The prothymosin α gene is specifically expressed in ectodermal and mesodermal regions during early postimplantation mouse embryogenesis

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Abstract Prothymosin α (ProTα) is a highly acidic nuclear protein, once believed to have an extracellular immunoregulatory role but more recently implicated in cell proliferation and/or differentiation. Several recent studies have revealed that ProTα mRNA is present during embryogenesis. However, these studies did not investigate the spatial distribution of ProTα mRNA in the embryo. Here we present a detailed study of the spatial distribution of ProTα mRNA during the early stages of postimplantation development (6.5–12.5 dpc) of the mouse. Three findings are of particular interest. First, ProTα mRNA levels increase during the early postimplantation stages (6.5–8.5 dpc) of mouse embryogenesis. Second, ProTα mRNA is not uniformly distributed in the mouse embryo, but is present in a spatially specific manner. Third, we have observed that the mouse ProTα gene is expressed almost exclusively in ectodermal and mesoderm-derived structures, and not in cells which give rise to the definitive endoderm.

Key words: Prothymosin α; Mouse development; Cell proliferation

1. Introduction

ProTα is a highly acidic 12.5 kDa polypeptide originally isolated from rat thymus [1,2] and subsequently detected in a wide range of organisms and tissues [3–5]. There is a high degree of homology among the ProTα and ProTα cDNAs of different species [6–11]. In humans, ProTα is encoded in a gene family composed of six members [8]. One of these genes, containing five exons, gives rise by alternative splicing to two ProTα mRNA species, while the other five genes appear to be pseudogenes [8]. Human ProTα gene has been localized to chromosome 2 [12].

It was once believed that ProTα function was involved in immune responses [3,13,14]. However, the presence of both the protein [15] and its mRNA [9,16] in a wide variety of tissues and organisms, the lack of a hydrophobic signal in the ProTα sequence [6–11] and the finding that ProTα mRNA is localized exclusively on free polysomes [8], are strong arguments against the possibility of an immunological role. On the other hand, a putative nuclear localization signal was detected in the ProTα cDNA sequence [17] and several experimental approaches [18–21] have provided evidence that this polypeptide has a nuclear site of action. Furthermore, there is good evidence to suggest that ProTα has a role in cell proliferation activity [7,9,14,22,23]. The fact that cells in which ProTα mRNA has been blocked with antisense oligomers cannot divide [24] strongly supports this theory. Recent findings indicate that the biological function of ProTα is in some way regulated by a phosphorylation mechanism which is highly dependent on cell proliferation activity [25–27].

Although ProTα mRNA levels during embryogenesis have been analysed before [9,22], there have been no studies of the spatial distribution of ProTα mRNA in the embryo. To further investigate the role of ProTα during development, we have used in situ hybridization to map the distribution of ProTα mRNA in the early postimplantation mouse embryo.

2. Materials and methods

2.1. Cloning of the mouse ProTα cDNA

To obtain the mouse ProTα cDNA, a mouse thymus cDNA library (Stratagene) was screened with a human ProTα probe at low stringency. To obtain the human ProTα probe, oligonucleotide primers complementary to nucleotides 295–315 (5′) and 370–390 (3′) of the human ProTα cDNA [7], flanked with additional 5′ nucleotides coding for the *Eco*RI restriction site, were used as primers in a standard PCR amplification reaction [28] using 5 μg of human genomic DNA (denaturation 95°C, 25 s; annealing 58°C, 30 s; elongation 72°C, 30 s; 40 cycles). The PCR product was gel-purified, extracted twice with phenol–chloroform, and ethanol-precipitated with 20 μl of glycogen as carrier. The precipitate was then resuspended and digested with *Eco*RI, and the resulting fragments inserted into the *Eco*RI site of pGEM7Zf(+) (Promega) using standard procedures [28]. The subcloned amplification product was sequenced to confirm its identity as human ProTα. A ^{32}P-labelled copy of the insert of this subclone was obtained using a random prime labelling kit (Pharmacia), and then used as probe to screen a mouse thymus cDNA library (Stratagene) at low stringency. Plaque lifts were hybridized in 5 × SSC, 10 × Denhart's, 0.5% SDS, 100 μg/ml salmon sperm DNA, at 65°C. Final wash conditions were 1 × SSC, 0.2% SDS at 55°C. Individual hybridizing clones were plaque-purified and phage inserts were subcloned in pBluescript (Stratagene) using the in vivo excision technique (Stratagene). The longest clone was sequenced on both strands by the dideoxy technique using the Sequenase enzyme (US Biochemical Corporation).

