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A molecular view of cardiogenesis

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Summary. Cardiac development involves a complex integration of subcellular processes into multicellular and, finally, whole organ effects. Until recently it has been difficult to investigate the genetic control of this organ level differentiation of the heart. The proliferation of molecular biology methodologies has provided mechanisms to directly investigate the control of these processes. This article focuses on molecular lines of research on two key areas in cardiac development: the regulation of expression of sarcomeric contractile and regulatory proteins, and atrial natriuretic factor. Molecular approaches are described which have allowed investigators to begin to determine the tissue and stage-specific expression of genes, to locate those genes in the genome, determine their sequences, and to directly investigate the mechanisms controlling their expression.
Key words. Cardiac development; embryogenesis; gene expression; complementary DNA; molecular methodologies; myocardial contractility; myosin; atrial natriuretic factor.

Introduction

Regulation of cardiac development involves a complex integration of subcellular processes into cellular and multicellular effects. The complex organ-level processes of cardiac looping, myocardial differentiation, septation, trabeculation, and valve formation must be controlled on the genetic level. Until recently it has been difficult to investigate the genetic control of these whole organ level processes. The proliferation of molecular biology methodologies has provided mechanisms to directly investigate the control of these processes. In recent years these methodologies have begun to be applied to developmental questions in higher vertebrates. However, because of their complex methodologies, these approaches have not been as extensively integrated into the research repertoire of cardiac developmental biologists as they could be.

This article will focus on molecular lines of research in two key areas in cardiac development: the regulation of expression of sarcomeric contractile and regulatory proteins, and the regulation of expression of atrial natriuretic factor, a major secretory product of cardiac myocytes which is involved in regulation of cardiovascular homeostasis. First, however, the major strategies in the molecular field will be outlined to provide the uninitiated cardiac developmental biologist with a primer for understanding molecular terminology, methodologies, and most importantly, the types of questions which can be answered with these approaches. It is not the purpose here to describe protocols; for this the interested reader is referred to excellent reference texts from which much of this outline is derived [2, 20, 23, 24, 40].

Molecular approaches allow investigators to determine the tissue and stage-specific expression of genes, to locate those genes in the genome, and determine their sequences. The newest approaches allow direct investigation of the mechanisms controlling the expression (or repression) of genes via action on regulatory portions of the gene. Using these methodologies, investigators can directly test which putative epigenic (extrinsic) factors are capable of regulating expression of specific genes, and how they accomplish this interaction with the genome. By building on such findings on a number of different cardiac-specific gene programs, we may be able to construct a pattern which translates to whole-organ effects.

Outline of molecular strategies

Molecular strategies yield results on structure and function on the gene, RNA, and protein product level. Approaches used to identify unique gene products on the protein level have been employed for some time. They include biochemical identification of the isolated protein on the basis of its molecular weight (MW) and charge differences in its native configuration, or MW alone (as determined by native and denaturing protein gel electrophoresis, respectively). Gross differences in amino acid sequence can be detected from the size of fragments

generated by proteolytic digestion with enzymes that cleave at specific amino acid sequences. In the last ten years, there has been an explosion of research results obtained from immunochemical identification of proteins by the use of polyclonal and monoclonal antibodies. Antibodies recognize short (generally 4–5) amino acid sequences termed epitopes. A polyclonal antibody will contain antibody molecules directed against a variety of epitopes, while monoclonal antibodies are each directed against a single antigenic epitope. It is now possible to have such antibodies commercially generated.

The power of antibodies, particularly in developmental studies, lies in their capacity to localize expression of molecules to specific cells (even when present in small numbers, such as conduction system cells) and to specific locations or structures within those cells. Cell-specific isoform expression has been utilized to follow development of atrial and ventricular regions of the heart[8,13,29,35,36,45]. Antibodies can then be employed to identify the specific protein after it has been electrophoretically separated from other proteins and transferred to a retentive nitrocellulose filter (Western blot). Antibodies can also be employed as tools to isolate the protein of interest by passing the crude extract through an affinity column to which the antibody is bound; the specific protein alone will be retained. This has been crucial in cardiac development studies in the isolation of conduction-system specific proteins[14]. In recent years, antibodies have come to serve a new and important role: they are being employed to isolate specific messenger RNA (mRNA) molecules or DNA fragments which code for the protein against which the antibody is directed (see below); they are also being used to *verify* that mRNA molecules or DNA fragments isolated by other means code for the protein of interest.

