

# BMP signaling regulates Nkx2-5 activity during cardiomyogenesis

Mina Jamali, Christina Karamboulas, Parker J. Rogerson, Ilona S. Skerjanc\*

Department of Biochemistry, Medical Sciences Building, The University of Western Ontario, London, ON, Canada N6A 5C1

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**Abstract** Nkx2-5 regulates the transcription of muscle-specific genes during cardiomyogenesis. Nkx2-5 expression can induce cardiomyogenesis in aggregated P19 cells but not in monolayer cultures. In order to investigate the mechanism by which cellular aggregation regulates Nkx2-5 function, we examined the role of bone morphogenetic protein 4 (BMP4). We showed that the expression of the BMP inhibitor, noggin, was sufficient to inhibit the induction of cardiomyogenesis by Nkx2-5 during cellular aggregation. Furthermore, soluble BMP4 could activate Nkx2-5 function in monolayer cultures, resulting in the formation of cardiomyocytes. Therefore, BMP signaling is necessary and sufficient for the regulation of Nkx2-5 activity during cardiomyogenesis in P19 cells. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Cardiomyogenesis; P19 cell; Embryonal carcinoma; Nkx2-5; Bone morphogenetic protein

## 1. Introduction

There are several families of transcription factors implicated in the processes of cardiac muscle commitment and differentiation, including the Nkx2, GATA, and MEF2 families. The *Drosophila* NK2 homeobox gene *tinman* is an obligatory mesoderm determination factor essential for subdividing the mesoderm into somitic, visceral and cardiac primordia [1]. Loss of *tinman* expression results in the loss of embryonic dorsal vessel and visceral muscles. In the mouse, the *tinman* homologue *Nkx2-5/Csx* is expressed in embryonic myocardial progenitors and in pharyngeal endoderm by 7.5 days post-coitus [2]. Mice lacking Nkx2-5 are defective in looping of the heart tube and in the expression of a subset of cardiac muscle-specific genes [3,4]. Nkx2-5 was shown to serve as a modest transcriptional activator in fibroblasts [5–7]. Optimal Nkx2-5 activity requires combinatorial interactions with other cardiac-restricted factors such as GATA4 and MEF2 [6–10].

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor  $\beta$  superfamily of signaling molecules and they mediate a variety of cellular events. In *Drosophila*, the BMP homologue, dpp, is secreted from the dorsal ectoderm and maintains *tinman* expression in the mesoderm [11]. Similarly in chick, BMP2 or -4 is expressed in tissues adjacent to the precardiac mesoderm and can induce

Nkx2-5 and GATA4 expression [12–14]. Conversely, disruption of BMP signaling with noggin or dominant negative receptors can prevent cardiomyogenesis in chick, *Xenopus*, and in P19CL6 cells [12,14–18]. Therefore, BMP/dpp signaling plays an important role in controlling cardiomyogenesis.

P19 cells are embryonal carcinoma cells capable of differentiating into a variety of cell types representative of all three germ layers in suspension culture with several chemical inducers (for review see [19]). The cardiac myocytes derived from P19 cells display the biochemical and physiological properties that occur during early embryonic development. Unlike P19CL6 cells and fibroblasts [18,20,21], P19 stem cells can support the induction of cardiomyogenesis by Nkx2-5, when aggregated in the absence of DMSO [22]. MEF2C and GATA4 have similar abilities in aggregated P19 cells [22,23]. Furthermore, using dominant negative or antisense approaches, both Nkx2-5 and GATA4 are essential for P19 cell cardiomyogenesis [24,25].

In the present study, we have examined the regulation of Nkx2-5 function by cellular aggregation. We have shown that the expression of the BMP antagonist noggin can inhibit the function of Nkx2-5 in aggregated P19 cells. Furthermore, the addition of soluble BMP4 could bypass the requirement for aggregation and induce cardiomyogenesis in monolayer cultures of P19 cells expressing Nkx2-5. These results indicate that BMP signaling is both necessary and sufficient for Nkx2-5 function.

