12-2001

Effects of a Serine 364 to Proline Mutation of the Connexin43 Protein in Transgenic Mice

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by

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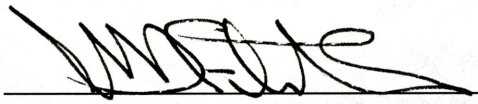
A Thesis submitted in partial satisfaction of
the requirements for the degree of
Master of Science in Anatomy

December 2001

Each person whose signatures appear below certifies that this thesis in their opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.



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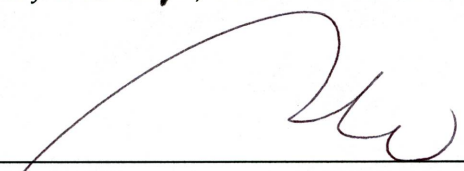

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ACKNOWLEDGEMENTS

Funding for this work was provided by NIH Grant: Fletcher-HL 53374, Veterans Administration Merit Review Grant: Fletcher-RDIS 0002, and Loma Linda University School of Medicine.

I wish to express special thanks to my advisor and chairperson Dr. William H. Fletcher for his constant and dedicated guidance, advice and support, without which this project would not have been possible. My gratitude to Dr. Paul J. McMillan, and Dr. Pedro B. Nava, my other committee members, for their advice, input and interest.

My appreciation to Dr. Chiranjib Dasgupta for his adroit expertise in molecular lab techniques and procedures. Particular love and thanks to Anna-Marie Martinez for her aid and support with all involved areas of this project from research, data collection to support in writing.

I wish to express my gratitude to Dr Robert H. Anderson for his invaluable knowledge and expertise in embryonic heart development and for his time. I would also like to thank the Animal Research Facility members at the Loma Linda Veterans Hospital for their time and efforts in the care of the many mice used in this research.

The transgenic mice were donated very generously. My thanks to Dr. Maithili Shah (Loma Linda University, CA) for the design of the transgene, to Dr. Cecilia Lo (University of Pennsylvania, PA) and to the NICHHD Transgenic

Mouse Development Facility at the University of Pennsylvania, Philadelphia for the creation of the founder colony of transgenic mice.

Last, but not least, I would like to thank my family for their motivational support, reassurances and bottomless source of love.

ABBREVIATIONS

Cx43	Connexin 43 protein
cDNA	Complimentary deoxyribonucleic acid
CMV	Cytomegalovirus
-COOH	Carboxyl group
dhfr	Dihydrofolate reductase
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
HLHS	Hypoplastic left heart syndrome
KPBS	PBS fortified with 50mM Potassium Chloride (KCl)
KO	Knockout
mRNA	Messenger ribonucleic acid
NEO	neomycin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKA	Cyclic-AMP-dependent protein kinase (protein kinase A)
PKC	Protein kinase C
RNA	Ribonucleic acid
RPM	Revolutions per minute
S364P	Serine 364 replaced with proline

SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscopy
TBS	Tris-buffered saline
TFA	Trifluoroacetic acid
VAH	Visceroatrial heterotaxia
WT	Wild type

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INTRODUCTION

Molecular genetics has been a popular subject for study in the last few decades, yielding an ever-expanding body of knowledge about the genome and new methods with which to obtain and interpret data for genetic analyses. The most interesting research has focused on the mechanisms of genetic mutations and its effect on human embryonic development. Among the most engaging research is the implication that genetics can play an integral part in a variety of diseases and disorders, including congenital heart disease (Grossfeld, et al. 1999).

Cardiovascular disorders which may be traced directly back to a genetic defect, familial or germ line, include cardiomyopathies, arrhythmias, connective tissue disorders, septation defects, laterality defects, outflow tract defects and both right and left sided obstructive lesions (Grossfeld, et al. 1999). These defects most often strike their victims during childhood, causing considerable pain and suffering not only to the patient, but also to the families who care for these children. In knowing that genetics is linked to a number of congenital diseases, it is imperative to gain a better appreciation and understanding of the mechanisms involved so that there is hope for the prevention and treatment of these devastating malformations.

Of particular interest, genetic defects have long been thought to be one of many contributors to visceroatrial heterotaxia syndrome (VAH), a critical developmental disorder of laterality with cardiac involvement which results in

neonatal death in the most critical cases unless it is corrected by life-saving cardiac transplants. In addition to laterality defects in the heart, VAH patients often have defects in lungs, spleen, liver, where the organs may exhibit a variety of abnormalities. The normally asymmetrical organ may be on the wrong side of the longitudinal axis (ie. liver found on the left), reversed (ie. right lobe of lung has two lobes and left lobe has three), symmetrical (ie. both lungs have three lobes like the right lung) , absent or multiple spleen (ie. asplenia or polysplenia), or some combination of these defects.

Studies indicate that there have been examples of familial transmittance in VAH, pointing the proverbial finger to genetics as a potential causation factor. DNA sequenced from patients with VAH and other heart defects by our group indicates that some have a mutation or a cluster of mutations in connexin43 (Cx43), the gene that encodes for a gap-junction protein found in many communication competent cells including cardiac and neural tissues (Britz-Cunningham, et al. 1995, Dasgupta, et al. 2001).

Britz-Cunningham et al. first documented that five patients had a single-point mutation substituting serine 364 for protein (S364P) in the Cx43 gene (S364P-Cx43), some independently, others in conjunction with other mutations located in the carboxyl terminal coding domain of the same gene. These patients were diagnosed with a severe form of VAH syndrome in which complex heart malformations are combined with multiple visceral situs abnormalities, all of

which result from a failure to establish normal left/right asymmetry. Some patients also had splenic involvement. Subsequent cell-cell dye-transfer studies showed that cell lines transfected with the S364P-Cx43 mutant gene formed Cx43 channels that were abnormally regulated.

More recently, Dasgupta et al. used denaturing gradient gel electrophoresis (DGGE) on heart tissue samples from Hypoplastic Left Heart Syndrome (HLHS) patients who had received heart transplants. Out of 14 patients, eight children had identical Cx43 mutations involving two silent polymorphisms and two missense mutations where arginine was replaced by glutamine at positions 362 and 376. This mutant sequence is identical to the sequence of the Cx43 pseudogene, and suggests that a rare recombination may have occurred between the gene (on chromosome six) and pseudogene (on chromosome five) in these patients and contributed to HLHS. One of these patients also harbored the S364P-Cx43 mutation on one of its alleles.

The S364P-Cx43 mutation has been explored further through molecular and developmental studies (Britz-Cunningham, et al. 1995; Levin and Mercola 1998) and it has been determined that indeed it has the capacity to disrupt gap junctions on a cellular level, however, its effects on a germ-line in-vivo model and the direct affect of the mutation on embryonic cardiac development has never been reported. The justification for the creation and analysis of a

transgenic mouse line is clear. Would the S364P mutation of the Cx43 gene cause VAH and defects in heart development?

THE MORPHOLOGICAL METHOD

In contemplating the possibilities of the effects of the mutation, it is important to discuss how cardiac defects, if found, are to be identified and categorized. Unlike genetic data, which can be quantified, the diagnosis of VAH is an anatomic description that may be induced in a molecular or cellular model, but whose effects cannot be described or analyzed with such data. It must be evaluated and reported qualitatively in a whole animal model.

Criteria for the clinical diagnosis and pathological description of malformed hearts have had few changes in the last three decades. Not since the controversial writings of Van Praagh (Van Praagh, et al. 1980) and even earlier writings by Goor (Goor and Lillehei 1975) has significant progress been made to establish a uniform means of describing heart malformations. Although this does not affect the clinical outcome of patients with heart defects, it is difficult for surgeons to predict what pathology to expect when treating patients with complex and multiple heart defects, especially if these defects or syndromes are labeled with non-standardized language (Uemera, et al. 1995). How is medical science able to communicate if the descriptions and analyses of basic heart structure appear as a "Tower of Babel" in an age when scientists possess the technology to clone replacement heart tissue in a petri dish?

It appears as if the answer is something that has been often touted by scholars, but most eloquently said by artist, scientist and part-time philosopher Leonardo da Vinci, "Simplicity is the ultimate sophistication." Indeed, simplicity is the key.

Robert Anderson has fervently proposed in both his writings and lectures for the utilization of the "morphological Method"(Anderson and Becker 1997; Anderson, et al. 1999) first described by Lev (Lev 1954) and further elaborated by Van Praagh (Van Praagh, et al. 1964; Van Praagh, et al. 1980) and Goor (Goor and Lillehei 1975). The logic is simple. Describe what is seen grossly with a systematic approach that focuses on the general structures and its connections rather than the infinitesimal sub-components.

In the example of the description of the ventricles, normal or pathologic, the anatomy of interest can be broken down to its three parts: the inlet, the outlet, and the apical trabecular component. Using this basic principle, the concept of the "univentricular" heart is clearly erroneous and outdated, since hidden within the myocardium of such hearts a space can usually be found with identifiable trabeculations of the seemingly "missing" ventricle (Van Praagh, et al. 1980; Anderson and Becker 1997). Functionally, these hearts possess asymmetric characteristics of both right and left ventricles and should be described simply as such so that no misunderstandings can arise from the assignment of a fancy and poorly-descriptive name.

Setting basic criteria and using this morphologic approach for reporting the effects of the S364P-Cx43 mutation would be most advantageous. A picture may be worth a thousand words, but a good simple description is worth a million words! The easiest practice to detect and describe the effect of the mutation on cardiac development would be to follow the method of analyzing the quality of the left ventricle and its immediate connections to the rest of the cardiovascular system. Using the standardized procedure, the transgenic line when compared with a control line, can yield insightful data.

GAP JUNCTIONS REVISITED

GAP JUNCTIONAL STRUCTURE

Gap junctions are aggregates of channels through which adjacent cells exchange ions, metabolites, physiologic signals and molecules smaller than 1kD, without utilizing extracellular space (Murray and Fletcher 1984; Stagg and Fletcher 1990; Godwin, et al. 1993). The low-resistance of the channel facilitates the rapidity necessary for instantaneous electrochemical transfer from cell to cell.

Each gap junction channel is made up of 12 monomers of one or more types of connexin protein (Bruzzone, et al. 1996; Kumar and Gilula 1996). Six connexin protein subunits form one hexameric hemichannel (a connexon) in the cell membrane. Two opposing connexons, one from each contacting cell, form an aqueous, hydrophilic gap junction channel that is distinctly separated from extracellular space. The gap junctions were shown to directly mediate intercellular communication through the use of affinity purified polyclonal antibody to these gap junctions. The antibodies were injected into ionically coupled hepatocytes and effectively eliminated the transfer of fluorescent dye between adjacent cells. Importantly, when an antibody to gap junctions was injected into one cell of an eight cell frog embryo, all structures that should have developed from that cell were abnormally formed or missing (Warner, et al. 1984).

All vertebrates express multiple connexin genes. In the mammalian gap junction family, 17 connexin cDNA's have been identified, each with a high degree of homology with the others. All connexins have four membrane-spanning domains, two extracellular loops, and a cytoplasmic loop. Both the amino and carboxyl termini are located in the cytoplasm. The specific sequences of the connexin genes, especially that of the carboxyl terminus region (Van Kempen, et al. 1995), are also highly conserved between species.

GAP JUNCTIONS IN DIFFERENT TISSUES AND SPECIES

Interesting enough, the major differences in gap junctions are not between species but among tissue types. The amino acid sequence in the transmembrane and the extracellular loops of the protein are well-conserved between tissues and species. The differences reside in the amino acid sequences of connexins from different tissue types and are predominantly located in the cytoplasmic loop and the carboxyl tail (Van Kempen, et al. 1991), areas that are generally accepted as the gap junction regulatory domains.

Gap junctions are located in many tissue types, especially those that have cells that are joined to each other in physiological continuity. They can be found transmitting electrical impulses between cells within an organ or even between unrelated cells transmitting growth inducer or repressor chemicals from opposite sides of the developing tissues (Levin and Mercola 1999).

To date, connexin40, connexin43, and connexin46 have been identified in mammalian myocardial cells (Kanter, et al. 1992; Van Kempen, et al. 1995). Despite the small differences between each gene, each connexin protein forms uniquely different gap junction channels with specific electrical properties (Veenstra, 1992). Cx43, however, is recognized as the predominant protein found in the heart in a variety of species, including humans and all mammals evaluated. (Beyer, 1987; Fishman, 1990; Van Keppem, 1995). In animal models, the pattern of Cx43 distribution of the pig and mouse is the most similar to the human pattern (Van Kempen, et al. 1995). Cx43 proteins are directly responsible for the electrical coupling of myocardial cells and therefore are the primary determinant of axial longitudinal resistance (Van Kempen, et al. 1991).

REGULATION OF GAP JUNCTION CHANNELS

The specific method by which gap junctions control cellular communication is under continual investigation. There have been theories which examined the contribution of Ca^{++} and H^{+} changes in the cell to gap junction regulation, however these have not been proven to be physiologically relevant for most tissues. The only undisputed mechanism of Cx43 channel regulation is phosphorylation by protein kinases (Stagg and Fletcher 1990; Godwin, et al. 1993; Lampe, et al. 2000), although the known role and sites of phosphorylation depends on the tissue type, cell-cloning methods, pre-

experimental phosphorylation state or experimental conditions (Lampe, et al. 2000).

