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Original Article

Amphibian *in vitro* heart induction: a simple and reliable model for the study of vertebrate cardiac development

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ABSTRACT Amphibian embryos are an excellent model system for analyzing the mechanisms of vertebrate cardiogenesis. Studies of heart development in *Xenopus* have, for example, revealed that the inductive interaction of the heart primordia with the adjacent underlying endoderm and dorsal lip starts at the early stages of gastrulation. However, the molecular basis of those early inductive events and the genes expressed during the early phases of heart differentiation remain largely unknown. Amphibian blastula embryos contain pluripotent cells in their ectodermal region, called the "animal cap," which fortunately can be exploited for understanding a variety of organogenesis processes. Despite an enormous potential for analysis, the use of this system in cardiogenesis research has languished due to a lack of information concerning appropriate culture methods. Herein we report conditions for generating an *in vitro* heart induction system and present evidence from two types of *in vivo* transplantations, that the cultured heart rudiment can develop and function in the adult organism. It is expected that the fundamental principles established in this model system will provide a versatile research platform for a variety of organ engineering projects, including modifying *in vitro* organ growth with exogenous components (e.g. various growth factors) and developing methods for preparing tissue for transplantation.

KEY WORDS: *activin, animal cap, cardiogenesis, organ engineering, transplantation*

Introduction

Previous attempts at exploiting the convenience of the amphibian embryonic system as a model for vertebrate heart development have yielded mixed results.

Reproducibility as well as percentage yield for organ cultures has varied from experiment to experiment. As well, morphological and histological criteria have often been employed as the main assessment criteria for differentiation. Finally, accurate measurements of functional capacity of developed organ cultures have not always been performed.

Nevertheless, progress is being achieved on many aspects of amphibian heart organ culture including the identification of potential regulatory circuits which govern signaling between inducing and responding tissues. In *Xenopus*, for example, a role for the Wnt antagonists DKK-1 and Cresent has been proposed (reviewed by Schneider and Mercol, 2001). In this report we further refine the *Xenopus* organ culture system for cardiac differentiation, and provide protocols and results which enhance the suitability of this model system. A key step in the protocol, a dissociation/reaggregation procedure, is outlined and *in vivo* ectopic transplantation exercises are conducted to unambiguously assess the functional capacity of cytodifferentiated cultures.

Results

As the first phase of this study, we developed a completely new protocol for preparing a reliable *in vitro* heart induction system using *Xenopus* blastula animal caps (Fig. 1A). Dissected animal caps were dissociated into individual cells by treatment with calcium-free saline. The dissociated cells were then reaggregated by placing them in a standard saline solution containing calcium. During the latter process, cells were exposed to activin (10-1,000 ng/ml), a peptide growth factor (Asashima *et al.,* 1990) for 1-72 h. In the absence of