## 2. Materials and methods

### 2.1. Plasmid constructs

All expression vectors utilized the phosphoglycerate kinase (PGK; *pgk-1*) promoter to drive the expression of various cDNAs, as constitutive expression can be effectively achieved in P19 cells with this promoter. The constructs PGK-lacZ, PGK-puro, and B17 have been described previously [26,27]. PGK-noggin was created by subcloning a 950 bp fragment of mouse noggin (kindly provided by R.M. Harland, accession U79163) downstream of the *pgk-1* promoter.

### 2.2. Cell culture

P19 cells were obtained from the American Type Culture Collection (ATCC CRL-1825) and cultured as described previously [28,29]. Stable P19 cell lines expressing noggin, termed P19[noggin] cells, were generated using FuGENE 6 Transfection Reagent according to the manufacturer's protocol (Boehringer Mannheim) as described previously [25]. Cells were seeded 24 h before transfection with a total of 9  $\mu$ g of plasmid: 0.5  $\mu$ g of PGK-lacZ, 0.5  $\mu$ g of PGK-puro, 2  $\mu$ g of B17, and 6  $\mu$ g of PGK-noggin. Several high expressing clones were selected for further analysis.

Differentiation was induced as described previously [30–32]. Cells were aggregated for 4 days and then plated in 150 mm culture dishes (day 4) and harvested for RNA or fixed for immunofluorescence on day 6.

In order to determine the effects of BMP4 on the ability of Nkx2-5

\*Corresponding author. Fax: (1)-519-661 3175.

E-mail address: skerjanc@uwo.ca (I.S. Skerjanc).

**Abbreviations:** BMPs, bone morphogenetic proteins; MyHC, myosin heavy chain; PGK, phosphoglycerate kinase

to induce cardiomyogenesis, P19[control] and P19[Nkx2-5] cells were seeded at 100 000 cells on gelatin-coated coverslips in 35 mm dishes, grown in monolayer in the presence and absence of BMP4 (Genetics Institute, Cambridge, MA, USA) and fixed after 6 days.

### 2.3. Northern blot analysis

Northern blots were performed as described previously [33]. The probes used were: a 600 bp *Pst*I fragment from the human cardiac  $\alpha$ -actin last exon (PATA2; [34]), a 1 kb *Hind*III/*Bam*HI fragment of mouse BMP4 (generous gift from T.M. Underhill), a 2.4 kb *Xba*I fragment of GATA4 [35], a 1.6 kb *Eco*RI fragment of mouse *Nkx2-5* cDNA [2], and a 900 bp fragment of mouse *noggin* cDNA. All blots were standardized using a 750 bp *Eco*RI fragment of rabbit 18S cDNA.

### 2.4. Immunofluorescence

Cells were fixed in methanol at  $-20^{\circ}\text{C}$  for 5 min, rehydrated in phosphate-buffered saline for 15 min at room temperature, and then incubated with 50  $\mu\text{l}$  of a mouse anti-myosin heavy chain (MyHC) monoclonal antibody supernatant, MF20, as described previously (Ridgeway (2001) #2194). Immunofluorescence was visualized on a Zeiss Axioscope microscope with epifluorescence optics, images were captured with a Sony 3CCD color video camera, and processed using Northern Eclipse, Adobe Photoshop 5.5 and Canvas 7 (Deneba) software.

