

Pacemaker Development in Embryonic Rat Heart Cultured *in Oculo*

DIANE C. TUCKER, CANDACE SNIDER, AND W. T. WOODS, JR.

Department of Psychology [D.C.T.] and Department of Physiology and Biophysics [C.S., W.T.W.], University of Alabama at Birmingham, Birmingham, Alabama 35294

ABSTRACT. Conditions that cause pacemaker formation in the developing heart are poorly understood. Embryonic rat myocardium grafted into the anterior eye chamber of an adult rat provides a promising model system in which to study pacemaker development. Electrophysiologic mapping with two microelectrodes showed that each embryonic heart graft developed a primary pacemaker within the region of contact with the host iris. These single, primary pacemakers were found in the centers of graft-iris junctions both in grafts that originally contained the natural pacemaker (e.g. right atria and whole hearts) and in grafts that excluded the sinoatrial pacemaker region (*i.e.* ventricles and left atrial appendages). Pacemaker action potentials were recorded in the region identified by mapping as the origin of the impulse in 11 of 11 grafts. Action potentials recorded from surrounding working cells were similar to adult rat heart cells in maximum diastolic potential, overshoot, amplitude, and duration. In contrast, maximum upstroke velocity was consistently slower in grafts than in adult hearts. Beating of grafts slowed or stopped within 3 days after transplantation but resumed by 10–14 days at rates similar to those observed before dissection (265 ± 12), a pattern consistent with development of a new pacemaker *in oculo*. The graft-iris junction is the site of blood vessel and nerve ingrowth into the graft and it is a region of contact between differentiated embryonic myocardial cells and nonmyocardial (iris epithelial) cells. The roles of these three factors (vascularization, innervation, and surface contact) in establishing the pacemaker were examined using embryonic heart cultured both in the anterior eye chamber and *in vitro*. (*Pediatr Res* 23: 637–642, 1988)

Abbreviation

dpc, days postconception

During cardiac development, pacemaking and identifiable pacemaker cells arise early (1–17). The exact origin of this particular group of cells within the developing cardiac wall and how it becomes specialized into the sinoatrial pacemaker are unknown. Viragh and Challice (3, 17) suggested that the pacemaker begins as a group of undifferentiated cells in the loose mesenchymal tissue that contacts differentiated cardiac muscle

during the “looping” stage of development. Similar processes are observed when development of cardiac working muscle becomes induced by contact between “precardiac mesoderm” and endoderm both *in vitro* and *in situ* (5, 18–20). Assessment of the role played by contact induction in pacemaker cell differentiation has awaited development of a suitable model.

Patten's (21) proposal that the cardiac pacemaker originates in the ventricle has not been supported by electrophysiologic studies showing that rhythmic electrical activity first arises in the atrial region of embryonic chick heart, preceding both mechanical activity and histologically recognizable pacemaker cells (3, 6, 10, 11, 16, 17, 22, 23). The contractions observed first in the ventricular portion of the tubular heart (6, 9, 15, 21, 24, 25) result from sequential maturation of contractile rather than electrical activity; the hypothesis that the pacemaker relocates has been rejected by electrophysiologic studies (10, 11).

To develop a model in which pacemaker development can be monitored directly in embryonic cardiac tissue, we studied embryonic rat hearts grafted onto the iris in the adult rat. In the anterior eye chamber, fetal and embryonic rat heart tissue beats spontaneously for many months (26–28). Progressive changes in the beating rate of embryonic rat heart after grafting suggest that the original pacemaker system becomes suppressed and a new, stable one arises. We mapped electrophysiologic activity of embryonic heart grafted *in oculo* to determine pacemaker location.

Selective interventions in the anterior eye chamber environment (*e.g.* denervation) were combined with study of pacemakers established *in oculo* and *in vitro* to suggest a possible mechanism of natural pacemaker development.

METHODS

In oculo and in vitro culture. Heart rates were recorded in eight fetuses suspended in a 35° C physiologic solution with the uterine and fetal circulations intact. Uterine horns were removed aseptically from 19 ether-anesthetized Sprague-Dawley dams. Hearts were dissected from fetuses at 12 or 14 dpc (10–16 fetuses per dam) in sterile tris-Tyrode's solution (including glucose 0.1%; pH 7.4, 25° C). Fetal crown-to-rump length and heart dimension were measured with a micrometer ($\pm < 0.1$ mm) in a surgical microscope.