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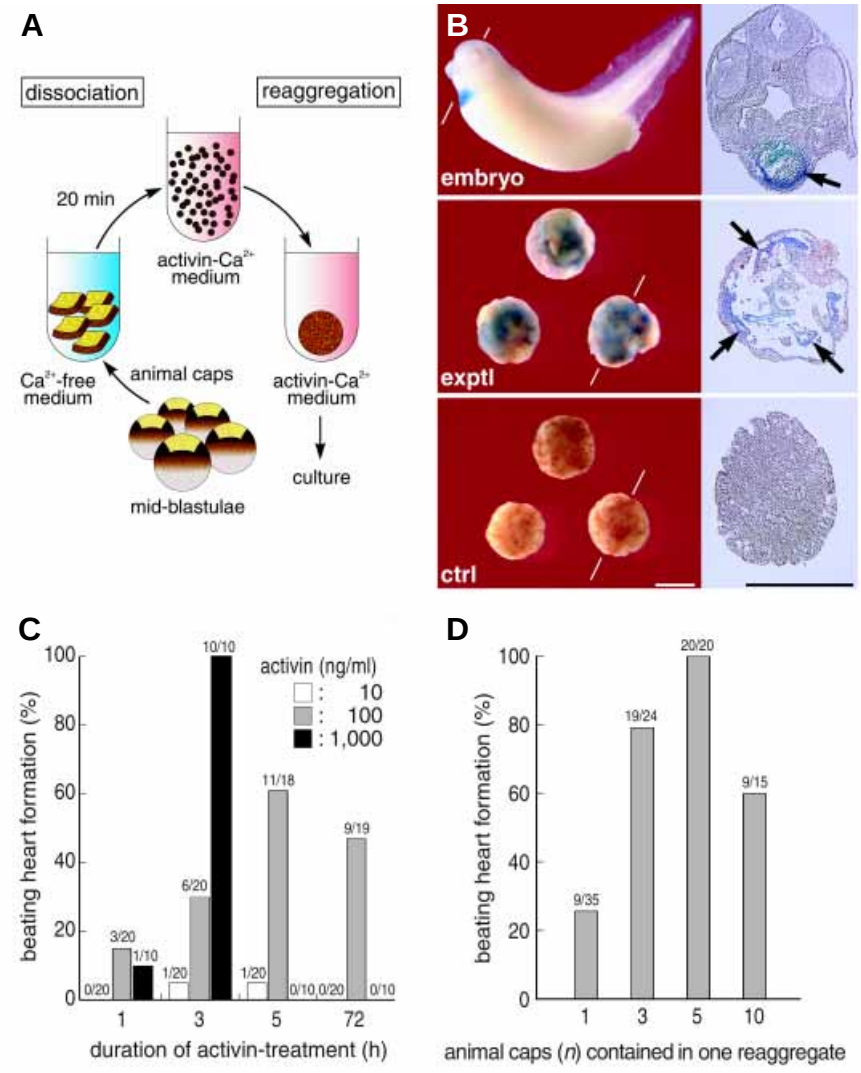

Fig. 1. *In vitro* heart induction system using embryonic pluripotent cells. (A) *The dissociation/reaggregation protocol for* in vitro *heart induction. The cell adhesion of mid-blastula animal caps were loosened in* Ca^{2+}*-free medium. Dissociated (by gentle pipetting) cells began to form a spherical reaggregate in the medium containing* Ca^{2+} *and an appropriate concentration of activin.* **(B)** Xenopus cardiac troponin I *(*XTnIc*), a marker gene for myocardial differentiation, expressed specifically in myocardium of a normal 3-day-old embryo (upper). The dissociated animal cap cells formed spherical reaggregates, regardless of whether they had been treated with activin. However, untreated reaggregates showed negative staining of the* XTnIc *signal after they were cultured for 3 days (lower).* XTnIc *signals were detected only in activin-treated reaggregates (middle). Note the* XTnIc *signals (arrows) at the tubular hearts on the transverse sections of the normal embryo and the activin-treated reaggregate. Scale bar, 0.5 mm.* **(C)** *Activin frequently induced beating hearts at concentrations higher than 100 ng/ml. The frequency of beating-heart formation reached 100% when the animal cap cells were treated with 1,000 ng/ml activin for 3 h. Each reaggregate was comprised of 10 animal caps (approx. 2,000 cells).* **(D)** *The number of cells contained in a reaggregate also affected heart formation. The dissociated cells were treated with 100 ng/ml activin for 5 h in this experiment. The frequency of heart formation reached 100% when 5 animal caps (approx. 1,000 cells) were contained in a reaggregate. The number on the top of each column refers to the number of reaggregates with beating hearts per the total number of reaggregates.*

activin, no tissue differentiation other than atypical epidermis was found in the reaggregates after 3 days of culture. Conversely, activin treatment (e.g., 100 ng/ml, for 5 h) caused the formation of beating tissue inside the thin epidermal vesicle. These tissues often showed tubular or globular morphology and contracted regularly (Supplementary Movie 1). Whole-mount *in situ* hybridization of the *XTnIc* (*Xenopus cardiac troponin I*) gene that is ordinarily expressed specifically in the mycardium of the heart (Drysdale *et al.*, 1994) revealed that these beating tissues were undoubtedly heart-like (Fig. 1B). These *in vitro*-induced hearts kept beating rhythmically for more than 2 weeks without a change in the culture medium. The heartbeat rate of the induced cultures depended on the ambient temperature, which is also true of the heartbeat rate in intact, normal embryos. For example, the heartbeat rates of the 1-week-cultured hearts increased about fivefold at 24°C (76 ± 5 bpm, n=5) compared to the rates at 5°C (16 ± 5 bpm). Electron microscopy revealed that the induced hearts exhibited a clear cytodifferentiation pattern typical of normal embryonic hearts. Myocardial cells with well-organized myofibrils joined at a junctional zone called the intercalated disc (data not shown).