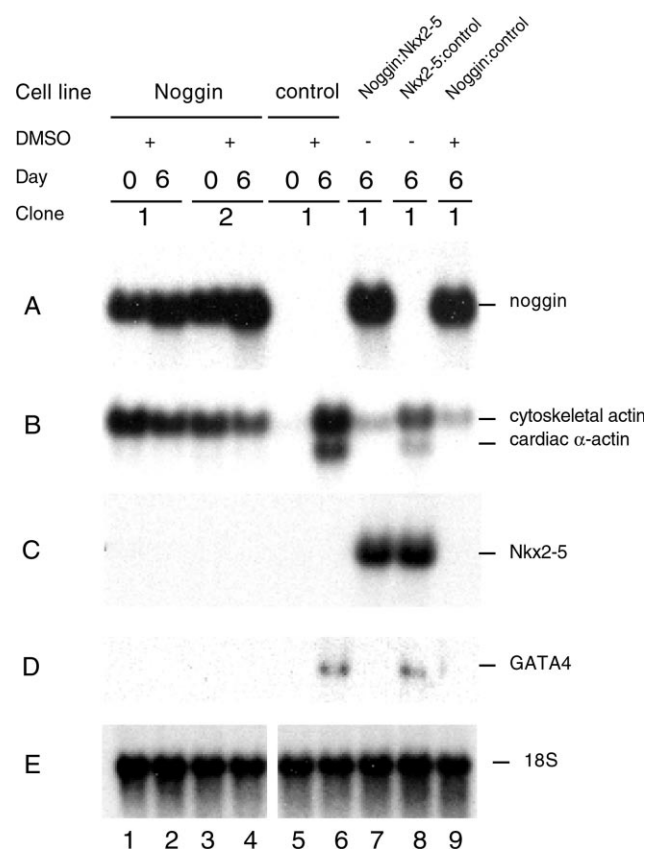


Fig. 1. Noggin inhibits DMSO- and Nkx2-5-induced activation of cardiac  $\alpha$ -actin and GATA4 expression. Two P19[noggin] cell lines, one P19[control] cell line, and a 1:1 mix of P19[noggin] and P19[Nkx2-5] cells were differentiated in the presence of DMSO. P19[Nkx2-5] cells were mixed 1:1 with P19[noggin] or P19[control] cell lines and differentiated in the absence of DMSO. Total RNA was harvested on day 0 and day 6 of differentiation, as indicated. Northern analysis was performed on 6  $\mu\text{g}$  of total RNA with probes for noggin (A), cardiac  $\alpha$ -actin (B), Nkx2-5 (C), GATA4 (D), and standardized using an 18S marker (E).

## 3. Results

Nkx2-5 can induce cardiomyogenesis in aggregated P19 cells but not in cells grown in monolayer [22]. In order to determine the mechanism by which cellular aggregation can regulate Nkx2-5 function, we examined the role of BMP2/4. BMP2/4 is necessary and sufficient to activate the transcription of Nkx2-5 and GATA4 [12,18]. BMP4 is also expressed by day 3 during the aggregation of P19 cells into cardiac muscle (data not shown).

To identify the muscle-specific transcripts regulated by BMP signaling in DMSO-induced cardiomyogenesis, Northern blots were performed on RNA isolated on days 0 and 6 from P19[noggin] and P19[control] cells (Fig. 1). High levels of the transfected noggin construct were expressed on both day 0 and day 6 in P19[noggin] cultures (Fig. 1A, lanes 1–4) but not in P19 control cultures (Fig. 1A, lanes 5 and 6). The lack of cardiac muscle in P19[noggin] cultures in comparison to control cultures was demonstrated by the lack of cardiac  $\alpha$ -actin and GATA4 expression on day 6 (Fig. 1B,D, lanes 2 and 4 compared to 6). Therefore, similar to results reported by others, inhibition of BMP signaling by noggin interferes with the DMSO-induced activation of cardiac  $\alpha$ -actin and GATA4.

