BMP-mediated inhibition of FGF signaling promotes cardiomyocyte differentiation of anterior heart field progenitors

Libbat Tirosh-Finkel[1],*, Amit Zeisel[2],*, Miriam Brodt-Ivenshitz[1],*, Ayelet Shamai[1], Zhong Yao[1], Rony Seger[1], Eytan Domany[2] and Eldad Tzahor[1],†

SUMMARY

The anterior heart field (AHF) encompasses a niche in which mesoderm-derived cardiac progenitors maintain their multipotent and undifferentiated nature in response to signals from surrounding tissues. Here, we investigate the signaling mechanism that promotes the shift from proliferating cardiac progenitors to differentiating cardiomyocytes in chick embryos. Genomic and systems biology approaches, as well as perturbations of signaling molecules, in vitro and in vivo, reveal tight crosstalk between the bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) signaling pathways within the AHF niche: BMP4 promotes myofibrillar gene expression and cardiomyocyte contraction by blocking FGF signaling. Furthermore, inhibition of the FGF-ERK pathway is both sufficient and necessary for these processes, suggesting that FGF signaling blocks premature differentiation of cardiac progenitors in the AHF. We further revealed that BMP4 induced a set of neural crest-related genes, including *MSX1*. Overexpression of Msx1 was sufficient to repress FGF gene expression and cell proliferation, thereby promoting cardiomyocyte differentiation. Finally, we show that BMP-induced cardiomyocyte differentiation is diminished following cranial neural crest ablation, underscoring the key roles of these cells in the regulation of AHF cell differentiation. Hence, BMP and FGF signaling pathways act via inter- and intra-regulatory loops in multiple tissues, to coordinate the balance between proliferation and differentiation of cardiac progenitors.

KEY WORDS: Anterior heart field, Cardiogenesis, BMP, FGF, Organogenesis, Chick

INTRODUCTION

Heart formation encompasses an orchestrated series of cellular events; even subtle alterations in this process can lead to serious cardiac disorders. Congenital heart disease, the most common of all birth defects in humans, arises from abnormalities in the early stages of cardiogenesis. The vertebrate heart is formed from two mesoderm populations or 'heart fields', termed the first and second heart fields, which arise from common origins and express both distinct and overlapping molecular markers (Black, 2007; Buckingham et al., 2005). In the chick, the anterior heart field (AHF) is located in the distal pharyngeal mesoderm of branchial arches 1 and 2 that contributes to the right ventricle and proximal outflow tract, while secondary heart field (SHF) marks the pharyngeal mesoderm (splanchnic mesoderm) in posterior branchial arches, caudal to the outflow tract, that gives rise to the most distal part of the outflow tract (Waldo et al., 2001). Hence, the arterial pole of the heart contains cardiac progenitors from both the AHF and SHF.

AHF cells are cardiac progenitor cells located outside the heart that contain multipotent progenitor cells that differentiate into myocardial, endocardial and smooth muscle cells, in both chick and mouse models (Kelly et al., 2001; Mjaatvedt et al., 2001; Nathan

et al., 2008; Tirosh-Finkel et al., 2006; Verzi et al., 2005). The coordinated differentiation of SHF/AHF cells is thought to be under tight spatial and temporal control, yet the molecular details of this dynamic step are not clear. In addition, the nature of the signal that keeps these progenitor populations in an undifferentiated state is unknown.

The transition from progenitors to differentiated cells is crucial for successful organogenesis. Many signaling pathways were shown to influence cardiac progenitor cell proliferation and differentiation. For example, recent studies have shown that the Wnt/β-catenin pathway plays distinct roles at various stages of cardiac development by triggering the renewal and expansion of cardiac progenitors, and blocking their differentiation (for a review, see Tzahor, 2007). BMP and FGF signals are initially required for cardiac specification, and later for the differentiation of cardiac progenitors in chick and frog embryos (Olson and Schneider, 2003). Loss-of-function studies in mice underscored the essential roles of the BMP and FGF signaling pathways in cardiogenesis (Rochais et al., 2009), although the precise dynamics of these signaling pathways during early heart development is often masked when analyzed by means of conventional genetic loss-of-function experiments.

BMP signaling pathway promotes the specification of the AHF mesoderm into the cardiac rather than into the skeletal muscle lineage (Tirosh-Finkel et al., 2006). Furthermore, BMP signaling is crucial to the differentiation of SHF/AHF progenitors in the chick (Tirosh-Finkel et al., 2006; Waldo et al., 2001). More recently, it was demonstrated that BMP-Smad1 signaling negatively regulates second heart field proliferation in the mouse (Prall et al., 2007). FGF signaling, too, plays multiple key roles during heart development, such as induction of cardiogenesis, proliferation,

[1]Department of Biological Regulation, Weizmann Institute of Science, Rehovot 76100, Israel. [2]Department of Physics of Complex Systems, Weizmann Institute of Science, Rehovot 76100, Israel.

*These authors contributed equally to this work
†Author for correspondence (eldad.tzahor@weizmann.ac.il)

septation and alignment of the outflow tract, which affect both the first and second heart fields (Alsan and Schultheiss, 2002; Hutson et al., 2006; Ilagan et al., 2006; Kelly et al., 2001; Park et al., 2008; Reifers et al., 2000; Zhang et al., 2008).

In this study, we explored the signals that control the transition from progenitor to differentiated cells during cardiogenesis. To achieve this, we used a wide range of bioinformatics and developmental methods ranging from genome-wide transcriptome analyses and combinatorial perturbations of signaling molecules in chick embryos, both in vitro and in vivo. Our results demonstrate a temporal order in signaling mechanisms in which BMP signals induce cardiomyocyte myofibrillogenesis by blocking FGF signaling within the AHF niche. We further show that FGF-ERK signaling inhibits the differentiation of AHF progenitors. We also found that BMP4 induced a set of neural crest-related genes, and that cranial neural crest cells are required for the BMP-dependent cardiomyocyte differentiation. Finally we overexpressed the BMP4 target gene Msx1 in vitro and in vivo, and show that it is sufficient, by itself, to induce cardiomyocyte differentiation and beating in AHF explants. Taken together, these novel findings suggest that the BMP-MSX-FGF signaling network in the AHF niche regulates cardiac progenitors by crosstalk between the endoderm, ectoderm and neural crest cells.

MATERIALS AND METHODS
Eggs, embryos and explant culture assays
Fertilized white eggs were incubated for 2-3 days at 38.5°C in a humidified incubator to reach stage 10 (Hamburger and Hamilton, 1992). AHF explants were dissected as described [previously termed SpM (Tirosh-Finkel et al., 2006)]. RT-PCR analysis was performed either immediately, or after 3, 12 or 24 hours in culture on a collagen drop covered with 0.5 ml of dissection medium (10% fetal calf serum, chick embryo extract 2.5% and pen/strep 0.5% in αMEM medium) in a four-well plate. In some experiments, Human recombinant BMP4 (Sigma, 200 ng/ml), recombinant Fc-Noggin protein (500 ng/ml, R&D Systems), FGF3 or FGF8 (200 ng/ml, R&D Systems), or the inhibitors SU5402 (20 μM) (Calbiochem), U0126 (30 μM) or SB203580 (30 μM) were added to the dissection medium.

RT-PCR
Complementary DNA (cDNA) was synthesized from DNase-treated total RNA, using an M-MLV reverse transcriptase-mediated extension of random primers (Promega). The cDNA product was amplified using different sets of primers directed for cardiac and skeletal muscle markers (sequences are available upon request).

BrdU assay for explant culture
Explants were incubated with the dissection medium for 20 hours, and BrdU was added at a final concentration of 10 μM for an additional 45 minutes. To stop the reaction, explants were fixed in 4% PFA, and BrdU incorporation was assessed by immunofluorescence staining.

Whole mount in situ hybridization
Whole-mount in situ hybridization was performed as previously described (Tirosh-Finkel et al., 2006).

Chick embryo manipulations
Implantation of cell aggregates was carried out according to Tirosh-Finkel et al. (Tirosh-Finkel et al., 2006). For bead implantation experiments, heparin-acrylamide beads (Sigma) were incubated with human recombinant BMP4 (100 ng/μl), FGF8 (500 ng/μl), Noggin (1 μg/μl) or SU5402 (10 mM) on ice for 2 hours. Control beads were soaked in carrier protein (0.1% bovine serum albumin or DMSO). Beads were inserted into cultured embryos using tungsten needles. Embryos were returned to the incubator for an additional 24 hours, and then fixed in 4% paraformaldehyde (PFA) and processed for the in situ hybridization analysis. DiI (Molecular Probes) labeling experiments were described (Tirosh-Finkel et al., 2006). To label AHF cells, embryos were placed

ventral side up in new culture plates, incubated for an additional 24 hours, fixed with 4% PFA and cryo-sectioned. For protein translation experiments, CHX (10 μg/ml) was added to the AHF explants at the indicated times. Dorsal neural tube ablation was performed at stage 8, as previously described (Rinon et al., 2007; Tzahor et al., 2003), and ablated embryos were developed to stage 10, at which time AHF dissection was carried out.