Previous studies indicated that activin's inducing effects on animal caps depend on both concentration and treatment time (Asashima *et al.*, 2000; Ariizumi and Asashima, 2001; Ariizumi *et al.*, 1991; Asashima *et al.*, 1999). We therefore determined the optimal conditions for activin action on *in vitro* heart induction. The effect of treatment time at three activin concentrations on reaggregates comprised of cells (approx. 2,000) from 10 animal caps was examined (Fig. 1C). Three hours at 1,000 ng/ml yielded beating hearts in all (100%) of the cultures, while treatment with 100 ng/ml for 5 h yielded beating hearts in 60% of the cultures. The number of cells contained in a reaggregate also affected the success rate for beating hearts (Fig. 1D). Reaggregates of 5 animal caps (approx. 1,000 cells) yielded complete heart induction by treatment with 100 ng/ml of activin for 5 h. These data validate our procedure as a reliable method for using multipotent amphibian cells in the study of heart induction *in vitro*.

Next, we performed RT-PCR analyses using several marker genes to evaluate the significance of the "dissociation/reaggregation" step to this *in vitro* heart induction system. Following treatment with activin (100 ng/ml), striking differences in the early gene expression pattern were recognized between the undissociated animal caps and reaggregates (Fig. 2A). The expression levels of an early neural marker, *XSox2*, and mesodermal markers (*Xbra*, *XWnt-11*, and *XNot*) were constantly maintained in the intact animal caps, whereas those of *XGATA-4* (an early marker for both heart and anterior endoderm [Jiang and Evans, 1996]) and endodermal markers (*XHex* and *XSox17*β) sharply decreased starting at 9 h after the initial treatment with activin. Conversely, early expression of

neural and mesodermal markers (up to 5h) and prolonged expression of *XGATA-4* and *Xhex* were observed when the animal caps were subjected to the dissociation/reaggregation step. An early heart-field marker *XNkx2.5* (Tonissen *et al.*, 1994) gradually increased its expression level starting at 5 h after the beginning of activin treatment.

The differences in the gene expression patterns became clearer when those animal cap explants were cultured for 3 days (Fig. 2B). Following treatment with activin, the undissociated animal caps still expressed neural and mesodermal markers. Expression of heart-specific marker genes was entirely absent in those tissue samples. In contrast, animal cap tissue that was first dissociated, then treated with activin, and finally reaggregated expressed neither neural nor mesodermal markers. Instead, it expressed heart field markers *XNkx2.5*, *XGATA-4*, and *XTbx5* (Horb and Thomsen, 1999) or cardiomyocyte markers *XMHCα* (Logan and Mohun, 1993), *XTnIc*, and *XANF* (*Xenopus* atrial natriuretic factor [Small and Krieg, 2000]). The expression of *endodermin*, a pan-endodermal marker gene, was induced in the animal cap cells that received activin treatment alone, irrespective of dissociation/reaggregation. These results clearly indicate that the dissociation/reaggregation protocol is the key step for achieving heart differentiation in activin-induced animal cap tissue.

In order to determine whether these *in vitro*-induced hearts are functionally active, two types of transplantation experiments (Fig. 3A), based on protocols devised by Grunz (1999), were performed. In one type of manipulation, replacement transplantation, a fragment of a reaggregate of activin-treated animal cap cells was used as a donor. Donor reaggregates were precultured in saline for 1 day prior to transplantation into late neurula recipient embryos. Removal of the heart primordia from neurulae (control: no donor transplant tissue) resulted in development of edema and death within several days or at most 2 weeks. When an *in vitro*-induced heart rudiment was transplanted to the site from which the original heart primordium had been removed, 74% (217 of 294) of experimental embryos developed normally for 5 days (Fig. 3B). The donor heart began to contract 1 day after transplantation, which corresponded to the same time at which the normal embryonic heart in untreated normal embryos begins beating. Cell-lineage analyses using fluorescent dyes revealed that the donor hearts contained a few blood cells derived from the recipient (data not shown). Almost all embryos (210 of 217) could not suppress the onset of edema thereafter and died within 2 weeks. One of the 7 remaining embryos survived for 76 days after the transplantation and reached developmental stage 56 (Nieuwkoop and Faber, 1956). However, it did not succeed in metamorphosing into adult.

