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Research Article

Enhancement of vertebrate cardiogenesis by a lectin from perivitelline fluid of horseshoe crab embryo

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Abstract. Cardiac myocytes are the first cells to differentiate during the development of a vertebrate embryo. A wide variety of molecules take part in various steps in this process. While exploring biologically active molecules from marine sources, we found that a constituent of perivitelline fluid from embryos of the Indian horseshoe crab can enhance growth and differentiation of chick embryonic heart. We have purified the factor and identified the cardiac promoting molecule to be a novel lectin. We show that this molecule influences cardiac development by increasing the number of cells constituting the heart and by modulating the expression of several cardiac development regulatory genes in chick embryos. Using mouse embryonic stem cells we show that the cardiac myocyte-enhancing capacity of this molecule extends to mammals and its effects can be blocked using methylated sugars. This molecule may prove to be an important tool in the study of cardiomyocyte differentiation.

Keywords. Vertebrate heart development, lectin, enhancement of differentiation, chick embryo, mouse ES cells, perivitelline fluid, horseshoe crab embryo.

Introduction

During vertebrate embryonic development, heart formation involves a series of tightly regulated molecular and morphogenetic steps, with complex interactions between different cell types and regulatory molecules, including extracellular matrix components, and growth and transcription factors. In early chick embryo, cells destined to contribute to the heart are localized to the posterior epiblast [1]. Prior to gastrulation, these cells migrate rostrally to occupy a position in the primitive streak just caudal to Hensen's node around the Hamburger Hamilton (HH) stage 3 [2] and, by HH stage 4, pass through the primitive streak into mesoderm and migrate anteriolaterally to form the bilateral anterior lateral plate mesoderm [3]. Specification of these cells to cardiac lineages begins while the cells are still in the primitive streak [4] and continues through late gastrulation as the cells reach the anterior lateral plate [5] to form bilateral heart-forming regions (HFRs). Soon after their specification, cardiac precursors converge along the ventral midline of the embryo to form a linear, beating heart tube. Differentiation of cardiac myocytes is concomitant with organogenesis of the heart [6].

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Endoderm of the anterior lateral plate secretes signaling molecules like insulin, insulin-like growth factor II, activin and fibroblast growth factors [7]. These molecules promote survival of specified premyocardial cells as well as differentiating myocytes. Bone morphogenetic proteins (BMPs) play an inductive role [8], while BMP antagonists like noggin and chordin block cardiogenesis [6, 9]. BMP2, in combination with FGF2, is able to induce the expression of definitive cardiogenic markers in non-cardiogenic mesodermal cells [10].

In response to the cardiogenic signals, competent mesodermal cells destined to cardiomyocyte lineage express homeobox gene *Nkx2.5*, an early marker for cardiomyocyte differentiation [11]. Nkx2.5 cooperates with zinc finger transcription factor of the GATA family to activate cardiac gene expression [12]. GATA factors play an essential role in cardiac tube formation [13]. Expression of structural proteins like myosin marks the terminal differentiation of the precursors into mature, functional myocytes. Mature cardiac myocytes express either ventricular- or atrial-specific myosin molecules depending on their diversification fate either to ventricular or atrial lineage.

The embryonic extracellular matrix, which is comprised of glycosaminoglycans, glycoproteins, collagens, and proteoglycans, is believed to play multiple roles during heart morphogenesis [14]. An anterior-posterior concentration gradient of fibronectin on the endodermal surface of the mesoderm-endoderm interface in early chick embryo has been implicated in the rostral migration of precardiac mesoderm cells [15, 16]. At HH stage 5, during directional movement, the precardiac cells use the fibronectin gradient in a haptotactic manner to actively migrate across the substratum [15]. Further, during heart compartmentalization, adhesion molecules such as cadherins and fibronectins and associated intracellular signaling pathways coordinate morphogenesis [16].

In the present report, we have used chick embryo explants cultured *in vitro* and mammalian murine embryonic stem (ES) cells to help understand cardiogenesis during early development. We have shown that, in chick embryo a component of perivitelline fluid (PVF) from the embryo of an Indian horseshoe crab (*Tachypleus gigas* Müller), a marine arthropod, influences cardiac development in a dramatic manner by enhancing the size of the heart while the rest of the embryo is barely affected. When cultured gastrulating chick embryos were allowed to grow in presence of appropriate amount of PVF of horseshoe crab embryos of stage 19, we found a significantly larger heart in treated embryos. The cardiac development-enhancing activity of PVF resided with a lectin with a relative molecular mass of 2.7×10^4, made of 221 amino acid residues, which was found to be identical to that of Limulus lectin L6 of Japanese horseshoe crab *T. tridentatus* [17]. Moreover, the enlargement of heart was found to be due to an increase in the cell number that constituted the heart and was not a hypertrophic response. In fact, several of the cardiac regulatory molecules were also modulated by this molecule. Here we report, for the first time, a factor that enhances exclusively the cardiac development.

To determine whether the remarkable cardiac development-promoting activity of this lectin also extends to mammals, we treated mouse ES cells with this factor during differentiation. Since the ES cells are pluripotent, they take up several independent fates including that of cardiac myocytes during differentiation [18] and the hallmark of cardiomyocytes, the rhythmic pulsating activity, distinguishes these cells among the differentiating heterogeneous cell population. When the ES cells were allowed to differentiate in presence of the lectin from PVF, the number of beating clusters was increased indicating enhancement in cardiomyogenesis. Taken together, these results underscore a cardiac development-promoting principle from the PVF with activity extending from aves to mammals.

Materials and methods

Collection of PVF. Naturally fertilized Indian horseshoe crab (*T. gigas* Müller) stage 19 embryos [19] were collected from the Bay of Bengal at Balasore (Lat. 21° 27'N, Long. 87° 04' E), Orissa, India, and transferred to the laboratory. The eggs were thoroughly cleaned with chemically reconstituted sterile, chilled seawater and transferred to a sterile plastic centrifuge tube with a minute hole in its base. This was in turn placed in a sterile intact centrifuge tube. The vitelline membrane of the embryos was pierced with a needle and immediately the tube duplex was centrifuged at 5000 rpm, 10 min at 4°C. The liquid in the outer tube was collected and aliquoted and stored at –70°C as PVF. Protein content was estimated by UV spectroscopy and Bradford's assay [20].

