was assessed. Similar to FBS treatment, incubation of MEFs with BMP-4 for 60 min induced marked phosphorylation of Smad1/5/8 in all three genotypes (Fig. 7). Levels of phospho-Smad1/5/8 were higher in BMP-4-treated grem1−/− cells compared to wild-type, but, similar to data obtained with FBS treatment, the fold-change induction did not differ significantly between the two groups (Fig. 7). These data suggest that reductions in the cellular level of Grem1 do not significantly alter serum and BMP-4-mediated Smad1/5/8 phosphorylation in mouse embryonic fibroblasts.

![Fig. 6.](image)

**Fig. 6.** FBS-induced changes in Smad1/5/8 phosphorylation in MEF cells. MEFs from wild-type, grem1+/− and grem1−/− were grown in complete medium (NS), serum deprived (S) overnight and then re-exposed to medium containing 10% FBS for 60 min as described in Methods. (A) Protein lysates (10 μg) were separated on 10% SDS-PAGE and probed by Western blotting with anti-phospho-Smad 1/5/8 and β-actin as loading control. Shown are representative Western blots for n = 3 experiments. B. Band intensities were determined using Scion image software. The intensity ratios of phospho Smad 1/5/8/β-actin are shown. C. Fold changes in the intensity of phospho Smad 1/5/8/β-actin from vehicle to FBS-treated conditions was calculated. Data were plotted as mean +/− SEM. Statistical significance was determined using one-way ANOVA with post-hoc Tukey-Kramer multiple comparison test, *p<0.05, **p<0.01, and Student’s unpaired t-test (C, p = 0.1).
did not dramatically alter the baseline level of p44/42 ERK phosphorylation. Deletion of grem1−/− did not contribute to increased cell proliferation and migration detected in grem1−/− cells.

**4. Discussion**

This study aimed to identify the effect of Grem1 on fibroblast proliferation and signalling in vitro. Previous data demonstrated both positive and negative roles for Grem1 in the regulation of cell proliferation. Using grem1−/− mouse embryonic fibroblasts, we showed that complete ablation of Grem1 expression increased cell growth and proliferation in the presence of absence of growth factors. Modest reductions in BMP-4-stimulated Smad1/5/8 phosphorylation were detected in grem1−/− cells. Consistently, only small changes in Smad-regulated genes such as Id1 and PAI-1 were detected in grem1−/− cells. Finally, levels of p42/44 ERK phosphorylation were reduced in response to serum-stimulation in the absence of Grem1, suggesting that the signalling pathways driving the elevated grem1−/− cell growth do not involve either canonical Smad1/5/8 signalling or the non-canonical ERK pathway downstream of BMPs.

Using MTT assay as a readout of cell growth and scratch wound assay as an indicator of cell migration, MEFs from grem1−/− were found to have higher proliferative activity. Grem1−/− cells also had higher amounts of cell death as assessed by propidium iodide staining in serum-free medium compared to wild-type and grem1+/− cells (data not shown). There were no major differences in all three cell types when cell cycle analysis was performed, suggesting that deletion of Grem1 did not induce any alteration in the progression through the cell cycle.
G1/S and G2/M checkpoints (Fig. 2). One interpretation of these data is that reduced levels of Grem1 may be increasing the actual rate of progression through the cell cycle, therefore not altering the percentage of cells in each phase but still contributing to increased proliferation. Grem1 has previously been implicated in the regulation of cell proliferation. In vascular smooth muscle cells, Grem1 increased cell growth in normal medium, and inhibited BMP-driven cell migration [16]. Similarly, Grem1 reversed BMP-4 induced inhibition of proliferation in cultured basal cell carcinoma cells [18]. In contrast, overexpression of Grem1 decreased the growth of normal rat fibroblasts and CHO cells [28]. Similarly, over-expression of Grem1 in an osteoblastic tumour cell line reduced proliferation through transcriptional increases in p21Cip1 in a pathway independent of BMP signalling [29]. Data from our group indicate that depletion of Grem1 in vivo was associated with reduced proliferation of kidney epithelial cells in response to hydronephrosis resulting from ureteral obstruction (Curran et al., submitted). In contrast, in human kidney mesangial cells, transfection of Grem1 inhibited FBS-induced cell proliferation (C. Godson, personal communication). To our knowledge, our data is the first examination of in vitro cell proliferation in the absence of Grem1, as opposed to experiments employing recombinant Grem1 [14,15], Grem1 over-expression [16,17] or Grem1 shRNA [20]. Taken together, these data point to cell-type specific roles for Grem1 in the regulation of cell proliferation. In addition, the temporospatial changes in both Grem1 and its target BMPs are likely to also determine whether Grem1 can drive or inhibit cell proliferation in each specific cellular context.