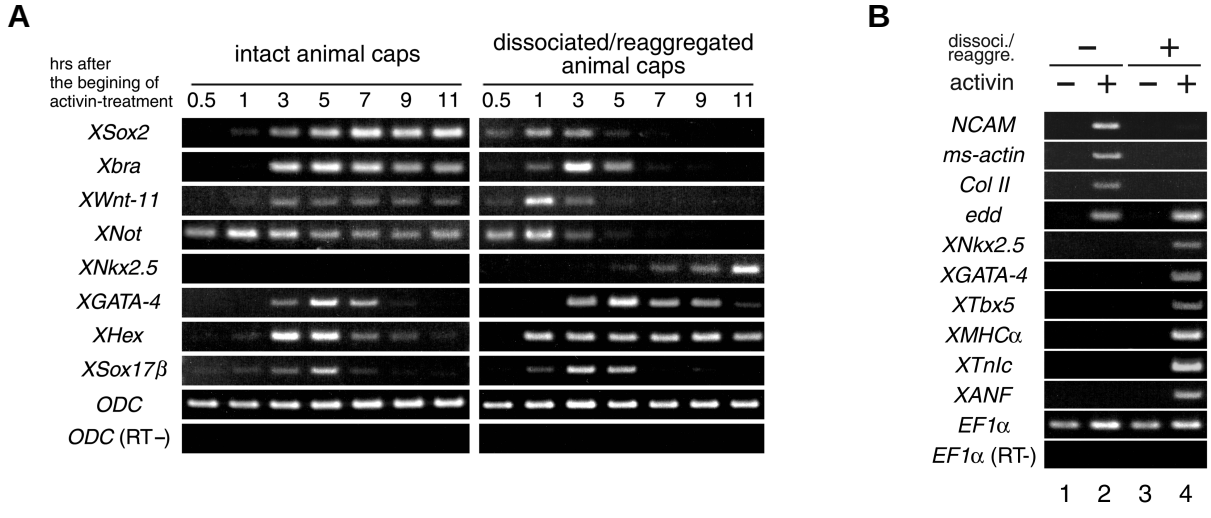

Fig. 2. Gene expression patterns of the *in vitro*-induced heart rudiments. (A) *Comparison of early gene expression patterns between the intact (undissociated) and dissociated/reaggregated animal caps after activin treatment (100 ng/ml). The left-hand column indicates the time course of gene expressions in the intact but activin-induced animal caps. Neural and mesoderm differentiation markers (*XSox2 *and* Xbra/XWnt-11/XNot*, respectively) expressed constantly when the animal caps were treated with activin for more than 3 h. Conversely, the expressions of* XGATA-4 *(a marker for both heart and anterior endoderm),* XHex *(for anterior endoderm), and* XSox17β *(for pan-endoderm) disappeared by treating with activin for more than 9 h. Note that* XNkx2.5, *an early heart-field marker, was never detected in these samples. The right-hand column shows the profile of gene expressions in the animal caps after the combined activin and dissociation/reaggregation treatments. In contrast to the profile in the left-hand column, the expressions of the neural and mesoderm markers were faded out by exposure to activin for more than 5 h. The expressions of* XGATA-4 *and* XHex *were maintained for at least 9 h after the beginning of activin treatment. Note that* XNkx2.5 *was initially detected at 5 h after the beginning of activin treatment and gradually increased its expression level.* ODC*, loading control; (RT-), negative control lacking reverse transcriptase.* **(B)** *Gene expression patterns in the intact or reaggregated animal caps cultured for 3 days. Non-activin-induced cells expressed none of the markers even after they were subjected to the dissociation/reaggregation protocol (lanes 1 and 3). Activin treatment (100 ng/ml, 5 h) induced the expression of the pan-neural (*NCAM*), muscle (*ms-actin*), and notochord (*Col II*) markers in intact animal caps cultured for 3 days (lane 2). No heart-specific marker was detected in these samples. By contrast, the reaggregates consisting of activin-induced cells exclusively expressed all of the heart-differentiation markers, such as* XNkx2.5, XGATA-4, XTbx5, XMHCα, XTnIc, *and* XANF *(lane 4). The expression of* endodermin *(*edd*), a pan-endoderm marker, was induced by activin treatment, irrespective of dissociation/reaggregation (lanes 2 and 4).* EF1α*, loading control; (RT-), negative control lacking reverse transcriptase.*