Viral injections
To infect the AHF in vivo, concentrated retroviruses RCAS-mMsx1 and RCAS-GFP were injected into the head mesoderm of stage 8-9 chick embryos. Retrovirally infected chicks were then harvested at stage 18, and subjected to immunohistochemistry or in situ hybridization analyses.

Immunohistochemistry
For paraffin wax-embedded sections, embryos were fixed in 4% PFA, embedded in paraffin wax and sectioned at 10-15 μm using a Leica microtome. For frozen sections, explants were fixed with 4% PFA, left overnight in 30% sucrose embedded in OCT and sectioned at 10 μm. Sections were blocked with 5% whole goat serum in 1% bovine serum albumin in PBS, prior to incubation with primary antibody. Antibodies: Myomesin 2, 1:40; MF20 (MHC), 1:1; tropomyosin, 1:40; G3G4 (Brdu antibody), 1:100; pHis3, 1:400 (Santa Cruz); Caspase 3, 1:40 (Cell Signaling), pERK, 1:50; 3C2, 1:5. Secondary antibodies: Cy2, Cy5 or Cy3-conjugated anti-mouse or rabbit IgG (Jackson ImmunoResearch Laboratories), 1:100.

Data analysis
mRNA expression levels were measured at 0, 3, 12 and 24 hours for both control and BMP4-treated AHF cells. Total RNA was isolated and hybridized on Affymetrix GeneChip Chicken Genome Arrays. CEL files were normalized according to the 'MAS5' algorithm, using the Affymetrix Expression Console. The value 5 (log base 2 scale) was used as the threshold for detection level. Noise in the expression levels was estimated based on the two duplicate samples taken at t=12 hours, for both control and BMP4-treated cells. Sets of significantly and differentially expressed genes (SDEG) with 5% false discovery rate (FDR) were identified. Distinct SDEG with different time-dependent expression profiles in control versus BMP4-treated AHF cells were found (483 genes, at 30% FDR). Fig. S1 in the supplementary material shows the expression heatmap of these genes, which were divided into seven clusters. The genes in each cluster are listed in Table S1 in the supplementary material.

The microarray data can be downloaded from the Gene Expression Omnibus under 'BMP-mediated inhibition of FGF signaling promotes cardiomyocyte differentiation of anterior heart field progenitors' (accession number GSE19698).

RESULTS
BMP4 induces expression of a wide array of myofibrillar sarcomeric genes
In this study, we sought to investigate the molecular mechanisms underlying the transition of cardiac progenitors in the AHF niche into differentiated cardiomyocytes in the heart. We have previously characterized explants dissected from stage 10 embryos with cardiogenic potential (Tirosh-Finkel et al., 2006). These cells contribute to the distal pharyngeal mesoderm of the first two branchial arches (Nathan et al., 2008); thus, we refer to them as AHF explants. Administration of BMP4 induced beating of AHF-derived cardiomyocytes after 16-24 hours of culture (Tirosh-Finkel et al., 2006). To understand the molecular mechanism through which BMP4 induces beating, we performed a genome-wide microarray screen, using the Affymetrix GeneChip Chicken Genome Array, to measure gene expression (at time courses 0, 3, 12 and 24 hours) in naïve and BMP-treated AHF cells (Fig. 1A and see Fig. S1 in the supplementary material). An unsupervised analysis for distinct temporal expression profiles revealed ~400 genes, grouped into seven
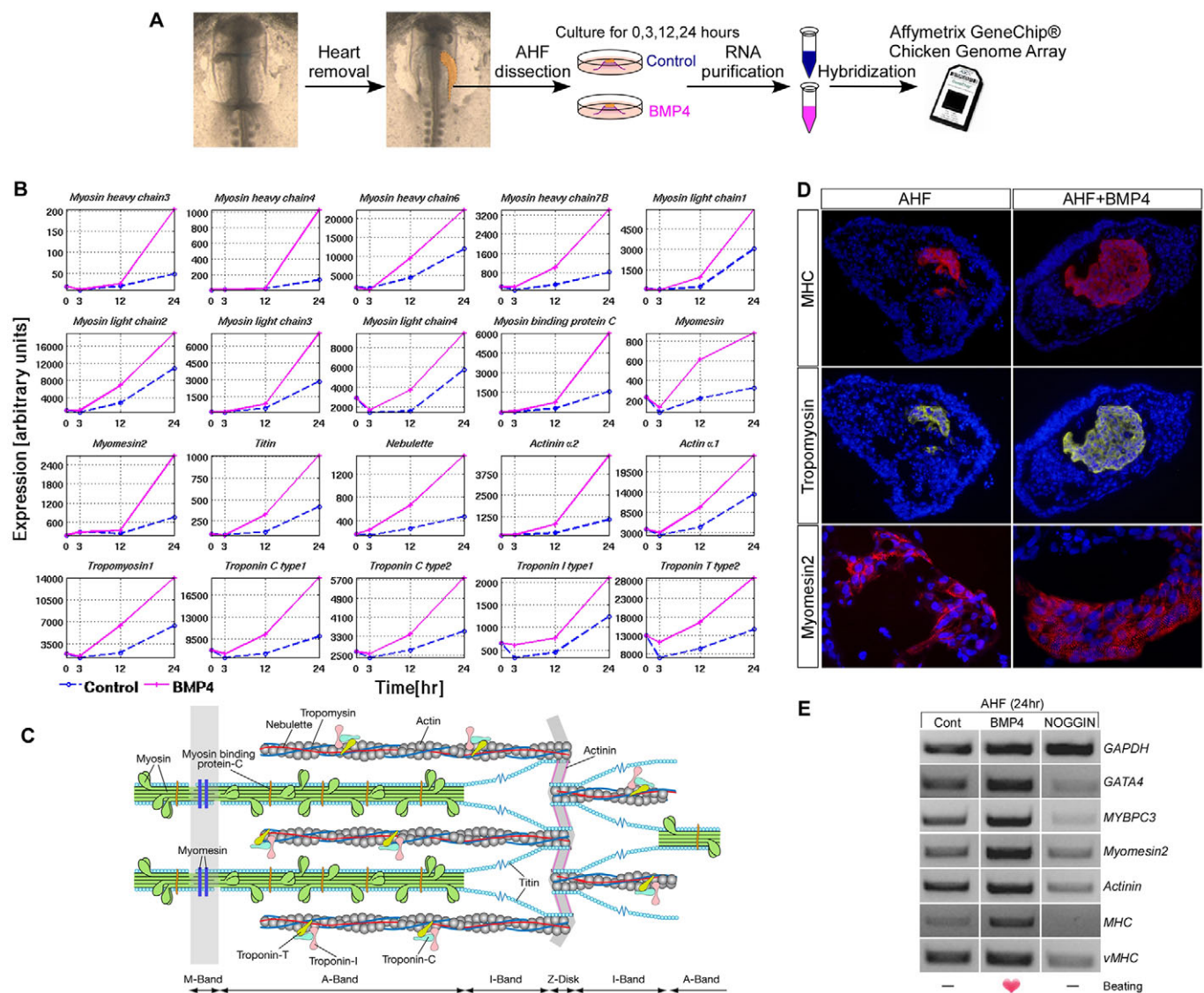
Fig. 1. BMP4 induces global myofibrillar sarcomeric gene expression in AHF progenitors. (**A**) The dissection of stage 10 AHF explants (orange) cultured at various time points in the presence or absence of BMP4. Following RNA purification, a microarray screen was performed. (**B**) Microarray data showing a variety of sarcomeric genes in control (broken blue lines) and BMP4-treated (pink) samples. (**C**) Structural model of a sarcomere incorporating the genes found in the array. (**D**) Immunostaining for the indicated sarcomeric proteins in AHF explants treated with or without BMP4 for 24 hours. (**E**) RT-PCR analysis for the indicated sarcomeric genes of AHF explants treated with either BMP4 or Noggin.

clusters, the profiles of which significantly differed between control and BMP4-treated cells. Particularly noteworthy was the enrichment of myofibrillar sarcomeric genes that were strongly upregulated by BMP4 in cluster 3 (see Fig. S1 and Table S1 in the supplementary material).