BACKGROUND ON CONNEXIN43 AND THE S364P MUTATION

CONNEXIN 43 GENE

The connexin43 gene, located on chromosome six, contains 15,000 base pairs in which exon 2 encodes for a 43-kD gap-junction protein with 382 amino acids in humans. The topographic structure of Cx43 is like that of the other members of the connexin family (Kumar and Gilula, 1996).

Humans also carry a Cx43 pseudogene on chromosome five that shares 97% homology with Cx43 (Fishman, et al. 1991). Although the pseudogene is processed, there is no evidence that a translated protein is produced (Dasgupta, Martinez et al. 2001).

It is interesting to note that although Cx43 is expressed mainly in the heart, it is also found in neural, ovarian, uterine, renal, bone and vascular tissues. In fact Cx43 gap junctions are found in tissues derived from all three embryonic germ layers. cDNA libraries for Cx43 have been found across species and tissues from humans to frogs.

REGULATION OF Cx43

Cx43 has a short half-life of one to two hours (Beardslee, et al. 1998). Short half-lives are common in proteins rich in PEST sequence residues: proline, glutamic acid, serine and threonine (Darrow, et al. 1995). Cx43 has two such PEST-like regions between residues 272-285 and 327-340 (Laird, et al. 1991). Due to the high turnover rate, minor disturbances in the regulation of Cx43 could

have a significant impact on the amounts and function of Cx43 in normal cardiac tissue function(Darrow, et al. 1995) as well as cardiac remodeling (Beardslee, et al. 1998).

Cx43 is phosphorylated on serine and threonine residues, based on two-dimensional gel electrophoresis but the specific residues modified are uncertain (Laird, et al. 1991). All potential phosphorylation sites are in the cytoplasmic carboxyl-terminal region of the Cx43 protein (Kanter, et al. 1992; Darrow, et al. 1995).

Lampe et al. recently reported that the phosphorylation of Cx43 is critical throughout the life cycle of the gap junction. Phosphorylation is one identifiable regulator of the post-translational trafficking of the Cx43 protein to the plasma membrane, oligomerization of Cx43 proteins into connexons and assembly of gap junctional structures as well as the communication properties of single Cx43 channels. Specifically, phosphorylation by active protein kinase C (PKC) appears to decrease intercellular communication and this has been associated with phosphorylation of serines 368 and 372. It has also been reported that phosphorylation of a single connexin protein affects the behavior of the entire gap junction channel. This confirms the importance of the carboxyl tail regulatory domain of a single connexin and its effect on the tissue at large (Lampe, et al. 2000).

Mutations with frequencies as low as 8-10% can have dramatic effects on the regulation of the gap junction channel. Thus, even one altered protein out of the 12 that oligomerize to make up a gap junction channel can significantly alter the function of the gap junctions (Dasgupta, et al. 2001).

When critical serines are replaced, Cx43 channels have severely altered cell-cell communication properties due to the interruption of cyclicAMP (cAMP)-dependent protein kinase or protein kinase C-dependent phosphorylation sites (Stagg and Fletcher 1990; Godwin, et al. 1993). In-vitro studies with cells expressing the S364P Cx43 mutated protein also show decreased cell-cell communication (Britz-Cunningham, et al. 1995).

CONNEXIN 43 IN EMBRYONIC DEVELOPMENT

Cx43 is expressed in the early chick blastoderm in a circumferential pattern, which is integral to left-right (LR) patterning in early development (Levin and Mercola 1998). The addition of physical interruptions (slits) between the two sides of the blastoderm's axis resulted in disruption of LR symmetry, indicating that normal axial development could very well be dependent upon gap junctional communication across the midline. In situ hybridization assays of various connexins in early stage embryos also indicated that Cx43 is present during this early stage. Further tests with antibody to Cx43 showed that the Cx43 protein directly affected LR symmetry in more than half of the embryos. This work challenges former theories, which suggest that the two sides of the

developing embryo are independent of each other (ie, there is no communication between them.)

Further evidence of the importance of Cx43 to normal development was seen in studies using the Xenopus amphibian model where gap junctional communication is found as early as 8-cell stage embryos (Levin and Mercola 1998). The Xenopus studies demonstrated that cross-midline gap junctional communication is important for left-right patterning. Disruption in early stage (4-8 cell) dorsal blastomeres with H7, a chimeric connexin construct that disrupts gap junctional communication, caused heterotaxia in 29% of the embryos. Likewise, expression of S364P-Cx43 by early stage dorsal blastomeres also caused heterotaxia in 23% of the blastula, which is statistically similar to the heterotaxia caused by H7. Importantly, control animals injected with normal Cx43 protein had no significant incidence of heterotaxia. This set of experiments proved that the S364P-Cx43 protein has strong dominant-negative properties in early embryonic development and impairs channel conductance that results in the interruption of the embryo's laterality axis.

CONNEXIN 43 IN THE HEART

The expression of Cx43 in the heart varies during embryogenesis. In the murine heart, Cx43 is expressed in all myocardial cells and has been identified in the distal conduction system from the distal segment of the bundle branches to the purkinje fibers (Van Kempen, et al. 1995; Van Kempen, et al. 1996). It is

apparently lacking in the sinoatrial node or the atrioventricular node (AVN) at all stages of embryonic development or in the adult. The pattern of distribution is similar in humans although there are two notable differences. First, the density of Cx43 in human ventricles is greater than that of the mouse ventricles. Second, Cx43 is present more extensively in the conduction system of the adult human heart. In addition to the areas described in the murine conduction system, Cx43 can also be found in the proximal bundle branches and the atrioventricular bundle that immediately follows the Cx43-negative AVN (Van Kempen, et al. 1995).

In the rat model (Van Kempen, et al. 1996), Cx43 protein is initially found in the free anterior and lateral walls of the atria and throughout the trabeculae of the ventricles in d13-14 myocardium (equivalent to d11 in the murine model). During d16-20, it appears in the previously incomplete interatrial septum. It has also been noted that there is a progressive accumulation of the Cx43 protein as the embryo matures. (Van Kempen, et al. 1991)

The fact that Cx43 is absent in the pacemaker nodal cells of the heart is interesting. Cx43 has long been thought to influence intraventricular conduction velocity. In fact, the emergence of gap junctions in murine myocardial cells coincides with the initial onset of true contractions, around the eighth day of the mouse's gestation period of 21 days (Navaratnam, et al. 1986; Van Kempen, et al. 1991).

Cx43 is detected in mouse as early as the 8-cell stage (Stone 1995) and likely plays a role in organogenesis. Observation of homozygote Cx43 gene knockout mouse embryos (Reaume, et al. 1995) has shown that the absence of Cx43 in the heart does not affect the ability of heart muscle to beat. A separate study attempted to measure the rate of conduction in the Cx43 knockout mice (Guerrero, et al. 1997), but conduction rates of the homozygote knockouts could not be accurately determined. However, recordings of the ventricular epicardial conduction in heterogyzote neonates revealed that their paced beats were 30% slower than wild type neonates. It appears that while Cx43 is not crucial for cardiac conduction, it still has an impact on myocardial impulse propagation.

Experiments altering the regulation of Cx43 have produced a variety of cardiac malformations as well as other developmental abnormalities, especially in tissues of neural crest origins. Studies examining the under-expression of Cx43 used Cx43 knockout mice (Reaume, et al. 1995). Although there was an absence of detectable Cx43 protein and a significant reduction in junctional communication, there was not a complete absence of cell-cell communication. The embryos were also capable of surviving, at least prenatally. Postnatally, few homozygotes were born live and all homozygotes died within minutes to hours post-birth with cyanosis, labored breathing and a swollen abdomen.

Autopsy of the dead pups revealed a swelling in the region overlying the right ventricular outflow tract. Compared to the blood-filled cavity in normal

pups, the region underlying this swelling in the knockouts was filled with intraventricular septae myocytes of normal myotubular structure. It is likely that these swellings obstructed the right ventricular outflow tract leading to cyanosis and subsequently death in the neonate pups. The only other gross and histological cardiac defect found in these embryos was hypoplastic right ventricle. When CMV driven-Cx43 over-expressor transgenic mice were crossed with the Cx-43 KO animals, there was partial rescue of the fatal phenotype with some pups surviving for two weeks after birth (Ewart, et al. 1997). The implication of the knockout's decreased viability and potential rescue indicates that normal Cx43 is essential to heart development.

Similar studies mimicking the overexpression of Cx43 in murine embryos have demonstrated increased gap junctional communication in tissues of cardiac and neural crest origins. (Ewart, et al. 1997; Lo, et al. 1997) The transgenic mice embryos have cardiac abnormalities similar to those with under-expression of the same gene. The embryos show evidence of pulmonary stenosis, exaggerated interventricular cleft, swelling overlying the right ventricular outflow region, a thickened inter-ventricular septum, abnormal coronary vasculature, and in some cases a defect resembling an apical ventricular aneurysm which is sometimes seen as a complication of acute myocardial infarctions in humans. But unlike the general hypotrophic ventricular myocardium of knockout mice, these embryos displayed hypertrophy of the ventricular myocardium. In addition to the

cardiac defects, the embryos also exhibited non-cardiac abnormalities, including neural tube defects, disruption of peripheral nerves development and decreased viability.

Although the precise mechanism is still under investigation, it is clear that Cx43 is integral to cardiac development and function in many species of animals. It is also clear that any insufficiency of the protein due to a mutated structure could have drastic effects on the development and, potentially, the function of the heart.

BACKGROUND OF THE S364P MUTATION

The S364P-Cx43 mutation is not an isolated event in nature. The protein for avian Cx43 carries this serine to proline substitution as part of its regular sequence although avian hearts with gap junctions proteins encoded from this genetic sequence are considered to be "normal" for these species. No mammals share this characteristic with avians, as Cx43 is highly conserved within mammals.

How can it be that birds have a normal, functional heart with a gene sequence that has so far been found only in humans in conjunction with grave heart malformations and laterality defects? It is important to note that the "normal" avian heart structure is very different from that of the human and the predominant avian cardiac connexin is not Cx43, but Cx42, a homologue of mammalian Cx40. Cx40 is present in all mammalian heart in varying amounts

but its expression appears to be repressed by Cx43 in late embryogenesis (Van Kempen, et al. 1995).

In humans, the S364P-Cx43 mutation could be an acquired mutation or a familial one (Dasgupta, Martinez et al. 2001). It is a rare mutation and in the case of one infant, the S364P-Cx43 mutation was found as one of three separate alleles for the Cx43 gene. Such mosaicism is rarely the result of an inherited genetic defect.

The introduction of the S364P mutation in the Cx43 protein appears to be a simple point mutation, however the nature of the amino acid as well as its location has the potential to alter the protein in a way that would render it physiologically nonfunctional. For example, the introduction of proline in a position that is normally occupied by a serine may alter the secondary structure of the protein. It is well known that proline produces a near 180° turn between adjacent amino acids, which could introduce a dramatic kink in the structure of the protein.

This S364P mutation may also affect the tertiary structure of the protein. In molecular models of the Cx43 protein, the 364 position is critically located in the cytoplasmic tail region, which as mentioned earlier, is the regulatory region of Cx43 gap junction channels. This model suggests that the cytoplasmic domains may interact with each other, controlling the opening and closing of gap junction channels. Since the introduction of a proline in the 364 position may

produce a kink in the secondary structure this molecular deformity may not allow normal interactions between adjacent carboxyl tail domains. Whether this would hold the gap junction in a chronically open or closed position is under investigation, but it is well known that any disruption in protein regulation could have adverse effects on the embryo, where regulation and timing of developmental events is key for normal heart formation.

THE DEVELOPMENTAL ANATOMY OF THE MURINE HEART

Early experiments studying cardiac dysmorphogenesis relied on pathologic studies of fully developed abnormal hearts. The focus was placed upon the end result rather than the early development of these malformations because little was known about the normal process of heart formation beyond gross morphological changes (Kirby 1990). More recently, there has been increasing interest in using mice as a model for development and the embryogenesis of murine systems has been documented (Kaufman 1995). Specific focus on heart development has been slower, but the large scale of the project has limited individual studies to focus on short periods of cardiac organogenesis or individual segments (ie. the development of atrioventricular structures from days 10 to 15) rather than the whole heart (Webb, Brown et al. 1998). The recent surge of cardiac research using genetically altered mice has necessitated documenting normal heart development that would afford a closer look at the process for comparison. What is known about normal mouse heart development is concentrated around days 8.5 to term and is spotty at best.

HEART DEVELOPMENT IN THE MOUSE

At day 8.5, the heart is still in the form of a primitive tube formed by the fusion of the two embryonic dorsal aortae. This tube has several expansions representing the future locations of the different chambers and their connections. As mentioned earlier, the heart begins to beat during this stage.

By day 9.5, the tubular heart has begun to loop to the right although it is still unseptated (Webb, Brown et al. 1998). This curvature directs the atrioventricular canal towards the space where the left ventricle will develop. The future septi are marked internally by the beginnings of the endocardial cushions. Externally the area of the interventricular foramen is delineated by an interventricular groove. Within the primitive ventricle, the myocardium of the future left ventricle is more developed compared to the future right ventricle.

By day 10.5, the atria are separated by the primary atrial septum. The ventricles are slightly separated by growth of the muscular ventricular septum from the apical region of the heart, although in some sections the ventricles appear to be one common chamber. The proximal ventricular outflow tracts are not completely separated.