Treatment of stage 4 chick embryo explants with whole PVF/PVF Fraction VII. Freshly laid fertile eggs of white Leghorn chicken were incubated at 37°C for 18 h, so that they could develop to HH stage 4 [2]. These embryos were cultured using New's single ring technique [21]. Embryos were treated with either PVF or PVF Fraction VII at appropriate concentrations determined as previously described [22]. In brief, 100 µl Pannet Compton (PC) saline [23] containing appropriate amount of test chemical was carefully

placed inside the ring in such a way that the cultured embryo encircled by the ring is completely bathed in the test solution. Controls received equal volume of plain PC saline. After 30 min at room temperature for diffusion, the cultures were transferred to 37°C for 6–36 h, depending on the developmental stage at which the effects were studied. At the end of the treatment period, embryos were observed under dissection microscope for overall morphological effects of the treatment and then processed as required for the planned downstream processing.

Embryos developed to HH stage 10 [2] at the end of 18 h of post-culture incubation and to HH stage 14 [2] by 36 h. By stage 10, a beating heart tube is formed, which bends to the right of the embryo at stage 14 [2]; an extensive network of blood vessels develops and the looped heart pumps significant amount of blood through these [23]. Embryos at these stages were fixed in acetic alcohol and stained with hematoxylin-eosin. Morphological features and overall development of treated embryos were compared to those of controls (PC saline treated).

After 18 and 36 h of treatment, embryos were fixed for histological studies in Bouin's fixative and 5-μm paraffin sections were stained with hematoxylin-eosin. Serial sections mounted in DPX were studied and compared between control and treated embryos.

Gel exclusion fast performance liquid chromatography (FPLC). An aliquot of 0.5 ml crude PVF was subjected to gel exclusion FPLC (Amersham Pharmacia) using two 30-cm pre-packed columns of Superose 12. Phosphate buffer (10 mM; 13.4 ml 0.5 M Na_2HPO_4, 3.3 ml 1 M NaH_2PO_4 and 8.5 g NaCl in 1000 ml, pH 7.4) was used as an eluent and 0.5-ml fractions were collected at a flow rate of 0.5 ml/min for a total elute of 60 ml. AUFS and chart speed used for the chromatography were 0.1 unit each.

Fractions that eluted under the same peak were pooled and concentrated using Centricon columns (Millipore, USA). Protein content was estimated by UV spectroscopy. Fraction VII was found to be the most prominent eluate, and 10 μg protein from Fraction VII was subjected to 12.5% SDS-PAGE to determine the size and heterogeneity of the constituent protein.

Amino acid sequence analysis of constituent of Fraction VII. The single band found in Fraction VII of PVF on SDS-PAGE was excised and submitted to W. M. Keck Biomedical Mass Spectrometry Laboratory of Biomolecular Research Facility, (University of Virginia Health System, Charlottesville, USA) for identification of the protein by amino acid sequencing.

Cardiac cell count. Entire hearts from cultured stage-matched untreated embryos and those treated with 20 ng Fraction VII for 24–32 h were dissected out in live condition using extremely fine glass needles. These were transferred to chilled Dounce homogenizer, and homogenized using a loose piston in nucleus extraction buffer (TENM2; sucrose buffer: 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 15 mM triethanolamine, pH 7.6, 0.3 M sucrose) to extract intact nuclei [24]. Ethidium bromide (2 ng/ml final concentration) was added to the homogenate, which was then incubated on ice for 20 min. Neubauer's chamber was charged with appropriately diluted homogenate and fluorescent nuclei were counted under UV light.

Whole mount *in situ* hybridization. Chicken *Brachyury* [25] cDNA cloned in *pBS* vector was a kind gift from Prof. J. C. Smith (Cambridge, UK). The recombinant plasmid *pcBRA9* was linearized with *Xba*I and transcribed using T3 RNA polymerase in presence of DIG-labeled UTP to get antisense transcript of 350 bases. Chicken *noggin* [26] cDNA cloned into pBS vector (*cNOG*) was a gift from Dr. J. Cooke (London, UK). Recombinant plasmid *pcNOG* was linearized with *Sac*II and transcribed using T7 RNA polymerase in presence of DIG-labeled UTP to generate antisense transcript of 1.3 kb. Chicken ventricular myosin heavy chain1 (*VMHC1*) [27] cloned in pGEM 4Z was a gift from Prof. D. Bader (Tennessee, USA). The recombinant plasmid pc*VMHC1* was linearized with *Nde*I and transcribed using T7 RNA polymerase in presence of DIG-labeled UTP to get antisense transcript of 3 kb.

For whole mount *in situ* hybridization, the protocol described by Nieto *et al.* [28] was followed for all genes except for c*VMHC1*, where protocol of Wilkinson [29] was used. Stage 4 embryos with or without treatment with 20 ng fraction VII were incubated for 6–36 h until they reached HH stage 5, 7, 9/10 or higher, and were fixed in 4% paraformaldehyde overnight at 4°C. Subsequently these were dehydrated through the grades of PBS:methanol and stored at 100% methanol at –20°C for not more than a week. Prior to hybridization, embryos were rehydrated, permeablized with 10 μg/ml proteinase K, re-fixed in 4% paraformaldehyde containing 0.2% glutaraldehyde. After equilibrating in hybridization mix overnight at –20°C followed by incubation at hybridization temperature for 6 h, embryos were hybridized overnight at suitable temperature with antisense DIG-labeled riboprobe. Nonspecifically bound probe was eliminated during stringency washes and using RNase treatment. Hybridized probe was detected using alkaline phosphatase-tagged anti-DIG antibody after blocking the nonspecific protein binding sites

Table 1. Primer sequences used for quantitative real-time RT-PCR for stage 4 embryos.

Gene	Primer sequence	Annealing temperature (°C)
GAPDH	F-AGGTGCTGAGTATGTTGTGG-3' R- ATATCATCATACTTGGCTGGT-3'	55
GATA5	F-CGGCTTATTCCCACGACTCC-3' R-TGCTCTCAAAGTGTCCGGCC-3'	57.6
Nkx2.5	F- AGACAGAGGAAGAGGAGGAA-3' R- TTGCAGGCAGGGCTGTTGT-3'	58.7
AMHC1	F- CCGCACCACAGAAGACCAGAT-3' R- GGAGGAGCACTTGGCATTGAC-3'	65
VMHC1	F- TCTTATATCTGGGAGCCAGG-3' R- GCTACAAACACCAAGCAGAG-3'	61.2
Noggin	F- ATCTAATCGAGCACCCGGAC-3' R- GCGATGATGGGGTACAGGAT-3'	62.3

with 10 % fetal calf serum. Excess antibody was washed off and specific antigen-antibody complexes were localized using BCIP-NBT color reaction.