During early embryogenesis, myocardial cells contain disorganized sarcomeres and an immature electrophysiological system, whereas more differentiated cardiomyocytes are characterized by an increase in sarcomeric organization and electrophysiological maturation – the two components that must be in place for emergence of contractions (Martin-Puig et al., 2008). A search among the transcripts that showed significant upregulation between untreated and BMP4-treated AHF explants did not reveal any genes associated with cardiac action potential or with gap junctions (see Fig. S2 in the supplementary material; data not

shown). However, we did observe marked (2-6 fold) induction of 20 sarcomeric genes in BMP4-treated AHF cells (Fig. 1B). BMP4 induced the expression of sarcomeric genes (e.g. Titin, Myosin-binding protein C, Myomesin, Actinin and others; Fig. 1B) that encode for thick myosin myofilaments, as well as sarcomeric components that facilitate formation of the sarcomeric structure by associating with both Actin and Myosin. Induction of sarcomeric genes by BMP4 was verified by RT-PCR (Fig. 1E and Fig. 3; data not shown). Furthermore, BMP4 induced myofibrillar organization as shown by immunofluorescence staining for the sarcomeric proteins Myosin Heavy Chain (MHC), Tropomyosin and Myomesin2 (Fig. 1D).

In the absence of ectopic BMP4, it is likely that endogenous *BMP2* and *BMP4*, which are expressed in AHF explants (Tirosh-Finkel et al., 2006) promote the upregulation of sarcomeric genes.
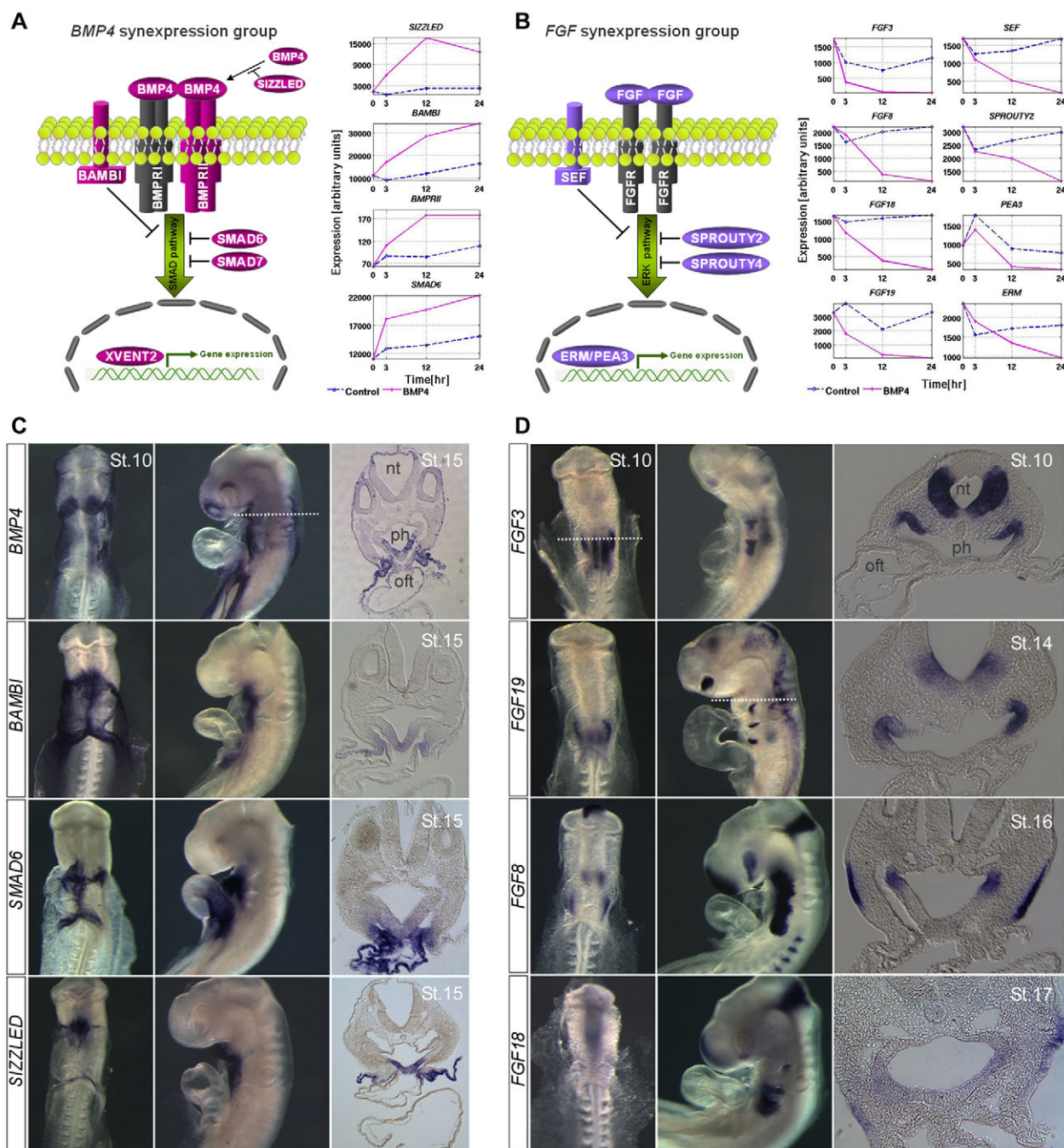
Fig. 2. BMP4 suppresses the FGF synexpression group and induces its own synexpression group. (**A**) Microarray data set showing that BMP4 induces its own synexpression group: a set of genes that share a complex 'spatial' expression pattern and function in the same signaling pathway (depicted in the model). In general, members of synexpression groups are believed to function as a negative-feedback mechanism. (**B**) BMP4 blocks the FGF synexpression group. (**C,D**) In situ hybridization screen, in whole-mount and sectioned embryos, for the BMP4 (C) and FGF (D) synexpression groups at the indicated stages of cardiogenesis in the chick. Left, ventral view; middle, lateral view; right, transverse sections at the indicated axial level (marked by the broken line).

To further explore this possibility, we treated AHF explants with either BMP4 or its antagonist Noggin. Relative to untreated AHF explants, BMP4 induced sarcomeric gene expression, as shown by RT-PCR, while Noggin treatment blocked their expression (Fig. 1E). Taken together, these results suggest that BMP signaling plays a crucial role in the transition of cardioblasts into beating cardiomyocytes by globally increasing myofibrillar gene expression and facilitating sarcomeric protein architecture.

BMP4 induces the expression of its synexpression group and suppresses that of the FGF synexpression group

Next, we searched the microarray dataset to gain further insights into the molecular mechanisms underlying BMP-induced cardiomyocyte differentiation. We first noticed the upregulation of *BAMBI*, *SMAD6*, *BMPRII* and *Sizzled*, members of the BMP4 synexpression group (Fig. 2A and see Fig. S3A in the
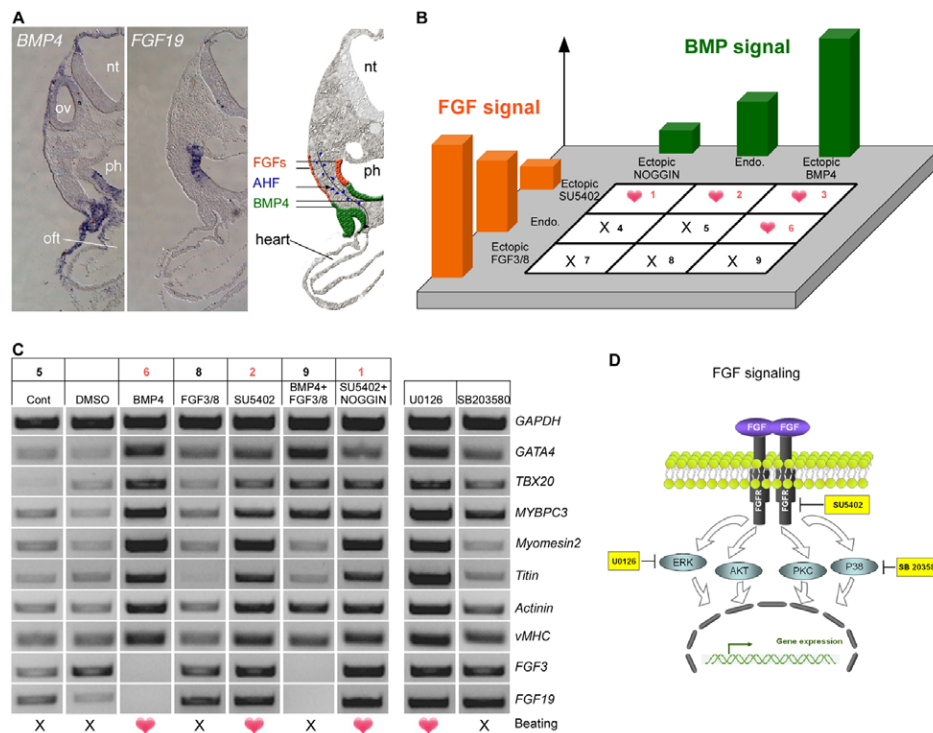
Fig. 3. Inhibition of the FGF-ERK signaling pathway is sufficient and necessary to promote cardiomyocyte differentiation and beating.
(**A**) Model of the mutual expression pattern of FGF and BMP4 synexpression groups. Left panels show two consecutive sections subjected to in situ hybridization for *BMP4* and *FGF19.* On the right, an illustration of AHF cells migrating towards the heart, initially receiving FGF signals and later receiving BMP signals nearer the heart. (**B**) Schematic matrix depiction of experiments performed under nine distinct combinations of BMP and FGF signals: increased (ectopic BMP4 or FGF3/8), intermediate (endogenous) and reduced (by ectopic Noggin or SU5402, respectively). Crosses mark experiments in which no beating was observed in AHF explants; a red heart indicates beating. (**C**) RT-PCR analysis of the combined influence of BMP and FGF signaling pathways on cardiac lineage markers, myofibrillogenesis and FGF family members. The colored numbers in the top row identify the corresponding experiments, as shown in B. (**D**) Model showing the targets of the different pharmacological inhibitors within the FGF signaling pathway. The results presented in B,C reflect at least five independent experiments.