By day 11.5, the tubular heart has looped and chamber formation is clear (Van Kempen, et al. 1996). In the atria, the second interatrial foramen is visible, but the definitive rim of the future oval fossa (the fossa created by the postnatal closure of the foramen ovale) has not yet formed (Webb, et al. 1998). Endocardial cushions are beginning to fuse in the atrioventricular septal area. There is also newly derived mesenchyme, formed from the fusion of primary atrial septum mesenchyme and atrioventricular endocardial cushions, separating the subaortic and subpulmonary outlets.

By day 12.5, a fold of the atrial wall has formed the cranial rim of the future oval fossa and the atrial septum is anatomically complete with the exception of the foramen ovale, which remains patent until birth (Kaufman 1995). The ventricular septum continues to grow to separate the left and right ventricles. The pulmonary infundibulum has begun to appear regionally, but its origins are mesenchymal, not muscular.

By day 13.5, the atria continue to grow and the complex atrioventricular septal area is coming together at this stage. The membranous and muscular components of the interventricular septum are in intimate proximity, but they may not be fused (Webb, et al. 1998).

By day 14.5, most mesenchymal areas of the heart are becoming muscularized. The atria have completely extended around the outflow tracts, but are thin and moderately trabeculated. The right and left ventricular walls are of equal thickness, although the volume capacity of the left ventricle is slightly larger. The papillary muscles and chordae tendinaeae cannot be readily distinguished. The two part interventricular septum (cranial membranous component from the extension of the bulbar ridges and the caudal muscular component) is completely fused (Kaufman 1995). The pulmonary veins are small in the developing embryo and are still unlikely to be seen microscopically at this stage (Icardo and Sanchez de Bega 1991). The tricuspid valve is missing the septal leaflet, but the mitral valve flaps are complete (Webb, Brown et al.

1998). The aortic valve has lowered to the level of the other valves (through a continual shortening of the subaortic component of the outflow tract).

At this stage, the heart is anatomically complete. Further heart development is limited to the growth and expansion of the structures and myocardium in the individual chambers. Blood can be identified in all the chambers, but due to the presence of the shunt through the ductus arteriosus, more blood is seen in the right-sided chambers.

ESTABLISHING LATERALITY IN THE HEART

The heart is the first organ to establish left/right asymmetry in the developing embryo (Levin and Mercola 1998). Research on the anatomy of defects of laterality has been done on mice homozygous for the inversus viscerum (*iv*) gene (Icardo and Sanchez de Bega 1991) and has given some insight to the morphological characteristics evident in visceral heterotaxy.

Using the same principles established by Van Praagh and modified by Anderson, the laterality of the heart should be established by looking for the most constant component of the structure, the one characteristic that is always present regardless of the severity of heart malformations. The attribute should also be identified without a qualitative comparison with the arrangement of the symmetrically opposite structure (Anderson, et al. 1998). Laterality of the ventricles should be determined by the character of the apical trabeculations. The right ventricle has coarse apical trabeculations with obvious large

trabeculations on its septal surface. On the other hand, the left ventricle displays fine criss-crossing apical trabeculations and its septal surface is smooth instead of trabeculated.

In humans, the morphological characteristics that differentiate the right from the left atria are obvious and can be found by comparing the complexity of myocardium and shape of the atrial appendages. Murine hearts lack atrial appendages and thus the means for a clear and quick method of determining atrial right and left-handedness. As an alternative, closer examination of the atria under light microscopy or SEM at the level of the AV junction is done (Icardo and Sanchez de Bega 1991). The right atrium appears more globular than the left and has a blunt inferior border. The left atrium is triangular and unciform with a comparatively thinner lower border. With a little extra work, the laterality of the chambers of the murine heart can be elucidated.

THE EXPERIMENTAL MODEL AND HYPOTHESIS

THE EXPERIMENTAL MODEL

It is established that the S364P mutation does occur in some human infants with visceroatrial heterotaxia (VAH) syndrome and that the expression of the S364P-Cx43 mutation disrupts cell-to-cell communication and/or laterality in development. What has not been explored is whether the mutation by itself is enough to be a causative factor for VAH. The next logical step is to analyze the affect of the S364P-Cx43 mutation on cardiac development in a transgenic mouse line to clarify the role of this defect in heart malformations.

HYPOTHESIS

The serine to proline point mutation of codon 364 site in the connexin43 gap junction gene results in developmental malformations of the heart in the murine model that may mimic those found in humans (Britz-Cunningham, et al. 1995; Dasgupta, et al. 2001).

MATERIALS AND METHODS

TRANSGENIC ANIMALS

The transgenic mice were created by Dr. Cecilia Lo at the NICHHD Transgenic Mouse Development Facility at the University of Pennsylvania, Philadelphia from constructs designed by Dr. Maithali Shah of Loma Linda University.

The transgenic construct generated is Cx43-CT/PRO/CMV43/dhfr (figure 1). This CMV promoter-driven expression vector contains the human connexin43 sequence in which Ser364 was replaced with proline. A 600 bp fragment from the 3′ untranslated sequence of the dihydrofolate reductase (dhfr) gene from *Toxoplasma gondii* was inserted upstream of the SV-40 poly A insert. The dhfr sequence served as a tag for PCR-based genotyping of embryos.

After qualitative identification of successful insertion, the microinjection quality DNA was sent to the transgenic facility, where the founder colony of transgenic mice was made for the present study.

ANIMAL HOUSING

The animals were housed in the Animal Research Facility at the Jerry L. Pettis Memorial Veterans Affairs (VA) Medical Center. The mice are individually marked with a numbered metal ear tag. The breeder males are housed individually in small cages while females are housed individually or in small groups of no more than four per cage. Pregnant females are kept in

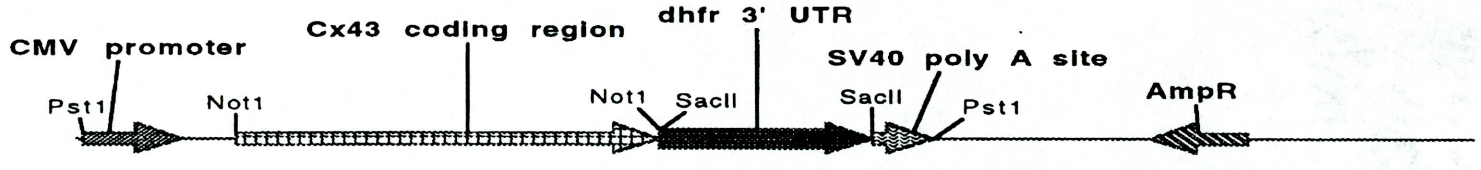

Figure 1: Diagram of construct for the CMV driven S364P transgene called Cx43-CT/PRO/CMV43dhfr.

individual cages so that their pups would be accountable and left undisturbed. The cages, food and water are changed twice a week.

The mice are placed on standard research-grade rodent chow diets while pregnant dams and nursing mothers are fed higher fat breeder diets after confirmation of copulation and maintained until the pups are ready to be weaned. All animal studies were approved by the Animal Subjects committees of Loma Linda University and the Jerry L. Pettis VA Hospital using NIH guidelines.

FOUNDER COLONY MATING PLAN

Heterozygote founder mice (WT/S364P-Cx43) were inbred to produce homozygote (S364P-Cx43/S364P-Cx43) and heterozygote (WT/S364P-Cx43) carriers whose genotypes cannot be distinguished with PCR due to the preservation of the WT gene on chromosome six in this non-targeted mutation. Part of the paradox of using transgenic animals is that the location and number of insertions of the S364P-Cx43 transgene is never known and is always transmitted alongside a copy of the WT allele. Breedings would yield heterozygote or homozygote transgenics that are never "true" heterozygotes or homozygotes.

These qualitatively genotyped carrier mice were bred with heterozygote knockout mice (WT/KO) to produce a variety of progeny. The mice with positive PCR products for both the mutation marker and the knockout marker

are designated as (S364P-Cx43/KO) and are reserved as parents and inbred for embryo harvesting. These matings would yield three varieties of genotypes; homozygote transgenics (S364P-Cx43/S364P-Cx43), homozygote knockouts (KO/KO) and heterozygote hybrids (S364P-Cx43/KO). Homozygote transgenic (S364P-Cx43/S364P-Cx43) embryos were harvested for the present study. Any (WT/WT) offspring were humanely destroyed.

Wild type C57/B6 mice were used as controls. Founder mice were ordered from Harlan® Laboratories and used as the breeders for control embryos. The controls were numbered, housed and bred in the same manner and location as the experimental cohorts.

DNA GENOTYPING

All founder mice and their progeny are genotyped in order to select suitable candidates for the breeding. At the time of weaning, the mice are numerically tagged and approximately 1cm of tail is sectioned and processed for DNA.

The tail clip to be used for animal genotyping is cleaned with isopropyl alcohol and then placed in a sterile Eppendorf tube. To the labeled tube, 700µL of 100µg/mL proteinase K in Lazatin lysis buffer (appendix I) is added. The cap of the tube is covered with an additional layer of parafilm to prevent opening and desiccation during the heated incubation. The tail clip in buffer solution is then placed in a 56°C incubator and gently shaken overnight.

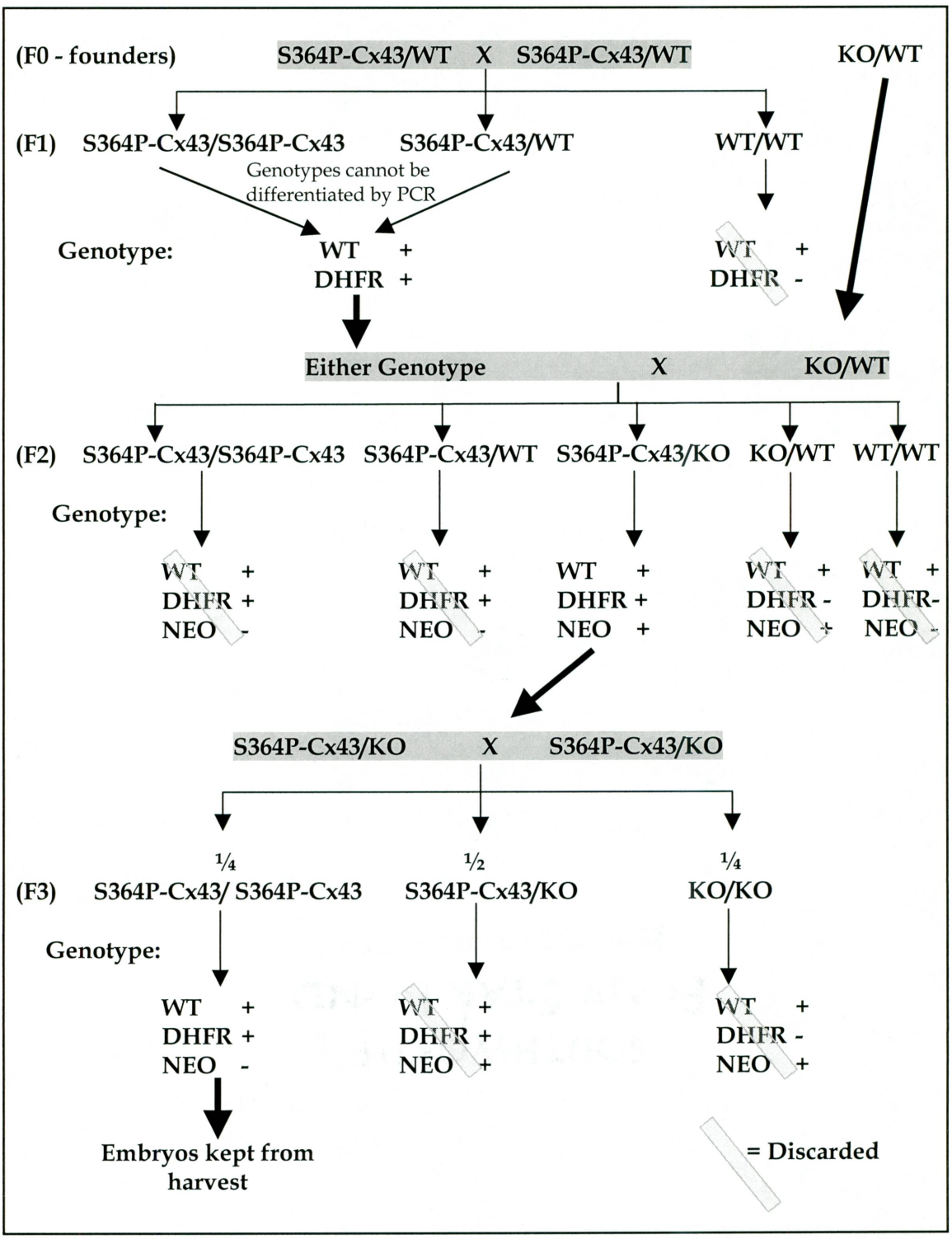

Figure 2. Breeding scheme between the founder colonies (S364P-Cx43/WT & KO/WT) and the subsequent generations. The resultant liter genotypes are determined by PCR amplification.

The next morning, the tail should be dissolved with only a few pieces of hair present. If it has not dissolved, it should be re-incubated with a fresh batch of proteinase K. After incubation the tube is centrifuged for 20 minutes in 4°C at 14,000rpm. Immediately after spinning, the tube is removed carefully so that the hair pellet is not disturbed and placed on crushed ice until the next step.

The supernatant (including the clear sticky substance [DNA], but not the hair pellet) needs to be transferred to a new tube as soon as possible with a sterile pipette tip. Add 500µL of phenol: chloroform (1:1), cover and gently shake by hand for three to five seconds. Centrifuge the tube again for two minutes in room temperature at 4,000rpm until two clear phases can be discerned.