Quantitative real-time RT-PCR. Stage 4 embryos were treated with 20 ng PVF Fraction VII for 6–24 hours till the comparable control embryos reached stage 5, 7, 9/10–12. The prospective HFR/forming heart tubes/well-formed beating heart tubes, depending on the stage of harvest, were carefully dissected out. The tissue was homogenized in TRIzol reagent (Sigma) and total RNA was isolated according to the manufacturer's protocol. RNA was separately extracted from the rest of the embryo without the heart/HFRs. Isolated RNA (4 µg) was used for cDNA synthesis and 2 µl cDNA was used for a 25-µl real-time gradient PCR reaction (Bio-Rad iCycler) using 2X iQ SYBR Green Supermix (Bio-Rad) and primers (Table 1). The conditions used were as follows: Initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 1 min, annealing for 1 min and extension at 72°C for 1 min and final extension of 72°C for 10 min. The threshold cycle (C_t) values were recorded and the fold change calculated according to the formula: Fold change $= 2^{-(\Delta(\Delta C_t))}$. The quality of the amplified DNA and the appropriate size was verified by electrophoresis of the PCR product on a 1.5 % agarose gel in 0.5× Tris borate EDTA.

Maintenance of ES cells and differentiation into cardiomyocytes. The murine ES cell line D3 was maintained with or without feeder layer as described [30]. In brief, the ES cells were grown in DMEM supplemented with 15 % FBS, 1× nonessential amino acids, 1× L-glutamine, 1× penicillin streptomycin (all from Invitrogen), 0.007 % 2-mercaptoethanol (Sigma) and 1000 U/ml LIF (ESGRO; Invitrogen). Cells were passaged every 48 h by enzymatic dispersion using 0.25 % trypsin EDTA (Invitrogen). We followed the method of hanging drop culture [30] for 2 days (0–2 days) followed by 3 days in suspension culture for differentiating the ES cells into cardiomyocytes, and the medium was same as that used for ES cell culture but without LIF supplementation. This resulted in the formation of three-dimensional embryoid bodies (EB). The EBs were plated on day 5 on gelatin-coated 48-well dishes (Corning) with a single EB per well and 12 EBs were plated for each set of experiment and control. The culture medium was changed every alternate day. The cardiac differentiation of EBs was monitored at different stages of development and the number of beating EBs and beating clusters per EB were counted to assess the efficacy of differentiation.

Influence of PVF on cardiomyogenesis. To determine the effect of PVF Fraction VII on cardiomyogenesis and to discern the effective time window of action, the medium was supplemented with 300 ng/ml Fraction VII at different time periods (0–2 days, 2–5 days and day 5) during differentiation, and cardiomyogenesis was monitored. Same amount of PBS was used in control samples. In another set of experiment, we dialyzed the Fraction VII against the culture medium to nullify the effect of PBS and used it at different time periods. Additionally, various concentrations (30 ng/ml, 300 ng/ml and 3 µg/ml) of Fraction VII were added to the medium during early stage of differentiation (0–2 days) to assess the optimum concentration required for cardiomyocyte induction. Same amount of DMEM was used in control set of experiment.

RNA isolation from EBs, reverse transcription and quantitative real-time PCR. Total RNA was harvested using TRIzol (Sigma) from control and PVF Fraction VII-treated EBs at different stages of differentiation and treated with RQ1 DNase (Promega) to remove residual DNA contamination. Purified RNA (3 µg)

Table 2. Primer sequences used for quantitative real-time RT-PCR for embryoid bodies (EBs).

Name	Primer sequences	Annealing temperature (°C)
β-Actin	F-AGCCTTCCTTCTTGGGTATGG-3' R-ACCGATCCACACAGAGTACTTGC-3'	58
Brachyury	F-CCTATGCGGACAATTCATCT-3' R-TACCATTGCTCACAGACCAGA-3'	56
αMHC	F-GTGAGCGGCGCATCAAGG-3' R-GCTGGAGAGGTTATTCCTCG-3'	56
βMHC	F-ACCAACCTGTCCAAGTTC-3' R-GGCTCCAGGTCTGAGGG-3'	56
Noggin	F-GGCGGCCAGCACTATCT-3' R-GACCACAGCCACATCTGT-3'	56
Chordin	F-TGGAGACCAAGCCTCAGC-3' R-TCTGAGCCATAGAATCCC-3'	56

was used for first strand cDNA synthesis using MMLV reverse transcriptase (RT, Promega) following the manufacturer's recommendations. Real-time PCR was performed using SYBR green (Bio-Rad) chemistry. The PCR was performed in an optical thermal cycler (Bio-Rad) and the data were normalized with β-actin (primers are given in Table 2). The data are presented as mean ± SEM (standard error of mean) using three replicates. The *t*-test was performed to measure the significance (*p* value) among the groups: $*p<0.05$; $**p<0.01$.

PVF fraction VII-sugar competition experiment. To determine whether the mechanism of action of PVF fraction VII could be competed by sugars, galactose and mannose were used for its binding. Glucose was used as a sugar control. The 0.9 % (w/v) solution of sugar (glucose, galactose or mannose) was used at a dilution of 1:10 with complete medium. Thus, the final concentration of galactose and mannose was 0.09 %, while the final concentration of glucose remained high because glucose was already present (4.5 g/L) in the complete medium. The sugars and PVF fraction VII (300 ng/ml) were mixed in the medium and incubated for 15 min at room temperature and then added to the cells during differentiation. In another set of experiment methyl α-galactose and methyl α-mannose were used at the same concentration (0.09 % final). The methylated sugars are believed to have higher affinity for lectins than normal sugars. All the treatments with sugar were done at 0–2 days of differentiation. Cardiac differentiation and beating cluster counting were done as above on day 10 of differentiation.

Results

Promotion of heart development in chick embryo by whole PVF. Gastrulating chick embryos treated with 30 μg whole PVF caused enlargement of heart in 83 % of the developing chick embryos after 18 h of incubation. In these embryos, brain development, axis elongation and somite number were comparable to those of controls. PVF at 3 μg concentration also caused enlargement of embryonic heart but was less effective (57 % of the embryos with enlarged heart; Table 3).