supplementary material). Synexpression groups designate sets of genes that share a complex 'spatial' expression pattern, are simultaneously up- or downregulated, and are thought to function in the same biological process (Niehrs and Pollet, 1999). The BMP4 synexpression group, first identified in *Xenopus* embryos, is believed to function as a negative-feedback mechanism to attenuate BMP4 signaling (Karaulanov et al., 2004). We suggest that *Sizzled*, which was strongly induced by BMP4 in both in vitro and in vivo analyses (Fig. 2A; see Fig. S3A,E in the supplementary material) and is a known antagonist of BMP signaling in Zebrafish and *Xenopus* (Lee et al., 2006; Muraoka et al., 2006), belongs to this group. In summary, BMP4 induces its own synexpression group that presumably functions as a negative-feedback mechanism.

As the FGF signaling pathway plays well-established positive roles during cardiac development, we also used our microarray data to examine changes in this pathway. Surprisingly, four FGF family ligands (*FGF3*, *FGF8*, *FGF18* and *FGF19*) were significantly downregulated by BMP4 (Fig. 2B). In addition, *SEF*, *Sprouty2*, *PEA3* and *ERM*, all members of the FGF synexpression group (Brent and Tabin, 2004; Kovalenko et al., 2006), were downregulated by BMP4 treatment in AHF explants (Fig. 2B; see Fig. S3B in the supplementary material). Collectively, our gene expression data reveal that BMP4 efficiently blocks FGF signaling in differentiating AHF explants.

To verify the relevance of these two sets of gene families during AHF development, we determined their expression patterns using in situ hybridization in chick embryos (Fig. 2C,D). Members of the two synexpression groups were expressed during AHF development in a mutually exclusive manner: BMP4 synexpression group members were expressed in the ventral pharynx, adjacent to the cardiac outflow tract (Fig. 2C), whereas members of the FGF synexpression group displayed a redundant expression pattern in the dorsal pharyngeal endoderm and in the overlying ectoderm (Fig. 2D; see Fig. S3C in the supplementary material). The mutually exclusive relationship of the two synexpression groups is demonstrated by the expression of *FGF19* and *BMP4* in serial tissue sections (Fig. 3A). Interestingly, in the chick FGF ligands are almost not expressed in the AHF mesoderm unlike their expression in the mouse (Watanabe et al., 2010). Based on analysis of their expression patterns, we propose that AHF mesoderm cells that migrate underneath the pharynx into the heart are regulated by a tight crosstalk between endoderm/ectoderm-derived FGFs and BMP4, to enable the differentiation of AHF progenitors within the heart (Fig. 3A).

Inhibition of the FGF-ERK signaling pathway is both sufficient and necessary to promote cardiomyocyte differentiation and beating

Beating of cardiomyocytes is a readily visible readout for their differentiation. To establish the conditions under which cardiomyocyte differentiation/beating is observed, we undertook a
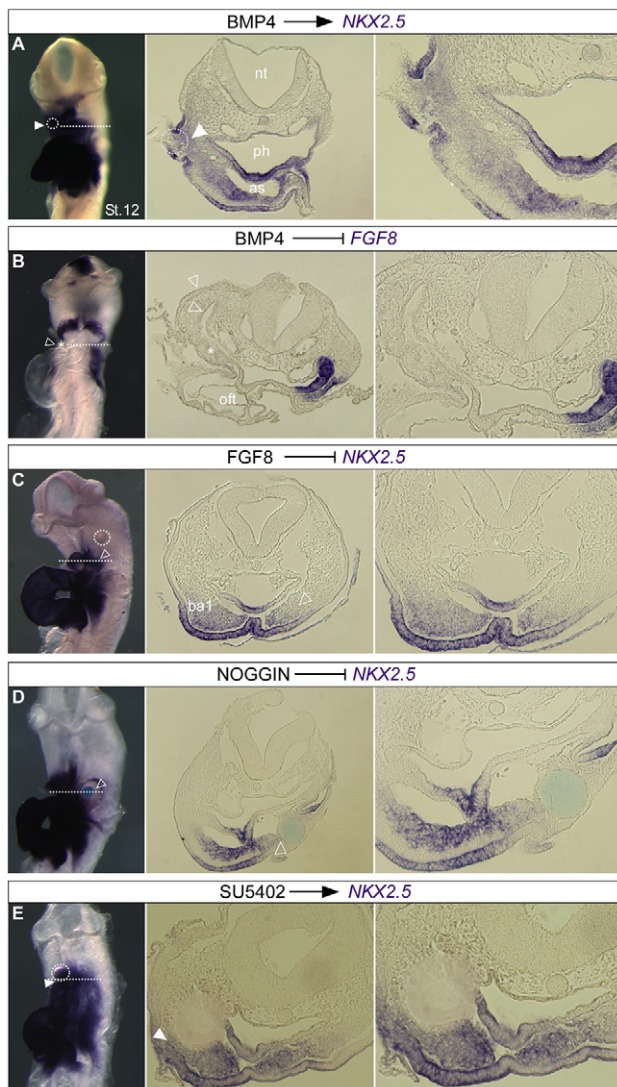
Fig. 4. BMP and FGF signaling pathways play opposing roles during AHF development in vivo. (**A-E**) In vivo implantation of beads soaked with the different signaling molecules (indicated in the rectangle above each experiment) into the mesoderm of stage 10 chick embryos. In situ hybridization for the indicated genes (marked in purple) was performed following overnight culture of these embryos (~stage 12). Left panels show ventral views; middle and right panels show transverse sections of 10× and 40× magnification, respectively. The plane of sectioning and the location of the bead are marked with broken lines or circles; white arrowheads represent induction, whereas black arrowheads represent repression of the indicated genes. In B, HEK-293 cells overexpressing BMP4 were implanted (marked with an asterisk). All of the in vivo experiments were repeated at least three times; representative results from most of the embryos are shown. nt, neural tube; ph, pharynx; as, aortic sac; oft, outflow tract.

systematic combinatorial approach to perturb the BMP and FGF signaling pathways (Fig. 3B). Each pathway was placed in one of three states: endogenous, inhibited (by adding Noggin for BMP signaling, and SU5402 for FGF signaling) or stimulated (by adding the corresponding ligand). First, BMP4 induced AHF differentiation/beating (experiments 6 and 3 in Fig. 3B), in a

manner that was indistinguishable from the administration of SU5402 (experiment 2). Moreover, inhibition of FGF signaling (by SU5402) induced beating even when the endogenous BMP signal was dampened by Noggin (experiment 1), whereas in the presence of either FGF8/FGF3, Noggin or both, no beating was observed (experiments 8, 4 and 7, respectively). Importantly, in the combined presence of FGF8 and FGF3, BMP4-induced beating was blocked (experiment 9). The phenotypic results (beating) of the different combinatorial manipulations (Fig. 3B) were consistent with the expression levels of various genes measured using RT-PCR (Fig. 3C).