Host rats (males, 4 wk old) were anesthetized with chloral hydrate (3.5 mg/kg intraperitoneal) and 1% topical amide and 1% methyaltropine were applied to constrict the iris. Each excised tissue was drawn into a beveled pipette and injected into the anterior eye chamber through a corneal slit. Neosporin ophthalmic ointment was applied to prevent infection. Corneas appeared healed within 1 wk. Host rats exhibited normal weight gain and no excessive grooming or scratching around the eyes. Sizes and rates of beating were measured twice weekly in whole embryonic hearts ($n = 8$), atria ($n = 61$), ventricles ($n = 39$), left atrial appendages ($n = 12$), and right sinoatrial tissue samples ($n = 12$) that were cultured in anterior eye chambers.

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Correspondence and reprints Diane C. Tucker, Ph.D., Department of Psychology, Campbell Hall, University of Alabama at Birmingham, Birmingham, AL 35294.

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Thirty-one hearts were removed aseptically from 12-day-old embryos and transferred to Falcon Primaria organ culture dishes containing 1.0 ml Dulbecco's modified Eagle's medium (Irvine Scientific, Santa Ana, CA). Dishes were stored in a water-jacketed, humidified 5% CO₂ incubator (Forma 3158) at 37° C. Media were replaced every 72 h for at least 15 days. Beating explants were observed in a Nikon SMZ 10 stereomicroscope to guide cell impalements with microelectrodes as described below. Visible contractions were observed whenever action potentials were recorded.

Embryonic atria cultured in sympathetically denervated eye chambers. Sympathetic innervation to one anterior eye chamber was interrupted permanently by removal of the ipsilateral superior cervical ganglion. Previous studies have demonstrated that norepinephrine content of atria cultured in denervated eye chambers was substantially lower than in innervated grafts (*i.e.* 0.17 ± 0.07 cf. 5.68 ± 1.09 ng/implant) (28).

Pacemaker mapping. Host rats were anesthetized with α -chloralose (60 mg/kg, intraperitoneal) and intubated via tracheotomy. During ocular surgery, ether was added to the inspired air to eliminate twitches of ocular muscles. Rats were secured to a frame that elevated the nose 40° from horizontal and rotated the head to expose the anterior chamber for microdissection. Cornea lying over the graft was excised.

A polypropylene cylinder (10 mm across, 8 mm high) was attached to the periocular skin with cyanoacrylic adhesive to form a 0.5 ml perfusion chamber through which 35° C rat physiologic solution was pumped at 5 ml/min. Rat physiologic solution contained in mmol/liter 130 Na⁺, 6.3 K⁺, 112 Cl⁻, 25.0 HCO₃⁻, 2.60 HPO₄⁻, 1.40 Ca⁺⁺, 0.64 Mg⁺⁺ and 5.6 glucose and was equilibrated with 95% O₂/5% CO₂; pH was maintained at 7.4 (29). Microelectrodes (2.5 molar KCl filled) were guided into single cells to record action potentials as described using the World Precision Instruments KS-700 Dual Microprobe System (New Haven, CT) (see Refs. 30 and 31). The first derivative of upstroke velocity was displayed via differentiator W.P.I. Inc. (New Haven, CT) (30, 31). By recording with two movable microelectrodes from all exposed surfaces at 10 to 50 μ interelectrode distances, sites of impulse initiation were determined and impulse spread was mapped as previously described in excised fragments of perfused canine myocardium (30, 31). External stimuli were delivered to cultured tissue via bipolar silver electrodes from a Grass S4 stimulator.

Statistical analysis. Analysis of variance was used to compare the initial tissue dimensions and rates of beating between groups in each study. Repeated measures analysis of variance was used to compare changes in beating rate of different tissue types over the period of study. Followup comparisons were made using Newman-Kuel's tests. Nonparametric statistics evaluated the incidence of spontaneous beating as a function of tissue type or culture condition.