To investigate whether BMP signaling is essential for Nkx2-5 activity, P19[noggin] cells were mixed with P19[Nkx2-5] cells in equal proportions, aggregated in the absence of DMSO for 6 days, and examined by Northern blot analysis. While differentiated P19[Nkx2-5]:P19[control] co-cultures expressed cardiac  $\alpha$ -actin and GATA4, aggregated P19[Nkx2-5]:P19[noggin] and P19[noggin]:P19[control] co-cultures did not (Fig. 1B,D, compare lane 8 with lanes 7 and 9). These results indicate that noggin expression inhibits both DMSO- and Nkx2-5-induced cardiomyogenesis. As expected, cultures of P19[Nkx2-5]:P19[noggin] and P19[Nkx2-5]:P19[control] cells expressed similar levels of transfected Nkx2-5 (Fig. 1C, lanes 7 and 8) and P19[noggin]:P19[Nkx2-5] and P19[noggin]:P19[control] co-cultures expressed similar levels of transfected noggin (Fig. 1A, lanes 7 and 9). Therefore, BMP signaling is essential for the ability of Nkx2-5 to activate the transcription of cardiac  $\alpha$ -actin and GATA4.

In order to confirm the results of Northern blot analysis, cultures were stained by immunofluorescence with the anti-MyHC antibody, MF20. In the presence of DMSO, P19[control] cells efficiently differentiated into MyHC-positive cardiomyocytes (Fig. 2D). When P19[noggin] cells were mixed with P19[control] cells for 6 days with DMSO, the expression of noggin inhibited the DMSO-induced cardiomyocyte formation (Fig. 2B), in agreement with the results from Northern blot analysis. Furthermore, Noggin expression was shown to interfere with Nkx2-5-induced cardiomyogenesis, as these mixed cultures did not form any myocytes by day 6 in culture compared to P19[Nkx2-5] cells mixed with P19[control] cells (Fig. 2F,H, respectively). No cardiomyogenesis was observed in the absence of DMSO in P19[noggin]:P19[control] co-cultures (data not shown). These findings suggest that BMP signaling is essential for Nkx2-5 function.

These results suggest that cellular aggregation may be responsible for initiating the BMP signaling cascade that subsequently regulates Nkx2-5 activity. If this is true, then expression of BMP should bypass the requirement for cellular aggregation. In order to test this hypothesis, monolayers of

