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Cardiovascular Development and Survival During Gestation in the TS65DN Mouse Model for Down Syndrome

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Abstract

The Ts65Dn mouse model for Down syndrome (DS) exhibits many phenotypes seen in human DS. Previous research has revealed a reduced rate of transmission of the T65Dn marker chromosome in neonates. To analyze potential fetal loss, litters from trisomic females at 10.5dpc through 14.5dpc were genotyped. No significant differences from the expected Mendelian ratio were found in transmission of T65Dn at any stage. Cardiovascular defects found in trisomic neonates are associated with formation of pharyngeal arch arteries. Vessel tracing was used to identify anomalies in 10.5dpc, 11.5dpc, and 13.5dpc embryos. Comparison of trisomic versus euploid embryos injected with India ink revealed delay and abnormality in cardiovascular development in trisomic embryos at each stage. Through the analysis of transmission rate and cardiovascular development in embryonic mice, we learn more about prenatal mortality and the origins of cardiac abnormality in the Ts65Dn mice to assist in understanding cardiovascular malformation associated with DS.

Keywords

Down syndrome model; cardiovascular development; prenatal survival; pharyngeal arch arteries

Introduction

Down syndrome (DS) results from triplication of human chromosome 21 (*Hsa21*). This autosomal aneuploidy occurs in approximately 1 of 800 live births whereby normal patterns of development are disrupted by dosage imbalances (Shapiro, 1997). DS patients exhibit multiple physical and neurological abnormalities with phenotypes occurring in various combinations as a result of single or multiple dosage-sensitive genes and coincidental effects stemming from genetic over-expression of *Hsa21* (Korenberg *et al.*, 1994, Roper and Reeves, 2006). Approximately half of DS neonates suffer from congenital heart defects (Ferencz *et al.*, 1989). These defects can be fatal within one year of birth for approximately 70% of DS fetuses diagnosed with congenital heart malformations *in utero* and left untreated (Wessels *et al.*, 2003). Intracardiac anomalies such as atrioventricular septal defects, tetralogy of fallot and mitral valve prolapse occur at greater rates in DS than in the general population (Haak *et al.*, 2002; Barlow *et al.*, 2001).

Due to the complications that often arise *in utero*, pregnancies with DS fetuses are likely to end in spontaneous abortion. Statistical estimates of fetal loss due to DS differ considerably due to variation in data selection, study design, and data treatment by different research

groups (Spencer, 2001), thereby creating a wide margin for differential mortality before birth. In a study of mothers who elected not to terminate identified DS pregnancies, 35% of all pregnancies identified in the second trimester and 100% of pregnancies identified before 14 weeks of gestation ended in spontaneous fetal loss (Hook et al., 1995). In another study, predictions of overall spontaneous fetal loss in DS pregnancies found lower rates of 24% in the second trimester (Morris et al., 1999). Although identification of trisomic fetal loss during pregnancy is limited by the timing and accuracy of DS prenatal detection and the subpopulations studied, high rates of fetal loss found in association with DS indicate developmental anomalies related to the disorder are frequently fatal early in embryonic development.

To study the mechanisms by which dosage imbalance disrupts development, the segmental trisomic Ts65Dn mouse model with a third copy of approximately half of the *Hsa21* orthologs is currently used for many studies (Moore and Roper, 2007). Reported transmission rates of the Ts65Dn marker chromosome range from 20% (Liu et al., 2003) to 38.6% (Davisson et al., 1993). However, transmission rates of 50% approximately halfway through gestation indicate a 1:1 Mendelian ratio of transmission demonstrating normal chromosomal segregation during oogenesis (Roper et al., 2006). Furthermore, the trisomy is transmitted to only 44.7% of pups of Ts65Dn mothers at birth (Williams et al., 2008), a small but significant reduction from the expected 50%, suggesting differential mortality of embryos during the second half of development. By 3-4 weeks after birth, transmission rates drop to 33% - 37% (Paz-Miguel et al., 2001; Roper et al., 2006; Williams et al., 2008), indicating decreasing survival rates with age. The Ts65Dn mice share many of the phenotypes seen in human DS. These phenotypes include cognitive anomalies associated with brain regions affected in DS (Holtzman et al., 1996; Reeves et al., 1995; Baxter et al., 2000), craniofacial abnormalities (Richtsmeier, et al., 2002), and cardiovascular defects (Moore, 2006). At birth, approximately 15% of Ts65Dn neonates exhibit both vascular and intracardiac defects including right aortic arch (RAA), interrupted aortic arch, and atrial and ventricular septal defects (Williams et al., 2008). Cumulatively, these findings suggest that problems occurring past the midpoint of gestation contribute to reduced survival rates of the Ts65Dn fetuses and young mice.