2.2. Mouse embryos

Following cervical dislocation of pregnant females, the conceptuses were removed and dissected free of decidua in phosphate-buffered saline (PBS). Noon of the day of plug was taken to be 0.5 days post coitum (dpc). Dissection of postimplantation embryos was as descibed by Cockroft [29].

2.3. Ribonuclease protection analyses (RPA)

Total RNA was isolated from embryos by the acid guanidinium thiocyanate–phenol–chloroform technique [28]. RPA was performed with the RPAII Ribonuclease Protection Assay kit (Ambion). ^{32}P-labelled antisense RNA probes were obtained using the Boehringer RNA transcription kit from pBluescript plasmids containing a 372 bp *Dpn*I–*Mae*II fragment of the mouse thymus ProTα cDNA. In each assay, 1×10^{10} cpm of riboprobe was used.

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Abbreviations: ProTα, prothymosin α; dpc, days post coitum; RPA, RNase protection assay.

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2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Whole-embryo RNA was purified and reverse-transcribed as per [30]. The resulting cDNA was amplified for 60 cycles of PCR and separated by electrophoresis in agarose [28]. Oligonucleotides complementary to the 171–203 (5′) and 489–508 (3′) nucleotides of the mouse ProTα cDNA [11], flanked with additional 5′ nucleotides coding for the *Eco*RI restriction site, were used as primers in PCR. The PCR product (PT133) was subcloned and sequenced as described in section 2.1.

2.5. Whole mount in situ hybridization

Whole mount in situ hybridization was done as per Wilkinson [31] with 10-h incubation. The signal was detectable within 20 min. Photographs were taken under an Olympus SHZ zoom stereo microscope with a darkfield stand.

3. Results and discussion

3.1. Isolation of a mouse ProTα cDNA clone

This article presents a detailed study of the spatial distribution of ProTα mRNA during early postimplantation development of the mouse (6.5–12.5 dpc). As a first step we isolated a mouse thymus ProTα cDNA. Oligonucleotide primers complementary to nucleotides 295–315 and 370–390 of the human ProTα cDNA [7], which span exon 3, were used as primers in a standard PCR amplification using human genomic DNA. The 100 bp amplification product was subcloned and the insert of the subclone was used as a probe to screen a mouse thymus cDNA library. The 1.3 kb insert of the identified clone was subcloned and sequenced. Comparison of the sequence of the mouse thymus cDNA clone with the sequence of the murine ProTα previously isolated [11] confirms that the isolated clone encoded the mouse ProTα homologue (data not shown).

3.2. ProTα expression in early postimplantation embryos

We were interested in determining if ProTα is involved in early events in the developing mouse embryo. To achieve this objective, ProTα mRNA levels during the early postimplantation stage (6.5 to 12.5 dpc) were examined by RPA (Fig. 1A). ProTα transcripts were first detected at 7.5 dpc and were present in the subsequent stages studied; no transcripts were detected at 6.5 dpc by this method (Fig. 1A). However, since we

Fig. 2. Spatial distribution of ProTα transcripts in early postimplantation mouse embryos (6.5–10.5 dpc). Unless otherwise stated, embryos are oriented so that anterior is at left and dorsal is at the top of page. (A) Hybridization labelling of a 6.5 dpc whole-mount embryo. ProTα mRNAs are present in the ectoplacental cone (EC) and in a small patch of epiblast cells at the anterior of the streak (arrow). An embryo hybridized with a sense ProTα riboprobe is shown as a control. (B) Hybridization labelling of a 8.5 dpc whole-mount embryo. ProTα mRNAs are widely distributed and are present at particularly high density in the cephalic mesenchyme (CM), presomitic mesoderm (PM) and intraembryonic mesoderm (M). An embryo hybridized with a sense riboprobe is shown as a control. (C,D) Hybridization labelling of a 9.5 dpc whole-mount embryo with sense (C) and antisense (D) ProTα riboprobes. High densities of ProTα mRNA were detected in the prosencephalon (PE), in the branchial arches (BA), in the forelimb bud (FB) and in the posterior somites (arrowheads). No transcripts were detected in the region overlying the midbrain or in the heart (H). (E,F) Higher magnifications of the branchial (E) and tail (F) regions of the 9.5 dpc whole-mount embryo shown in (D). (G) A control 10.5 dpc embryo hybridized with sense ProTα probe is shown to indicate the intensity of background staining. (H) Hybridization labelling of a 10.5 dpc whole-mount embryo. Transcripts are abundant in many areas of the brain, and are present at particularly high densities prosencephalon (PE), in the tail bud (TB), branchial arches (BA), the apical region of the forelimb bud (LB) and the dorsal border of the neurocoel (NE). Arrowheads point to the somites. Scale bars indicate 280 μm (A), 390 μm (B), 270 μm (C,D), 100 μm (E,F), 330 μm (G,H).