Fractionation of PVF, and characterization and identification of Fraction VII. On gel exclusion FPLC, PVF was separated into seven distinct fractions (Fig. 1A) of which, Fraction VII (PVF Fraction VII) had the maximum amount of protein (3 μg/μl). When subjected to 12.5 % SDS-PAGE under reducing condition, it revealed a single species of protein of about 27 kDa (Fig. 1B). We used PVF Fraction VII for subsequent experiments. The sequencing analysis of purified PVF fraction VII revealed a 221-amino acid protein with the sequence:

VQWHQIPGKLMHITATPHFLWGVNSNQ-
QIYLCRQPCYDGQWTQISGSLKQVDAD-
DHEVWGVNRND-
DIYKRPVDGSGSWVRVSGKLKHVSASGY-
GYIWGVNSNDQIYKCPKPCN-
GAWTQVNGRLKQIDGGQSMVYGVNSA-
NAIYRRPVDGSGSWQQISGSLKHITGSGL-
SEVFGVNSNDQIYRCTKPCSGQW-
SLIDGRLKQCDATGNTIVGVNSVDNIYRSG

Homology search on this sequence using BLAST showed it to be 100 % identical to *Limulus* lectin L6, a lipopolysaccharide-binding antibacterial protein from Japanese horseshoe crab *T. tridentatus* [17].

Promotion of heart development, angiogenesis and hematopoiesis by Fraction VII in gastrulating chick embryo. PVF Fraction VII was found to harbor the cardiac enhancing capacity. At 200 ng concentration, about 90 % of the embryos showed significantly larger

Table 3. Whole mount studies of whole perivitelline fluid (PVF)-treated embryos.

	Control embryos	Treated embryos
30 µg	(*n*=25)	(*n*=29)
No. of somites (mean ± SD)	9.4±1.63	11.6±1.3
Heart	19 Normal (76%) 1 Underdeveloped (4%) 5 Not developed (20%)	24 Large (83%) 5 Normal (17%) 0 Not developed
Brain	19 Normal (76%) 6 Underdeveloped (24%)	28 Normal (97%) 1 Underdeveloped (3%)
3 µg	(*n*=25)	(*n*=28)
No. of somites (mean ± SD)	9.04±1.4	10.46±1.72
Heart	20 Normal (80%) 4 Medially located (16%) 1 Underdeveloped (4%)	16 Large heart (57%) 7 Normal (25%) 5 Underdeveloped (18%)
Brain	20 Normal (80%) 5 Underdeveloped (20%)	22 Normal (79%) 6 Underdeveloped (21%)

Table 4. Morphological studies of embryos treated with PVF Fraction VII.

	Control embryos	Treated embryos
20 ng/embryo	(*n*=53)	(*n*=62)
No. of somites (mean ± SD)	10.32±1.7	10.18±2.19
Heart	46 Normal (86.79%) 3 Underdeveloped (5.66%) 4 Not developed (7.5%)	2 Normal (3.22%) 51 Large heart (82.2%) 9 Convoluted (14.5%)
Brain	42 Normal (79.2%) 8 Underdeveloped (15.1%)	50 Normal (80.6%) 12 Underdeveloped (19.3%)
200 ng/embryo	(*n*=8)	(*n*=11)
No. of somites (mean ± SD)	9.875±2.16	10.77±2.43
Heart	7 Normal (87.5%) 1 Underdeveloped (12.5%) 0 Large heart	1 Normal (9%) 10 Large heart (90.9%) 0 Underdeveloped heart
Brain	7 Normal (87.5%) 1 Underdeveloped (12.5%)	5 Normal (45.4%) 6 Underdeveloped (54.5%)

heart, but a little more than 50% of the embryos showed retarded brain development (Fig. 1C, D; Table 3). However, at 20 ng of Fraction VII, more than 80% treated embryos showed a significantly larger heart, with a small proportion of embryos (less than 15%) showing a very long and more convoluted heart tube (Table 4). Interestingly, the enlarged heart resulting from treatment appeared to be functioning normally. To study the arrangement and structures of chambers in these hearts, a set of treated embryos were serially sectioned and stained (Fig. 1E–R). These studies revealed that in treated embryos there was a significant enhancement of cardiac chamber space, although the wall thickness of the heart was comparable to that of controls (Fig. 1E–R). Other tissues like brain, neural tube, notochord and somites remained largely unaffected.

Some embryos (*n*=22) were allowed to develop for further 36 h undisturbed after treatment with 20 ng PVF Fraction VII. The treated embryos showed not only a dramatically enlarged heart but also apparently more extensive angiogenesis and hematopoiesis as compared to stage-matched controls. The enlarged heart of the treated embryos pumped apparently larger amount of blood through visibly more intricate and extensive network of blood vessels (Fig. 1S, T); however, a quantitative assessment of the blood flow and the amount of blood will be necessary before drawing more accurate conclusions.

Enhancement of *noggin* expression in PVF Fraction VII-treated chick embryos. HH stage 4 embryos treated with 20 ng PVF Fraction VII and incubated at 37°C for either 2 or 20–24 hours as well as corresponding controls were subjected to *in situ* hybridization using antisense riboprobe for *noggin.* After 2 h of incubation with or without PVF Fraction VII, the majority of the embryos were still at stage 4.