The concentrations of Noggin, SU5402, ectopic FGF and ectopic BMP are extrinsic variables (e.g. under direct experimental control) that reflect continuous values, although they were used here as binary (on/off) variables. In the AHF explant cultures, the biologically relevant variables that control differentiation/beating are FGF signal strength (FGFss) and BMP signal strength (BMPss). Signal strength is defined as the actual magnitude of the signal within the cell, a feature that is controlled by the extrinsic variables, as well as by other biological factors (e.g. receptor numbers, members of the BMP and FGF synexpression groups, and the like). In addition, FGFss and BMPss are coupled by cross-regulation between the two signaling pathways (note the discussion in Fig. S4 in the supplementary material, which depicts a 'beating diagram' in terms of the FGF and BMP signal strengths). Strikingly, our experimental results indicate that inhibition of FGF signaling is both sufficient and necessary for the differentiation/beating of AHF cells in vitro (Fig. 3B,C). Accordingly, we suggest that inhibition of FGFss below a certain level, either by SU5402 or by BMP4, constitutes the key parameter in the differentiation of cardiomyocytes (see Fig. S4 in the supplementary material).

Depending on the cellular context, the FGF stimulus can activate distinct signaling pathways such as ERK, AKT, PKC and P38 (Fig. 3D). To probe the precise molecular mechanism by which pathways downstream to FGF signaling suppress AHF differentiation, we used pharmacological inhibitors of the ERK (U0126) and P38 (SB203580) pathways. Treating AHF explants with these inhibitors demonstrated that inhibition of the FGF-ERK signaling pathway was sufficient to promote cardiomyocyte differentiation and beating, whereas inhibition of P38 did not (Fig. 3C,D).

FGF signaling represses cardiogenesis in the AHF in vivo

We then tested the effects of BMP and FGF signaling on AHF cells in vivo (Fig. 4). Implantation of BMP4-soaked beads into the head mesoderm of stage 9-10 chick embryos induced ectopic expression of the cardiac marker *NKX2.5* in the first branchial arch (BA1), whereas Noggin-soaked beads repressed *NKX2.5* expression in the distal part of BA1 (Fig. 4A,D, respectively). In order to validate the in vitro finding that BMP4 blocks FGF signaling, we used both BMP4-expressing HEK-293 cells and BMP4 beads. Indeed, BMP4 efficiently blocked *FGF8* expression in the dorsal region of the pharyngeal endoderm (Fig. 4B; data not shown). To demonstrate the inhibitory action of FGF signaling on cardiogenesis, we implanted either FGF8 or SU5402 beads adjacent to the AHF of stage 9-10 chick embryos. Whereas FGF8 repressed *NKX2.5* expression in distal BA1 (Fig. 4C), SU5402 induced its expression (Fig. 4E). Hence, our in vivo findings indicate that BMP and FGF signaling pathways play opposing roles during cardiogenesis within the AHF niche.
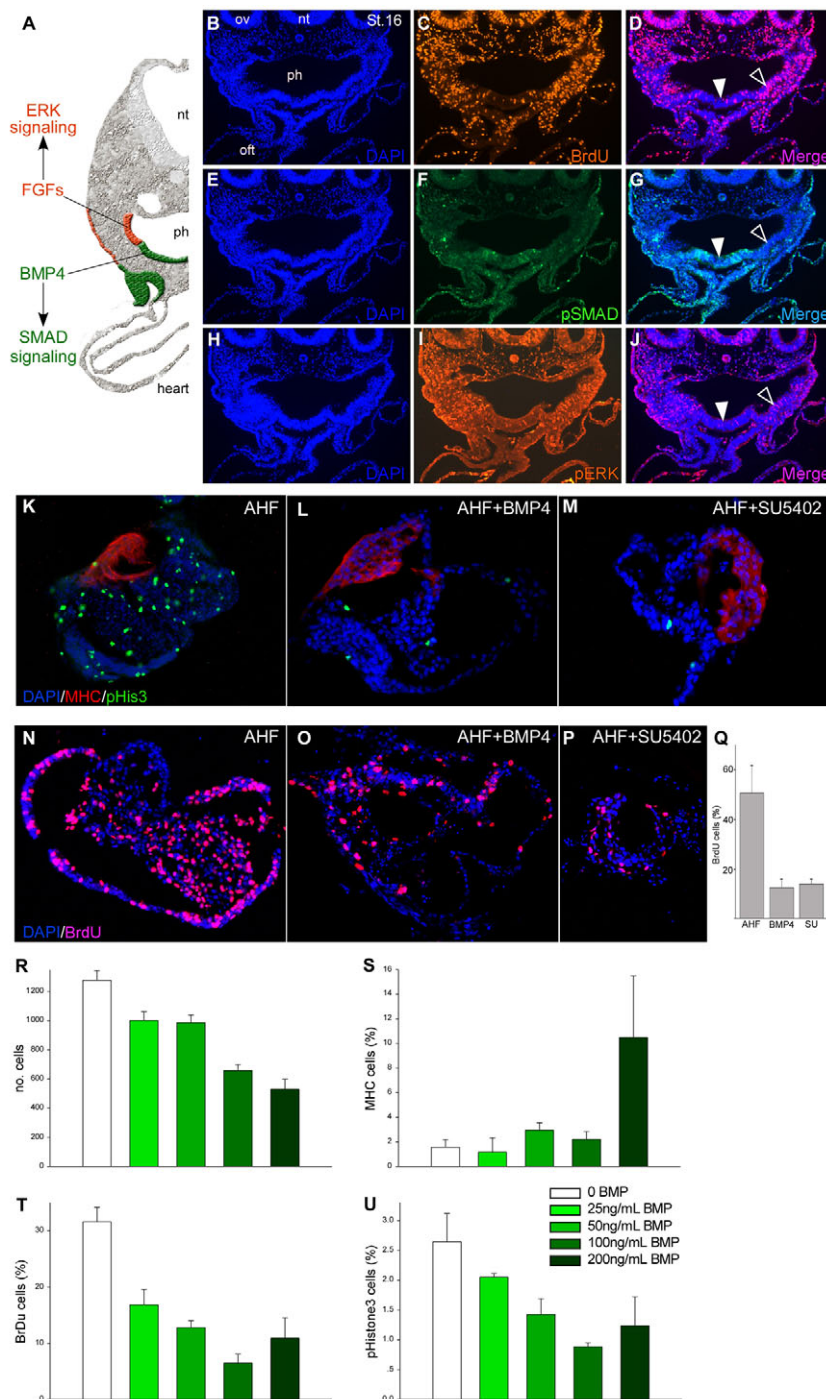
Fig. 5. BMP-mediated FGF inhibition correlates with reduced proliferation. (**A**) Model showing the tight linkage between the FGF-expressing domain and ERK signaling, as well as BMP4 expression and SMAD signaling. (**B-J**) Immunostaining of transverse sections of stage 12 embryos with the indicated antibodies. White arrowheads represent BMP-derived p-SMAD staining, whereas black arrowheads correspond to the FGF/BrdU/p-ERK proliferative domain. (**K-P**) Proliferative analysis by immunostaining for the indicated antibodies of AHF explants treated with either BMP4 or SU5402. (**Q**) Quantification of the results in N-P. (**R**) Increasing concentrations of BMP4 affect AHF cell number after 24 hours. (**S**) Percent MHC-positive cells. (**T**) BrdU-stained cells. (**U**) pHistone3 positive cells. nt, neural tube; ph, pharynx; ov, otic vesicle; oft, outflow tract.

BMP4 and inhibition of FGF signaling decrease cell proliferation in the AHF

The FGF-ERK signaling pathway is widely known as a potent inducer of cell proliferation. Given the distinct expression patterns of the FGF and BMP4 synexpression groups (Fig. 2), we compared the proliferative status within the AHF niche and the expression patterns of FGF and BMP4 synexpression groups (Fig. 5A). Immunofluorescence staining for pERK in transverse sections of stage 12 embryos was correlated with the FGF synexpression domain in the dorsal pharynx, whereas pSMAD staining, a readout for BMP signaling, overlapped with the BMP4 synexpression domain in the ventral pharynx (Fig. 5B-J).

Notably, BrdU staining, which is indicative of proliferating cells, broadly overlapped with the FGF expression domain and pERK staining. This analysis revealed that cell proliferation gradually decreases along the AHF adjacent to the cardiac outflow tract, where BMP4 is expressed. Furthermore, both BMP4 and SU5402 strongly reduced cell proliferation in AHF explants, as evidenced by p-Histone H3 (Fig. 5K-M) and BrdU staining (Fig. 5N-Q).

