Growth Factors in Axolotl Cardiac Induction

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Introduction. The amphibian heart develops from embryonic mesoderm. Embryonic explant studies have demonstrated that amphibian cardiac development requires an inductive interaction. The timing of cardiac induction varies among amphibian species: it is completed by the end of gastrulation in *Xenopus laevis* (Sater and Jacobson, 1989), while it is not completed until mid-neurula stages in *Ambystoma mexicanum* (Smith and Armstrong, 1990). This delay in cardiac induction makes the axolotl a particularly useful animal for the study of heart development. The probable source of a cardiac inducing substance in urodeles, as determined by explant experiments, is the pharyngeal endoderm (Fullilove, 1970). This inducing substance has not yet been identified.

Peptide growth factors are thought to be involved in a variety of inductive interactions in amphibian development. Localization experiments have demonstrated that many growth factors are highly expressed throughout embryonic development (reviewed in Whitman and Melton, 1989). A variety of *in vitro* and *in vivo* experiments using *Xenopus laevis* have suggested that fibroblast growth factor (FGF), activin, and Wnt may be important regulatory molecules in mesoderm induction (Amaya et al., 1991; Thomsen et al., 1990; Sokol et al., 1991). Growth factors have also been found to affect the morphogenesis of a variety of tissues *in vitro*. Given the evidence suggesting the regulatory function of growth factors in embryonic development, the possibility that growth factors could stimulate cardiac development in mesodermal explants was investigated (Muslin and Williams, 1991).

Results. Initially, the timing of cardiac induction in axolotls was re-examined using neurula-stage embryos. Pre-cardiac mesoderm with underlying ectoderm was isolated from embryos and maintained in hanging-drop cultures in Modified Steinberg's Solution (MSS), pH 7.4, with added antibiotics (Jacobson and Duncan, 1968; Armstrong and Malacinski, 1989). Some mesoderm was cultured in the presence of anterior pharyngeal endoderm. Embryos were staged according to the system of Schreckenberg and Jacobson (1975). Explants were observed daily for the presence of beating heart tissue using a compound microscope. Isolated pre-cardiac mesoderm derived from early-neurula (stage 14) embryos rarely formed beating cardiac tissue in culture (6/232, 3%), while mesoderm derived from late-neurula (stage 18) embryos frequently formed beating tissue in culture (10/15, 67%). If anterior endoderm was added to isolates of pre-cardiac mesoderm taken from early-neurula embryos, the rate of heart formation increased markedly (see Table 1).

To test the ability of growth factors to stimulate beating cardiac tissue formation in explants, pre-cardiac mesoderm isolated from early-neurula embryos was used. Isolates were incubated in MSS in the presence of various growth factors and were then placed in hanging drop cultures. Growth factors tested included basic FGF, platelet-derived growth factor BB (PDGF), epidermal growth factor (EGF), transforming growth factor beta1 (TGF-β1), insulin, retinoic acid, and PIF (murine activin A). Two growth factors, PDGF and TGF-β1, appeared to increase beating heart formation in mesodermal explants. This increase was statistically significant in both cases by Chi-square analysis (see Table 1). Mesodermal explants exposed to TGF-β1 or PDGF had a delayed onset of beating compared to explants with added endoderm.

The ability of TGF-β1 to respecify posterior mesodermal cell fate was next examined. When early-neurula posterior mesodermal explants were incubated in the presence of TGF-β1, beating heart formation was not detected (0/23 explants).

Growth factors may also regulate cardiac development by inhibiting heart formation. Previous studies have demonstrated that co-culturing pre-cardiac mesoderm with neural tissue inhibits beating heart tissue formation (reviewed in Jacobson and Sater, 1988). Given that FGF inhibits skeletal muscle development in certain cell lines (Vaidya et al., 1989), we theorized that bFGF may play an inhibitory role in cardiac induction. To test this idea, we incubated early-neurula pre-cardiac mesodermal explants in the presence of both TGF-β1 and bFGF. Explants exposed to both

growth factors did not exhibit an increase in beating heart formation over control explants (see Table 1), in contrast to explants exposed to TGF-β1 alone. The possible inhibitory effect of bFGF was also tested using mesodermal explants derived from mid-neurula (Stages 15-17) embryos that formed beating heart tissue in culture in 37% of cases (13/38). When such explants were exposed to bFGF, beating heart formation decreased significantly (5/38, 13%).