There are limitations to analysis of gene expression purely on the basis of the gene product. Some of these limitations have been overcome by employing DNA methodologies. In addition, DNA methodologies have allowed *direct* examination of questions of regulation of gene expression that have only been indirectly assessible via the final protein product. At the base of these molecular approaches is the generation of molecular probes: fragments of DNA (or RNA) which can be hundreds of nucleotides (NT) in length. To generate DNA probes, one can start with mRNA, isolate and characterize that which codes for a particular protein, and manufacture DNA sequences complementary to it (cDNA). A single protocol can be used to separate total mRNA from total RNA, by relying on the fact that only mRNA has a poly-A^+ (poly-adenylated) tail (which will bind to complementary oligo-dT sequences immobilized on a sepharose bead column). Any of several approaches can be employed to isolate specific mRNAs: selection of appropriately sized mRNA for the MW of the protein, immunoprecipitation of mRNA with an already proven antibody to its protein, or hybridization (complementary base pairing) with existing cDNA probes for a closely related protein (which requires only about 50 base pairs in common for hybridization). The selected mRNA can then be characterized if necessary by in vitro translation of its protein product. Translation systems derived from lysed cells such as rabbit reticulocytes provide all the cell machinery necessary to translate any foreign mRNA in vitro, and are commercially available. The translated protein can then be assayed by gel electrophoresis or antibody binding to verify that the right mRNA has been isolated.

Complementary DNA (cDNA) probes can then be generated from the mRNA. Using reverse transcriptase (a viral enzyme which transcribes DNA from RNA), a single-stranded DNA copy is first made. A second DNA strand is generated by causing the DNA to loop back on itself. The DNA loop is cut by S1 nuclease (an enzyme which cleaves only the single-stranded DNA contained in the loop), leaving a typical double-stranded DNA molecule. Since a cDNA molecule is a copy of the mRNA, and not of the genomic DNA from which the message was transcribed, it contains only the exons (which code for the protein), and the non-translated sequences at either end of the mRNA (which are utilized in processing the protein molecule). The cDNA does not contain the introns (non-coding sequences which are present in eukaryotic DNA between the exons), or the flanking regions of the DNA (to either side of the mRNA sequences) which regulate expression of the gene (termed promoters and enhancers; see below). Nevertheless, the extensive sequence homologies that cDNA probes contain with the genomic DNA are more than sufficient for hybridization (base pairing).

The cDNA probes can then be cloned. This capacity to generate *large* quantities of a DNA probe is at the base of the proliferation of recombinant DNA methodologies. The cDNA fragment is ligated into a commercially available cloning vector (bacterial plasmid or phage) which replicates the foreign DNA along with the vector's DNA after transfection (insertion) into bacterial hosts (typically *E. coli*). The plasmids are engineered with 'restriction sites' for insertion of this foreign DNA after cleavage by restriction enzymes (bacterial enzymes which cleave DNA or RNA at specific bases). The plasmids also contain inserts which impart antibiotic resistance, which are used to select successfully transfected colonies.

Alternatively, one can start with total genomic DNA (now commercially available for a number of different species), cleave it into fragments with restriction enzymes, and clone the fragments as above. This approach requires growth of extensive numbers of clones to insure that DNA fragment of interest will be cloned. It is productive if a specific probe is already available, such as antibodies, purified mRNA, or other related cDNA probes, which can be used to select the colonies.

There are several approaches available for screening to select clones of interest. In colony hybridization (nucleic

acid hybridization), transfected colonies are transferred to filter paper, cells lysed, the immobilized DNA denatured to single-strand form, and hybridized to an existing cDNA probe which is labelled with a radioactive marker for detection (a modified Southern blot procedure). Alternatively, genomic DNA fragments can be electrophoretically separated, transferred to nitrocellulose, and hybridized with the same radiolabelled probe (the original Southern blot procedure). When hybridization probes (antibodies, specific mRNAs, cDNA) are not available, the cDNA can be cloned into a bacterial 'expression vector' next to an active bacterial 'promoter'. The promoter will cause the cDNA to be transcribed (see below), and cDNA can then be identified by the protein products it produces. After screening, colonies of interest can be recovered from original plates and subcloned to yield purified cDNA probes, and to generate enough cDNA for subsequent uses.