RESULTS

Changes in spontaneous beating observed after implanting in oculo. After implantation, pacemaker rates fell to zero or slowed transiently. Thirty-eight of the 61 atria from embryonic rat hearts (12 dpc) grafted into anterior eye chambers stopped beating within 3 days. Spontaneous rates in the remaining atrial grafts were slower than recorded *in situ* before dissection (164 ± 11 versus 267 ± 8 bpm) (Fig 1). After 10 days *in oculo*, 91% of atrial grafts had resumed spontaneous beating [259 ± 13 bpm, a rate not different ($p > 0.05$) from the 265 ± 12 bpm recorded before dissection (see Figs. 1 and 2)]. In both atrial and ventricular grafts, beating rates increased significantly during the first 2 wk *in oculo* ($p < 0.05$).

Beating rate of an independent sample of atrial ($n = 20$) and ventricular ($n = 15$) grafts was measured biweekly to determine whether the beating rate difference observed during the first month *in oculo* would be maintained (see Fig. 3). Profile analysis indicated differential change in beating rate of atria and ventricle

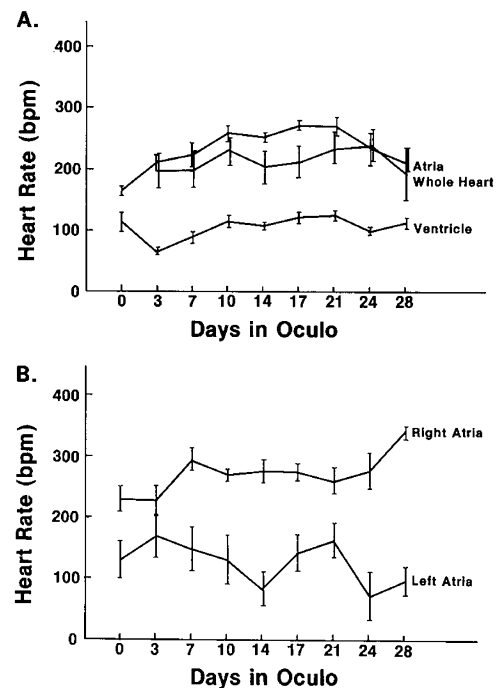


Fig. 1. Spontaneous beating rate of grafts. A, spontaneous rates of beating measured in 30 atria, 23 ventricles, and eight whole hearts (from 12 dpc embryonic rats) implanted into anterior eye chambers. B, spontaneous rates of beating measured in left atrial appendage and right sinoatrial region grafts from 14 dpc embryonic hearts grafted into the anterior eye chamber. Data are plotted as mean \pm 1 SEM.

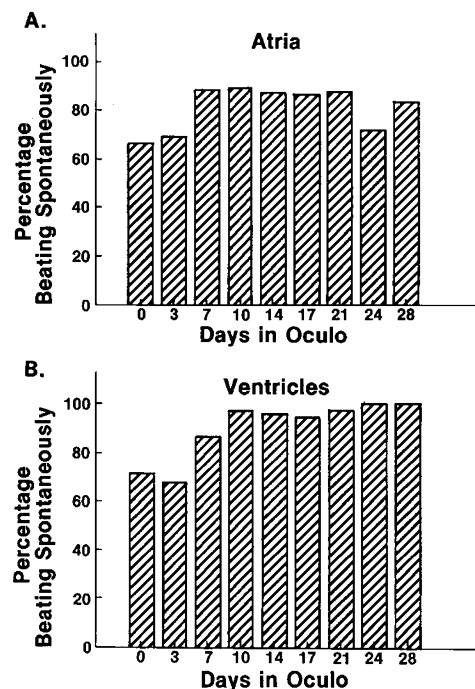


Fig. 2. Percentage beating spontaneously. The bar graphs show the percents of 44 atrial (A) and 40 ventricular (B) grafts that continued to beat spontaneously on specified days after implantation into anterior eye chambers.

graft [$F(3,24) = 6.33$; $p < 0.005$], with atria decreasing in rate between 2 and 8 wk whereas ventricles increased in beating rate. By 6 wk after grafting there was no significant difference in beating rate of atrial and ventricular grafts.