Discussion. Previous studies have suggested that cardiac development in urodeles is dependent on the release of a signal (inducer) from anterior endoderm which directs mesoderm to differentiate into myocardium (Jacobson and Sater, 1988). Cardiac induction is completed by mid-neurula-stages in the axolotl (Smith and Armstrong, 1990). We have tested the ability of various growth factors to enhance heart formation in early-neurula mesodermal explants. Two growth factors, PDGF and TGF-β1 were found to stimulate beating heart formation in such explants. In contrast, basic FGF was found to inhibit heart formation in pre-cardiac mesodermal explants. Although our data does not prove that these factors are involved in the regulation of heart formation *in vivo*, all three of these factors have been found in neurulating amphibian embryos. One interpretation of our data is that TGF-β1 and PDGF mimic the effects of a natural inducer molecule, stimulating a pattern of gene expression which leads to cardiac development. Alternatively, it is possible that TGF-β1 and PDGF merely enhance cardiac gene expression in tissue already committed to form heart.

In this study, mesodermal explants were observed for the presence of beating heart tissue. The onset of rhythmic contractions is a late manifestation of cardiac induction. It is possible that other growth factors tested stimulate cardiac development but do not direct sufficient protein synthesis to allow rhythmic beating to occur. An early and highly specific marker of cardiac differentiation would be useful to further delineate the process of cardiac induction.

Another limitation of this study is that pre-cardiac mesoderm cannot be easily isolated until early-neurula-stages. Any inductive interaction that might occur prior to neurulation can not be fully investigated using this methodology. The disruption of various growth factor signalling pathways in blastula- and gastrula-stage embryos will be helpful to further examine the process of cardiac development in amphibians. This can be accomplished by injecting mRNA (encoding mutant

Table 1
Effect of Growth Factors

	+ Growth Factor No. Beating/Total (%)	Control No. Beating/Total (%)
St. 14 Mesoderm	---	6/232 (3%)
+ Insulin	0/25 (0%)	2/25 (8%)
+ bFGF	0/23 (0%)	1/23 (4%)
+ Retinoic Acid	1/29 (3%)	0/29 (0%)
+ PIF (activin)	1/14 (7%)	0/14 (0%)
+ TGF-β/+ bFGF	2/27 (7%)	1/27 (4%)
+ EGF	7/32 (22%)	2/32 (6%)
+ PDGF BB	12/29 (41%)	0/29 (0%)
+ TGF-β1	20/34 (59%)	0/34 (0%)
+ Anterior Endoderm	---	12/19 (63%)

The effect of added growth factors on beating heart formation in stage 14 mesodermal explants. Porcine insulin (600 ng ml^{-1}), human recombinant basic FGF (50 ng ml^{-1}), retinoic acid all-trans (1 μM), PIF (1:3 dilution), human recombinant EGF (50 ng ml^{-1}), human recombinant PDGF BB (50 ng ml^{-1}), or human recombinant TGF-β_1 (30 ng ml^{-1}) were added to stage 14 anterolateral mesodermal explants at the time tissue was removed from embryos. Paired control explants were incubated in salt solution alone. All explants were observed for 30 days for the presence of beating cardiac tissue. The uppermost row represents pooled mesodermal explants incubated in control solutions. The lowest row represents mesodermal explants with anterior endoderm included. Beating heart formation in explants was usually detected within 14 days of culture.

growth factor receptors) or antibodies into 1- or 2-cell embryos (see Amaya et al., 1991). These experiments are currently ongoing in several laboratories.

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