We next determined how increasing concentrations of BMP4 affect the proliferation/differentiation states of AHF explants. Evidently, cell proliferation (number of cells, BrdU and p-Histone H3) was reduced in a dose-dependent manner, reaching a
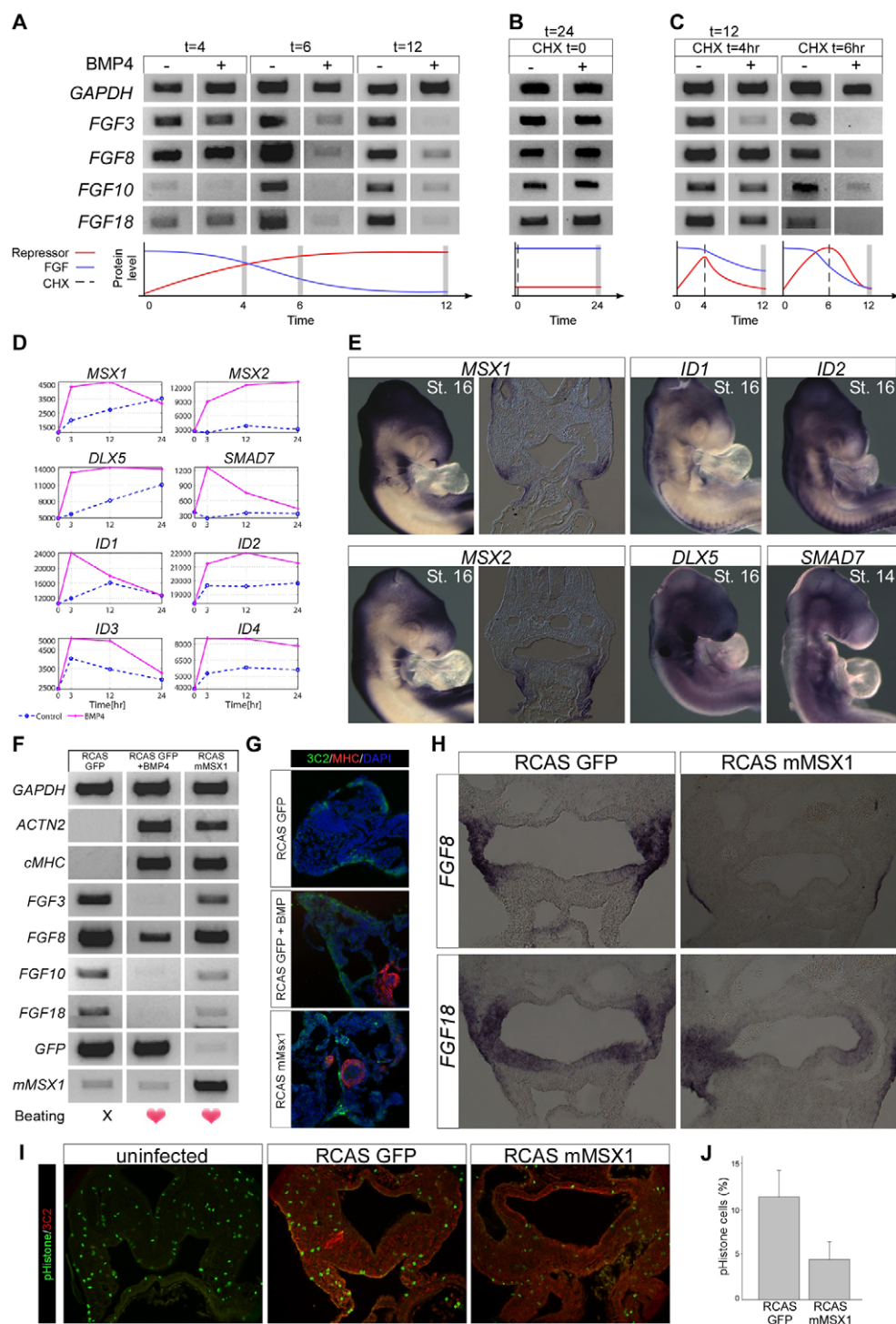
Fig. 6. BMP-MSX signaling represses FGF genes and promotes AHF differentiation non cell autonomously. (**A-C**) RT-PCR analysis of FGF gene expression in control and in BMP4-treated AHF explants, in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX). 't' indicates the number of hours after BMP4 treatment (A); or the time of CHX application (B,C). The graphs below each PCR panel present schematically the expression levels of the induced repressor and the FGF genes, with gray vertical bars marking the times of the experiments and broken vertical lines indicating the times at which CHX was introduced. (**D**) Microarray dataset showing that BMP4 induces neural crest related genes prior to myofibrillogenesis. (**E**) In situ hybridization analysis of some of the genes in D. (**F**,**G**) RT-PCR (F) and immunostaining (G) results of AHF explants infected with control retroviruses (*RCAS GFP*), with or without BMP4, compared with AHF explants infected with the mouse *RCAS Msx1* retroviruses. (**H-J**) In vivo misexpression of control retroviruses (*RCAS GFP* or *RCAS mMsx1*) in the head mesoderm injected at stage 8-9 and analyzed at stage 17 (after 48 hours). Transverse sections subjected to in situ hybridization for *FGF18* and *FGF8* (H) or stained with antibodies against p-Histone and the viral protein 3C2 (I). (J) Quantification of the data from multiple embryos (*n*=5).

maximum response at 100 ng/ml. By contrast, the number of MHC⁺ cells was significantly increased only at 200 ng/ml, suggesting a threshold (ON/OFF) phenomenon (Fig. 5R-U). Taken together, we suggest that FGF-ERK signaling maintains a pool of cardiac progenitors within the dorsal domain of the AHF by promoting their proliferation, thereby blocking their differentiation. By contrast, BMP4-mediated inhibition of FGF signaling within the ventral domain of the AHF reduces proliferation and promotes differentiation, as these progenitors reach the anterior pole of the heart.

BMP4-MSX signaling represses FGF genes and promotes AHF cardiomyocyte differentiation

In order to obtain a mechanistic insight into the inhibition of FGFs (*FGF8*, *FGF3*, *FGF18* and *FGF19*) by BMP4 and the subsequent differentiation of AHF cells, we sought first to define the temporal window during which this inhibition takes place. Accordingly, AHF explants were cultured in the presence of BMP4 for different time intervals. The expression pattern of the FGF genes was significantly downregulated within the first 6 hours (Fig. 6A). This inhibition could be produced either through
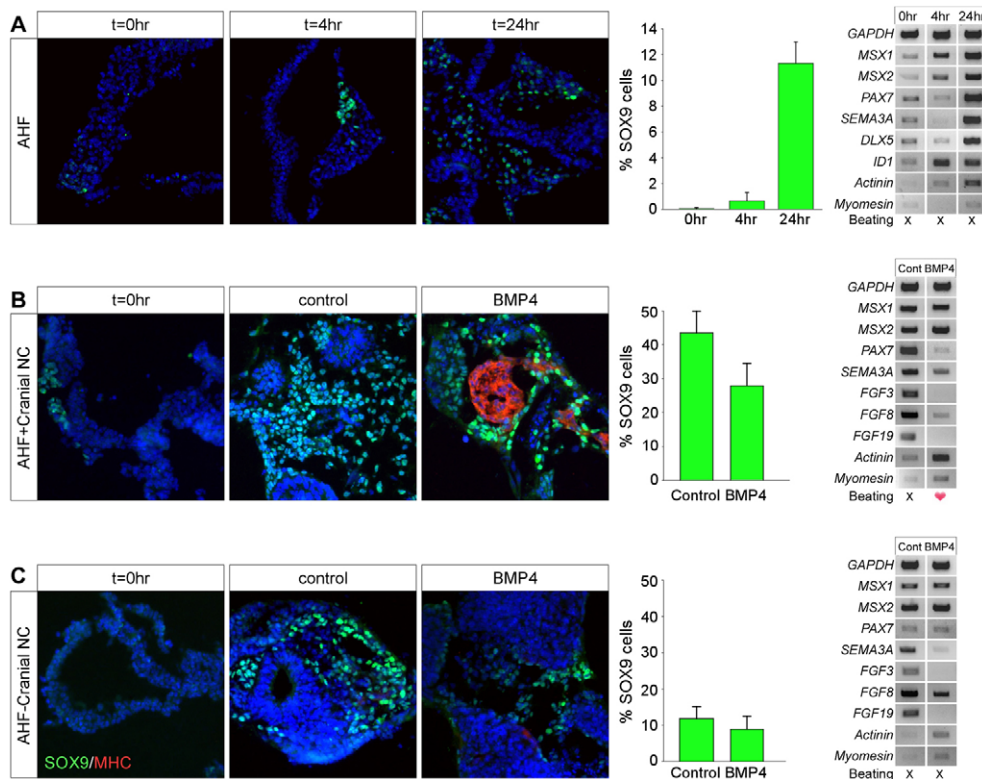
Fig. 7. Neural crest cells are present within the AHF niche and contribute to the BMP-induced cardiomyocyte differentiation. (**A**) Immunostaining for SOX9 in AHF control explants at the indicated time, its quantification (graph) and RT-PCR analysis of neural crest markers in the AHF explants. (**B**) Immunostaining for SOX9 and MHC in AHF and BMP4-treated explants, quantification of SOX9 cells (graph), and RT-PCR of neural crest, FGF and sarcomeric markers. (**C**) Same as in B, but for cranial NC-ablated embryos.