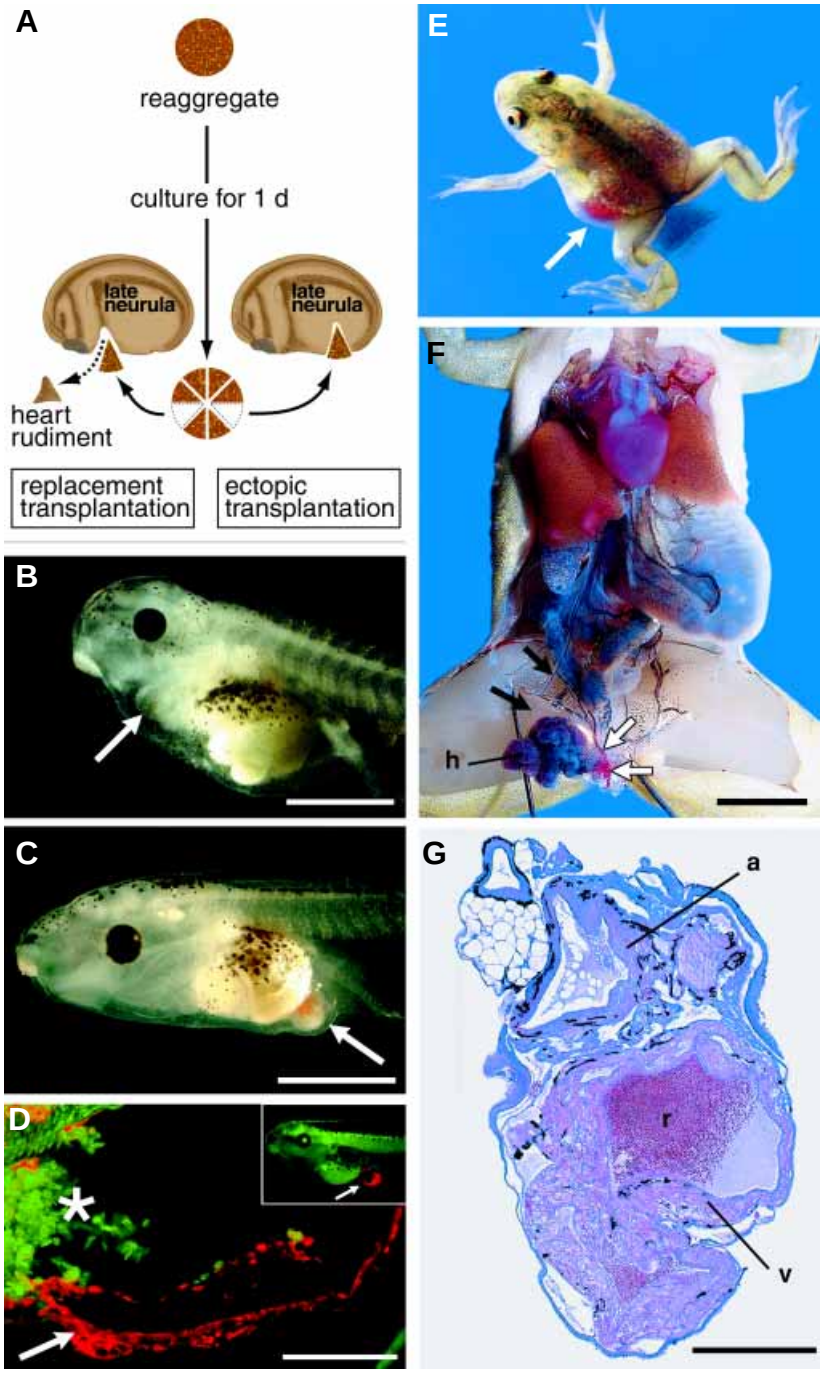

Fig. 3. *In vivo* transplantation of the *in vitro*-induced heart rudiments. (A) *Two types of* in vivo *heart transplantations. Following preculture for 1 day, a fragment of reaggregate was transplanted as donor tissue into late neurulae. The original heart rudiment of the host embryo was replaced with a donor heart rudiment in replacement transplantation, while a donor fragment was inserted into a slit made in the host's abdomen in ectopic transplantation.* **(B)** *Embryos that received a replacement transplantation of an* in vitro*-induced heart developed normally for 5 days. The substitute heart (arrow) was beating regularly and contained a small amount of host blood cells in its interior. Scale bar, 1 mm.* **(C)** *Embryos which received an ectopic transplantation developed normally without edema formation. The ectopic heart (arrow) contained a large number of red blood cells derived from the host embryo. Scale bar, 1 mm.* **(D)** *Cell-lineage analysis of an ectopic transplantation revealed that the ectopic heart (red, arrow) contained blood cells (green, asterisk) of the host. The external view of the embryo is shown in the upper right. Scale bar, 0.1 mm.* **(E)** *A young frog which was ectopically transplanted with an* in vitro*-induced heart rudiment at the neurula stage.* In vitro*-induced hearts remained in the recipients' left abdomen even after they had metamorphosed into frogs. An ectopic heart (arrow) filled with red blood cells can easily be seen through the thin skin.* **(F)** *Example of internal anatomy of an 1-year-old frog with a well-developed ectopic heart. The ectopic heart (h) adjacent to the host's intestine was incorporated into the host's vascular system. The blood from the host's mesenteric artery (black arrows) was flowing into the host's anterior abdominal vein (white arrows) via the ectopic heart. Scale bar, 5 mm.* **(G)** *Histological section of an ectopic heart. The heart can be divided into two chambers, a thin atrium (a) and a thick ventricle (v), based on the thickness of myocardium. r, red blood cells; scale bar, 0.5 mm.*

