Int. J. Dev. Biol. 40: 715-718 (1996)

Activin treated urodele ectoderm: a model experimental system for cardiogenesis

TAKASHI ARIIZUMI[1], SHINJI KOMAZAKI[2], MAKOTO ASASHIMA[1] and GEORGE M. MALACINSKI[3]*

[1]*Department of Biology, The University of Tokyo, Tokyo, Japan,* [2]*The Second Department of Anatomy, Saitama Medical School, Saitama, Japan and* [3]*Department of Biology, Indiana University, Bloomington, USA*

ABSTRACT The tissue interactions which comprise the inductive phenomena associated with urodele heart morphogenesis are relatively well understood. In order to take full advantage of the experimental potential of this system formulation of an *in vitro* tissue culture system would be very helpful. Herein are described conditions for culturing *Cynops pyrrhogaster* early gastrula ectoderm tissue in the presence of the peptide growth factor activin. Two-week old explant cultures frequently displayed beating heart-like rudiments within. The beating frequency was measured and the extent to which cytodifferentiation mimicked normal heart differentiation assessed. Both measurements provided optimistic assessments which should encourage further exploitation of this model system.

KEY WORDS: *urodele heart induction, activin-treated heart, amphibian heart culture, urodele cardiogenesis, newt heart development*

Introduction

Amphibian heart development provides favorable features for studying morphogenesis and differentiation. The induction phenomenon it exhibits has been well-characterized (reviewed by Jacobson and Sater, 1988; Neff *et al.*, 1996). Included among the best-known features are the following. Anterior pharyngeal endoderm contacts prospective cardiogenic mesoderm and stimulates morphogenesis and differentiation. This is a long process, which is initiated in urodeles certainly by the stage of neurulation, and likely much earlier. The tissue displacements and morphogenesis (e.g., folding) displayed during cardiogenesis are profound and easily monitored. Differentiation can be easily assayed, since the cardiac muscle proteins are well-characterized. And cytodifferentiation is relatively easy to recognize, as will be noted in the data described below. Nevertheless, progress on understanding urodele cardiogenesis — especially at the molecular level — has, in the opinion of many researchers, stalled. The molecular analysis of inductive events and the identification of genes expressed during the earliest phases of differentiation remain largely unknown.

An advance which should facilitate progress in understanding the molecular biology of cardiac induction and further our knowledge of early gene expression events is described herein: The development of a newt ectoderm culture system, which when treated with the peptide growth factor activin, exhibits a substantial array of the cytodifferentiated and functional properties of a naturally *(in vivo)* developing heart. With this experimental system it is possible that progress in some of the refractory areas of amphibian cardiogenesis research will finally be attainable.

Preparation of cultures

Cynops pyrrhogaster embryos were obtained from hormone-stimulated females (Ariizumi and Asashima, 1995) and staged according to Okada and Ichikawa (1947). At stage 11 (early gastrula; approx. 2.3 mm dia), the jelly coat and vitelline membrane were manually removed in Holtfreter's solution (HS; pH 7.4). Ectodermal sheets (1.0x1.0 mm) were cut and treated with an activin solution for 1 h (Fig. 1). As a control, they were cultured in HS containing 0.1% bovine serum albumin for the same period. After washing in two changes of HS with gentle pipetting, they were transferred to 3% agar-coated culture dishes filled with activin-free HS ("isolation culture"). Some of them were sandwiched between 2 sheets of ectoderm (1.5x1.5 mm) obtained from other early gastrulae ("sandwich culture"). After 2-5 weeks culture in HS (20°C), they were examined histologically (Ariizumi and Asashima, 1995) as will be described below.

An external view of a 2-week-old isolation culture which contains a beating heart-like rudiment inside is illustrated in Figure 2A. The formation of heart rudiment was confirmed in a histological section (Fig. 2B). At 2 weeks, the sandwich cultures formed trunk-tails, with the beating-heart rudiment inside (Fig. 2C,D). Approximately 30% of activin-treated ectoderm explants in the isolation-culture mode developed such a beating-heart rudiment. In the sandwich-culture mode, approx. 50% of activin-treated explants developed a beating heart. In both modes, control cultures never exposed to activin developed as atypical epidermis (100%) and

Abbreviations used in this paper: HS, Holtfreter's solution.