Grem1 has been shown to bind and inhibit BMP2, BMP-4 and BMP7 in the process of limb development and in fibrosis of the lung and kidney [10,12,30,31]. In addition, recent data has shown that Grem1 acts as an agonist at the VEGFR2 receptor, triggering angiogenic sprouting of endothelial cells [15]. Mechanistically, Grem1 can inhibit BMP-induced Smad1/5/8 phosphorylation in lung and kidney epithelial cells [20,32]. Both basal and BMP-4-triggered pSmad1/5/8 levels were higher in grem1−/− MEFs compared to wild-type, suggesting that this may contribute to elevated cell proliferation in these cells (Fig. 7). However, the levels of pSmad1/5/8 target genes such as Id1 and PAI1 were not significantly changed in grem1−/− cells compared to wild-type, suggesting that this altered Smad phosphorylation may not be sufficient to alter Smad4-dependent gene expression or cell proliferation (Figs. 8, 9). The levels of phospho-ERK, a major driver of cell division, were surprisingly lower in grem1−/− MEFs compared to wild-type (Fig. 10). Given the increased cell proliferation and scratch-wound repair capacity of grem1−/− cells versus wild-type, it is difficult to understand how cells with higher growth rates would display lower levels of pERK. Investigating our conditions and MEF cell lines further, we found that after serum stimulation, p44/42ERK phosphorylation was reduced in grem1−/− versus wild-type MEFs (Fig. 10). This was an important observation as the ERK pathway is a critical driver of cellular proliferation [33]. Our data suggest that the increase in proliferation in our grem1−/− cells may occur independently of ERK activation. One interpretation of the data is that in contrast to inhibition of BMP-mediated Smad1/5/8 phosphorylation, Grem1 drives ERK phosphorylation in these cells. Supporting this idea, experiments in subendothelial microvascular cells has indicated that Grem1 acts directly to activate ERK signalling in a method independent of BMP activity [14]. Thus, lower pERK levels detected in grem1−/− MEFs may be due to a reduced Grem1-induced drive downstream of VEGFR2. Future experiments using inhibitors of VEGFR2 signalling will be required to further test this hypothesis.
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**References**


**Fig. 10.** Deletion of *grem1* results in reduced ERK phosphorylation after serum stimulation. MEFs from wild-type (open bars) or *grem1* −/− (shaded bars) were non-starved (NS), serum deprived (S) overnight and then re-exposed to medium containing 10% FBS (FBS) for 60 min as described in Methods. Protein lysates (10 μg) were separated on 10% SDS-PAGE and probed by Western blotting with anti-phospho p44/42ERK, total ERK or β-actin as loading control. A. Representative changes in phospho-ERK upon serum starvation and FBS addition in all 3 genotypes. B. Representative Western blot image of 3 independent experiments showing increased pERK upon serum exposure. C. Band intensities were determined using Scion image software and the intensity ratios of pERK:total ERK was determined. D. The fold change in the intensity of pERK:total ERK from serum-starved to FBS-stimulated was calculated. Data were plotted as mean±SEM. Statistical significance was determined using one-way ANOVA with post-hoc Tukey–Kramer multiple comparison test, ‘p<0.05, ***p<0.001, and unpaired t-test, ##p<0.01.