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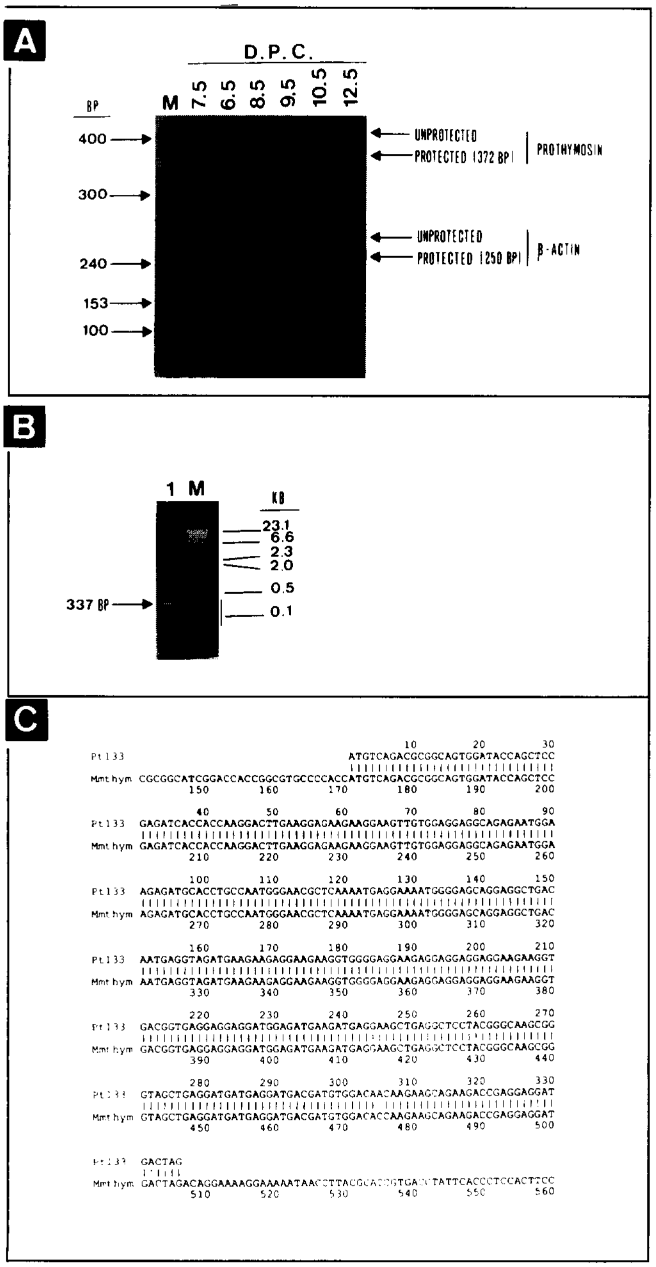