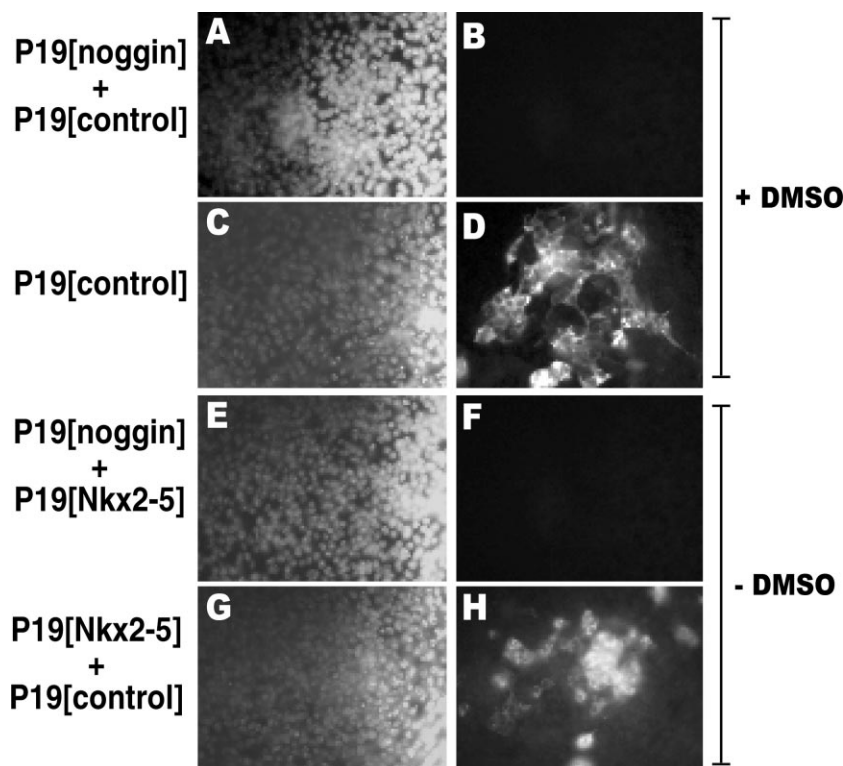


Fig. 2. Expression of noggin inhibits both DMSO- and Nkx2-5-induced cardiomyogenesis in P19 cells. Immunofluorescence using an anti-MyHC antibody, MF20, was carried out on day 6 of differentiation in the presence of DMSO for P19[control] cells (C, D) and P19[control] cells mixed with P19[noggin] cells (A, B). Immunofluorescence with MF20 was also performed on day 6 of differentiation in the absence of DMSO for P19[noggin] cells mixed with P19[Nkx2-5] cells (E, F) and P19[Nkx2-5] cells mixed with P19[control] cells (G, H). The corresponding Hoechst stain for nuclei is shown (A, C, E, G; observed magnification 400 $\times$ ).

P19[control] and P19[Nkx2-5] cells were grown in the presence of various concentrations of BMP4 for 6 days. P19[Nkx2-5] monolayers treated with 10 ng/ml and 50 ng/ml (data not shown) as well as 100 ng/ml BMP4 (Fig. 3D) showed substantial cardiomyocyte formation after MF20 staining compared to similarly treated P19[control] monolayers (Fig. 3C) and P19[Nkx2-5] cells grown in the absence of BMP4 (Fig. 3B).

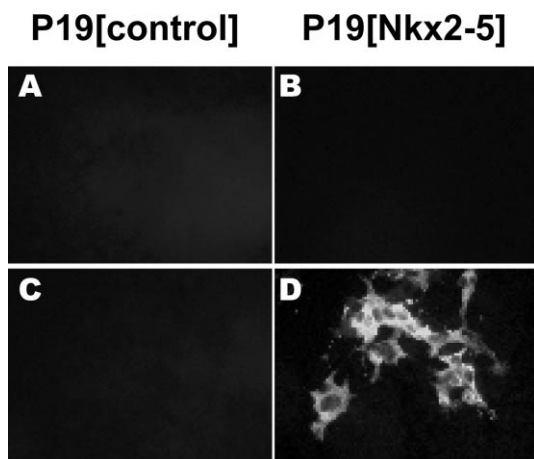


Fig. 3. Monolayers of P19[Nkx2-5] cells show enhanced differentiation in the presence of BMP4. P19[Nkx2-5] (B, D) and P19[control] cells (A, C) were grown in monolayer in the presence of 0 (A, B), and 100 ng/ml (C, D) BMP4. Cells were fixed after 6 days of growth and reacted with the anti-MyHC antibody, MF20 (observed magnification 400 $\times$ ).

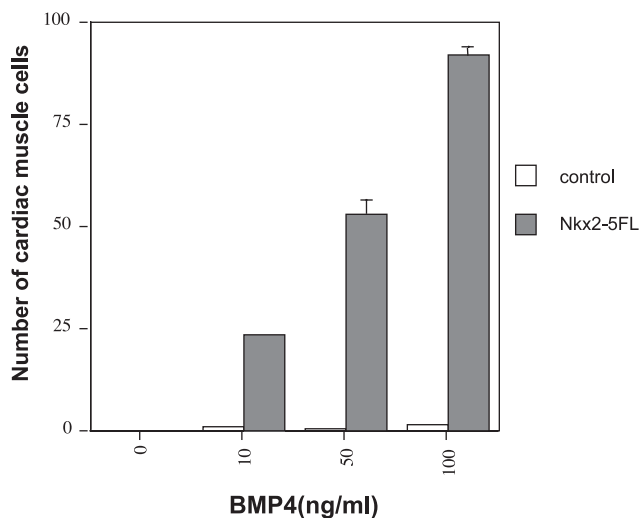


Fig. 4. Treating P19[Nkx2-5] cells in monolayer with an increasing concentration of BMP4 results in an increase in the number of MyHC-positive cells present. The number of MyHC-positive cardiomyocytes on each confluent coverslip was counted ( $n=3$ ) for P19[control] cells (white column) and P19[Nkx2-5] cells (black column) in monolayer after treatment with BMP4 as indicated for each column. Bars represent standard error of the mean for each column.