***Address for reprints:** Department of Biology, Indiana University, Bloomington, IN 47401, USA. Fax: 812.855-6705. e-mail: malacins@indiana.edu

0214-6282/96/$03.00

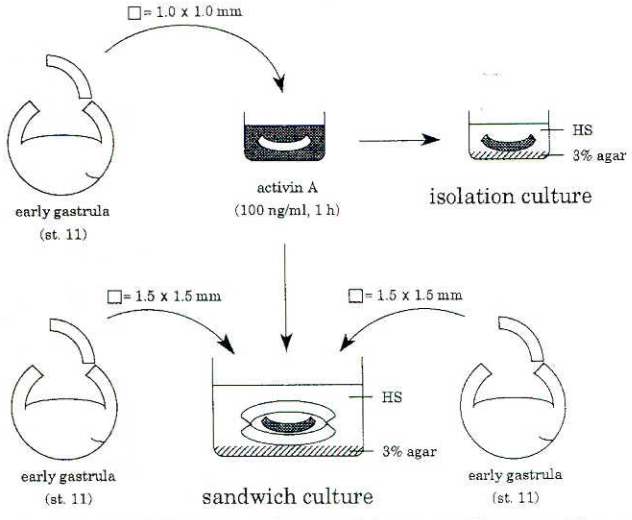

Fig. 1. Protocol for preparing activin-treated newt *(Cynops pyrrhogaster)* cultures for cardiogenesis studies. *The human recombinant activin A employed in this protocol was obtained by purifying the culture supernate of CHO cells which express the human inhibin ßA chain gene (Murata et al., 1988). This activin gives a single peak in reversed phase HPLC and a single band in silver-stained SDS-page in both reducing and non-reducing conditions. It was dissolved in HS at a final conc. of 100 ng/ml. 0.1% bovine serum albumin was added to this solution to retard adsorption of activin A to glass or plastic surfaces.*

never formed a beating heart (Fig. 2E,F). The heartbeat in 2-week-old isolation cultures was counted at various temperatures from 5°C to 30°C (Fig. 3). It was regular, and displayed the temperature-dependent frequency expected of a normal, beating newt embryonic heart (Taguchi et *al.*, 1989; Uehara *et al.*, 1989). These beating characteristics were observed in virtually all of the cases scored as "beating heart." That is, a beating heart was the sole criterion for scoring an explant culture as positive for heart development. Five-week-old sandwich cultures with a beating heart, examined with the transmission electron microscope, exhibited the cytodifferentiation pattern expected of a functioning heart, as illustrated in Figure 4.

Future prospects and critique

Since the protocols described herein employ "function" as the criterion for successful explant differentiation, there can be little doubt or uncertainty about the extent to which newt ectoderm responds to activin treatment. Clearly, 50% of the sandwich-culture ectoderm explants develop into beating hearts. It is likely that the other 50% display heart differentiation to one extent or another, but never develop to a fully functional state. That is, if less rigorous criteria (such as the presence of one of the myosin, actin or other muscle-specific proteins) were employed, the scoring result would likely show more "positives" than the 50% observed in the present studies.

Using *Xenopus* in the protocol diagrammed in Figure 1, we have never detected a beating heart in any of the *Xenopus* explants (Ariizumi and Asashima, 1994). The induction properties of activin on newt ectoderm are different from those on *Xenopus* ectoderm: the dose-dependent mesoderm induction of activin shown on *Xenopus* ectoderm (Ariizumi *et al.*, 1991) is not clearly observed in newt ectoderm. The activin-treated newt ectoderm differentiates solely into yolk-rich tissues, which are identified as endodermal tissues because of their histological characteristics, and the frequency of mesoderm differentiation is relatively low (Ariizumi and Asashima, 1995). In the sandwich culture, these tissues often induce complete trunk-tails composed of central nervous system and axial mesoderm (Ariizumi and Asashima, 1995; Fig. 2C,D in the present report). These results agree with those of the previous report using presumptive pharyngeal endoderm immediately above the blastopore of newt early gastrulae (Hama e*t al.*, 1985). Differentiation of the heart rudiment during normal development is known to depend on the influence of anterior pharyngeal endoderm (Jacobson and Sater, 1988). Although it has not been confirmed that the yolk-rich endodermal tissues induced by activin in newt ectoderm are identical to anterior pharyngeal endoderm, the presence of these tissues seems to be requisite for the formation of beating hearts. Thus, it appears that for *in vitro* studies the urodele might be a more amenable amphibian model system.