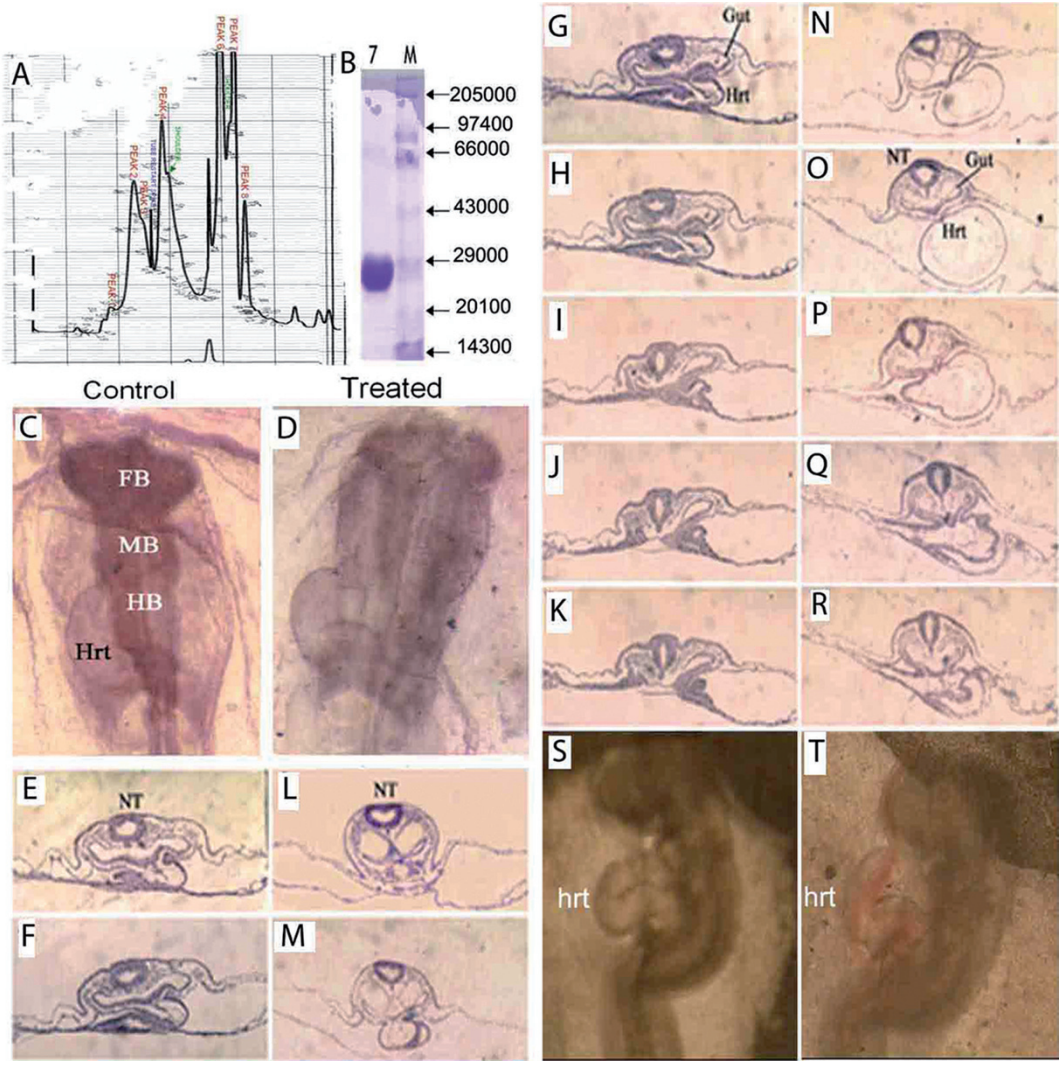

Figure 1. Purification and effects of perivitelline fluid (PVF) Fraction VII on cultured chick embryo explants. (*A*) FPLC profile of whole PVF. (*B*) On SDS-PAGE, the seventh fraction (Fraction VII) showed a single band of 27 kDa (track marked 7). When Hamburger Hamilton (HH) stage 4 chick embryos were grown in presence of 20 ng PVF Fraction VII for about 24 h (*D*), majority had significantly enlarged hearts as compared to control embryos (*C*). In the histological studies, serially sectioned treated embryo (*L–R*) clearly showed extremely large heart as compared to normal heart of a control embryo (*E–K*). Note comparable thickness of heart wall in controls and treated embryos. Cardiac development enhancing effect of PVF Fraction VII was more obvious after about 50–54 h of incubation (*T*) when compared to controls (*S*). FB: forebrain, HB: hindbrain, Hrt: Heart; MB: midbrain, NT: neural tube.

Controls showed standard pattern of expression of *noggin*, with Hensen's node staining the darkest followed by the cells of the primitive streak, while the area pellucida showed diffused, uniform staining (Fig. 2A). Treated embryos showed enhancement of staining for *noggin* in all the regions, *i.e.*, Hensen's node, primitive streak and the whole of area pellucida, but the overall pattern of distribution of transcript, indicated by the relative intensity of staining, remained comparable to controls (Fig. 2B).

After 20–24 h of post culturing incubation, embryos reached stage 10 or 11 where the heart is functional. At this stage, in untreated embryos, *noggin* was seen, if at all, uniformly expressed in the entire beating heart at a barely detectable level, while in the cells of neural tube and nascent brain, higher amount of *noggin* was seen (Fig. 2C, D). PVF Fraction VII treatment led to a significantly higher amount of *noggin* expression, if not *de novo* expression, in the entire heart (Fig. 2E, F).

Alteration of *VMHC-1* expression in PVF Fraction VII-treated chick embryos. HH stage 4 embryos treated with 20 ng PVF Fraction VII and corresponding controls, incubated at 37°C for 22–36 h (stage 11–13), were subjected to *in situ* hybridization using antisense riboprobe for *VMHC-1*. Control embryos showed normal expression of *VMHC-1* with intense staining exclusively in the ventricular myocytes

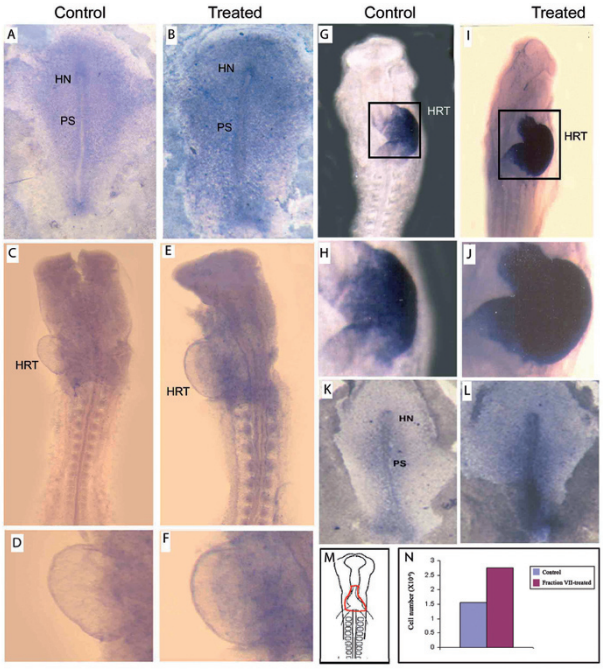

Figure 2. Molecular mechanism of action of PVF Fraction VII. On whole mount *in situ* hybridization, HH stage 4 embryo treated with 20 ng PVF for 2 h showed enhanced expression of *noggin* (*B*) as compared to untreated control embryo (*A*). After about 24 h of treatment, *noggin* expression could be faintly, but clearly, seen only in the heart of a treated embryo (*E, F*), while the heart of the control embryo (*C, D*) was almost completely devoid of *noggin* expression. Similarly, 20 ng PVF Fraction VII-treated embryos showed enhanced expression of *VMHC1*. After 24 h of treatment, in the resultant enlarged heart, a larger area expressed *VMHC1* (*I, J*) as compared to the normal pattern (*G, H*). Normal expression pattern of pan mesodermal marker *Brachyury* was localized to the primitive streak in controls (*K*), while this was more intense and extended to the cells surrounding the posterior primitive streak after 2 h of treatment with 20 ng PVF Fraction VII (*L*). When the total numbers of cells making up the entire heart (*M*, shown in red) of control and treated embryos were compared, it was obvious that the PVF Fraction VII treatment caused almost doubling of cell number in the heart (*N*). HN: Hensen's node, HRT: heart, PS: primitive streak.