There are a number of ways in which these probes can be employed. The methodology that may be of most interest to developmental biologists is the use of cDNA (or RNA) probes for in situ hybridization [24]. This is the process of detection of nucleic acids (NA) within intact cells by hybridization of labelled NA probes with homologous sequences within the cell. RNA and DNA probes can both be used, and mRNA or DNA sequences can be detected. The hybridization provides a longer sequence homology than that obtained with monoclonal antibodies, and thus a greater degree of specificity. The other advantages of in situ hybridization lie in greatly increased sensitivity (detection of only a few copies per cell is possible), and the capacity to use a range of tissue preparation methods, including formalin-fixed paraffin or plastic embedded tissues. This results in superior retention of ultrastructure as compared to that in unfixed, frozen sections (often required for retention of antigenicity when using antibody probes). Radioisotopic labelling methods are utilized for maximal sensitivity, but non-isotopic labels can also be employed if quantitation of signal or great sensitivity are not required.

Another major avenue of research which has been opened up by molecular methodologies is that DNA can be directly sequenced and the gene and its regulatory regions characterized when the genes of interest have been isolated by the above methods. Because of the methodologies involved, nucleotide sequencing is a practical approach, whereas generation of the amino acid sequences for which the nucleotides code is impractically slow for all but the smallest proteins. DNA is initially sequenced by a procedure referred to as 'restriction mapping', since it involves breaking DNA into large fragments (hundreds of base pairs in length) with restriction enzymes (RE) (bacterial enzymes which each recognize different target DNA sequences, usually 4–8 base pairs in length). Fragments are sized by electrophoretic separation. Then a map of RE cut sites can be constructed by further digesting the fragments generated by one RE with other REs, and comparing the overlapping fragments to generate a total map. At any step in this process the fragments which contain the gene of interest can be identified by Southern blot analysis (DNA fragments transferred to nitrocellulose are hybridized with radiolabelled mRNA). The final steps in sequencing involve 'reading' the individual NT sequence from these DNA fragments by chemical (Maxam-Gilbert) or enzymatic (Sanger) protocols. Pieces about 300 NT in length can be sequenced this way; results can be combined by sequencing overlapping fragments. For example, the chemical protocol involves radiolabelling DNA fragments at one end so that only the part of the fragment containing the label will be detected, treating the fragments in 4 separate aliquots with 4 reagents that destroy one of 4 NTs, and then 'reading' the resulting sequences from the bottom of the gel (the smallest fragments contain the NT closest to the end label). By then comparing the DNA sequence with that of the protein, one can delineate coding regions. The capacity to sequence genes has meant that functional roles of different parts of genes can now be directly assessed without having to rely on genetic mutants. It is possible to introduce specific mutations or deletions in specific regions of the known gene sequence, and assess the change in function of the gene and its product.

These methodologies are now being applied to analysis of transcriptional control mechanisms. Researchers are uncovering the mechanisms whereby activation (or repression) of genes is controlled via promoter/enhancer regions of DNA. This type of analysis is crucial to understanding how tissue-specific expression of genes is controlled, including control of transitions in this expression with development or functional challenges, and the precise nature of the factors which can cause this expression. The promoter is a NT sequence required for initiation and regulation of rate of transcription of mRNA from the DNA template. It is located immediately upstream from (preceding) the start site of transcription. Enhancer sequences are less well understood, but appear to assist in promoting gene transcription; they can exert this effect on promoters located upstream or downstream from them. Utilizing the same cloning and fragmentation techniques previously described, the putative regulatory regions of DNA located upstream from the gene can be isolated. One can further delineate regulatory regions by scanning for 'consensus' sequences which appear in most regulatory regions. (The most common are dubbed 'CAAT' and 'TATA' boxes on the basis of their content of C, A, and T sequences). Such analysis has been assisted by computer data banks of known gene sequences. These regulatory regions can then be assayed for their ability to promote expression of specific genes, utilizing a functional assay system referred to as the CAT assay. The putative control regions of the DNA are spliced into an expression vector immediately preceding (upstream to) the bacterial chloramphenicol acetyltransferase (CAT) gene, and this 'construct' is then transfected into

cultured cells of the tissue of interest. The bacterial CAT gene is not present in eukaryotic cells, so its expression relies on the presence of an active promoter preceding it. Only if the promoter region can be activated by factors within the host cell with the CAT gene be expressed. When the CAT gene is expressed, the product will acetylate radiolabelled chloramphenicol, which can be distinguished by radioautography from the non-acetylated form. Mutations or deletions from these putative regulatory sequences can then be progressively introduced to isolate the functional regions within the overall DNA sequence. By transfecting other cell types than the cell of origin of the promoter, it is possible to determine whether they contain regulatory factors which will also activate the promoter.