These observations are most likely explained by the incomplete connection of the donor heart with the host's main blood vascular and pronephric systems (Chan *et al.*, 1999).

In the other type of manipulation, ectopic transplantation, an activin-induced animal cap reaggregate was transplanted into a slit made in the posterior abdomen of a recipient neurula embryo. Fifty percent (276 of 547) of the recipients developed normally through morphogenesis except for exhibiting an ectopic beating heart (Fig. 3C). Both host and ectopic hearts began to contract at 1 day after transplantation. Large numbers of red blood cells were easily recognized through the thin envelope of the ectopic heart (Supplementary Movie 2). As in the replacement transplantations, cell lineage analyses revealed that these blood cells were derived from the host embryo (Fig. 3D). Further, we have succeeded in raising through metamorphosis 29% (80 of 276) of these ectopic transplants into adults. In one remarkable case, the donor heart hypertrophied at the abdomen of the host frog and thus its beating could be externally monitored (Fig. 3E and Supplementary Movie 3).

Additional analyses were carried out by dissecting 33 of 80 recipients (aged approximately 1 year) at random. A well-developed ectopic heart integrated with the host's blood vascular system was readily observed in all samples (Supplementary Movie 4). The ectopic heart, located adjacent to the host's intestine, was beating at nearly the same rate as the host's heart. Clamping the host's anterior abdominal vein caused the expansion of the ectopic heart, revealing blood circulation between the ectopic heart and the host's blood vascular system. A detailed analysis of a video recording of the internal anatomy of one such specimen indicted that blood was circulating from a branch of the host's mesenteric arteries to the ectopic heart (Fig. 3F). The blood from that ectopic heart flowed into the host's anterior abdominal vein. Examination of histological sections revealed that the ectopic heart could be divided into at least two chambers (atrium and ventricle) based on the thickness of the myocardium (Fig. 3G).

