J. Anat. (1990), **168**, pp. 257–263
With 4 figures
Printed in Great Britain

Looping of chick embryo hearts *in vitro**

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(*Accepted* 21 *August* 1989)

INTRODUCTION

In all vertebrates above and including the Elasmobranchii, the heart is the earliest organ to deviate from bilateral symmetry. This occurs via the process known as 'looping' or 'bending', during which the heart rudiment comes to lie on the left hand side of the body. Despite the apparent simplicity of this process, and its importance in establishing the basic morphology of the developing heart, the underlying mechanisms have not yet been completely elucidated. In medical textbooks, looping is often ascribed to the fact that the venous and arterial ends of the heart are fixed by the walls of the pericardial cavity and elongation of the heart therefore necessarily leads to flexion (Moore, 1982; Sadler, 1985), a view probably derived originally from Patten (1922). By contrast, modern research studies on heart looping have generally attributed this process to intrinsic factors (see review by Manasek, 1983). The experimental basis for this view is that hearts isolated from the embryo before looping begins, and cultured in a variety of environments, are reported to be capable of undergoing normal looping. Early studies (reviewed and extended in Bacon, 1945) indicated that isolated heart rudiments from amphibian embryos have a certain capacity to curve *in vitro*. However, the key reference appears to be that of Butler (1952). In this work, heart rudiments were isolated from early chick embryos and cultured in hanging drop preparations in Ringer's salt solution. Looping was observed to proceed normally at the stages tested. Similar studies were subsequently carried out by Orts Llorca & Gil (1967) and Castro-Quezada, Nadal-Ginard & De La Cruz (1972). These findings have been taken to rule out extrinsic factors as the primary cause of looping (Stalsberg, 1970; Manasek, 1983). However, although the work of Butler is frequently cited in this regard, upon study it reveals a number of surprising deficiencies. It has only been published as an MA thesis, and is correspondingly difficult to obtain. The technique of hanging drop culture imposes surface tension strains on the explant, and the medium employed appears primitive in the light of current organ culture techniques. Most crucially, however, the majority of explants were made after the time at which looping had been initiated *in vivo*. At Stage 9 (stages according to Hamburger & Hamilton, 1951) chick heart *anlagen* are as straight as they ever are; by Stage 10, looping has patently begun. Butler states that 22 explants were made in total. 15 of these were from between Stages 11 to 13. The remaining 7 are described as being prepared from embryos of '9 to 12 somites'. Embryos with 7, 8 or 9 somites belong to Stage 9, and all have straight hearts: embryos with 10, 11 or 12 somites belong to Stage 10, and have hearts which have commenced looping. Since the exact stage distribution of embryos within Butler's experimental group is not given, it must be presumed that at least some of these hearts had begun to loop at the time of

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explant. The subsequent study by Orts Llorca & Gil (1967) also described the embryos as being of Stages 9 and 10, but did not give any experimental numbers at all, while Castro-Quezada *et al.* (1972) employed embryos at Stage 10 (i.e. certainly after looping has begun) for isolation experiments. Manasek (1983) states that Butler's experiments were repeated successfully, but gives no experimental details or numbers. It is therefore unclear from the literature how many unlooped hearts were used in these studies, and it is possible that the number is small. Since it is arguable that a heart which has begun to loop *in vivo* under the influence of external factors may well *continue* to loop *in vitro*, it is crucial to the argument that looping is intrinsic, to establish that a significant number of pre-looping hearts can indeed loop *in vitro*. In order to demonstrate this point unequivocally, we therefore decided to remove hearts from embryos of 6 somites onwards (i.e. Stage 8: the point of heart rudiment fusion in chick embryos) and to record the exact somite number of each donor embryo. An organ culture technique was employed which allowed direct observation of several hearts simultaneously.

MATERIALS AND METHODS

Fertilised chicken eggs (Muirfield Hatcheries, Kinross) were incubated blunt end up at 38 °C for 36 hours in a Marsh automatic incubator (Lyon Electric Co, San Diego, USA).