Applications of molecular approaches: molecular basis of myocardial contractility

One area of cardiac development in which molecular approaches have played a big role is the biochemical differentiation of the types of myocardium. Expression of different isoforms of contractile and regulatory proteins provides the major biochemical basis for the plasticity of contractile status, and has been extensively studied in neonatal and adult cardiac muscle. However, there is little understanding of the functional contribution of the normal pattern of isoform expression during cardiogenesis, and virtually nothing is known of the capacity of the embryonic heart to respond to challenges by altering its contractile protein phenotype. Recently a number of studies have documented the normal isoform pattern in the embryonic avian heart [8, 12, 13, 29, 35, 41, 43, 45] but only a few studies have examined the mammalian heart during the embryonic period [4, 36]. Little information exists on the factors which regulate this expression during cardiogenesis, but studies on gene regulation in postnatal and cultured cardiac muscle [16, 17, 19, 28] have begun to provide insight into potentially common mechanisms.

The diversity of isoforms expressed in cardiac (and skeletal) muscle, and the changes in this expression which can occur in response to functional challenges, were first detected on the basis of protein analysis. Biochemical assessment of molecular weight and charge differences of myosin isoforms has been combined with localization of isoforms to specific cell types by antibodies. Major advances have subsequently been made by directly assessing the mRNAs transcribed for those proteins, and identifying and sequencing some of the genes from which they are transcribed. Localization of expressed genes by in situ hybridization with cDNA probes has helped to elucidate the specific patterns of expression. Most recently, analysis has begun of the factors controlling the expression of these genes via analysis of promoter regions using expression vector systems.

This research has demonstrated that most of the sarcomeric proteins in higher vertebrates are members of multi-gene families. There are multiple genes for the contractile protein myosin, both for its heavy chain (HC) and light chain (LC) subunits, which are expressed in cardiac muscle [6, 11, 18, 19, 31, 37]. The contractile protein actin and the regulatory proteins troponin (T, I, C) and tropomyosin have cardiac and skeletal muscle specific isoforms, but do not have multiple isoforms *unique* to cardiac muscle. However, during embryonic cardiogenesis, some of these isoforms are co-expressed in both cardiac and skeletal muscle, resulting in multiple isoform expression [1, 5, 25, 26, 37, 41]. In higher vertebrates only myosin light chain and troponin-T isoforms are generated by differential splicing of mRNAs from a common gene [42]; in all other contractile and regulatory proteins a different gene product has been shown to be the result of expression of a different gene.

In the case of myosin HC expression, expression of different isoforms is correlated with functional differences. The higher the ATP utilization of an isoform, the less energy efficient the isoform, but the faster the rate of contractility it generates [3, 27, 32]. The role of myosin LC isoform switches has yet to be elucidated, although its isoform composition does influence access to calcium. Similarly, a role for switches in regulatory proteins has yet to be defined.

Regulation of isomyosin HC expression is tissue-specific, and is regulated differently in each muscle type during development and in response to functional challenges. Atrial, ventricular, and conduction system myocytes all have been shown to have a distinct myosin phenotype at some stage of development in both avian and mammalian hearts. Myosin HC alpha predominates in atria of mammals of all ages, while myosin HC beta predominates in the fetal ventricle, to be replaced totally by HC alpha in smaller mammalian hearts [4, 9, 10, 16, 18, 21, 22]. Functionally, mammalian myosin HC gene expression is less responsive in atrial than ventricular cells to hormonal or hemodynamic challenge [16, 28]. In the bird, atria and ventricles express distinctly different isoform complements from the onset of differentiation, but this complement differs in the embryo from the adult [6, 8, 12, 13, 29, 31, 35, 43, 45]. This isoform expression has been considered to be unmodulatable by extrinsic factors, although there has not been enough investigation in this area.

Conduction tissue has been shown to have a distinct myosin phenotype in both mammalian and avian hearts [7, 14, 30, 35], although there is still a great deal of controversy over the exact nature of the phenotype, as well as how early in embryonic development the cells can be discriminated from surrounding myocardium by this biochemical marker.