Electrophysiologic mapping of pacemaker location in oculo. In

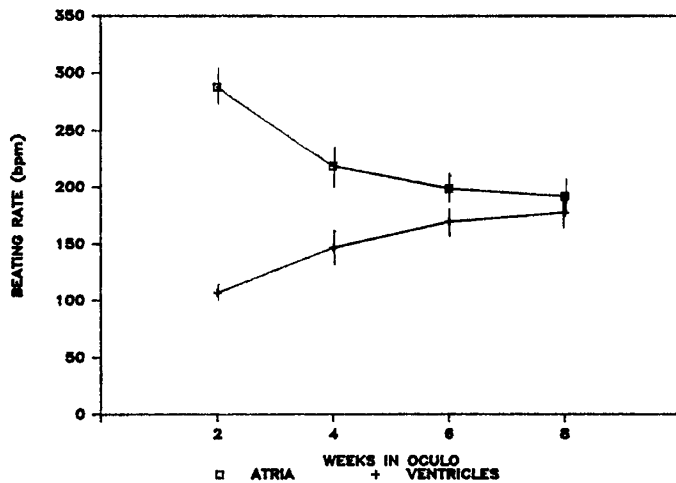


Fig. 3. Beating rate of atrial and ventricular grafts. Beating rates measured from atrial and ventricular grafts during the first 8 wk *in oculo*. Atrial grafts beat significantly faster than ventricular grafts during the first month *in oculo*; beating rates did not differ at either 6 or 8 wk.

oculo embryonic heart tissue assumed a round shape flattened somewhat against the iris. The origin of the graft impulse was located by the two electrode technique and confirmed visually. Regardless of the orientation of the tissue when grafted, the area of earliest depolarization was always near the center of the junction between the graft and iris. Mapping of impulse spread showed that impulse conduction propagated nearly symmetrically from the pacemaker region to the edge of the graft as shown in Figure 4. Slices were removed serially from seven grafts and the sites of earliest activation mapped using simultaneously recorded action potentials. Recordings were made from five to 10 sites on both exposed surfaces of the graft before and after removal of the slices as illustrated in Figure 4. Excised slices remained excitable to external stimuli while suffused but they revealed no spontaneous electrical activity. Neither rate nor conduction pattern were altered until 95% of the graft was removed, suggesting that the pacemaker lay within the muscle tissue adjacent to the iris. Excision of the identified pacemaker region resulted in cessation of all spontaneous contractile and electrical activity in all cases.

Pacemaker action potentials were recorded in the region identified by mapping as the origin of the impulse in 11 of 11 transplants (see Fig. 4). Action potentials recorded from cells surrounding this region differed mainly in upstroke velocity (>20 v/s, $p < 0.05$) and the absence of phase 4 depolarization. Figure 5 and Table 1 compare action potential characteristics of *in oculo* and adult rat heart cells. *In oculo* and adult rat heart cells were similar in maximum diastolic potential, overshoot, amplitude, and duration; maximum upstroke velocity was consistently lower for *in oculo* heart cells than for adult cells in arterially perfused hearts (see Table 1; Fig. 5).

Most isolated ventricles (26 of 39 at 12 dpc) stopped beating after being implanted into the anterior eye chamber. However, by 14 days *in oculo*, 33 of 39 ventricle grafts were beating spontaneously (125 ± 8 bpm; see Figs. 1 and 2) at a rate slower than observed in atrial grafts at that time. Electrophysiologic mapping of three ventricle grafts localized the pacemaker to the center of the graft-iris junction in each.

Four of 12 left atrial grafts from 14 dpc fetal hearts beat spontaneously (124 ± 47 bpm) by 10 days *in oculo* (see Fig. 1) at a rate not different from ventricular grafts. The beating rate of left atrial grafts was monitored for only the first 4 wk *in oculo*. The left atrial appendages were electrically quiescent when first separated from their corresponding right sinoatrial regions in the dissection dish because their original sinoatrial (right atrium) pacemakers were intentionally excluded. Right atrial grafts beat