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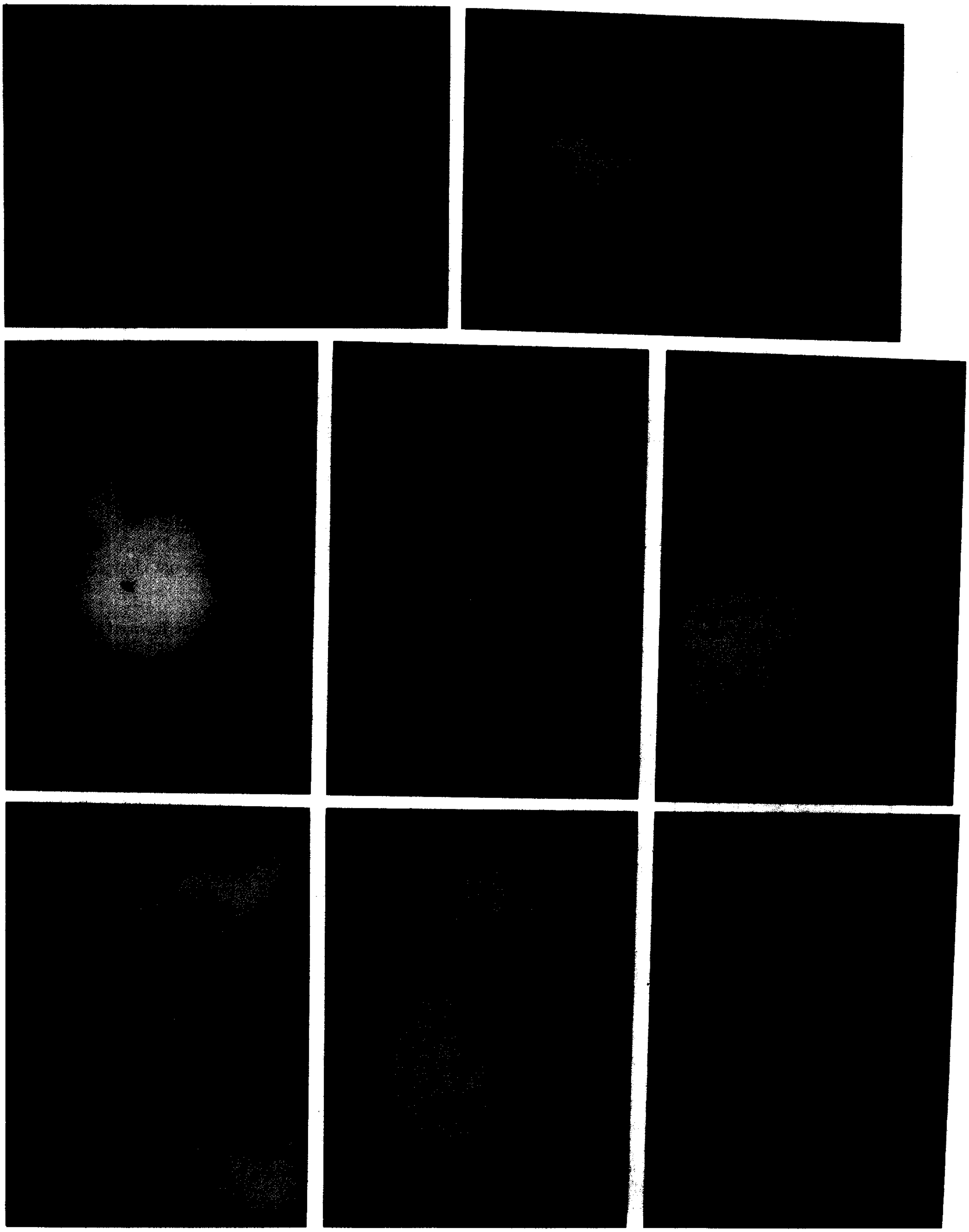
Fig. 1. ProTα RNA levels in postimplantation mouse embryos. (A) 10 μg of total RNA from embryos at the day of gestation indicated was analysed by RPA using the 372 bp *Dpn*I–*Mae*II fragment of the mouse thymus ProTα cDNA. A β-actin riboprobe was used for control of RNA integrity and loading. (B) RT-PCR analyses of ProTα mRNA levels in 6.5 dpc embryos. RT-PCR was done as described in section 2. Lane 1 corresponds to 20 pg of 6.5 dpc embryo RNA. The RT-PCR product (PT133) was purified and subcloned as described in section 2. (C) Comparison of the sequence of the RT-PCR product obtained in (B) and the sequence of the mouse ProTα cDNA [11]. The 336 bp region of overlap shows 99.7% sequence homology.



suspected that RPA is not sufficiently sensitive to detect very low levels of ProTα mRNA, we used RT-PCR, (which can detect as few as 10–100 transcripts in a single cell [30]), to check the RPA result. A ProTα signal was detected in 20 pg of 6.5 dpc embryo RNA (Fig. 1B). The RT-PCR product was subcloned and sequenced. Fig. 1C shows the sequence of the subcloned RT-PCR product alongside that of the mouse ProTα cDNA [11]. Sequence analyses confirmed that the RT-PCR product (PT133) obtained from 6.5 dpc embryo RNA was ProTα. Taken together, these results show that ProTα mRNA levels are very low at 6.5 dpc, increase between 6.5 and 8.5 dpc, and remain roughly constant between 8.5 and 12.5 dpc.