Single strength Alpha Eagle's medium (Flow Laboratories, Irvine, Scotland) was supplemented with 10% fetal calf serum (FCS), 50 iu/ml penicillin, 50 μg/ml streptomycin and 20 mM HEPES buffer (all Flow Laboratories). Double strength medium was made up to contain twice the above amount of supplement and then diluted with an equal volume of 0·6% Bacto-Agar (Difco) in Analar water (BDH) at 40 °C, to produce an Agar medium mixture with the same final concentration as single strength medium. One hundred μl of Agar mixture was added to each of the centre 60 wells of a flat bottomed bacteriological grade 96-well plate (Sterilin). The outer wells were filled with single strength medium to inhibit drying out. Plates were then cooled to 4 °C for 15 minutes to allow the Agar mixture to set.

Culture on this Agar gel provides good nutrient and mechanical support for heart rudiments without the possibility of distortions arising due to adhesion of the heart to the substrate. Quantitative comparisons between this method and more conventional forms of organ culture are in progress in this laboratory.

Chick embryos were removed through the blunt end of the egg and placed in a sterile 30 mm bacteriological grade Petri dish (Sterilin) containing single strength medium. The embryos were staged, and embryos of 6 to 12 somites were prepared for heart removal.

Squares of Silastic elastomer were placed in 30 mm dishes and covered with single strength medium. A square of exposed photographic film was placed over the elastomer to provide contrast. The donor embryos were then pinned, endoderm side up, to the elastomer with 4 insect pins (00 gauge).

Electrolytically sharpened tungsten needles were used to remove the hearts from the embryos. First, the pericardial cavity was cut open either side of the heart in a cranial–caudal direction. Then the caudal attachments of the sinus venosi were severed. A needle was then passed behind the heart to sever the dorsal mesocardium and finally the cranial attachment of the heart at the truncus arteriosus was cut. A specially made tray of Tantalum foil was then used to transfer the heart to one of the wells of the previously prepared 96-well plate. Drawings were made of each heart and the plate transferred to a humid 37 °C CO_2-gassed incubator.

Twenty four hours later the hearts were examined and redrawn. Where looping of the hearts had occurred, it was quite unequivocal (Figs. 1–3). With a complex three-dimensional shape like the looped heart, attempts at defining the degree of looping were felt likely to give spurious exactitude to the results, and hearts were therefore simply scored as having looped or not looped. Selected hearts were photographed before and after culture using a Wild MPS51 photomicroscope system. One control heart before culture, and two experimental hearts after culture, were prepared for scanning electron microscopy. Both these experimental hearts had previously been photographed. Scanning electron microscopy was carried out as follows: hearts were fixed for 5 hours in 2·5% glutaraldehyde in 0·2 M sodium cacodylate buffer to which 0·6 g sucrose per 100 ml had been added. They were then washed in 0·08 M sodium cacodylate buffer, then dehydrated through an alcohol series to absolute alcohol. The specimens were transferred using wax-coated forceps to metal stubs where they were mounted with double-sided tape. Specimens were first placed in an Emscope SC500 sputter coater. The replicas were examined and photographed using a JEOL-35Cs scanning electron microscope system.

Spontaneous rhythmic contractions were observed in 22 of the heart cultures after 24 hours. Experiments on intact chick embryos (Manasek & Monroe, 1972) have demonstrated that contraction is not necessary for looping to occur.

RESULTS

A total of 36 hearts from Stage 8 to 10 were explanted; 23 of these were from Stage 8 and 9, and all of these were scored as being straight before culture. All experiments are listed in Table 1 by stage and somite number at time of explant.

Figure 1 shows a heart *in situ* before removal; Figure 2 shows the same heart after removal and Figure 3 shows this heart after 24 hours of culture. The heart was then prepared for scanning electron microscopy, and one of the resulting micrographs is shown in Figure 4.