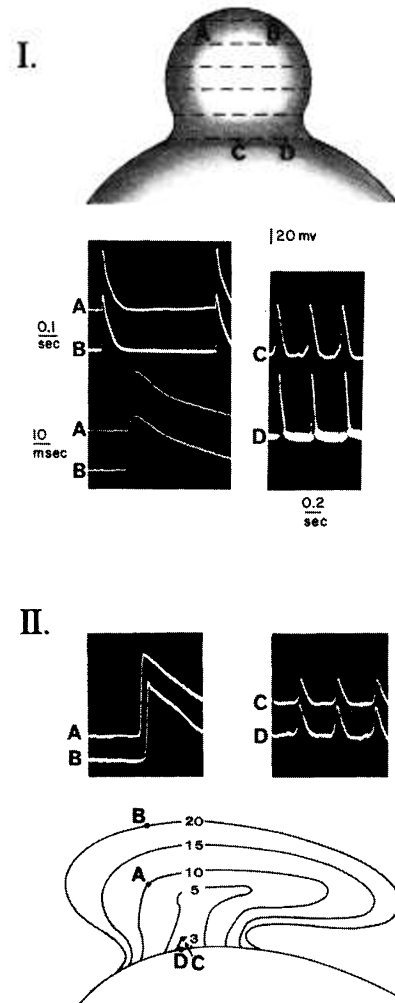


Fig. 4. Location of *in oculo* pacemaker. The diagrams show results of *in oculo* atrial impulse mapping studies A–D indicate impalement sites in each panel. I, serial slices (300 μ thick) were removed beginning in the distal portion of the graft to reveal the level at which the pacemaker was located. II, dual microelectrode impalements revealed sequence of excitation within each graft. Isochrones indicate times at which layers of a typical graft became activated. Point “D” within the junction between the graft and the iris was the origin of impulses and coincided with sites of vascularization and innervation.

at faster rates ($p < 0.05$) on days 0, 7, 10, 14, 17, 24, and 28 *in oculo*. Electrophysiologic mapping of these spontaneously beating left atrial grafts confirmed that their pacemakers were also located at the center of the graft/iris junction.

Denervation. Sympathetic denervation did not alter the pattern of spontaneous beating during the first 3 postnatal wk or the site of pacemaker location. Twenty-one of the 52 atria implanted in sympathetically denervated anterior eye chambers became quiescent within 3 day *in oculo*. All but three were beating spontaneously by 14 days *in oculo*. Mapping studies in four grafts localized the site of impulse initiation in sympathetically denervated grafts to the center of the graft-iris junction in each case.

Spontaneous beating of embryonic heart *in vitro*. Within 3 days, four of the 18 whole hearts (12 dpc) placed in organ culture dishes stopped beating; the remaining 14 slowed their beating rates from 70 ± 12 to 14 ± 8 bpm. Two days later, 16 of 18 were beating faster than at 3 days (44 ± 9 bpm). Measurements were made at room temperature (25° C) after removing dishes from the 37° C incubator. Electrophysiologic mapping and visual

observation of these explants confirmed that beating began at foci where viable cardiac cells contacted either the culture dish surface or the layer of fibroblasts that covered it.

DISCUSSION

Embryonic myocardial tissue growing in the anterior eye chamber was driven by a single primary pacemaker located within the junction between the graft and the host iris. Electrophysiologic studies localized the pacemaker of grafted tissue to the central portion of the graft-iris junction. In grafts containing the original pacemaker, beating stabilized by 10–14 days after grafting at a rate comparable to that observed before dissection.

A difference in the beating rate of atrial and ventricular tissue was expected based on the experiments of DeHaan (4) and LeDourain *et al.* (32) in which precardiac mesoderm explants destined to be atrium or ventricle were cultured separately. The atrial anlage beat more rapidly than the ventricular anlage. Our data suggest that the right atrium (containing the sinoatrial region) generates the most rapid pacemaker activity during the first weeks after grafting. When uncoupled from the right atrium early in development the left atrium develops spontaneous firing, but at a rate initially similar to that of ventricular tissue.

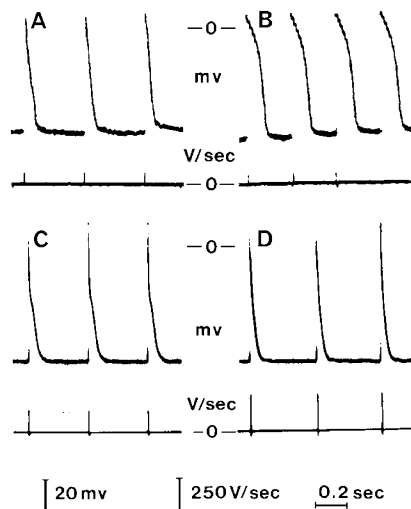


Fig. 5. Comparison of action potentials recorded from *in oculo* heart and isolated, perfused adult rat heart. Action potentials were recorded with microelectrodes. Spontaneously generated action potentials were recorded *in oculo* from atria (A) and ventricle (B) grafts *in oculo* for 6 wk. Right atrial (C) and right ventricular (D) action potentials are from the isolated right heart of a 2-month-old rat perfused through the coronary arteries.