The role of activin as a regulatory molecule in early amphibian embryogenesis has been well documented (reviewed by

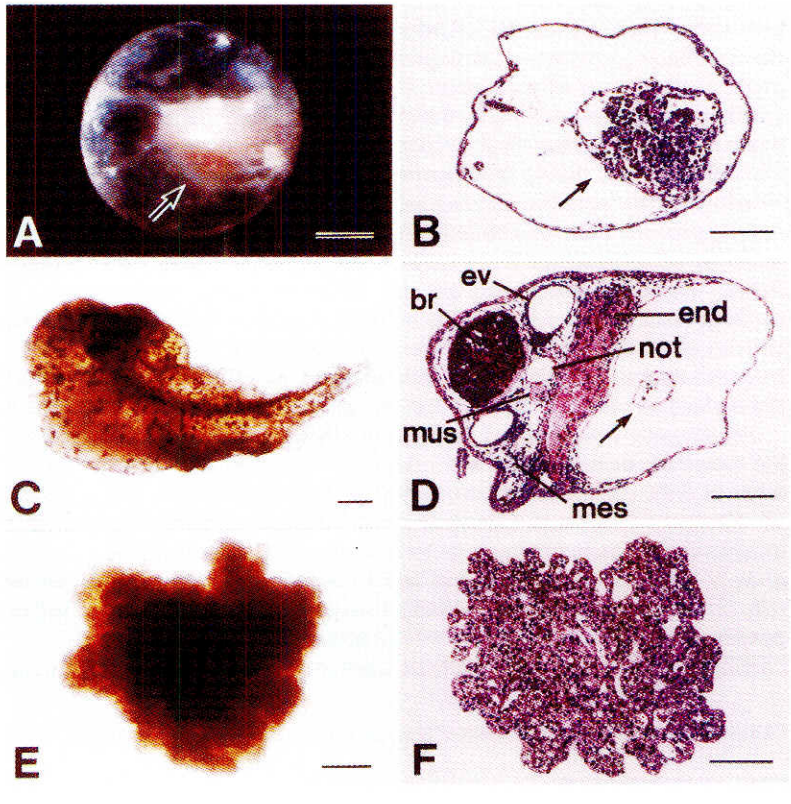

Fig. 2. External views and histological sections of 2-week old cultures. (A) *An isolation culture showing a beating heart-like rudiment in its interior* **(B)**. **(C)** *A sandwich culture showing trunk-tail formation, with a beating heart rudiment inside* **(D)**. **(E)** *Control cultures (both in the isolation- and sandwich-culture modes) never exposed to activin became wrinkled, and developed as atypical epidermis* **(F)**. *Arrow, heart rudiment; br, brain; end, endoderm; ev, ear vesicle; mes, mesenchyme; mus, muscle; not, notochord. Bar, 200 mm.*

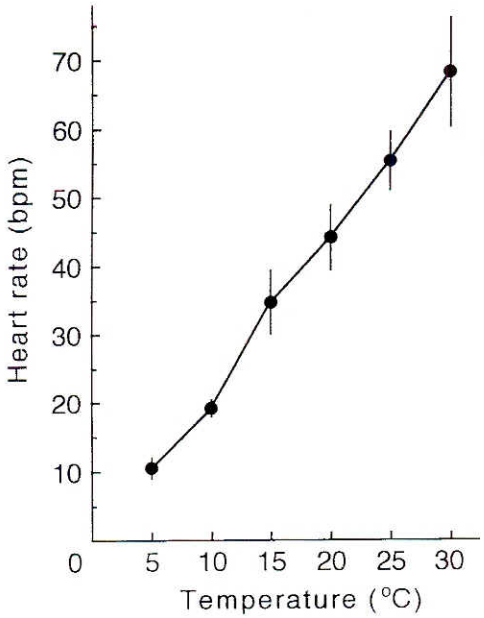

Fig. 3. Measurement of pulsation of heart rudiment developed from activin-treated ectoderm after 2-week culture period (in the isolation-culture mode). *The beat frequency was measured at various temperatures from 5°C to 30°C. Explants were incubated for 30 min at each temperature before heart rate was measured. Each point represents 4 explants with a beating heart. Bars show the S.E.*

Asashima, 1994): activin has a potent mesoderm-inducing activity on the *Xenopus* ectodermal explants (Asashima *et al.*, 1990) and induces almost all mesodermal tissues in a dose-dependent manner (Ariizumi *et al.*, 1991). Activin protein is present in the *Xenopus* early embryo maternally (Asashima *et al.*, 1991). Recent results reveal, however, that activin might act in combination with other regulatory molecules (e.g., other peptide growth factors) during cardiogenesis (Sugi and Lough, 1995; also see Muslin and Williams, 1991). A culture system such as the one described in this report should provide a good system for analyzing phenomena such as the role of multiple signal molecules in promoting cardiac induction. Not only could positive signal molecules be searched for, but as well signal molecules which act to inhibit the action of peptide growth factors such as activin could be identified with this system. We are of the opinion that it is highly unlikely that only "positive" signal molecules act during the course of an embryological induction. "Negative" signals which act to down-regulate the action of opposing forces likely, in our opinion, play a role in the complex circuitry of cardiogenesis. This activin/explant system should provide a useful bio-assay for identifying such additional regulatory molecules which comprise the mechanism(s) of cardiac induction.