As can be seen, all the hearts except one showed unequivocal looping. This heart was one of the earliest removed. In addition, it was noted at the time of the experiment that the heart had suffered some damage during removal, and failure to loop may have been due to this cause.

DISCUSSION

Heart looping is generally described as being an intrinsic process, on the basis of experiments in which isolated early heart rudiments loop when cultured *in vitro*. Examination of these experiments, however, reveals that most of them were carried out after the time at which patent looping had commenced *in vivo*. Although it appears that a minority of experiments may have been carried out before the onset of looping, it is not possible to estimate how many are in this category from the published information. Given that it is possible that looping initiated by external forces *in vivo* might well continue for mechanical reasons after isolation, this important question must therefore be said to remain in doubt. To resolve this problem, we have therefore explanted heart rudiments from times earlier than any previously reported and the results clearly indicate that hearts can indeed loop when isolated from external forces such as mechanical constraints. The currently accepted view, although not well founded, fortunately turns out to be correct. This is not, of course, to imply that heart development cannot be modified or stabilised *in vivo* by external forces. For instance, Lepori (1967) demonstrated that the direction of heart looping can be reversed by cutting the right cardiac splanchnopleure. Normal looping can be restored in these

Table 1. *Looping behaviour of hearts after explant*

Stage	Somite number	Number of experiments	No. unlooped after 24 hours	No. looped after 24 hours
8	6	4	1	3
9	7	2	0	2
9	8	4	0	4
9	9	13	0	13
10	10	9	0	9
10	11	2	0	2
10	12	2	0	2

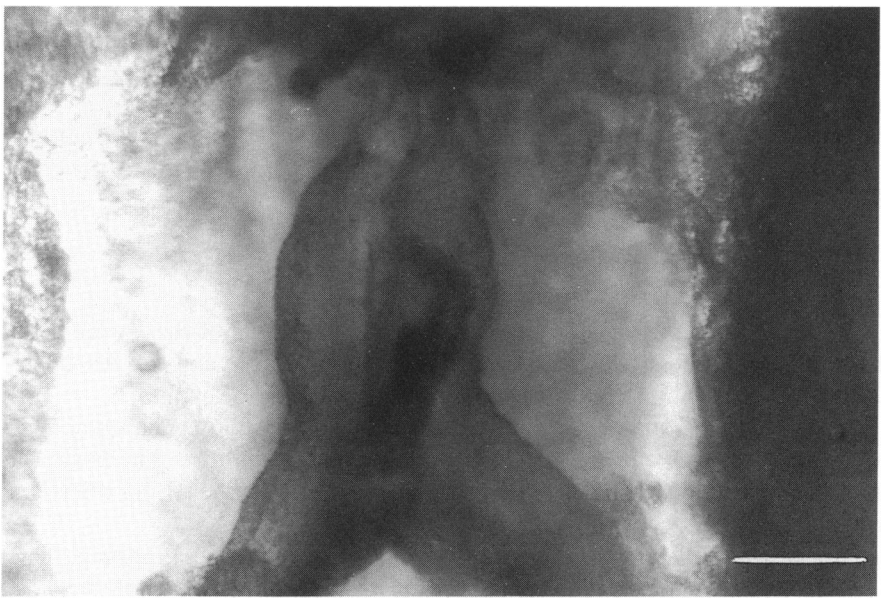

Fig. 1. Heart from a 9 somite donor chick embryo shown in ventral view before isolation. Scale bar represents 200 μm.

embryos by a compensatory cut to the left splanchnopleure (Castro-Quezada *et al.* 1972). Plainly, however, the primary force behind the looping is intrinsic. A number of views have been put forward to explain how this might occur. Lepori (1967) suggested that looping may be brought about by differential cell migration rates on each side of the embryo. Stalsberg (1970) proposed that differential rates of incorporation of precardiac material into the heart from each side could be responsible (Stalsberg & Dehaan, 1969). Manasek, Burnside & Waterman (1972) noted that there is a difference in cell shape between the concave and convex surfaces. Lacktis & Manasek (1978) implicated the dorsal mesocardium as having an important role in providing positional information. Manasek (1983) and Manasek *et al.* (1984) consider the overall implications of previous findings and advance a remarkable model which proposes that a handed helical arrangement of myofibrils in the myocardium might be responsible for regulation of a deformative force produced by the cardiac jelly, which is known to exert internal pressure on the myocardial envelope (Nakamura & Manasek, 1981). Cardiac jelly certainly changes in structure and composition during heart morphogenesis (Hurle, Icardo & Ojeda, 1980).