In order to quantitate results observed in Fig. 3, the number of cells expressing MyHC present on a coverslip was counted and the results of these counts are shown in a bar graph (Fig. 4). These findings indicate that increasing BMP4 concentrations to a maximum of 100 ng/ml results in an increase in the formation of Nkx2-5-induced cardiac muscle cells in monolayer. Since BMP4 was not able to function in control P19 cells in the absence of Nkx2-5, these data suggest that BMP4 is sufficient to activate Nkx2-5 function in P19 cells.

#### 4. Discussion

In the present study, we utilized the P19 cell culture system to examine the regulation of Nkx2-5 in cardiac muscle development. We identified BMP4 as a candidate for regulating Nkx2-5 activity during cellular aggregation due to its temporal pattern of expression during DMSO-induced cardiomyogenesis. We examined the role of BMP signaling by using the BMP antagonist, noggin. Stable P19 co-cultures of Nkx2-5 and noggin aggregated in the absence of DMSO failed to differentiate into cardiomyocytes. We also demonstrated that addition of BMP4 protein to P19 cells overexpressing Nkx2-5 in monolayer cultures bypasses the requirement for cellular aggregation and results in cardiomyogenesis. These results suggest that BMP signaling is both necessary and sufficient for the regulation of Nkx2-5 activity.

In mammalian systems, BMP signaling is both necessary and sufficient to activate the transcription of Nkx2-5 [12,18]. However, a role for BMP signaling in the regulation of Nkx2-5 protein function had not been determined previously. We present a model in which BMP signaling is required at two different stages of cardiomyogenesis. First, BMP signaling is essential for the expression of Nkx2-5 and GATA4 [12,18]. Second, BMP signaling is essential for the activation of Nkx2-5 function, resulting in enhanced GATA4 expression and cardiomyogenesis (this work).

We have shown that BMP4 is sufficient for regulating Nkx2-5 but, since noggin inhibits all BMP signaling, other BMP family members such as BMP2 may also be involved. In an alternate model, it is possible that the previously identified enhancement of Nkx2-5 and GATA4 expression via BMP signaling may simply reflect an activation of low levels of Nkx2-5 protein function, which could then upregulate GATA4 expression, resulting in cardiomyogenesis. The relevance of the regulation of Nkx2-5 function by BMP signaling to cardiomyogenesis in the developing embryo remains to be determined.

Since Nkx2-5 is constitutively expressed in P19[Nkx2-5] cells, the activation of Nkx2-5 function by BMP signaling likely occurs at a post-transcriptional level. Consequently, BMP signaling could result in an enhancement of Nkx2-5 translation, an increase in Nkx2-5 protein stability, or in changes to the post-translational modification of Nkx2-5 by the downstream effectors of BMP signaling, such as TAK1. Alternatively, BMP signaling could result in the induction of the expression of a cofactor that modulates Nkx2-5 function, such as GATA4 or possibly ATF-2 or Smads1/4 [18,36]. These pathways could involve direct interactions between Nkx2-5 and the Smads factors and/or a cross-talk with the TAK1 pathway.

In summary, Nkx2-5 function can be regulated by the bal-

ance of inhibition and activation of BMP signaling, leading to the induction of cardiac muscle-specific gene expression. By encompassing events from gastrulation to cardiomyogenesis, the P19 cell system is valuable for analyzing molecular mechanisms controlling early cellular differentiation.

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