Shortcomings are, however, associated with this urodele experimental system. First is of course the paucity of molecular markers for urodele cardiogenesis. Most amphibian studies have focused on *Xenopus* (e.g., Logan and Mohun, 1993; Cox and Neff, 1995). Perhaps it would be possible to use the available *Xenopus* cDNA markers (or even those from zebra fish [e.g., Stanier and Fishman, 1994] or *Drosophila* [e.g., Bodmer, 1995]) to screen appropriate urodele cDNA libraries. As well, as mentioned in Neff *et al.* (1996), it should be possible to perform differential display analyses to detect relatively low abundance gene products in early stages of cardiogenesis. Second is the observation that only 50% of the activin-treated sandwich-culture explants display a beating heart. Future experiments will be designed to improve that frequency. But as mentioned above, since a beating heart represents a highly complex differentiation program, it is likely that the other explants exhibit heart development to one extent or another. Considering both positive and negative features, it is likely that activin-treated urodele explants will be a useful model for a variety of studies on early heart development.

Acknowledgments

This research was funded, in part, by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

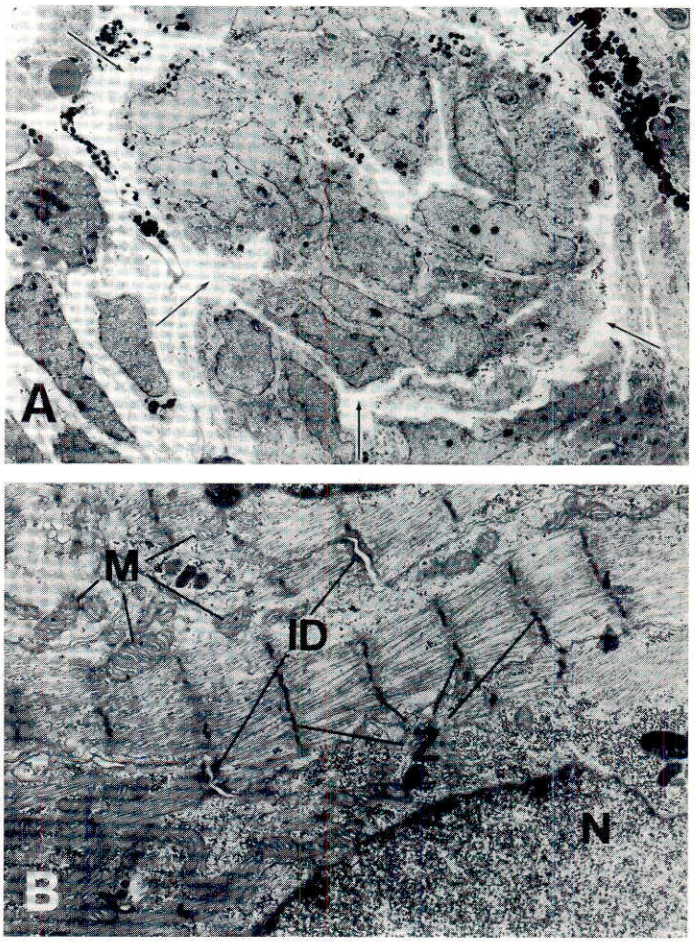

Fig. 4. Transmission electron microscopy of 5-week old activin-induced heart rudiments (in the sandwich-culture mode). (A) *A large block of myocardial cells (arrows) can be seen. These cells are well developed because yolk platelets are absent. x1,960.* **(B)** *Higher magnification view of a portion of a myocardial cell. The myofibrils are well organized and several intercalated discs (ID) have formed. M, mitochondria; N, nucleus; Z, Z band. x15,200. Specimens were fixed in 3% paraformaldehyde/2.5% glutaraldehyde/0.1 N cacodylate buffer (pH 7.4) for 1 d, and then washed in the buffer and post-fixed in 1.0% OsO_4 and buffer. They were then dehydrated and embedded in epoxy resin, sectioned, and double-stained with uranyl acetate and lead citrate.*

References

ARIIZUMI, T. and ASASHIMA, M. (1994). *In vitro* control of the embryonic form of *Xenopus laevis* by activin A: time and dose-dependent inducing properties of activin-treated ectoderm. *Dev. Growth Differ. 36:* 499-507.