Discussion

A variety of evidence has accumulated over the past several decades indicating that the heart develops similarly in all species. Furthermore, it is apparent that many congenital abnormalities in adult hearts will likely be best understood by studying the embryonic development phases of cardiac tissue (reviewed in Harvey and Rosenthal, 1998). Thus, we endeavored to build on earlier studies which helped define the conditions for optimal cardiogenesis in the model vertebrate organism, *Xenopus*.

Grunz (1992) succeeded in producing beating hearts *in vitro* by treating isolated *Xenopus* dorsal lips with Suramin, a polyanionic compound. The Suramin-treated dorsal lips formed secondary heart structures in the ectopic transplantation and acted as a substitute for the original heart in the replacement transplantation experiment (Grunz, 1999). In *Xenopus* early development, the heart primordia differentiate into hearts under the influence of the adjacent endoderm and dorsal lip (Nascone and Melcora, 1995). Neural tissues act in a negative fashion on this inductive interaction. Suramin is likely to change the fate of the dorsal lips from dorsal mesoderm to heart and suppress their neural inducing activity. Therefore, the heart differentiation of the Suramin-treated dorsal lip contains a complex and multi-step process, which is disadvantageous for analyzing the molecular mechanism of cardiogenesis *in vitro*, especially the earliest phase of the inductive interaction. In addtion, we previously reported the urodele newt animal caps developed into beating hearts by treating with a high concentration of activin (Ariizumi *et al.*, 1996). Approximately 30% of the animal caps formed beating hearts and the rate of formation reached 50% when they were co-cultured with intact animal caps. Those percentages were also imperfect as a model system for analyzing heart development *in vitro.* Thus, we embarked on the development of a more suitable system using *Xenopus* pluripotent cells that would be more advantageous to molecular biological analysis.

The data generated in this study provide a research platform for further analysis of cardiac development. Important features of this model system include the following: First, this heart induction system, using blastula-stage pluripotent cells, represents a simple yet reliable method that can be quickly and inexpensively exploited by a broad range of researchers. Most previous studies, especially those with mammalian embryos, have been undertaken with the burden of isolating the primordia from embryos, which are difficult to obtain and which require special expertise for the dissection and isolation of heart primordia. In the protocol described herein, the key step is the dissociation/reaggregation procedure, which is straightforward and readily accomplished. Whether activin itself is the actual inducer remains uncertain (Logan and Mohun, 1993; Schneider and Mercola, 2001). Most probably activin, especially at the relatively high concentrations employed in this culture system, is involved in a complex regulatory circuit that may include other peptide growth factors (e.g., BMP, FGF, etc.) functioning in the "induction" process (Lough *et al.*, 1996; Schultheiss *et al.*, 1997; Barron *et al.*, 2000; Shi *et al.*, 2000). Wnt signals, which likely act in both a positive (Pandur *et al.*, 2002) and a negative (Schneider and Mercola, 2001; Marvin *et al.*, 2001) fashion, also participate in the induction process. With this model system it should be possible to elucidate many of the details of the regulatory circuits, especially those involved in the earliest stages of the induction process (Lohr and Yost, 2000; Zaffran and Frasch, 2002).

Second, the demonstration that ectopic transplants develop sufficiently to participate in the development of the blood vascular system opens further avenues for experimentation. The transplanted heart primordium developed an atrium and ventricle connected with the host's blood vessels. To date we have not, however, succeeded in completely replacing the original heart with a transplanted primordium. Further improvement of the transplantation protocol (e.g., changing the stage at which transplantation is performed) will probably be the key to success. Since the fundamental mechanisms of heart development are evolutionarily conserved among vertebrates (Lyons, 1996; Mohun and Sparrow, 1997), the simple amphibian system described herein warrants exploitation, especially as a model for organ engineering.

Experimental Procedures

Embryos and activin solution

Embryos of the African clawed frog, *Xenopus laevis*, were obtained using standard procedures (Ariizumi *et al.*, 1999) and staged according to Nieuwkoop and Faber (1956). Holtfreter's saline (HS; 60 mM NaCl, 0.7 mM KCl, 0.9 mM $CaCl_2$, 4.6 mM HEPES, 0.1 g/l kanamycin sulfate, 0.1% BSA [A-7888, Sigma, USA], pH 7.6) was used as a medium in all experiments. A peptide growth factor, human recombinant activin A (a gift from Dr. Y. Eto [Central Research Laboratories, Ajinomoto Co. Inc., Japan]), was used as an inducer and dissolved in HS at appropriate concentrations (10-1,000 ng/ml).