(Fig. 2G, H). The rest of the embryo was completely devoid of staining. Amongst the PVF Fraction VII-treated embryos, a majority showed intense staining in large ventricles, while a few showed staining in the entire heart (Fig. 2I, J).

Modulation of *Brachyury* expression in PVF Fraction VII-treated chick embryos. Embryos treated with 20 ng PVF Fraction VII for 2 h along with controls were used for *in situ* hybridization using antisense riboprobe for *Brachyury*. In untreated embryos, the expression was prominently seen in the primitive streak, while the rest of the area pellucida was fairly clear of staining (Fig. 2K). In treated embryos there was a clearly enhanced staining for *Brachyury* along the primitive streak (Fig. 2L). Additionally, quite intense staining was seen in cells in the region starting from just caudal to Hensen's node that often blurred the borders of primitive streak posterior region.

Augmentation of cell number in the larger hearts formed due to Fraction VII treatment in chick embryo. Control (n=12) and Fraction VII-treated (n=12) embryos were used after 24–32 h of incubation. On average, the hearts of control embryos had 1.13×10^4–1.975×10^4 cells, whereas the enlarged heart of treated embryos consisted of 2.15×10^4–3.443×10^4 cells. Thus, the enlarged heart of a treated embryo comprised 175–190 % more cells (Fig. 2M, N).

Enhancement of some cardiac-specific genes and *noggin* by Fraction VII in chick embryo. At stage 5, among the genes studied, *GATA 5* showed the maximum fold increase in its transcription (more than 55 fold) upon exposure to PVF Fraction VII, followed by *noggin* in cells of the HFRs (42 fold), while in the rest of the embryo it was not significantly changed. Transcription of *Nkx2.5* was up-regulated by about 20 fold, while transcripts for structural proteins of cardiac myocytes like *VMHC1* and *AMHC1* were not much altered. At Stage 7, *GATA 5* expression level in treated embryos was similar to that seen in stage-matched controls. Increment in *Nkx2.5* and *noggin* were modest and surprisingly *AMHC1* showed the highest increment at a level of 18 fold. At stage 10, structural proteins showed maximum up-regulation at less than 20 fold, while *Nkx2.5* was enhanced by 17 fold, indicating that the jump in the expression levels of cardiac regulatory and structural genes are the maximum at this stage of development (Fig. 3D, E).

Concentration-dependent enhancement of ES cell differentiation into cardiomyocytes due to PVF Fraction VII exposure. Addition of the PVF Fraction VII during differentiation resulted in an increase in the number of beating clusters per EB (4.92±0.48 clusters per EB) (Fig. 3F, H, I) within 5 days of treatment as compared to the control (2.93±0.34 clusters per EB). The proportion of EBs beating in control (89.3±4.8 %) and the treated (95.2±4.8 %) set remained nearly identical because in the control set itself almost all the EBs showed pulsating activity (Fig. 3G). This enhancement was most significant when the treatment was given at early time point during differentiation (0–2 days: Fraction VII, 5.2±0.7; control, 2.97±0.4; Fig. 3F), whereas the control and treated samples showed comparable results when PVF Fraction VII was added at later stages (up to 5 days; Fig. 3H). Interestingly, at the intermediate time period (2–5 days), there was slight