Transfer the top aqueous layer to a clean and labeled Eppendorf tube with a sterile pipette tip and immediately add 700µL of cold isopropanol alcohol. Invert the tube until DNA forms into a thin white threadlike precipitate that sticks together and eventually pellets and attaches to the side of the tube. Carefully remove all liquid from the tube while keeping an eye on the DNA pellet. Allow the tube to air dry for a few minutes so that any remaining liquid is removed. Then, add 50-100µL of TE buffer to the tube to dissolve the pellet. Again, seal the cap with parafilm and incubate on a shaker in a 56°C incubator for at least one hour, or until the DNA pellet has completely dissolved into the solution. If necessary, more TE buffer can be added in 20µL increments followed by at least one hour of incubation. After the DNA is successfully dissolved in

solution it is ready to be used for PCR and must be stored in -20°C. Cold storage will keep the DNA fresh for several months.

PCR AMPLIFICATION

The genomic DNA was subjected to a single round of PCR using three sets of primers. The separate sets of primers made by Operon® laboratories were solubilized with 3-D H_2O to a 5pmol stock solution. The stock was stored in -4°C when not in use.

A wild type (WT) primer set was used to amplify the promoter region of the connexin43 gene (5'-IMR5, 3'-IMR3). The S364-P-Cx43 mutation was tagged with dHFR and necessitated the use of its own specifically designed primers (5'-TS47, 3'-TS28). The knockout was tagged with a neomycin sequence, and part of the 3' WT gene, which made use of both a neo primer and a WT primer (5'-neo5', 3'-IMR5).

Sigma® brand Taq polymerase kits, with Taq polymerase and buffers, were used for PCR amplification. Reaction mixes were made in duplicate with protocol developed by Dr. Lo (University of Pennsylvania, Philadelphia) with purified DNA, primers (both 3' and 5'), Dntp mix, 10X buffer, 25mM MgCl2, 3-D H_20, and approximately five units Taq polymerase per reaction tube (Appendix II). The PCRs were performed using Perkin-Elmer brand GeneAmp 2400 thermo cyclers.

PCR REACTION TIMES

For the WT and dhfr primers, 2μL of DNA was denatured at 94°C for two minutes. It was then amplified for 35 cycles, each composed of 75 seconds at 94°C, 60 seconds at 58°C, and 75 seconds at 72°C followed by 10 minutes of extension at 72°C. The PCR products are then kept at 4°C until further use.

For the longer KO neomycin primers, 2μL of DNA was denatured at 94°C for three minutes. It was then amplified in 41 cycles, each composed of one minute at 94°C, two minutes at 60°C, and five minutes at 72°C. Three minutes of extension time was maintained at 72°C. Again, the PCR products are kept at 4°C until further use.

PCR GEL ELECTROPHORESIS

The PCR products were prepared for gel electrophoresis by the addition of 2 μL of 6X loading dye (Promega® brand) to 10 μL of PCR product. Aliquots of 10μL were run in individual wells against 2 μL of 1 Kb DNA ladder (Life Technologies® brand), and similarly prepared positive and negative controls in a 1% aragose mini-gel (appendix I).

ANIMAL MATING

The pair of mice to be mated are placed in the vicinity of each other up to 2 days in advance in order to stimulate ovulation. On the day of breeding, the mice are placed in the same cage between 16:00 hours and 18:00 hours. The male is then removed the next morning. The copulation plug in the female must be

identified with a vaginal probe the next morning before it dissolves. The removal of the male and the investigation for the copulation plug is best accomplished between 06:00 hours and 10:00 hours. After identification of the plug, the female mouse is labeled, placed in its own cage and marked for change to the breeder diet.

EMBRYO AGE MEASUREMENT

The day of the identification of the copulation plug is denoted as day 0.5. For example, the 15th day is designated as day 14.5. The embryonic age is an approximation that could easily be off by +/- six hours. It has been known that embryos within the same litter can vary in age from several to 24 hours, but this will not affect the overall analysis of the data, as the hour-to-hour changes are minor and secondary to the laterality and morphology of the embryo hearts.

EMBRYO HARVEST

The Embryo harvesting should be performed at 12:00 noon in order to standardize the embryonic age. This is done under the assumption that the mice mate at 0:00 midnight on the evening of the breeding.

ADMINISTRATION OF ANESTHETIC

At the time of harvesting, the dam is anesthetized with 0.75mL of cold Avertin cocktail mix (Appendix I) by an intraperitoneal injection with special care given so that the embryos lying beneath the abdominal wall are not

disturbed by the needle. The injected dam is arranged in a supine position on clean paper towels until it is adequately sedated for surgery.

DISSECTION OF DAMS AND EXTRACTION OF EMBRYOS

When the dam is unconscious, the skin in the umbilicus region is lifted with tweezers and an incision is made through the skin and underlying muscle layers with sharp dissecting scissors. Through this slit incision, the abdominal wall is cut open in a V formation by bilateral incisions in a cranial direction to the rib cage. After cutting, the abdominal wall is deflected to reveal the bicornuate uterus containing multiple lumps: each lump representing an embryo surrounded by embryonic tissues.

The uterus is then removed from the dam by separation from its peritoneal attachments via the ovarian ligaments and the cervix. The arrangement and number of embryos on each side of the uterus is noted as it is removed. The entire chain of embryos is washed twice and bathed in KPBS (figure 3a). The dam is euthanized with an overdose of Avertin cocktail mix and set aside for proper disposal.

Under a low magnification dissecting scope, the embryos within the uterus are removed by cutting on one side of oval expansion (figure 3b). The removed outermost layer is the maternal uterine tissue while the undisturbed layer underlying this is the embryonic yolk sac. The individual embryos and the

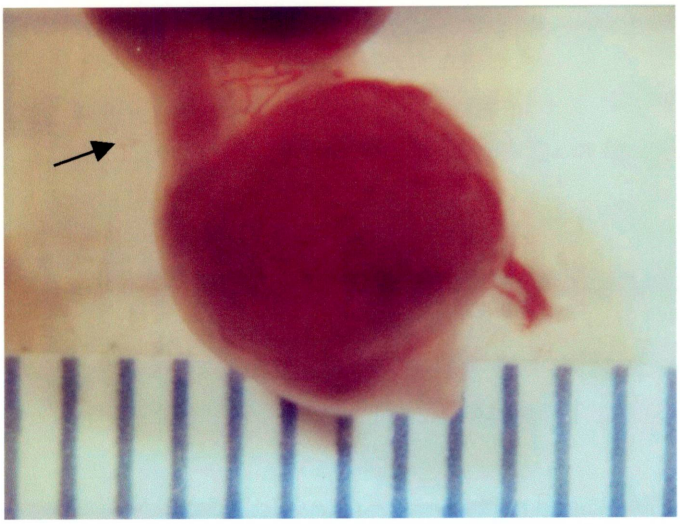

FIGURE 3. Embryo harvest

Figure 3a

The embryo (seen here magnified approximately 10X under a dissecting scope) is surrounded by the muscular uterus (arrow).

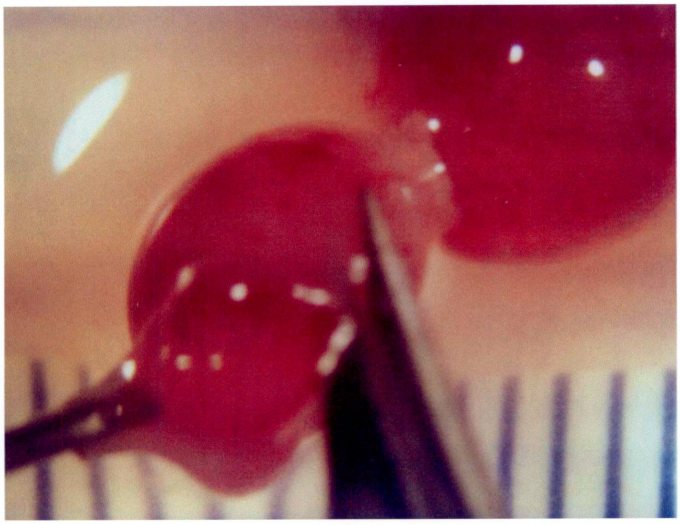

Figure 3b

The embryo is carefully dissected from the uterus with tweezers and spring scissors. Special care is given so that the embryonic sac is not punctured.

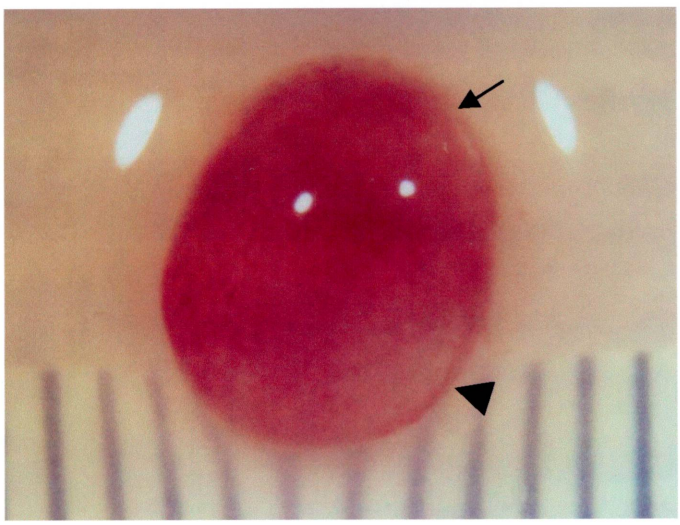

Figure 3c

After removal from the uterus, the embryo is seen here with the placenta (arrow) and a band of maternal tissue (arrowhead) with covers most of the embryonic sac.

yolk sac are gently separated from the uterine tissue and placed in a clean dish of KPBS (figure 3c).

The band of maternal tissue that surrounds the embryo is gently separated from the surface of the amniotic sac (figure 4a). The placenta and parts of the yolk sac intimately adjacent to it are cut from the embryos and discarded (figure 4b). The remaining yolk sac and the amniotic sac, now deflated and still surrounding the embryo, are separated from the embryo (figure 4c), rinsed in clean KPBS and placed in a labeled 1.5 mL micro test tube (Eppendorf) for DNA genotyping.

Each embryo is then photographed with a Polaroid camera fitted into the eyepiece of the microscope with a millimeter scaled ruler for reference. Embryos from days 12.5 through 14.5 are also weighed after gentle blotting with sterile tissue paper to remove excess moisture from the KPBS bath. Due to the delicate nature of the smaller embryos (days 9.5-11.5), the blotting and transferring of the embryos onto the scale would result in severe damage and this measurement was omitted in the interest of tissue preservation. After these measurements, the embryos are then re-suspended for an additional hour in KPBS to assure that when the heart ceases, it will be in the relaxed, uncontracted stage.

Embryo Preservation

After the timed suspension in the KPBS bath, the embryos are placed in individually labeled tubes or vials containing 10% formalin. They are set aside

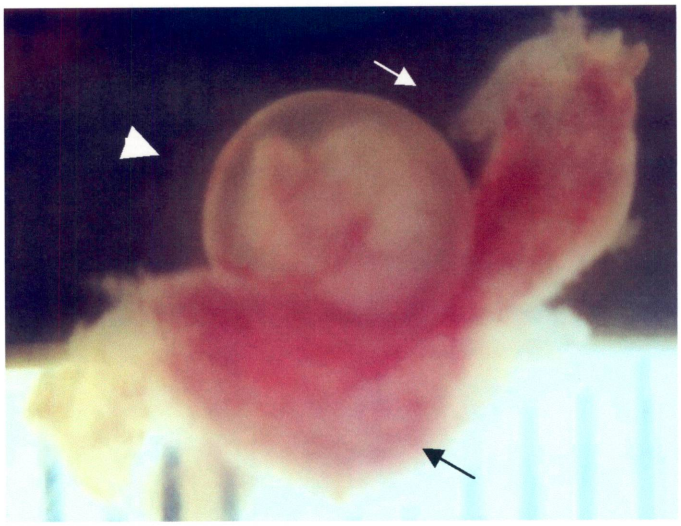

FIGURE 4. Dissection of Extraembryonic membranes

Figure 4a

After the band of maternal tissues is cut apart (white arrow), the embryonic sac (arrowhead) is revealed. The placenta is also more apparent (black arrow).

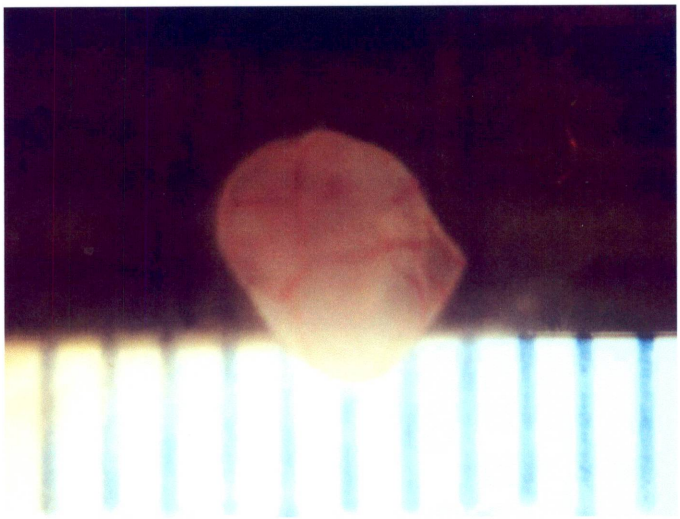

Figure 4b

After the removal of the placenta, and the adjacent tissues, all that remains is the embryo surrounded by a deflated chorion and amnion (the extraembryonic tissues).