Most intriguing for understanding the genetic basis of these biochemical differences is the *regional* heterogeneity in contractile protein expression in the embryonic and adult mammalian heart [9, 10, 12, 30]. In the neonatal and adult heart, this heterogeneity may be correlated with

different functional or mechanical demands on regions of the heart wall (endocardial versus epicardial surfaces, for example). In the embryonic heart, such differential effects may also apply, but only experimental testing will determine this.

The genetic basis for contractile protein regulation can be clearly seen in functional regulation. The effects of thyroid hormone (TH) in particular have been well documented: it acts directly via nuclear receptors to up-regulate expression of myosin HC alpha, and down-regulate expression of myosin HC beta. In the small mammal the postnatal increase in circulating TH levels causes rapid transition in the ventricles from the 'fetal' isoform (HC beta) to the 'adult' isoform (HC alpha) within a few weeks after birth[4, 18, 21, 28]. It is not known if TH has the same effect on the genome before birth, although it does have such an effect on fetal ventriculocytes in culture[16, 17]. A number of recent investigations have begun to uncover the nature of the regulatory regions with which TH interacts to up-regulate expression of HC alpha, and how the tissue-specific differences in expression are effected[16, 17, 19].

The sheer number of myosin genes, the tissue-specific and developmental-stage manner in which their expression is regulated, in combination with the clear functional significance of such regulation in the adult, make this an important system for investigation of genetic control of higher level differentiation processes during cardiogenesis.

Atrial natriuretic factor

The second area of research in cardiogenesis in which molecular techniques have recently yielded intriguing results is the expression of atrial natriuretic peptide or factor (ANF). ANF is a small cardiac peptide hormone. As its name implies, it functions in the maintenance of cardiovascular homeostasis via its effect on electrolyte (principally sodium) and fluid balance; it has a number of other effects which have yet to be fully elaborated, such as vasodilatory effects and modulation of function of the renin-angiotensin-aldosterone system[15]. For years it was known that the adult atria contained granules which could be used to morphologically identify atrial myocytes, but their contents were a mystery. In the last ten years there has been an explosion of research as their contents have been identified and characterized as ANF. Expression of ANF is tissue-specific, and is developmentally and functionally regulated. The major expression of ANF in the adults is in atrial myocytes; there is some expression in the ventricle[15]. Functional challenges (ventricular hypertrophy, glucocorticoids) can induce ventricular expression of ANF[34]. Most intriguingly, a number of recent mammalian and avian studies have shown than embryonic ventricles as well as atria synthesize ANF; both fetal tissues contain a high amount of mRNA for ANF. During the first postnatal week, atrial ANF levels increase, while ventricular levels fall. Interestingly, the ventricular expression has consistently been localized to sub-endocardial myocytes[33, 34, 38, 39, 44]. Thus, ANF expression is regulated in a highly tissue-specific manner during development.

The molecular signals involved in ANF expression have recently been investigated by assaying promoter function using the CAT assay[34]. By introducing increasingly smaller fragments of the promoter region of the ANF gene into an expression vector transfected into atrial and ventricular myocytes of different developmental stages, it was determined that the same promoter fragment which induced atrial ANF gene expression at all developmental stages contained all necessary information to promote ANF gene expression in fetal ventricular myocytes and to suppress this expression in neonatal ventricular myocytes, as well as to promote ANF expression in response to hormonal (glucocorticoid) stimulus. These data are consistent with modulation of ventricular ANF gene expression by positive transacting regulatory factors (factors extrinsic to the gene).

The functional significance of this fetal ventricular ANF expression remains to be determined. It has been proposed that there may be a common 'motif' regulating cardiac gene expression, since two regions within the ANF regulatory region show homology with the cardiac myosin HC alpha gene. Comparable sequence homology was not found with any other genes[34]. What makes this sequence homology particularly intriguing is the striking correlation between the sub-endocardial regional localization of ANF expression within the fetal rat ventricle[33, 38, 39], and the same recently described subendocardial localization of alpha-myosin heavy chain expression in the fetal rat ventricle[36]. These cells may also be, by their location, part of the future conduction system (which has been shown to express both HC alpha and ANF in the adult mammalian ventricle). Correlation of expression of these two myocardial-specific gene products to the same subset of ventricular myocardial cells is the kind of result obtainable by molecular methodologies which will allow us to build a complete picture of the control of cellular differentiation within the heart. Is such coexpression due to a distribution of discrete cell populations with committed programs of gene expression, or to (as yet undetermined) localized stimuli which differentially promote the expression of certain muscle-specific genes? If the latter is the case, the homologous sequences in the promoter regions of both genes could be part of the mechanism for coordinate expression of these genes.