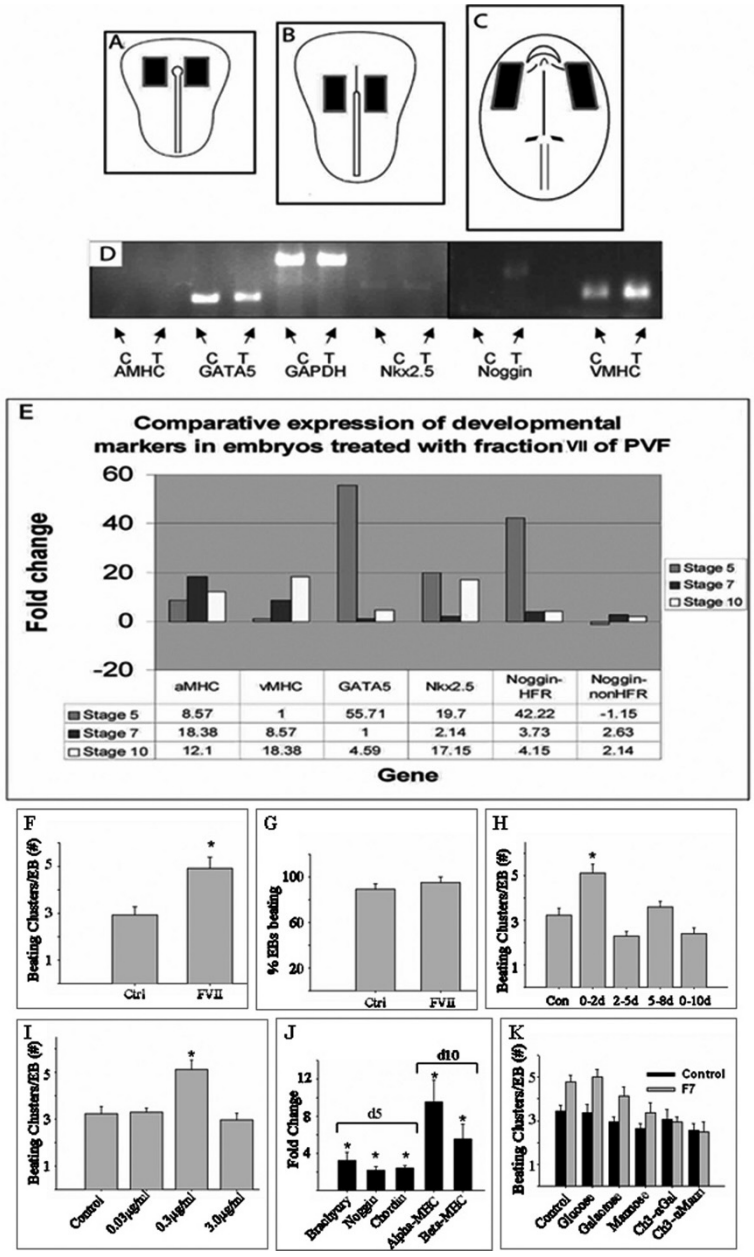

Figure 3. (*A–E*) Enhancement of expression of cardiac-specific marker genes due to treatment with 20 ng PVF Fraction VII. Embryonic regions known to contain cardiac progenitor cells according to published fate maps were excised from control (*C*) and treated (*T*) embryos at stages 4 (*A*), 5 (*B*) and 7 (*C*), and from fused cardiac tube at stage 10–12. (*D*) Total RNA was extracted and subjected to quantitative RT PCR using primers for the genes *AMHC1, GATA5, Nkx2.5, noggin* and *VMHC1*. House keeping gene *GAPDH* was used as loading control. (*E*) When the expression profiles of some these genes were compared across different stages, differential modulation of cardiac specific genes by PVF Fraction VII becomes obvious. (*F–K*) Induction of cardiomyocyte differentiation in mouse embryonic stem cells by PVF Fraction VII and its inhibition by methylated sugars. Treatment of mouse ES cells early (0–2 days of differentiation) with Fraction VII increased the number of beating clusters of cells per embryoid body (EB) (F) after10 days of differentiation, while the proportion of EBs beating in control and the treated set remained nearly identical because in the control set itself almost all the EBs showed pulsating activity (*G*). The cells were treated with Fraction VII during early time point of differentiation (0–2 days) and observed on days 5+5 (n=6). Fraction VII treatment was most effective in enhancing the frequency of beating clusters within each EB (*H*) early during differentiation (0–2 days), while delayed treatment was not so effective. The EBs were treated for different time points and observed after 10 days of differentiation (days 5+5; n=3). The most effective concentration for promoting cardiomyogenesis monitored at days 5+5 of differentiation was found to be 300 ng/ml (n=3) (*I*). Addition of PVF Fraction VII led to an early up-regulation of the expression of *noggin* and *chordin* (days 5+5; n=3) (J). There was a concurrent surge in the expression of genes which regulate both mesoderm formation (*Brachyury* in days 5 + 5; n=3), and cardiac myogenesis (αMHC and βMHC in days 5+10; n=3) in mouse EBs (*J*). The cardiogenic activity of PVF Fraction VII was partially blocked by the galactose and mannose sugars (days 5+5) (*K*). These sugars did not block the endogenous cardiac differentiation when added to the medium. However, when pre-incubated with Fraction VII, there was marginal impairment ($p<0.05$) in the inductive influence of Fraction VII by mannose when compared with that of Fraction VII alone or Fraction VII with glucose. Methylated sugars were found to be more potent in inhibiting Fraction VII activity ($p<0.05$) than their normal counterparts. Both methyl α-galactose (CH3-a-Gal) and methyl α-mannose (CH3-a-Mann) singly inhibited the endogenous cardiac differentiation when added to the medium (n=3–5).

attenuation in the cardiomyogenesis upon exposure to PVF fraction VII.

To assess the optimum concentration of PVF Fraction VII to get maximum enhancement of cardiomyogenesis, PVF Fraction VII at three different concentrations (30 ng, 300 ng and 3 μg/ml) was used at early stages of differentiation (0–2 days). The EBs were observed at day 5+5. The number of beating EBs as well as beating clusters per EB was counted. Among the concentrations studied, the PVF Fraction VII at 300 ng/ml PVF Fraction VII was found to be optimal (5.23±0.7 beating clusters/EB, Fig. 3I) and it not only expedited the cardiomyogenic onset but also promoted the differentiation to a significant extent (Fig. 3I). Higher concentration (3 μg/ml with 2.97±0.28 beating clusters/EB) or longer exposures (data not shown) did not aid cardiac differentiation.

Effect of PVF Fraction VII on development-specific gene expression in EBs. Since the PVF Fraction VII had a cardiogenic activity and could enhance cardiac differentiation in a time-dependant manner, the expression of mesoderm and cardiac markers in response to Fraction VII was measured (Fig. 3J). Interestingly, addition of Fraction VII (0.3 μg/ml) led to an increase in *Brachyury* expression in early differentiating EBs (day 5) as determined by quantitative real-time PCR (Fig. 3J). Consistent with the positive influence of Fraction VII, an increase in cardiac markers was also evident. Expression of cardiac-specific α-myosin heavy chain (*αMHC*) and *βMHC* was significantly increased (Fig. 3J). Taken together, Fraction VII expedited the process of mesoderm induction and subsequent cardiomyogenesis from the ES cells *in vitro.* Since Fraction VII increased the expression of *noggin* in chick embryos, we also estimated its expression level in treated ES cells. As seen in Figure 3J, addition of PVF Fraction VII enhanced the expression of both *noggin* and *chordin* at early stages (day 5) in differentiating EBs.

Inhibition of PVF-induced cardiac promotion in ES cells by sugars. To determine whether the effect of PVF Fraction VII (a lectin) could be blocked by sugars, galactose and mannose (two most common monosaccharides besides glucose) were selected, keeping glucose as control as it is required for cardiac differentiation from ES cells *in vitro* and is a normal constituent of culture medium. Compared to the glucose control, these two sugars did not cause an appreciable decrease in the number of beating clusters per EB (Fig. 3K). When methylated sugars (methyl α-galactose and methyl α-mannose, known to bind lectins more efficiently) were added to the differentiating media with PVF Fraction VII, cardiac enhancing activity of PVF Fraction VII was blocked, with the number of beating clusters reduced to 2.93±0.23 due to methyl α-galactose and 2.5±0.43 due to methyl α-mannose, as compared to 4.8±0.3 in control with PVF Fraction VII (Fig. 3K).

Discussion

In the present study, we investigated the participation of a novel molecule in the cardiac morphogenesis in chick embryo. We discovered that total PVF from stage 19 horseshoe crab embryos [19] can enhance axial elongation, somite differentiation, brain compartmentalization and cardiogenesis at 30 μg concentration. The most prominent of the observed effects was an extremely enlarged heart.

In an attempt to identify the active principle(s) that bring about such spectacular effects, we fractionated the PVF. On FPLC, PVF was separated into seven fractions with Fraction VII harboring the highest amount of protein. It contained a lectin with molecular mass of 27 kDa and Blast analysis showed that its amino acid sequence was identical to Lectin L6 from hemocyte lysate of Japanese horseshoe crab *T. tridentatus* [17]. Lectin L6 binds lipopolysaccharide from the outer membrane of Gram-negative bacteria with high affinity and act as a bacteriostatic agent to prevent infections in the crab [17].