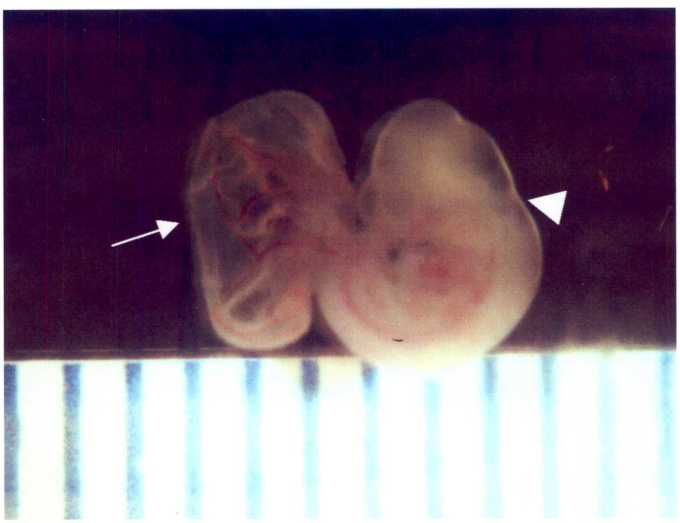

Figure 4c

The extraembryonic tissues (arrow) can be teased away to reveal the embryo inside (arrowhead). It is cut, washed and used for genotyping. The embryo is then measured, weighed and then preserved for microscopy.

for fixation overnight and may be kept in this solution until they are ready to be embedded, sectioned and stained for microscopy.

EMBRYO GENOTYPING

The extraembryonic membranes must be digested to separate the high-molecular-weight genomic DNA from other substances in the tissue. For this, 700 μL of proteinase K in Lazatin lysis buffer (100μg/mL) is pipetted into each tube containing tissues to be digested. The tubes are sealed with parafilm and are gently shaken overnight in an incubator set at 56°C.

The next day, the DNA is purified by adding approximately 500μL phenol:chloroform:isoamyl alcohol (25:24:1) to each tube. The tubes are shaken for a few seconds and spun down in a centrifuge for one minute at 4,000rpm. The top aqueous layer is removed with a pipette and placed in a clean newly labeled tube with 700μL of cold isopropanol and inverted gently a few times to precipitate the long thin glassy DNA strands. When the DNA strands form a pellet, remove the liquid from the tube, making sure to maintain eye contact with the pellet and to leave it on the side of the tube. 30 to 80μL of TE buffer is then added to the pellet to re-suspend the DNA. The tube is then incubated at 56°C for an hour or until the DNA has satisfactorily re-suspended in the TE buffer. The DNA can be used immediately for PCR analysis or kept frozen at -20°C indefinitely.

CONTROL EMBRYO HARVEST

The harvesting, extraction and preservation of the control litters were conducted in the same manner as the transgenic litters. The inbred wild type litters were not genotyped.

CONTROL EMBRYO SELECTION

For each gestational age, the length and weight data for two litters were compiled and the average and standard deviations were calculated. Three embryos from each gestational age were chosen for sectioning to represent the range of embryo sizes. One of mean size, one at one standard deviation above and one below the mean.

EMBRYO HISTOLOGY TECHNIQUE

Embryos slides were prepared by the Anatomy Department of Loma Linda University under the direction of John Hough.

The embryos were sectioned transversely in a cranial to caudal direction in 7μM intervals beginning at the level of the lower jaw where the aortic arch can first be identified, through the mid section of the liver, where no additional sections can be made through the heart. Every fifth section was kept for staining with hemotoxylin and eosin so that each of the serial sections in the final slides set are 35μM apart. In the smaller embryos, the orientation was difficult to establish during the embedding process. Their sections are not perfectly transverse; in some cases they are similar to coronal sections. The basic structure

of the heart and its connections, however, can still be discerned, evaluated and compared with the controls.

DATA CAPTURE

After the slides were prepared, they were photographed with a Zeiss® Universal Microscope and a 2.5X Nikon objective. A blue filter was used with the camera to improve the quality of the lighting from the tungsten microscope lamp.

DATA

CARDIAC FINDINGS

Embryos homozygous for the S364P-Cx43 mutation were recovered during embryo harvesting at the rate of zero to two embryos per litter. With the exception of one (to be described later), all embryos appeared externally healthy at the time of harvest. They had beating hearts and a cursory notation of the location of the liver (translucent through the thin developing integument) did not suggest any incidence of laterality defects.

Qualitative microscopic analysis of the slides from the S364P-Cx43 transgenic embryos from ages 11.5d to 14.5d indicate that there are no gross abnormalities in cardiac development. The control embryos were also 100% normal. Specific attention was paid to the development of the inflow tracts, outflow tracts and the myocardial quality of the ventricles, all of which revealed normal structural anatomy, and appropriate temporal development. Using the criteria set up by the morphological method, no identifying characteristics of visceroatrial heterotaxy could be found.

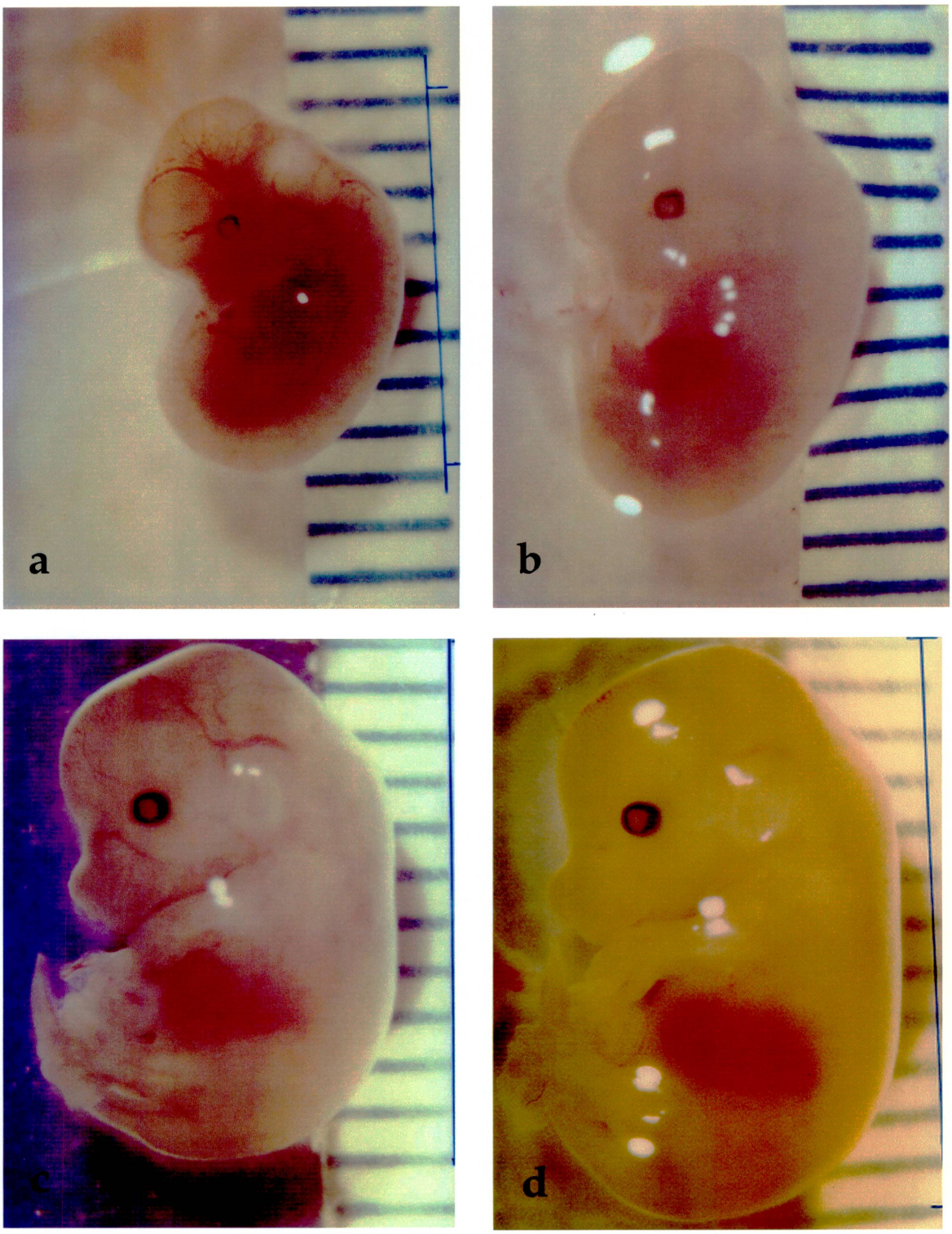

Figure 5. Normal Embryos were photographed against a millimeter scale for reference from ages 11.5d (figure 5a), 12.5d (figure 3b), 13.5d (figure 3c), and 14.5d (figure 3d).

S364P-Cx43 (d11.5)

S364P	Weight	Length
507.1		7.5
507.2		7
507.3		7.25
507.4		6.25
507.5		7.25
507.6		7.5
507.7		7.625
507.8		7.25
507.9		8
Average		7.291667
St. Dev.		0.484123

S364P	Weight	Length
509.1	0.0514	7.25
509.2	0.053	7.125
509.3	0.054	7.625
509.4	0.0541	8
509.5	0.0513	7.375
509.6	0.0644	7.875
509.7	0.0594	8
509.8	0.0494	7.75
509.9	0.0639	7.875
509.10.	0.057	7.875
509.11	0.0446	7.25
Average	0.054773	7.636364
St. Dev.	0.006019	0.328132

S364P	Weight	Length
TAverage		7.48125
TSt. Dev.		0.431634

WT (d11.5)

WT	Weight	Length
609.1		5.25
609.2		6.625
609.3		7.125
609.4		5.875
609.5		6
609.6		6.75
609.7		5.125
609.8		7
Average		6.21875
St. Dev.		0.772721

WT	Weight	Length
610.1		6.25
610.2		7
610.3		6.625
610.4		6.625
610.5		6.5
610.6		6.125
610.7		5.875
610.8		6.75
610.9		6.75
610.10.		7
Average		6.55
St. Dev.		0.368932

WT	Weight	Length
TAverage		6.402778
TSt. Dev.		0.588735

CONTROL	Weight	Length
Av. +1SD		6.991513
Average		6.402778
Av. -1SD		5.814042

Table 1. Length and weight statistics for age 11.5d S364P and WT embryos. Homozygote S364P embryos and controls selected for microscopy are shaded.

S364P-Cx43 (d12.5)

S364P	Weight	Length	BMI
505.1	0.1061	9.37	0.011323
505.2	0.1054	9.375	0.011243
505.3	0.1168	9.75	0.011979
505.4	0.1305	10.125	0.012889
505.5	0.1172	10.125	0.011575
505.6	0.113	9.8	0.011531
505.7	0.1106	9.9	0.011172
505.8	0.1115	9.875	0.011291
Average	0.113888	9.79	0.011625
St. Dev.	0.007977	0.291327	0.000572

S364P	Weight	Length	BMI
515.1	0.0967	9	0.010744
515.2	0.0976	10.25	0.009522
515.3	0.1016	9.375	0.010837
515.4	0.0702	8.25	0.008509
515.5	0.1066	9.5	0.011221
515.6	0.0889	9	0.009878
515.7	0.0968	9.25	0.010465
515.8	0.1091	9.375	0.011637
515.9	0.0963	9	0.0107
Average	0.095978	9.222222	0.01039
St. Dev.	0.011375	0.533187	0.000951

S364P	Weight	Length	BMI
516.1	0.091	9.125	0.009973
516.2	0.0823	8.75	0.009406
516.3	0.1003	9.5	0.010558
516.4	0.0819	8.875	0.009228
516.5	0.0928	9.125	0.01017
516.6	0.0884	9	0.009822
516.7	0.0882	9.5	0.009284
516.8	0.0806	9.25	0.008714
516.9	0.0668	9.125	0.007321
516.10.	0.0881	9.875	0.008922
516.11	0.0724	9.5	0.007621
516.12	0.0823	9.8	0.008398
Average	0.084592	9.285417	0.009183
St. Dev.	0.009015	0.352339	0.001008

WT (d12.5)

WT	Weight	Length	BMI
603.1	0.077	8.625	0.008928
603.2	0.0773	8.875	0.00871
603.3	0.0906	9.25	0.009795
603.4	0.0818	9.5	0.008611
603.5	0.0804	9.25	0.008692
603.6	0.0815	8.8	0.009261
603.7	0.0848	9.375	0.009045
603.8	0.0618	8	0.007725
603.9	0.0474	8	0.005925
603.10.	0.061	8.375	0.007284
Average	0.07436	8.805	0.008397
St. Dev.	0.013309	0.549975	0.001127

WT	Weight	Length	BMI
604.1	0.0941	9.875	0.009529
604.2	0.1007	9.5	0.0106
604.3	0.0793	9	0.008811
604.4	0.0864	9.125	0.009468
604.5	0.078	8.125	0.0096
604.6	0.0831	8.5	0.009776
604.7	0.0873	9.125	0.009567
604.8	0.0828	9	0.0092
604.9	0.0729	8.375	0.008704
Average	0.084956	8.958333	0.009473
St. Dev.	0.008462	0.551985	0.000558

WT	Weight	Length	BMI
TAverage	0.079379	8.877632	0.008907
TSt. Dev.	0.012245	0.541147	0.001038

CONTROL	Weight	Length
Av. +1SD	0.091624	9.418778
Average	0.079379	8.877632
Av. -1SD	0.067134	8.336485

S364P	Weight	Length
TAverage	0.096207	9.405
TSt. Dev.	0.01523	0.458727

Table 2. Length and weight statistics for age 12.5d S364P and WT embryos. Homozygote S364P embryos and controls selected for microscopy are shaded.