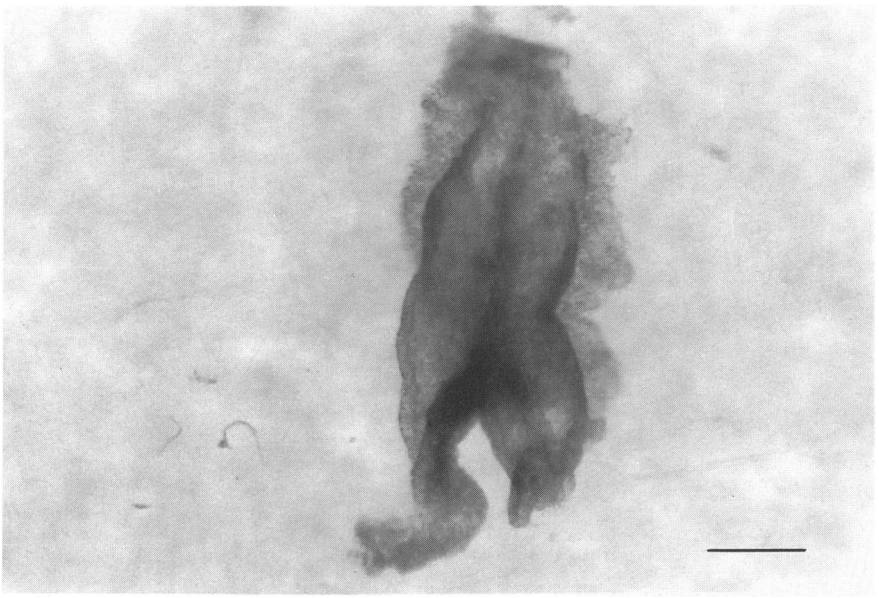

Fig. 2. Heart from the same embryo after isolation. Scale bar represents 200 μm.

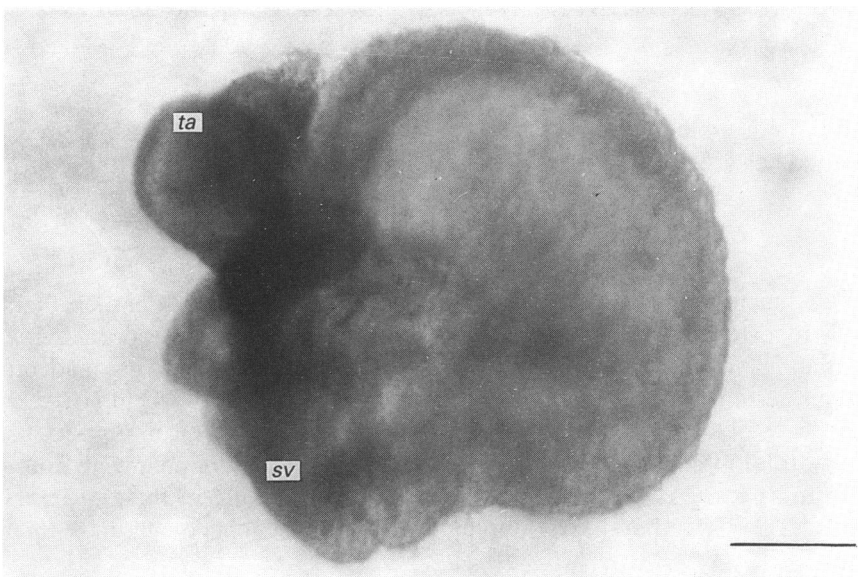

Fig. 3. Heart shown in Figs. 1 and 2 after 24 hours of subsequent culture. Marked looping has occurred. *sv*, original sinus venosi; *ta*, truncus arteriosus. Scale bar represents 200 μm.