3.3. Investigation of the spatial distribution of ProTα transcripts by whole-mount in situ hybridization

To further characterize the pattern of ProTα mRNA synthesis during postimplantation development, we investigated the spatial distribution of ProTα transcripts over the early postimplantation stage (6.5 dpc to 10.5 dpc) by whole-mount in situ hybridization. On the basis of previous reports, we expected ProTα to have a fairly uniform distribution, but our results revealed that mouse ProTα is expressed in a spatially specific manner in the early stages of mouse development. Only very low levels of ProTα transcripts were detected at 6.5 dpc (Fig. 2A), confirming the results of RT-PCR. At this stage, ProTα mRNAs were detected in a small patch of cells in the anterior part of the streak (Fig. 2A), and in extra-embryonic regions such as the ectoplacental cone, the chorion and the allantois (Fig. 2A). In 7.5 dpc embryos, low levels of ProTα mRNA were present in the non-streak embryonic mesoderm and in the presumptive head-fold (results not shown). By 8.5 dpc, ProTα mRNA was widely distributed (Fig. 2B) and present at particulary high density in the cephalic mesenchyme, the presomitic mesoderm and the intra-embryonic mesoderm. At 9.5 dpc, distribution of ProTα mRNA was extremely complex (Fig. 2D): transcripts were present at high levels in the first and second branchial arches, in the forelimb bud and in the midbrain (Fig. 2D,E), and at very high levels in the posterior somites and tail bud (Fig. 2D,F); however, no transcripts were detected in the region overlying the midbrain, in the optic eminence or in the heart (Fig. 2D,E). At 10.5 dpc, the pattern reached by 9.5 dpc basically persisted. Transcripts were present at high density in the maxillary component of the first branchial arch and in the mandibular, hyoid and visceral arches (Fig. 2H), and at very high density in the tail bud, the apical region of the forelimb bud and the dorsal border of the neurocoel (Fig. 2H). Transcripts were also observed in the telencephalon, midbrain and forebrain, as well as in the area surrounding the auditory vesicle (Fig. 2H). No transcripts were detected in the anterior somites (although low levels were detected in the dorsal border of the posterior somites) or in the heart bulge. The tail bud contains the remnants of the primitive streak [32]: the high level of ProTα mRNA detected in this area in 10.5 dpc embryos is thus in accordance with that observed in 6.5 and 7.5 dpc embryos. This is also the area in which 'secondary neurulation' takes place. During this process, and by means of extremely active cell division, an undifferentiated mesenchyme begins to organize dorsally and to cavitate, forming the neural tube of the tail [32]. Thus, the high density of ProTα transcripts in this area could reflect a relationship between the function of ProTα and the high rate of cell migration and proliferation. A similar conclusion is suggested by the distribution of ProTα mRNAs in the forelimb bud, were transcripts are present at high density in the apical ectodermal ridge and at lower density in the proximal core (Fig. 2D,E,H). In the developing mouse forelimb, the apical ectodermal ridge is an area of extremely intense mitotic activity which acts as a progress zone of undifferentiated mesenchyme [33]. Thus as in the tail bud, there is a close correlation between ProTα levels and cell migration/proliferation activity. The distribution of ProTα mRNAs in the branchial region of the head (Fig. 2D–F) likewise strongly supports a relationship between cell migration and ProTα function. In this area, the interface between the hindbrain neural plate and the surface ectoderm gives rise to neural crest cells which interact with other head tissues, thus contributing to a series of cranial ganglia and branchial arches [33]. The spatial organization of crest migration [33] parallels the gradient of ProTα observed in the branchial arches. Futhermore, it is important to emphasize that the distribution of ProTα observed in the branchial region of the head and in the tail and limb buds parallels that of the expression of a number of important regulatory genes [35–37].

In the course of this study we have observed that the mouse ProTα gene is expressed almost exclusively in ectodermal (forelimb bud, brain, branchial arches, neuroepithelium) and mesoderm-derived (tail bud) structures, and not in cells which give rise to the definitive endoderm. No expression was detected in endoderm-derived organs such as the heart. The functional implications of this observation are not clear and will be the subject of future studies.

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