S364P-Cx43 (d13.5)

S364P	Weight	Length	BMI
506.1	0.0582	8	0.007275
506.2	0.0882	9.375	0.009408
Average	0.0732	8.6875	0.008342
St. Dev.	0.021213	0.972272	0.001508

S364P	Weight	Length	BMI
513.1	0.1884	11	0.017127
513.2	0.2887	12	0.024058
513.3	0.194	11	0.017636
513.4	0.1615	10.0625	0.01605
513.5	0.1664	10.125	0.016435
Average	0.1998	10.8375	0.018261
St. Dev.	0.051598	0.792543	0.003298

S364P	Weight	Length	BMI
517.1	0.2869	12.1	0.023711
517.2	0.2424	12	0.0202
517.3	0.2305	11.75	0.019617
517.4	0.1496	10	0.01496
517.5	0.2053	11	0.018664
517.6	0.2175	11.75	0.018511
Average	0.222033	11.43333	0.019277
St. Dev.	0.045258	0.801041	0.002836

S364P	Weight	Length	BMI
TAverage	0.190585	10.78173	0.017677
TSt.Dev.	0.067859	1.224773	0.055405

WT (d13.5)

WT	Weight	Length	BMI
602.1	0.1418	11.125	0.012746
602.2	0.1261	10.125	0.012454
602.3	0.1286	10.25	0.012546
602.4	0.1407	10.625	0.013242
602.5	0.1322	10.375	0.012742
602.6	0.1456	10.7	0.013607
602.7	0.1486	10.875	0.013664
602.8	0.1487	10.5	0.014162
Average	0.139038	10.57188	0.013146
St. Dev.	0.008955	0.330432	0.000619

WT	Weight	Length	BMI
607.1	0.1525	10.75	0.014186
607.2	0.1658	11.5	0.014417
607.3	0.1736	11.5	0.015096
607.4	0.1335	10.375	0.012867
607.5	0.1536	10.625	0.014456
607.6	0.1225	10.125	0.012099
607.7	0.1492	10.625	0.014042
607.8	0.1597	10.875	0.014685
607.9	0.1616	10.75	0.015033
607.10.	0.1353	10.625	0.012734
Average	0.15073	10.775	0.013937
St. Dev.	0.015966	0.436208	0.001095

WT	Weight	Length	BMI
TAverage	0.145533	10.68472	0.013621
TSt.Dev.	0.014273	0.395577	0.036082

CONTROL	Weight	Length
Av. +1SD	0.159807	11.0803
Average	0.145533	10.68472
Av.-1SD	0.13126	10.28915

Table 3. Length and weight statistics for age 13.5d S364P and WT embryos. Homozygote S364P embryos and controls selected for microscopy are shaded.

S364P-Cx43 (d14.5)

S364P	Weight	Length	BMI
504.1	0.2647	12.5	0.021176
504.2	0.2918	12.44	0.023457
504.3	0.2887	13	0.022208
504.4	0.3935	13.5	0.029148
504.5	0.2829	12.9	0.02193
504.6	0.2813	12.375	0.022731
504.7	0.2954	12.875	0.022944
504.8	0.2896	12.625	0.022939
Average	0.298488	12.77688	0.023362
St. Dev.	0.039526	0.372673	0.106062

S364P	Weight	Length	BMI
510.1	0.2695	12.5	0.02156
510.2	0.2934	13	0.022569
510.3	0.3138	13	0.024138
510.4	0.2925	13	0.0225
510.5	0.3543	14	0.025307
510.6	0.3539	14.5	0.024407
510.7	0.3421	14.5	0.023593
510.8	0.3553	14.5	0.024503
510.9	0.3654	14.5	0.0252
510.10.	0.3324	14	0.023743
510.11	0.2703	12.5	0.021624
Average	0.322082	13.63636	0.023559
St. Dev.	0.035707	0.839372	0.001326

S364P	Weight	Length	BMI
TAverage	0.312147	13.27447	0.023457
TSt. Dev.	0.038199	0.797186	0.001829

WT (d14.5)

WT	Weight	Length	BMI
600.1	0.2003	12	0.016692
600.2	0.2034	12	0.01695
600.3	0.2376	12.5	0.019008
600.4	0.2297	12.5	0.018376
Average	0.21775	12.25	0.017776
St. Dev.	0.018684	0.288675	0.064722

WT	Weight	Length	BMI
601.1	0.2506	12.625	0.01985
601.2	0.2308	12	0.019233
601.3	0.1936	11.375	0.01702
601.4	0.2355	11.175	0.021074
601.5	0.2333	12.5	0.018664
601.6	0.2299	12.375	0.018578
601.7	0.2944	13.125	0.02243
601.8	0.2994	12.375	0.024194
Average	0.245938	12.19375	0.020169
St. Dev.	0.0353	0.650103	0.0543

WT	Weight	Length	BMI
Taverage	0.236542	12.2125	0.019369
TSt. Dev.	0.032876	0.54078	0.060793

CONTROL	Weight	Length
Av. +1SD	0.269417	12.75328
Average	0.236542	12.2125
Av. -1SD	0.203666	11.67172

Table 4. Length and weight statistics for age 14.5d S364P and WT embryos. Homozygote S364P embryos and controls selected for microscopy are shaded.

NEURAL FINDINGS

One case of abnormal neural development was documented in a transgenic embryo aged 12.5d. This embryo had persistence of the anterior neuropore and the associated anencephaly as well as unequal and underdevelopment of the eyes. This is evident by the grossly abnormal structure of the head. Although it was among the smallest of its litter, its length and weight were within normal limits for its age. These abnormalities were grossly documented but were not examined further. Sections of the heart of the embryo did not reveal any abnormal development. There were no gross neural abnormalities in any of the control litters.

EMBRYONIC SIZE DIFFERENCE

In comparing the weights and lengths between the S364P-Cx43 embryos and the controls, the transgenics are an average of 0.2-1mm larger than the control animals. This is insignificant because the controls are of the C57/B6 breed and are generally smaller than the mice used for the transgenic line.

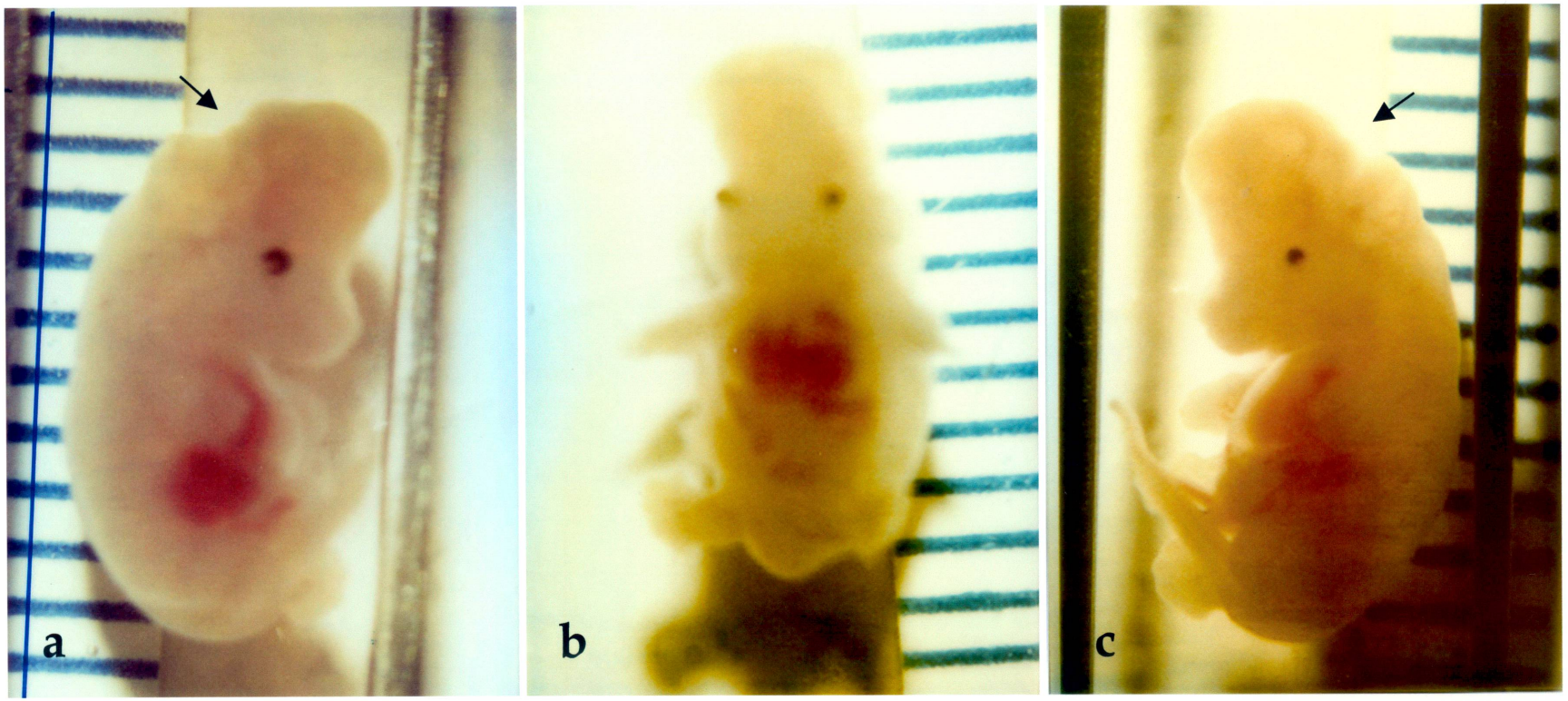

Figure 6. A S364P-Cx43 transgenic embryo (age 12.5d) is seen here from the right lateral (figure 6a), frontal (figure 6b), and left lateral (figure 6c) views. There is a defect in the rostral-dorsal midline with failure of closure of the anterior neuropore (arrows) which is associated with mild anencephaly. The eyes on both sides of the embryo are also asymmetrical and severely deformed.

ANALYSIS

S364P-Cx43 TRANSGENICS HAVE NORMAL CARDIAC DEVELOPMENT

Each of the transgenic embryos homozygous for the S364P displayed normal cardiac development. All the inflow tracts, outflow tracts, and chambers of the heart and its connections were normal in morphology. Blood was present in all chambers in varying quantities at all times, depending on the state of contraction at the time of death. Chronologically, the milestones of development in the heart were also normal. The atria and ventricles were morphologically distinct and complete on day 14.5. The interatrial septum was complete on day 12.5. The interventricular septum was complete on day 14.5. All the leaflets of the valves (save the septal tricuspid leaflet) were also formed and functional on day 14.5. All the embryos had beating hearts at the time of harvest.

S364P-Cx43 TRANSGENICS DO NOT AFFECT EMBRYONIC VIABILITY

Their survival to days 11.5 to 14.5 is equivalent with survival to the mid second trimester to the early third trimester in humans and is testament to the viability of these transgenic animals. The non-targeted S364P mutation does not interfere with development of the murine cardiac model up to day 14.5 of the 20 to 21 day long gestation period.