Conclusions

The results of biochemical and molecular investigations to date clearly indicate that atrial and ventricular myocytes express different gene programs from the outset of their overt differentiation, even though they initially begin this differentiation within the wall of a common tube subjected to common functional challenges, hormonal, metabolite, and oxygenation status on the gross level.

They display different membrane properties, including membrane receptors, ion channels and depolarization characteristics; different contractile protein phenotypes; and different secretory characteristics. The evidence concerning the origins and differentiation of cells belonging to the conduction system is in its beginning phase, aided by the fact that these cells have distinct patterns of contractile protein gene expression. The development of functional assays of the conditions for activation of a number of atrial and ventricular myocyte genes (promoter regions) will allow us to directly investigate the mechanisms regulating the differential expression of genes in atrial and ventricular myocytes during cardiogenesis.

We are now at a point where we can begin to determine in vivo which tissue-specific patterns of gene expression atrial and ventricular myocytes follow in early development, and in the face of extrinsic alterations in known developmental modulators such as hemodynamic flow and volume, or hormonal stimulus such as thyroid hormone levels. This is the first step in determining how the multiplicity of genetic programs specific to cardiac cell types and their stages of development are integrated to produce the complex differentials in growth which result in normal organogenesis.

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Development of the myocardial contractile system

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Summary. Recent studies regarding developmental changes in the myocardial contractile system from fetal, newborn, and adult animals are reviewed. From the data obtained so far, we conclude that in the early fetus myocardial contraction is mainly dependent on Ca which enters via the sarcolemma. Ca release from the sarcoplasmic reticulum is minimal. The role of the sarcoplasmic reticulum as a source of contractile Ca increases and the role of Ca influx across the sarcolemma in contractile system decreases with development.
Key words. Contractile system; fetus; premature myocardium; calcium; sarcoplasmic reticulum; contractile protein; sarcolemma.

Myocardial contractile function as a muscle

In the adult myocardium, a relatively small Ca influx across the sarcolemma induces the subsequent release of greater amounts of Ca from the sarcoplasmic reticulum (SR), which then activates the myofilaments[5]. Mitochondria also have a capacity to take up Ca, although it is unlikely that mitochondria regulate intracellular Ca normally. Thus the amount of Ca reaching myofilaments is dependent on the function of the subcellular organelles regulating cellular Ca concentration. Myocardial contractile function is largely dependent on the amount of Ca reaching the myofilaments and the quantity and quality of myofilaments.

Several investigators have studied postnatal changes in contractile function in the rat[3], cat[4], dog[23], and rabbit[24]. All these studies showed that the contractile force of the unit myocardium increases with development. The contractile function of the fetal heart, however, is less well known. According to the data of Sissman[30], the myocardium begins to contract at 3-somite-stage in the rat, 9-somite (about 8 days of gestation) in the rabbit, and Hamburger and Hamilton stage 10 in the chick embryo. The contractile function of these early embryonic hearts as muscles has not been studied extensively. Friedman[6] showed that contractile force in the near term fetal lamb is less than in the adult. This decreased contractile force in the premature heart was mainly attributed to the smaller amount of contractile protein per unit muscle. Developmental changes in intracellular Ca concentration have not been examined directly.

We studied developmental changes in the contractile system using the fetus at the 18th, 21st and 28th day of gestation (term: 31 days), newborn, 3–5-day-old, and adult New Zealand White rabbits[12,13]. In the rabbit, cardiovascular anatomy is established by approximately the 16th day of gestation. From the 18th day of gestation to birth, heart weight increased about 80 times (table 1). Using the isolated, arterially perfused ventricular or septal preparation[15–17,20], we measured the contractile

Table 1. Heart and body weights

Age	n	Body weight (g)	Heart weight (g)	Heart wt/body wt × 100
18-day fetus	8	1.25 ± 0.05	0.0043 ± 0.0006	0.34 ± 0.03
21-day fetus	8	2.9 ± 0.06	0.0132 ± 0.005	0.46 ± 0.03
28-day fetus	12	30 ± 0.9	0.136 ± 0.005	0.46 ± 0.01
5-day newborn	9	85 ± 5	0.34 ± 0.09	0.40 ± 0.05

Values are means ± SE. From Nakanishi et al.[12].