SMAD proteins, via the induction of a repressor, or through downregulation of an activator of FGF genes. To test whether the inhibition of FGF genes is caused by transcription and translation of a BMP4-induced target, we used the protein synthesis pharmacological inhibitor cycloheximide (CHX). Notably, FGF mRNAs were unchanged when BMP4 was added in the presence of CHX (Fig. 6B), indicating that BMP-mediated inhibition of FGF genes is likely to be indirect, via repressive activity of an intermediate protein, rapidly produced after BMP4 stimulation. Moreover, delayed applications of CHX into BMP4-treated explants demonstrated that the intermediate protein is translated and effectively inhibits FGF genes within 4-6 hrs after BMP4 treatment (Fig. 6C).

We therefore re-examined our dataset of AHF explants treated with BMP4 at 3 hours for potential repressors that were upregulated by BMP4 prior to the inhibition of the FGF genes. Interestingly, eight transcription factors, mostly known for their roles in neural crest cells, were significantly upregulated by BMP4 in this time-frame: *MSX1*, *MSX2*, *ID1-4*, *DLX5*, *SMAD7* (Fig. 6D,E). Because both MSX and ID genes have previously been shown to play important roles in second heart field development in mouse (Chen et al., 2007) and frog (Martinsen et al., 2004) embryos, we decided to focus on them for further gain-of-function analyses (Fig. 6F-J). Strikingly, overexpression of the mouse *Msx1* (*mMsx1*) using retroviral infection of AHF explants induced cardiomyocyte differentiation and beating along with an upregulation of the sarcomeric genes and MHC protein, comparable with that induced by BMP4 application (Fig. 6F,G). RCAS-mediated ID2 overexpression had no effect (data not shown). Importantly, FGF genes were downregulated in the Msx1-infected explants, albeit to a lesser extent compared with BMP4 application, which could be due to the delay in the production of the viral gene.

Next, we investigated the effect of Msx1 overexpression on FGF genes in vivo (Fig. 6H). Concentrated mouse *Msx1* viruses were injected into the head mesoderm of stage 10 embryos and analyzed after 48 hours at about stage 18. Both *FGF8* and *FGF18* were significantly reduced in the Msx1-infected embryos compared with the GFP control embryos (Fig. 6H). We then explored whether Msx1 could affect cardiomyocyte differentiation downstream to the FGF-ERK signaling. Indeed, Msx1 overexpression resulted in a significant decrease in p-Histone staining compared with control embryos (Fig. 6I,J). Taken together, we demonstrate that Msx1, like BMP4, can repress FGF genes in vitro and in vivo and promote robust cardiomyocyte differentiation in AHF progenitors.

BMP-mediated cardiomyocyte differentiation requires cranial neural crest (CNC) cells

The robust upregulation of cranial neural crest markers (Fig. 6D) prompted us to investigate the involvement of neural crest cells in AHF differentiation. In fact, it has been shown that FGF signaling (particularly FGF8) was elevated after neural crest ablation in chick embryos (Rinon et al., 2007; Waldo et al., 2005). In the chick, MSX1/2 are expressed in CNC cells (Fig. 6E), whereas in the mouse they have broader expression patterns in the AHF niche (endoderm, mesoderm ectoderm and CNC cells) (Chen et al., 2007). Therefore we speculated that MSX genes, which are strongly induced by BMP4, promote cardiomyocyte differentiation in a non-cell autonomous manner involving a crosstalk between the endoderm, ectoderm and CNC cells with the cardiac progenitors. We first analyzed cranial neural crest markers in AHF explants using RT-PCR and SOX9 immunostaining (Fig. 7A). SOX9 staining, as well as that of the cranial neural crest markers, *MSX1*, *MSX2*, *SEMA3*, *PAX7*, *DLX5* and *ID1*, were hardly detected in freshly dissected AHF explants. By contrast, a sharp increase in the levels of these genes and of SOX9 protein, was detected after 20
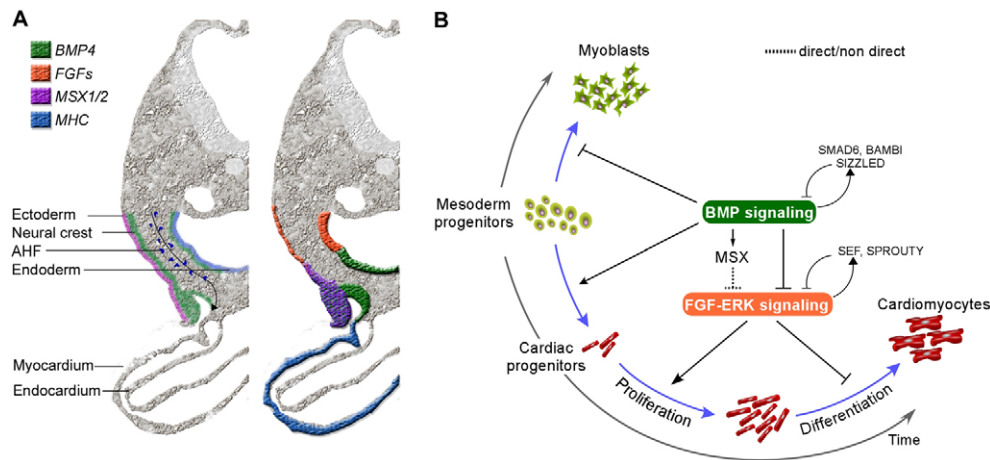
Fig. 8. BMP-MSX-FGF signaling network within the AHF regulates cardiac progenitors proliferation and differentiation by crosstalk between the endoderm, ectoderm and neural crest cells. (**A**) The AHF niche contains cardiac progenitors that are maintained in a multipotent and undifferentiated state by signals from the surrounding tissues such as the pharyngeal endoderm, ectoderm and neural crest cells, as depicted in the left section. A schematic drawing of the expression patterns of BMP, MSX, FGF and MHC molecules is shown on a similar section (right). (**B**) A model for the combinatorial, sequential effects of BMP, acting via MSX, on FGF-ERK signaling and the downstream effects on the specification, proliferation and differentiation of AHF cells during cardiogenesis. BMP signaling initially induces cardiac lineage specification (and blocks skeletal muscle specification). Subsequently, cardiac progenitors are exposed to high levels of FGF-ERK signaling, which facilitates the survival and proliferation of cardiac progenitors and blocks their premature differentiation. At later stages, during which AHF cells approach the cardiac outflow tract, BMP-MSX signaling represses FGF-ERK and AHF proliferation, promoting accurate deployment of differentiating AHF progenitors to the looping heart.

hours of culture. We conclude from this experiment that AHF explants promiscuously upregulated neural crest gene program in culture. Alternatively, the residual CNC cells in the explants can expand very rapidly.

To further investigate the role of CNC cells in the BMP-driven AHF differentiation, we performed CNC ablation in stage 8 chick embryos (Rinon et al., 2007; Tzahor et al., 2003). Despite the known regenerative ability of these cells, AHF explants that were dissected from CNC-ablated embryos failed to undergo BMP4-induced cardiomyocyte differentiation, clearly observed in control AHF explants treated with BMP4 (Fig. 7B,C). Interestingly, BMP4 is still able to block, albeit less efficiently, FGF gene expression in AHF explants dissected from CNC ablated embryos (Fig. 7C). Hence, cranial neural crest cells are required for the BMP-induced cardiogenesis in AHF explants, in agreement with the key roles of cranial and cardiac neural crest cells in the migration and differentiation of both secondary heart field (Hutson et al., 2006; Waldo et al., 2005) and cranial paraxial mesoderm (Rinon et al., 2007) progenitors.

DISCUSSION

The AHF contains a pool of cardiac progenitor cells that lies outside the linear heart tube, in the splanchnic mesoderm on the ventral side of the pharynx. Cardiac progenitors within the AHF are maintained in a proliferative and undifferentiated state by multiple signaling mechanisms that have thus far been poorly defined (Fig. 8A). Abnormal transition of proliferating cardiac progenitors into differentiating cardiomyocytes severely affects cardiac looping and outflow tract extension, processes that are generally associated with congenital heart disease. Our study reveals that the transition from cardiac progenitors to cardiomyocytes depends on dynamic input from the BMP and FGF signaling pathways in various tissues along the topography of the AHF niche (Fig. 8A,B). Although FGF-ERK signaling is initially required to maintain AHF progenitors in an undifferentiated state, BMP-MSX signaling, at the entrance to the

cardiac outflow tract, blocks the FGF-ERK pathway. In this study, we demonstrate that it is this key step that lies 'at the heart' of cardiomyocyte differentiation (Fig. 8B).