Pacemakers such as those identified with microelectrodes can be induced by trauma (*e.g.* serial sectioning), but in our grafts this appeared unlikely for the following reasons. First, action potentials resembling those of “working cells” of adult rat heart were recorded in the working cardiac muscle surrounding the pacemaker locus exposed by serial slicing of grafts (see Fig. 4). Second, after removal of slices above the implant-iris junction, we never observed pacemaker-like action potentials and earliest depolarization in tissue lying outside of the junctional region. Third, pacemakers were identified near the center of the attachment of the graft to the iris, whereas trauma-induced pacemaking should be equally likely in all portions of the incision and in distal portions of the remaining tissue. Fourth, conduction patterns in lower parts of the graft were unaffected by serial slices in upper parts, indicating that pacemaker shifts, if any, occurred over less than 200 μ .

Inasmuch as orientation of tissue during grafting was not systematic, the consistent location of *in oculo* pacemakers seems likely to be a consequence of either migration of the original pacemaker to this site or induction of a new pacemaker at the graft-iris junction. To distinguish between these possibilities, grafts that excluded the original sinoatrial pacemaker (*i.e.* ventricles and left atrial appendages) were studied. *In oculo*, these tissues established spontaneous beating that was driven by a single primary pacemaker located at the center of the graft-iris junction. These data suggest that the anterior eye chamber may contain elements sufficient to support pacemaker development in fetal myocardium. Studies presented herein do not exclude the possibility that the original pacemaker remains viable but is overridden by a new pacemaker located within the junction between the graft and the iris. Detailed electrophysiologic studies of grafts between 3 and 10 days after transplant would aid in clarifying this issue. Potentially important characteristics of the junction between the graft and the iris include ingrowth of autonomic nerves and blood vessels and contact of embryonic heart with the iris squamous epithelium.

Observation of sustained spontaneous activity emanating from the graft-iris junction in sympathetically denervated eye chambers suggested that sympathetic innervation is not required for establishment of the pacemaker at the graft-iris junction. Sympathetic innervation may, however, influence the pacemaker's rate of beating (28).

Additional factors that might influence establishment of a pacemaker at the graft-iris junction include the growth of large blood vessels into the graft and penetration of the graft by nerves that sprout from the iris. The timecourse of changes in beating rate observed *in oculo* appears to coincide with vascularization and innervation of grafts (26, 27, 33). As a first step toward evaluating the role of neural and vascular stimulation in pacemaker formation, embryonic hearts cultured *in oculo* were com-

Table 1. Action potentials recorded in working cells of excised, perfused adult rat hearts and in fetal rat hearts growing *in oculo**

Cell Type	Rate (bpm)	MDP (mv)	V _{max} (v/s)	Osht (mv)	Amp (mv)	APD ₂₀ (ms)	APD ₅₀ (ms)	APD ₈₀ (ms)	CV _{rel} (mm/s)
Adult atrial cells	158	-77	240	14	90	7	19	37	637
	± 57	± 6	± 50	± 5	± 6	± 4	± 9	± 16	± 426
	(30)	(30)	(31)	(30)	(31)	(31)	(30)	(29)	(19)
Adult ventricular cells		-80	230	9	89	6	13	28	853
		± 6	± 50	± 6	± 6	± 3	± 6	± 11	± 365
		(23)	(24)	(23)	(24)	(24)	(24)	(23)	(18)
<i>In oculo</i> atrial cells	183	-72	62	12	92		14		200
	± 37	± 9	± 12	± 6	± 8		± 10		± 100
	(26)	(26)	(26)	(26)	(26)		(26)		(10)
<i>In oculo</i> ventricular cells		-74	65	14	94		28		
		± 11	± 15	± 6	± 16		± 11		
		(18)	(18)	(18)	(18)		(18)		

* MDP, maximum diastolic potential; V_{max}, maximum upstroke velocity; Osht, overshoot; Amp, amplitude; APD₂₀, APD₅₀, APD₈₀, action potential durations at 20, 50, and 80% repolarization; CV_{rel}, 2-point relative conduction velocity.

pared with hearts placed in organ culture, an aneural and avascular environment. Spontaneous beating of embryonic hearts was maintained *in vitro* for more than 3 months, suggesting that neither blood vessel ingrowth nor innervation is required to support sustained spontaneous beating *in vitro*. Whether vascularization or innervation affect pacemaker formation *in oculo* has yet to be determined.