ARIIZUMI, T. and ASASHIMA, M. (1995). Head and trunk-tail organizing effects of the gastrula ectoderm of *Cynops pyrrhogaster* after treatment with activin A. *Roux Arch. Dev. Biol. 204:* 427-435.

ARIIZUMI, T., SAWAMURA, K., UCHIYAMA, H. and ASASHIMA, M. (1991). Dose and time-dependent mesoderm induction and outgrowth formation by activin A in *Xenopus laevis*. *Int. J. Dev. Biol. 35:* 407-414.

ASASHIMA, M. (1994). Mesoderm induction during early amphibian development. *Dev. Growth Differ. 36:* 343-355.

ASASHIMA, M., NAKANO, H., SHIMADA, K., KINOSHITA, K., ISHII, K., SHIBAI, H. and UENO, N. (1990). Mesodermal induction in early amphibian embryos by activin A (erythroid differentiation factor). *Roux Arch. Dev. Biol. 198:* 330-335.

ASASHIMA, M., NAKANO, H., UCHIYAMA, H., SUGINO, H., NAKAMURA, T., ETO, Y., EJIMA, D., NISHIMATSU, S., UENO, N. and KINOSHITA, K. (1991). The presence of activin (erythroid differentiation factor) in unfertilized eggs and blastulae of *Xenopus laevis*. *Proc. Natl. Acad. Sci. USA 88:* 6511-6514.

BODMER, R. (1995). Heart development in Dros*ophila* and its relationship to vertebrates. *Trends Cardiovasc. Med. 5:* 21-28.

COX, W.G. and NEFF, A.W. (1995). Cardiac myosin heavy chain expression during heart development in *Xenopus laevis*. *Differentiation 58:* 269-280.

HAMA, T., TSUJIMURA, H., KANÉDA, T., TAKATA, K. and OHARA, A. (1985). Inductive capacities of the dorsal mesoderm of the dorsal marginal zone and pharyngeal endoderm in the very early gastrula of the newt, and presumptive pharyngeal endoderm as an initiator of the organization center. *Dev. Growth Differ. 27:* 419-433.

JACOBSON, A. and SATER, A.K. (1988). Features of embryonic induction. *Development 104:* 341-359.

LOGAN, M. and MOHUN, T. (1993). Induction of cardiac muscle differentiation in isolated animal pole explants of *Xenopus laevis* embryos. *Development 118:* 865-875.

MURATA, M., ETO, Y., SHIBAI, H., SAKAI, M. and MURAMATSU, M. (1988). Erythroid differentiation factor is encoded by the same mRNA as that of the inhibin βA chain. *Proc. Natl. Acad. Sci. USA 85:* 2434-2438.

MUSLIN, A.J. and WILLIAMS, L.T. (1991). Well-defined growth factors promote cardiac development in axolotl mesodermal explants. *Development 112:* 1095-1101.

NEFF, A.W., DENT, A.E. and ARMSTRONG, J.B. (1996). Heart development and regeneration in urodeles. *Int. J. Dev. Biol. 40:* 719-725.

OKADA, Y.K. and ICHIKAWA, M. (1947). Normal table of *Triturus pyrrhogaster*. *Jpn. J. Exp. Morphol. 3:* 1-6.

STANIER, D.Y.R. and FISHMAN, M.C. (1994). The zebra fish as a model system to study cardiovascular development. *Trends Cardiovasc. Med. 4:* 207-212.

SUGI, Y. and LOUGH, J. (1995). Activin-A and FGF-2 mimic the inductive effects of anterior endoderm on terminal cardiac myogenesis *in vitro*. *Dev. Biol. 168:* 567-574.

TAGUCHI, M., UEHARA, M., ASASHIMA, M. and PFEIFFER, C.J. (1989). Development of the heartbeat during normal ontogeny and during long-term organ culture of hearts of the newt, *Cynops pyrrhogaster*. *Cell Differ. Dev. 27:* 95-102.

UEHARA, M., TAGUCHI, M., ASASHIMA, M. and PFEIFFER, C.J. (1989). Developmental physiology of cardiac contraction in the Japanese newt *in vivo* and *in vitro*. *J. Mol. Cell. Cardiol. 21:* 709-718.