BMP signaling promotes AHF myofibrillogenesis and beating

BMP molecules, like their homolog in flies [Decapentaplegic (Dpp)], have been demonstrated to be both sufficient and necessary for the differentiation of cardiac progenitors in both first and second heart fields or in embryonic stem cells (Behfar et al., 2002; Frasch, 1995; Prall et al., 2007; Schlange et al., 2000; Schultheiss et al., 1997; Shi et al., 2000; Tirosh-Finkel et al., 2006; Waldo et al., 2001). Previous studies in both chick and mouse models (Prall et al., 2007; Waldo et al., 2001) have demonstrated the negative effect of BMP signaling on the proliferation of progenitor cells within the AHF. How BMP attenuates cell proliferation was previously not clear. Our findings close this gap, by showing that differentiation-promoting/proliferation-attenuating BMP activity is mediated by inhibition of the FGF signaling pathway. Because the inhibition of FGF-ERK signaling also induced myofibrillogenesis, we conclude that the evolutionary conserved effect of BMP signaling on myofibrillogenesis is conceivably indirect, via inhibition of FGF signaling.

Opposing activities of the BMP and FGF signaling pathways during cardiogenesis

We suggest that BMP signaling affects AHF cells in two phases: it is initially required to 'lock' mesoderm progenitors into the cardiogenic lineage (Tirosh-Finkel et al., 2006) whereas, at later stages, during which AHF cells approach the cardiac outflow tract, BMP signaling represses AHF proliferation [our data and Prall et al. (Prall et al., 2007)] by blocking FGF signaling, to promote accurate deployment of AHF progenitors to the looping heart (Fig. 8B). Importantly, FGF signaling plays a key role in the survival and expansion of mesoderm-derived cardiac progenitors (cardioblasts)

within the AHF, consistent with recent genetic studies in the mouse (Ilagan et al., 2006; Park et al., 2008; Watanabe et al., 2010; Zhang et al., 2008). Our results indicate that, in addition, FGF signals, which are restricted to the pharyngeal endoderm and ectoderm in the chick, act to block the premature differentiation of AHF cells, highlighting the importance of blocking FGF signaling as a key step in the differentiation of cardiomyocytes (Fig. 8B). Interestingly, the inhibitory effect of the FGF pathway on the differentiation of AHF progenitors was masked because of the positive requirement of this pathway for the survival of cardiac progenitors. In line with our studies, Hutson et al. have uncovered similar inputs of the BMP and FGF signaling pathways on SHF progenitors by showing that FGF8 signaling is required to maintain the SHF in a proliferative undifferentiated state, while BMP is a strong myocardial differentiation signal (Hutson et al., 2010).

Proliferation versus differentiation

The relationship between proliferation and differentiation is a classic example of a biological yin and yang, the idea being that cessation of proliferation leads to differentiation. In most cells, proliferation is dependent on ERK signaling, which facilitates the transition through the early G1 phase of the cell cycle. Our data indicate that BMP treatment or the direct inhibition of the FGF-ERK pathway strongly suppressed AHF proliferation. Whether this step is accompanied by the induction of sarcomeric gene activator(s) or 'repression of a repressor' of these genes, is currently under investigation. It has been shown that the withdrawal of proliferation signals initiated premature muscle differentiation in somites (Amthor et al., 1999). We propose that proliferation-promoting signals act as suppressors of differentiation during embryogenesis.

Neural crest cells are involved in the BMP-FGF crosstalk within the AHF niche

The AHF niche (Rochais et al., 2009) contains mesodermally derived cardiac progenitors that are exposed to signals from the pharyngeal endoderm, ectoderm and neural crest cells (Fig. 8A). These tissues robustly express the FGF and BMP synexpression groups: the 'FGF zone', which is located at the dorsal edges of the pharynx and is highly proliferative (pERK$^+$ and BrdU$^+$), and the 'BMP zone' at the ventral pharynx near the outflow tract, which is characterized by a lower proliferative index (pERK$^-$ BrdU$^-$ pSMAD$^+$) (Figs 2 and 5). We suggest that any perturbation of the delicate balance of BMP and FGF signals within the niche should lead to abnormal heart development. Other signaling mechanisms undoubtedly feed into this complex regulatory system.

In this study, we also demonstrated that neural crest cells, are important players in the BMP-MSX-FGF signaling circuit. Ablation of the dorsal part of the cranial neural tube (Rinon et al., 2007), where CNC cells reside, abrogated the effect of BMP4 on cardiomyocyte differentiation (Fig. 7B,C). Neural crest ablation in the chick resulted in increased FGF8 signaling and elevated proliferation in the secondary heart field (Hutson et al., 2006; Waldo et al., 2005) and cranial paraxial mesoderm (Rinon et al., 2007). Taken together, these studies suggest that both cardiac neural crest (interacting with SHF progenitors) and cranial neural crest cells (in the AHF niche) buffer proliferative signals (presumably FGFs) secreted from the endoderm and ectoderm in order to promote myocardial and myogenic differentiation, as well as migration into the outflow tract and branchial arches, respectively. Other examples of non-cell autonomous roles of cardiac/cranial neural crest in the regulation of the AHF niche were demonstrated in mice lacking either *Smad4* (Jia et al., 2007) or the

BMP receptor *Alk2* (Kaartinen et al., 2004) in neural crest cells. Both of these mouse models revealed abnormal differentiation of AHF/SHF progenitors and severe OFT defects. Furthermore, these studies are consistent with a BMP-dependent signaling mechanism involving neural crest cells that regulate AHF/SHF progenitors.

Using gain-of-function approaches in vitro and in vivo, we showed that MSX1 promotes robust cardiomyocyte differentiation and beating along with downregulation of FGF genes (Fig. 6). These findings suggest that MSX1 functions between the BMP and FGF genes to promote cardiomyocyte differentiation (Fig. 8B). In line with this, genetic studies in mice in which Msx genes have been shown to play an important role in the second heart field: double knockout of Msx1/2 resulted in increased proliferation of the second heart field niche (Chen et al., 2007). Importantly, the expression of *Msx1/2* in the mouse is seen in the ectoderm, endoderm, mesoderm and neural crest cells, whereas in the chick they seem to be restricted to the neural crest. How exactly Msx1 represses FGF gene expression is currently not clear. Because we observed a sharp upregulation of CNC markers in our explants, it could be that MSXs are induced in the FGF-expressing cells and block their transcription directly. Alternatively, a more plausible scenario is a non-cell autonomous loop in which BMP signaling induces MSX expression in CNC cells, which in turn secrete another signal(s) that suppresses FGF expression. We have tested retinoic acid as a candidate for such a mechanisms. We found that although retinoic acid is able reduce *FGF8* RNA in vitro, it is not sufficient to block expression of other FGF genes or to induce cardiomyocyte differentiation by itself (data not shown).

Universal cross-regulation of the BMP and FGF signaling pathways

Our study uncovered mutually exclusive expression patterns of BMP and FGF synexpression groups within the AHF, presumably acting as self-regulatory, internal negative-feedback loops in the BMP and FGF signaling pathways, and providing another level of regulation that could compensate for variations in interconnectivity among them (Benazet et al., 2009). Our study on cardiogenesis, and that of Benazet et al. on limb patterning (Benazet et al., 2009), suggests that the crossregulation between different signaling pathways is more robust than intra-pathway self-regulation. The crosstalk between the BMP and FGF signaling pathways has been widely documented in diverse biological settings (Benazet et al., 2009; Bilican et al., 2008; Huang et al., 2009; Maatouk et al., 2009; Pajni-Underwood et al., 2007; Weisinger et al., 2008). Moreover, it has been shown that the BMP-FGF crosstalk regulates the epicardial versus myocardial lineage switch at the inflow pole of the heart although this occurs without any apparent effect on the proliferation of these cardiac progenitors (van Wijk et al., 2009).

The signals regulating progenitor cell number and their differentiation capacity during embryogenesis may also regulate progenitor cell number postnatally, during normal or pathological processes, or during in vitro cardiogenesis using embryonic stem (ES) and induced pluripotent stem (iPS) cells. Our findings highlight the challenges inherent in directing the differentiation of these cells into cardiomyocytes, as manipulation of these processes involves not only a thorough understanding of the interacting signaling pathways, but also the ability to accurately control the temporal order in which they are brought into play.

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