The observation that *in oculo* pacemakers were always located within the region of contact between the embryonic heart tissue and the iris may support the interpretation of Viragh and Challice (3, 17) that contact between differentiating cardiac tissue (*i.e.* cells that are beginning to express myofibrils) and nonmyocardial cells induces embryonic heart cells to fire spontaneously and to specialize into pacemaker cells. *In oculo*, the iris epithelium may provide the nonmyocardial substrate that modifies development of embryonic heart cells as does contact with loose mesenchymal tissue during maturation *in situ*.

If contact between embryonic heart muscle and nonmuscle surfaces induces differentiation of cells into pacemakers, the question arises why the more peripheral cells that may contact the iris distant from the site of firm attachment do not also become pacemakers. In skeletal muscle (34) and in neuronal circuits (35) activity stabilizes the current state of differentiation or connectivity. If a similar process operates in embryonic heart tissue, differentiation of a small group of myocardial cells into pacemakers might maintain electrical activity that would inhibit pacemaker cell differentiation in other portions of the cardiac tissue. Alternatively, pacemaker function in one part of the graft could suppress or mask pacemaker activity in other cells (*e.g.* by overdrive suppression) (36).

Culture of embryonic rat heart *in oculo* provides a growing, pacemaker-driven model of cardiac development that remains electrophysiologically stable for many weeks (>20 wk). Wildenthal (37) reported dissociation between beating of atria and ventricles from whole fetal mouse hearts (E-19 to E-21) after several days in organ culture. The beating rate of atria was faster than the ventricular beating rate for only the first several days in organ culture; the atrial beating rate then often fell below that of the ventricles. This suggests that the sinoatrial pacemaker did not remain viable when hearts from fetuses during late gestation were explanted into organ culture. In contrast, Mauer (38) reported regular, spontaneous, and rapid beating of fetal mouse hearts (E-13 to E-22) for at least 5 to 7 days in organ culture. Fetal mouse hearts in organ culture maintained biochemical stability for several weeks but did not grow and deteriorated slowly (36, 39). In contrast, myocytes in embryonic hearts grafted *in oculo* continue to divide and become binucleated after several weeks. Myocytes differentiate from fetal morphology into adult-like heart cells with plentiful and well-organized myofibrils, mature intercalated discs, and prominent Z lines. However, valves do not form and the chambers of the embryonic heart are not maintained *in oculo*, except perhaps as sinusoids, making the *in oculo* culture system inappropriate for studying these aspects of cardiac morphogenesis. The *in oculo* culture system does provide a growing, differentiating, and pacemaker-driven model of cardiac development appropriate for studying controls of myocyte growth and differentiation, including pacemaker development; organ culture of embryonic heart does not support comparable growth and development.

Dissociated fetal or newborn cardiac muscle cells beat spontaneously when placed in cell culture (7). However, they organize into isopotential, homogeneous aggregates rather than regionally differentiated fetal cardiac tissue. Mechanisms underlying the spontaneous beating of fetal and neonatal cardiac cells in culture could be related to the sustained, pacemaker-driven activity observed when embryonic heart is cultured *in oculo*, especially because nerves and blood vessels are also unnecessary for the spontaneous beating of myocytes placed *in vitro*.

The spontaneous beating of embryonic heart cultured *in oculo* is driven by a pacemaker that is always located at the center of

the junction between heart and iris. This was observed even when the natural pacemaker was intentionally excluded from the grafted tissue (*i.e.* left atrium or ventricle). Migration of the natural pacemaker to the implant-iris junction in cardiac tissue that contained a pacemaker before grafting is unlikely but has not been excluded. Gradual migration of the original pacemaker toward a new site would not, however, correlate with the relatively abrupt and pronounced changes in rate observed consistently in atrial grafts. Taken together, observations reported herein using both *in oculo* and *in vitro* culture of embryonic heart suggest that a new pacemaker may be induced in embryonic heart in the *in oculo* culture system. The *in oculo* culture system is a promising model system in which to study conditions that support and inhibit pacemaker development.

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