S364P-Cx43 TRANSGENICS DO NOT HAVE DEFECTS OF LATERALITY

The normalcy of the hearts effectively rules out expression of the S364P transgene as a direct cause of visceroatrial heterotaxy as the laterality of the

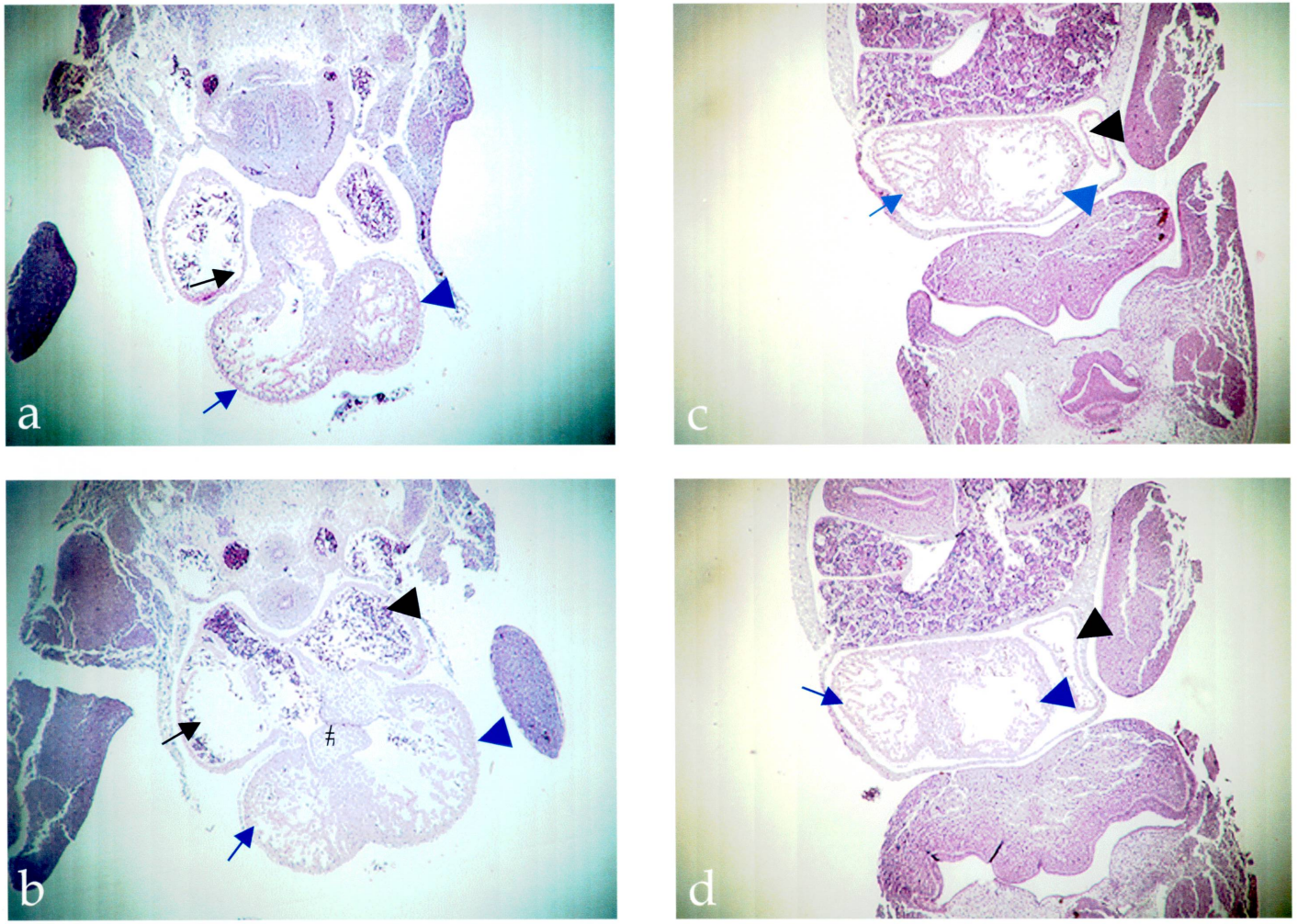

Figure 7. Slides of 11.5 day old S364P-Cx43 embryos (figures 7a and 7b) and WT embryos (figures 7c and 7d). The transgenic embryo was sectioned from rostral to caudal. Due to orientation difficulties, the WT embryo was sectioned from frontal to dorsal. Structures seen here include the right atrium (black arrow), left atrium (black arrowhead), right ventricle (blue arrow), left ventricle (blue arrowhead), and the developing tricuspid and mitral valves (#).

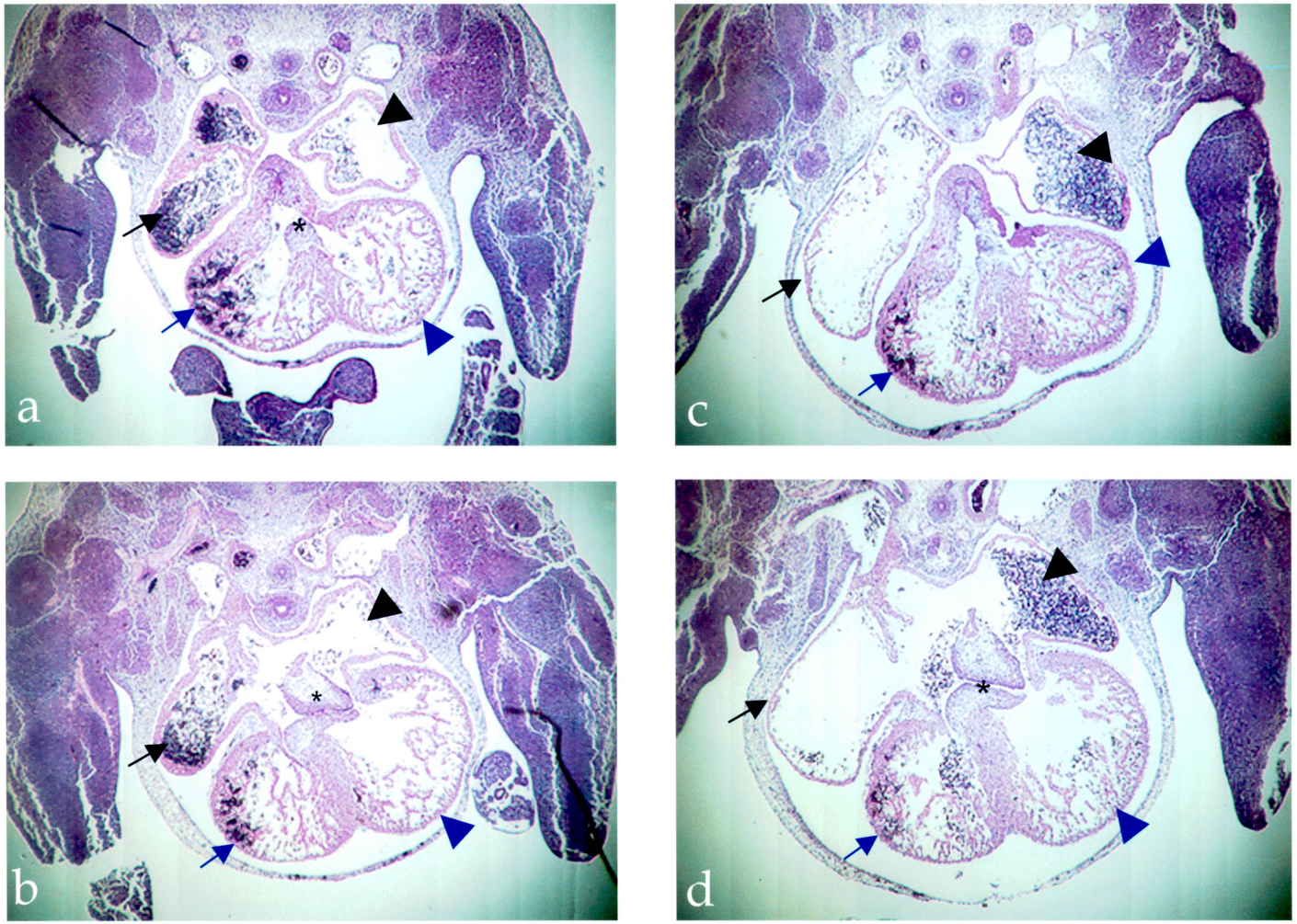

Figure 8. Sections of S364P-Cx43 (figures 8a & 8b) and WT (figures 8c & 8d) of d12.5 embryos. Structures seen here include the right atrium (black arrow), left atrium (black arrowhead), right ventricle (blue arrow), left ventricle (blue arrowhead), and the developmentally normal interventricular septal defect (*).

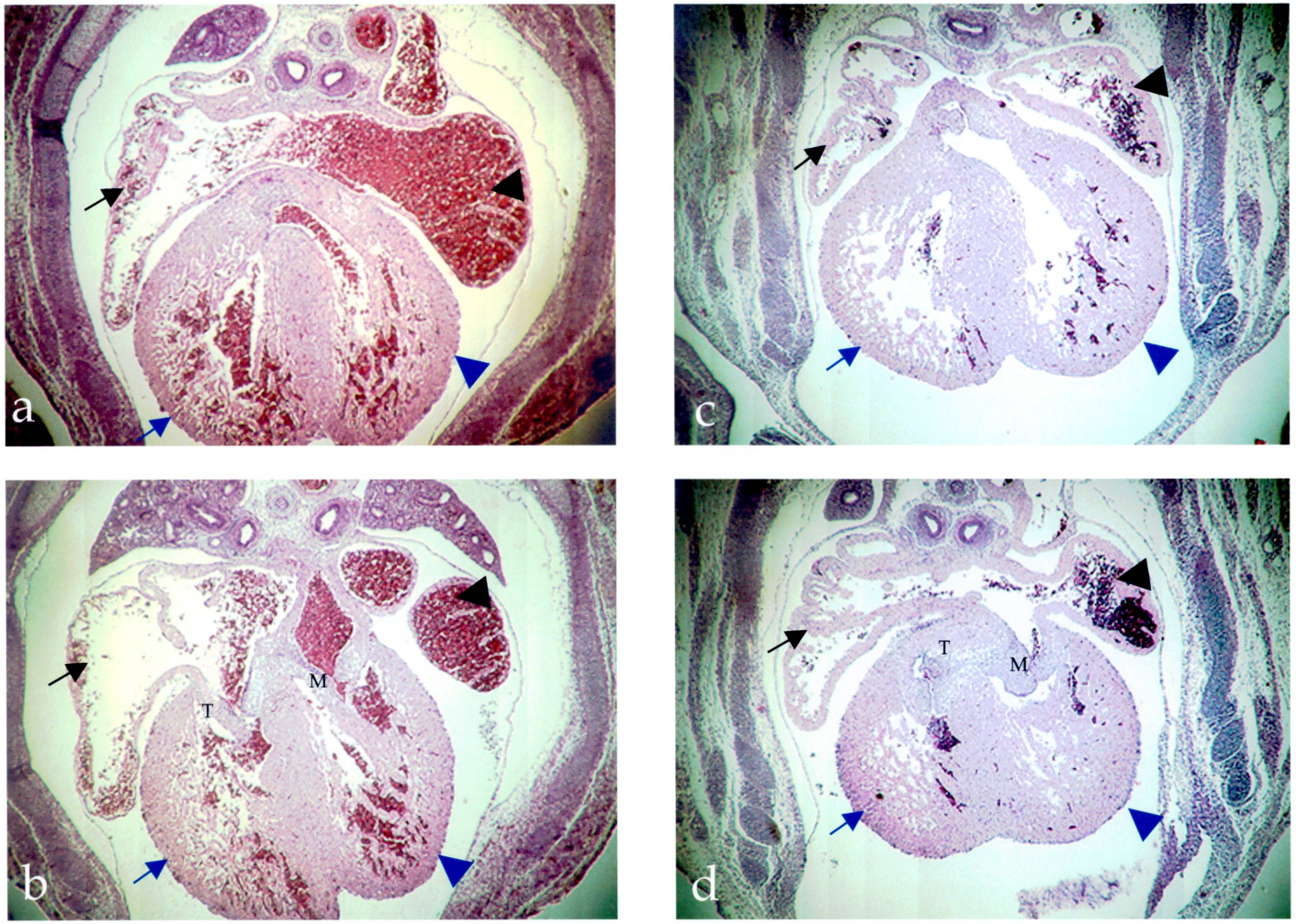

Figure 9. Sections of S364P-Cx43 (figures 9a & 9b) and WT (figures 9c & 9d) of d13.5 embryos. Structures seen here include the right atrium (black arrow), left atrium (black arrowhead), right ventricle (blue arrow), left ventricle (blue arrowhead), and the tricuspid (T) and mitral valves (M).

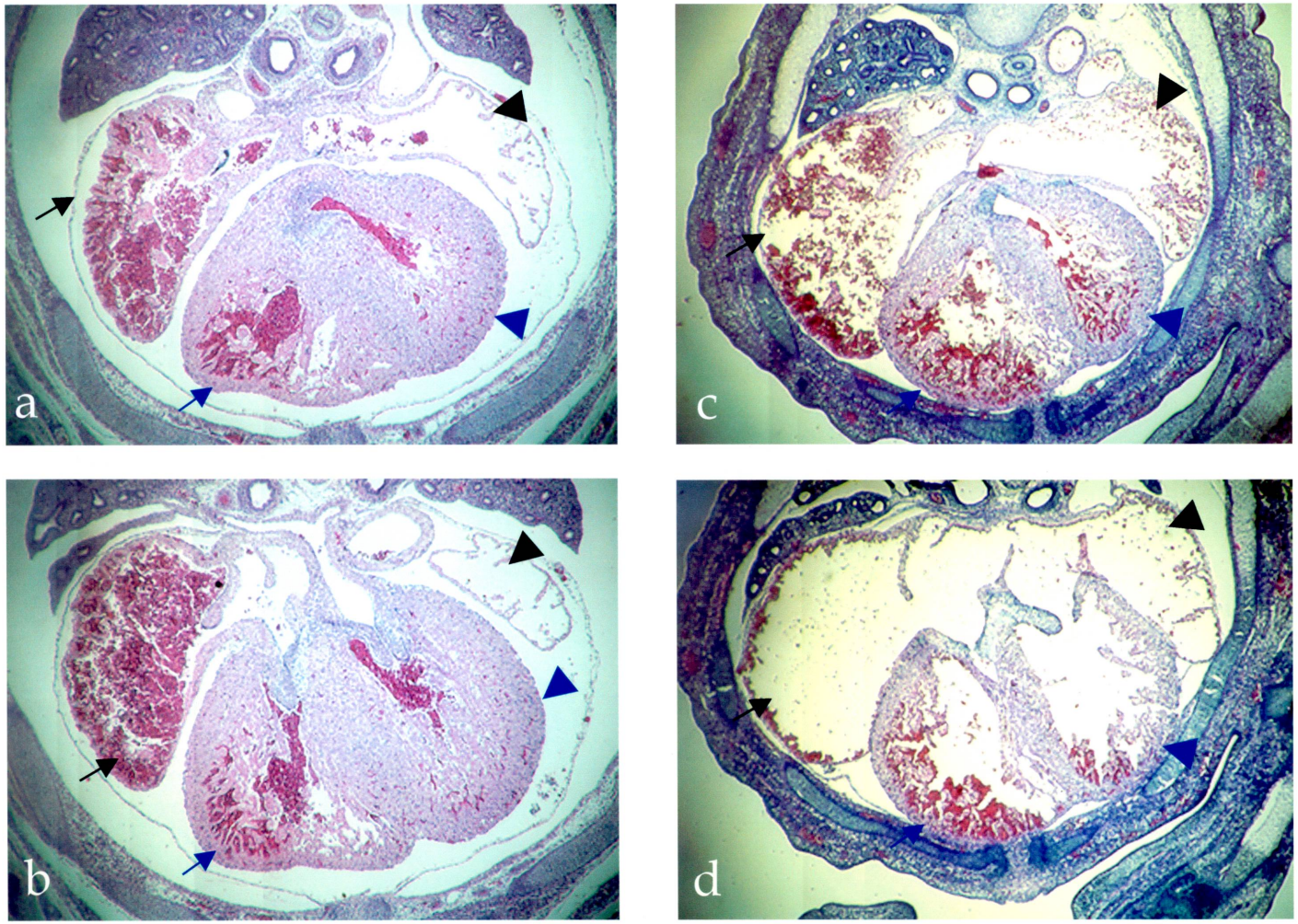

Figure 10. Sections of S364P-Cx43 (figures 10a & 10b) and WT (figures 10c & 10d) of d14.5 embryos. Structures seen here include the right atrium (black arrow), left atrium (black arrowhead), right ventricle (blue arrow), left ventricle (blue arrowhead), and the tricuspid (T) and mitral valves (M). The ventricular septal defect is now completely closed.

individual chambers is determined early in organogenesis. Examination of the trabeculations of the ventricles and the shapes of the atria revealed normal laterality. The position of the liver was grossly noted to be normal and the splenic conditions were not evaluated in the embryos.