In vitro *heart induction protocol*

The animal cap region was squarely removed from the mid-blastula stage embryo (st. 8) using tungsten needles. It was 0.8 mm x 0.8 mm in size and contained 205 ± 32 cells (n = 10). The following procedure, named the dissociation/reaggregation protocol, was performed in 96-well plates with round bottoms (SUMILON; MS-309UR, Sumitomo Bakelite, Japan). A maximum of 10 animal caps were bathed in 100 μl of Ca^{2+}-free HS for 20 min to loosen their cell adhesion. After being exchanged for 100 μl of activin solution (e.g., 100 ng/ml, dissolved in HS containing Ca^{2+}), cells were dispersed by gentle pipetting. The newly formed spherical reaggregates were transferred into 200 μl of HS after they had been left in the activin solution for a defined period (e.g., 5 h).

In vivo *heart transplantation protocol*

After being precultured in HS for 1 day, the reaggregate prepared as described above was divided into small pieces and transplanted as a donor tissue into the sibling neurulae (st. 20). In replacement transplantation, the original heart primordium (with the surrounding epidermis) of the host embryo was replaced with a fragment of reaggregate. In ectopic transplantation, a donor fragment was inserted into a slit made in the host's abdomen. The transplanted embryos were kept in 10% HS for the first 4 days, and raised in dechlorinated water until they metamorphosed to adults. To trace the lineage of donor tissue in the transplanted embryos, embryos were injected at the two-cell stage with a total volume of 10 nl of 1% fluorescein-dextran-amine (FDA; D-1820, Molecular Probes, USA) or 1% Texas Red-dextran-amine (TRDA; D-1863, Molecular Probes, USA). Animal caps dissected from TRDA-labeled blastulae were used for making donor heart rudiments, while FDA-labeled neurulae were used as recipients. The 4-day-old larvae were examined under an epifluorescence microscope. Fluorescent photographic images were computer-composed using photo-retouching software.

Gene expression analyses and histology

Whole-mount *in situ* hybridization was performed according to Harland (1991). Probes were generated using DIG RNA labeling mixture (Boehringer Mannheim) and color was developed by BCIP/NBT. In the RT-PCR analysis, total RNA isolation and RT-PCR were performed as previously described (Yokota *et al.*, 1998). Sequences of the oligonucleotide primers are available

from the authors on request. Transplanted embryos and ectopic hearts were fixed in Bouin's fluid for 12 h, dehydrated in a graded series of ethanol, cleared in xylene, embedded in paraffin, and cut into 8-µm thick sections. Sections were stained with Delafield's hematoxylin/eosin.

Acknowledgements

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Electronic Supplementary Material for this paper, consisting of four "QuickTime" movies is available at the following address: http://www.ijdb.ehu.es/abstract.0306/esm1.htm

Supplementary Movie 1. An *in vitro*-induced beating heart. Animal cap tissues developed into beating hearts after combined activin and dissociation/reaggregation treatments. These induced hearts kept beating rhythmically for more than 2 weeks without a change in the culture medium.

Supplementary Movie 2. A tadpole with a secondary heart (ventral view). The recipient neurula embryo developed into a tadpole with a double heart after the ectopic transplantation of the *in vitro*-induced heart rudiment. The ectopic heart of this 5-day-old tadpole is filled with a large number of red blood cells.

Supplementary Movie 3. An adult frog with a secondary heart. This frog was ectopically transplanted with an *in vitro*-induced heart rudiment at the neurula stage. The recipient embryo metamorphosed into a frog normally except for exhibiting an ectopic beating heart.

Supplementary Movie 4. Internal anatomy of a frog with an ectopic heart. This frog is the same as shown in Fig. 3F. When the host's anterior abdominal vein was clamped the ectopic heart expanded with a large amount of blood.

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