Endocardial cells also appear to display a degree of polarisation, which may contribute to the looping phenomenon (Icardo, Ojeda & Hurle, 1982). Colchicine does not affect the initial looping process (Icardo & Ojeda, 1984), so presumably this is independent of the action of microtubules.

Since we confirm here that the process of looping is indeed intrinsic, it may be that important clues can be gained from the study of heart rudiments of genetic strain of mice in which heart looping is abnormal (Layton, 1978; Layton & Manasek, 1980).

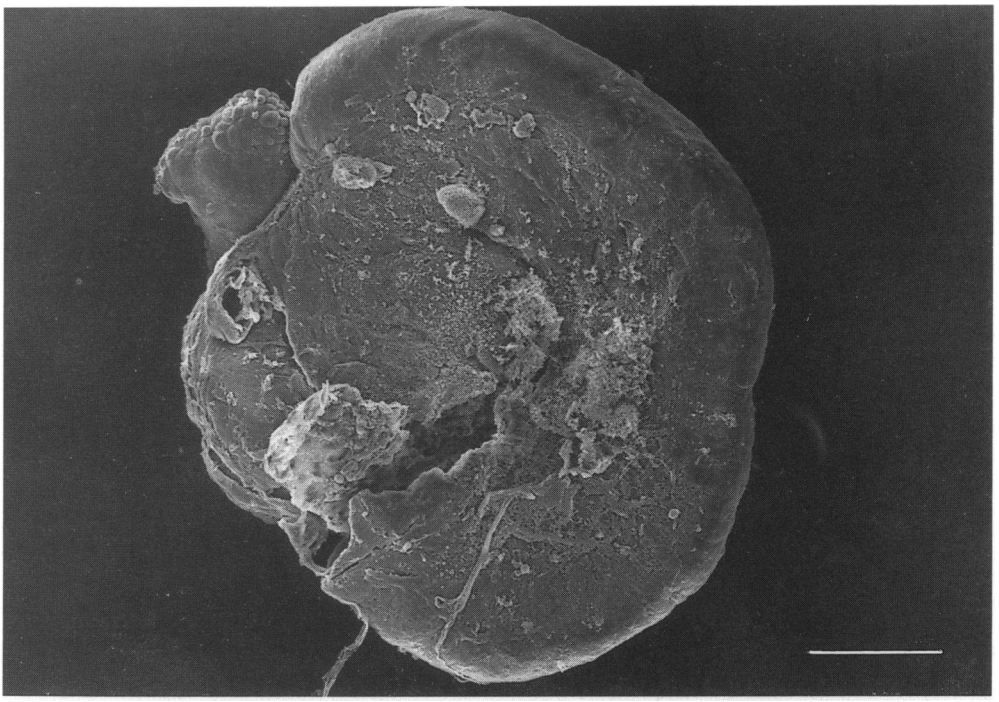

Fig. 4. Scanning electron micrograph of heart shown in Fig. 3. Scale bar represents 100 μm.

Certainly, the simple test system we describe may make it possible to devise experimental tests of a number of likely candidate mechanisms for the control of looping.

SUMMARY

Heart rudiments were removed from chick embryos at times earlier than any previously reported, and cultured *in vitro*, in order to test the widely reported view that looping is an intrinsic process. This view is based on experiments that are inadequately reported in certain key details. An organ culture technique was employed which combined ease of observation with good nutrient and mechanical support for the explants. A total of 23 hearts from stages before the onset of looping were examined. Hearts from as early as the six-somite stage looped normally in these experimental conditions. The view that heart looping is an intrinsic phenomenon is therefore confirmed.

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