This conclusion is made only with the understanding that such a non-targeted mutation does not replicate the genetic condition in the human patients. In addition, the transgene is driven by a CMV promoter while normal Cx43 is driven by endogenous physiologic promoters.

HE S364P TRANSGENE MAY DISRUPT NEURAL CREST DEVELOPMENT

In a case of the single 12.5d old S364P transgenic embryo, there was an obvious anterior neuropore closure defect. This is a phenomena that was never seen in more than eight litters of normal control mice. Closure of the anterior neuropore occurs early in embryogenesis and a defect is associated with anencephaly and other cranial defects. The eyes of the embryo were also malformed. They were smaller in size than the control embryos and there was also a difference in size between the left and right eyes.

The nature of neural tube defects are similar to that of embryos in which Cx43 overexpression is driven by the CMV promoter (Ewart, et al. 1997). In embryos overexpressing Cx43, there was selective disruption of neural crest cells. Some of those embryos had a persistent open neural tube in the area of the midbrain/anterior hindbrain. Some also had dysgenesis of the cranial nerve,

including a reduced oculomotor (III) nerve. While the combination of defects observed in the S364P embryo are not identical to those found in embryos overexpressing Cx43, they are similar enough to suggest that the neural crest cells were indeed disrupted to the point of causing grossly observable pathology.

MORBIDITY AND MORTALITY OF THE EMBRYOS

The morbidity and mortality of the mutations were not documented in this study, as all the embryos used were harvested prenatally. The breeding of the founder colony yielded live litter sizes that were comparable to their wild type counterparts. Had the transgenic litter sizes been smaller, this would not have posed a problem as transgenic female mice are notorious for high rates of infertility and low-yield litters. One uncontrollable problem was that the mothers were left alone with their newborn pups and pups born dead or pups who died within hours of birth are often cannibalized long before their existence could be added to the litter tally. The hour of birth is random, and often around midnight. It would have been impossible to monitor the birth of each of the dozens of litters.

USE OF THE MORPHOLOGICAL METHOD

In the process of evaluating thousands of slides, the "Morphological Method" proved to be extremely useful and precise. Strict comparisons between the transgenes and the controls would have been difficult, as the embedding orientation and consequently the serial sections of the mice were not identical

and would not have been useful for comparative data. The study focused on the identification of defects of laterality and by following the protocol outlined by Anderson et. al, time was spent gathering only the pertinent data on the right and left-handedness of the cardiac chambers and its connections.

RISKS OF USING THE TRANSGENIC MODEL

Due to time and monetary constraints, non-targeted mutations were inserted into transgenic mice created for this study. The associated risks for this method vary and can be unpredictable making the maintenance and evaluation of the transgenic animals prone to errors that must be explored, but can never be fully predicted or appreciated. As long as potential errors and variances are expected and carefully documented when they are realized, the benefit of using these models would be to yield a basic set of explorative data in a few months and for a fraction of the cost of a targeted knock-in mutation. In a manner of speaking, results from this simplified research would provide the justification for a larger knock-in study. Such justification would be imperative, as the creation of a knock-in founder colony alone would involve two to three years of lab work as well as a six-figure price tag.

The colony used in the S364P mutation study was created using a transgene carrying the single-point mutation marked with a dHFR tag. This transgene was randomly inserted into the founder mice's DNA without the possibility of determining either the number of copies inserted or the location of

these insertions. Crude qualitative indicators, such as the viability and the reproductive health of the transgenic mice are used to make sure that little to no disruptions of any important genes occurred. It is also important to note that the use of a transgenic line necessitates the understanding that any defects found in the data could have been a direct result of the random insertion of the transgene.

CIRCUMVENTING THE PROBLEMS OF GENOTYPING THE TRANSGENICS

One problem associated with the breeding of these non-targeted transgenic animals is the issue of genotyping. Genetic markers used to amplify the normal wild type connexin43 gene produce bands on gels for both wild type and transgenic PCR preparations because the wild type gene remains undisturbed in the transgenic model. Due to the consistent presence of the wild type band it is impossible to use this method to determine if the animal is a heterozygote or homozygote carrier of the mutation, as both would have one band with both the wild type primers and the dHFR tagged primers. The time consuming and laborious process of back breeding can help to distinguish heterozygotes versus homozygotes but it would be extremely inefficient to use this method for genotyping.

To circumvent this problem, knockout mice were introduced as intermediate breeders in the breeding for homozygote embryos since it is impossible to carry both the knockout gene and the mutated gene on the same

allele. In the last step, breeding two animals, each with a separate knockout allele and transgenically mutated S364P allele would produce embryos with three genotypically different types of offspring for embryo harvesting which are distinguishable by PCR genotyping methods: homozygotic for the S364P-Cx43 mutation, homozygotic for the connexin43 knockout and heterozygote combination of the two (see figure 2 in the materials and methods section).

The introduction of an altered allele, the KO, is not without its risks. Although the gene is comparatively small, it is still possible that recombination could occur during meiosis and affect subsequent generations.

FUTURE STUDIES

Future experiments can focus on the effects of the S364P-Cx43 mutation on neural crest development. The mutation has already been shown to affect xenopus embryogenesis, perhaps it will also significantly affect the murine model and humans. This should also be conducted with the targeted knock-in mutation and could lead to insightful information about the role of the mutation in embryogenesis.

Cx43 is undisputedly the most important connexin involved in the development and viability of the cardiac system. Mutations in the gene should be explored to the fullest extent since congenital heart defects should be avoided if at all possible. Although the S364P-Cx43 mutation is rare, the fact that it has been found in a number of non-related human cardiac transplant patients

indicates that it may be a causative factor. The non-targetted mutation did not show conclusive evidence linking the mutation to disease, however, it is only one of many methods of transgenic study.

The best method of evaluating the effects of the S364P-Cx43 mutation is the development of a gene-targeted knock-in line. This would provide an in vivo model that more closely resembles the human genomic defect. A knock-in transgenic line would also be superior to the non-targeted mutation because the knock-in utilizes the endogenous promoters and repressors present in the normal Cx43 gene. Thus, the timing and tissue distribution of gene expression should be identical to that of wild-type Cx43.

CONCLUSION

The introduction of a serine to proline point mutation at the 364 position of the connexin43 gene in the transgenic murine model did not produce morphologically identifiable cardiac defects in the developing embryos from the ages of 11.5 to 14.5 days in utero (equivalent to late second trimester in the human fetal gestation period). The selected transgenic embryos were compared with normal controls using a morphological approach, where qualitative analysis was used to compare and contrast the inlet, outlet and trabecular apical components of the left ventricle. The only notable deformity is a single case of anencephaly and associated optical dysgenesis in an embryo, age 12.5d. Although the mutation can be held suspect for the failure of closure of the anterior neuropore, other molecular and breeding conditions cannot be ruled out as inducers of the deformity.

The S364P mutation of the Cx43 gene, first reported by Britz-Cunningham (Britz-Cunningham, et al. 1995), was found in patients with visceroatrial heterotaxia syndrome who exhibited a variety of laterality defects in addition to those of the heart. The failure of finding signs of left ventricular hyperplasia in the transgenic embryos cannot exclude this mutation as a causative factor. The transgene was not specifically targeted, nor was the location of the insertion(s) and its disruptive potential identified. In addition, the ages of the embryos may

have been too young for anatomical defects, other than those of laterality, to manifest grossly.

It would be prudent to repeat the investigation using knock-in mice so that the S364P-Cx43 mutation would more closely mimic the molecular genetic situation identified in the human patients. The observation of later-stage embryos as well as neonatal morbidity and mortality may also prove to be useful in determining the effect of S364P on cardiac development and embryogenesis.

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APPENDIX I - TEST SOLUTIONS

1M Tris-HCl, pH 8.5

12.1g Tris base
80 mL 3-D H_2O

Mix the above in a 100mL flask. Then adjust the pH to 8.5 with 12N HCl drop wise. Finally, q.s. to 100mL with 3-D H_2O.

10% SDS

10g SDS
100mL 3-D H_2O

In a 100mL bottle, add 10g SDS. Then, q.s. to 100mL with 3-D H_2O and mix gently until SDS dissolves. Store in room temperature.

1X TBS

100 mL 10XTBS
900 mL 3-D H_2O

Mix and store covered in room temperature.

1% ARAGOSE GEL

1xTBE (from 10x stock)
1% Agarose (1g/100ml 1xTBE)
5ug/ml Ethidium bromide (10µL/100mL 1xTBE)

Combine the above listed ingredients and microwave until the agarose has dissolved into solution (approximately 20 seconds to one minute.) Pour 40mL of hot gel into each minigel tray and insert the well combs. Cover and let the gel settle and harden in room temperature for at least an hour before use.

POTASSIUM FORTIFIED PHOSPHATE BUFFERED SALINE (KPBS)

KCl (50mM in solution)
NaCl
5.51g Na_2HPO_4
0.70g NaH_2PO_4
4 L 3-D H_2O

Mix the above ingredients using sterile techniques. Adjust pH to 7.2-7.4 with 1N HCl. Filter through a 0.22 µ pore vacuum bottle top filter.

AVERTIN ANESTHESIA COCKTAIL
(Source: Dr. Cecilia Lo, U. Pennsylvania)

AVERTIN STOCK

10 g 2,2,2 Tribromoethanol

10 mL 2-meth-2-butanol

Mix using aseptic techniques and store in amber colored 1.5ml micro test tubes (Eppendorf) for protection from light in approximately 1ml aliquots. Freeze the stock aliquots at -20°C.

COCKTAIL MIX

100 µL of AVERTIN STOCK

4 mL PBS

Prepare fresh by mixing ingredients in a foil wrapped blood tube for protection from light using aseptic techniques. Store on ice until use.

LYSIS BUFFER FOR TAIL CLIPS AND YOLK SACS

(Source: Bien Lazatin, U. Pennsylvania)

BIEN LAZATIN STOCK BUFFER

50mL 1M Tris HCL (pH 8.5)

5mL 500mM EDTA

10mL 10% SDS

20mL 5M NaCl

415mL 3-D H_2O

Mix and autoclave the final solution. May be stored in room temperature.

100 µg/mL STOCK PROTEINASE K

100µg Proteinase K

1 mL Lazatin Lysis buffer (see above)

Using aseptic techniques, mix and store in opaque 1.4 mL micro tubes (Eppendorf) at -20°C.

COCKTAIL MIX

Add and mix 1µL of stock proteinase K per 1mL of stock buffer. Mix must be prepared fresh and used immediately.

APPENDIX II - PCR PRIMERS AND REACTION MIXES

PRIMER SEQUENCES

WT: IMR5: **5′** - ACT TTT GCC GCC TAG CTA TCC C - **3′**;
IMR3: **5′** - CCC CAC TCT CAC CTA TGT CTC C -**3′**

Dhfr: TS47: **5′** - TGT CAC TGT AGC CTG CCA GAA CAC TT - **3′**;
TS28: **5′** - GTC AAT CAG AAC GAA AGC GAG - **3′**

KO: neo5′: **5′** - GCT TGC CGA ATA TCA TGG TCG C - **3′**;
IMR5: **5′** - ACT TTT GCC GCC TAG CTA TCC C - **3′**

PCR REACTION MIX

3′ Primer	2.5 µL
5′ Primer	2.5 µL
dNTP Mix	4 µL
10X buffer	5 µL
25mM $MgCl_2$	4 µL
Taq Enzyme	0.25 µL
Dist. H_2O	29.75 µL
Total Mix	50 µL