At a 20 ng concentration, PVF Fraction VII could induce cardiac enhancement in a majority of the embryos, while other features like neural tube, notochord and gut from the treated embryos were comparable to controls. This clearly shows that we have been able to isolate, identify and characterize the molecule responsible for promoting cardiac development amongst a mixture of molecules in the PVF. Histological analysis showed that the enlarged heart from treated embryo enclosed a very large chamber space, while the chamber walls were of normal thickness, comparable to those of untreated control embryos. This indicates that the heart enlargement is more likely to be due to cardiac hyperplasia rather than due to hypertrophy. To confirm the likelihood of cardiac hyperplasia, we compared the total number of nuclei in the untreated and treated hearts. For this, the entire hearts from the bulbus cordis to the sinoatrial region were cut and pooled; the cells were macerated and nuclei stained with ethidium bromide. We counted the fluorescent nuclei under UV to estimate the total number of cells per heart. Treated hearts comprised almost twice the number of cells as compared to control hearts confirming that the heart enlargement was indeed due to a larger number of

cells taking the cardiac fate and not because of larger sized cells.

When the embryos were allowed to grow in presence of Fraction VII for longer, the enlargement of heart was even more obvious in comparison to matched control. Further, there was a significant enhancement of angiogenesis and hematopoiesis. This action of PVF Fraction VII became obvious only after the prolonged exposure.

Interestingly, not only the number of cells increased due to Fraction VII treatment, but the expression profiles of several cardiac-specific regulatory and structural genes were also altered in both chick embryo and mouse ES cells as seen in real-time RT PCR studies. In PVF Fraction VII-treated chick embryo *GATA 5*, a zinc finger transcription factor prominent in precardiac mesoderm [31] showed the maximum enhancement of expression. The precise role of the GATA family of molecules in cardiac myogenesis is not known, but studies on *GATA5* null mutant embryonic mice indicate that it is probably important in early heart tube formation [32]. Its over expression early in development may be one of the causes of cardiac promotion in Fraction VII-treated chick embryos. Expression of *NkX2.5*, a homeobox marker of the early stages of the heart field [11], was also significantly increased in the embryos due to treatment with Fraction VII. Overexpression of *Nkx2.5* in frog and zebrafish leads to larger than normal hearts in otherwise normal embryos [33]. This suggests that the propensity to become heart was controlled, at least in part, by achieving a threshold level of *Nkx2.5* and that raising the border zone level by external supply brought a larger number of cells to threshold [32]. This rationale might also hold true for embryos treated with Fraction VII. If the lectin in Fraction VII enhances the *NKx2.5* level through a yet-unknown mechanism, it could lead to the present phenotype of larger heart in an otherwise normal embryo. Maximum enhancement of *GATA 5* and *Nkx2.5* might be because these families of genes cooperate with each other to activate cardiac gene expression [12] and also regulate each other's expression through mutually reinforcing positive feedback loops [34]. One of the most intriguing observations of this study is enhancement of *noggin* transcription selectively in the HFR due to Fraction VII treatment in both intact embryo and ES cells. Classically, *Noggin* is known to promote neural fate by inhibiting BMP signaling [35, 36]. Similarly, the expression of *Chordin*, another inhibitor of BMP was also significantly enhanced in treated mouse ES cells. A study by Yuasa *et al.* [37] using ES cells and mouse embryos had shown that transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation. Our results with a phenomenal upsurge of *noggin* expression (more than 40 fold in chick embryo) specifically in the HFR within a few hours of treatment with Fraction VII at stage 4, followed by almost normal levels during subsequent development (about 4 fold at stages 7 and 10), may reflect this phenomenon. This surge of *noggin* expression might be another, possibly primary, cause of cardiac enhancement. Slightly elevated expression of *noggin* seems to continue even in fully functional heart at stages 10–11 of the treated embryos as seen in *in situ* hybridization. Similarly, an enhancement in the expression of two inhibitors of BMP, *noggin* and *chordin* was noted in mouse ES cells. This hinted at the possibility that Fraction VII-mediated suppression in BMP signaling during early differentiation might be facilitating mesoderm induction and subsequent cardiomyogenesis in both chick embryo and mouse ES cells. Overall enhancement of *Brachyury* expression, a marker for mesodermal cells [38], might be because of a greater number of cells taking the cardiac mesodermal fate. The higher amount of transcripts for cardiac-specific MHCs that encode structural proteins and are markers for cardiac myocyte differentiation obviously occurred because larger numbers of cells were recruited to the cardiac lineage.

We visualize the events after treatment with PVF Fraction VII in the following manner. Treatment of embryos somehow leads to stimulation of *noggin* expression significantly in the HFR. This, in turn, leads to extended cardiac differentiation [37] as seen from increased *GATA* and *Nkx2.5* expression. The end result of these events is a larger amount of heart tissue as seen from the significantly higher number of total cells making up the heart and the increased expression of *VMHC1* and *AMHC1*. If these really are the events that occur in the chick embryo, the same should work in mouse ES cells as an experimentally created short burst of *noggin* expression leads to cardiac differentiation in that system [37]. Indeed that is what we found when we examined the effects of PVF Fraction VII on *noggin* (and *chordin*) expression and the subsequent cardiomyocyte differentiation in mouse ES cells. Transient inhibition of BMP signaling by its inhibitors appears to be a crucial step in this phenomenon.

Enhanced recruitment of mouse ES cells into beating myocyte clusters due to treatment with PVF Fraction VII demonstrated that the cardiac enhancing effect of this molecule was not confined to chick but extended to mammalian cells as well. Fraction VII induces cardiomyogenesis in ES cells *in vitro* and the effect was more specific and pronounced upon exposure of cells to Fraction VII during the onset of differentiation compared to that at later stages, indicating a sensitive

period for its cardiac-enhancing potential. Blocking of cardiogenic capacity of Fraction VII with methylated sugars, which have a high affinity for lectins, indicated that the sugar/glycoprotein binding feature of this lectin was indeed involved in cardiac promotion. Modulation of expression of a whole range of genes starting from the basic mesodermal marker like *Brachyury* to the terminal differentiation markers like myosins indicates that this molecule might be intervening at the very beginning of the pathway leading to the formation of cardiac myocytes, probably at the time when the cells are specified to cardiac lineage.

We strongly believe that this molecule will prove to be an important tool in the study of cardiomyocyte differentiation and plan to further elucidate the molecular mechanism of its action.

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