The origins of many of these abnormalities are associated with the development of the pharyngeal arch arteries (PAAs), the precursors of the great thoracic arteries. Maturation of the arterial system begins early in mammalian development. Originally, the PAAs arise from the aortic sac to form a symmetric set of arches branching out to a dorsal aorta on either side of the body (Figure 1). At six weeks of human gestation and 10.5dpc in mouse, the 1st and 2nd arches form and then degenerate, leaving the 3rd, 4th, and 6th arches (Bard and Kaufman, 1999). Symmetry of the PAAs is then lost as the left dorsal aorta and left 3rd arch artery thicken while the right dorsal aorta regresses. The 3rd, 4th, and 6th arch arteries contribute to portions of the adult arterial system (Figure 1) such as the common carotid arteries (3rd), the aortic arch, brachiocephalic trunk and right subclavian artery (RSC) (4th), and the ductus arteriosus and root of the pulmonary arteries (6th). Through the development and loss of these structures, by 56 days of human development and 15.5dpc in mouse development, the adult thoracic arterial system forms.

The 5th arch artery, a transient structure that normally appears and degenerates shortly after six weeks of human embryonic development, can be found in approximately 50% of embryos (Khan and Nihill, 2006). Identification of a 5th arch artery and associated ring structures in mouse is variably reported (Bard and Kaufman, 1999, Hiruma et al., 2002), as the vessel may only be transiently patent with latex casting and ink injection methods. However, studies have identified patients with rare cases of persistent 5th arch artery (Donti et al., 1997; Lee et al., 1999; Stevenson and Hall, 2006). The persistence of the 5th PAA

may result in alterations in blood flow during cardiac development and lead to cardiovascular malformations. Blood flow patterns in the arch arteries are sensitive to changes within the developing heart and extraembryonic vessels. Hogers *et al.* (1997) found malformations in PAA development such as persistence of the 4th arch artery and left aortic arch (instead of the expected right aortic arch in chicks) after clipping the vitelline veins. Abnormalities in septation and development of the heart chambers may disrupt blood flow and contribute to malformation in arch arteries and vice versa.

Cardiovascular abnormalities and reduced survival rates in Ts65Dn neonates (Moore, 2006; Williams *et al.*, 2008) predict developmental malformations such as increased incidence of abnormal PAA development in Ts65Dn embryos. In a related DS model with perinatal lethality, trisomy 16 embryos demonstrate severe cardiac defects *in utero*, including abnormalities in PAA formation such as absence of one or more PAAs (Villar *et al.*, 2005). The expected transmission rate of the T65Dn chromosome midway through development (Roper *et al.*, 2006), but a decrease in transmission at birth (Williams *et al.*, 2008), suggest differential mortality of trisomic embryos during organogenesis but before birth. By analyzing trisomic transmission rates in embryonic Ts65Dn mice at 10.5dpc, 11.5dpc, 12.5dpc, 13.5dpc, and 14.5dpc, we expected to see a trend of differential mortality with decreased transmission rates and decreased litter size in later stages.

Materials and Methods

Animals

B6EiC3sna/A-Ts(17¹⁶)65Dn (Ts65Dn) female mice were mated with B6Eic × C3Sn F1 male mice (The Jackson Laboratories, Bar Harbor, ME) to create a Ts65Dn breeding colony to maintain the outbred backcross necessary for Ts65Dn transmission and maintenance. Mice were housed with controlled temperature and humidity, a 12-hour light/dark cycle, and food and water *ad libitum*. Animal protocols were approved by the Institutional Animal Care and Use Committee. For this study, 54 litters (301 total embryos) staged at 10.5dpc, 11.5dpc, 12.5dpc, 13.5dpc or 14.5dpc were collected and analyzed. Ts65Dn dams ranged from 3 to 10 months of age at the time of dissection. Embryos in each litter were not always at the same stage, but were staged according to the most developed embryo in the litter. Implantation sites and resorbing embryos were sometimes found in the uterus. In order to analyze transmission rates, information regarding embryo stage, litter size, and genotype were collected for all embryonic litters.

Gross Morphology and Dissection

Ts65Dn dams between 3 and 10 months of age were mated with F1 males. After proper gestation time, females were euthanized by cervical dislocation. During dissection, each embryo was isolated from its placenta at the umbilical vessels and placed into a fresh dish of Hank's Balanced Salt Solution (HBSS) (Cambrex Bio Science, Inc.). Blood pooled on the bottom of the dish from the umbilical vessels was collected in order to genotype each embryo. After isolation from the uterus, embryos were staged by physical appearance according to Theiler staging outlined by Baldock *et al.* (1998). Digital images were taken of each embryo using an Olympus DP10 to measure crown-rump length (mm) using Image J software (Rasband, 1997-2008).

Genotyping

Embryos were genotyped using interphase fluorescence *in situ* hybridization (FISH) as described by Moore and colleagues (1999). Embryonic blood was collected after placental separation to prevent maternal blood contamination. Implantation sites and resorbing

embryos not genotyped were excluded from analysis. Researchers were blind to genotype during phenotypic analyses.

Transmission Rate

Data from embryonic litters at 10.5dpc, 11.5dpc, 12.5dpc, 13.5dpc, and 14.5dpc were collected to determine if the expected rate of 50% transmission was maintained during different stages of development (54 litters, 301 embryos). To identify any decreased transmission of the T65Dn chromosome, each litter of embryos was categorized first for stage then by litter size.

India Ink Injections

During dissection, embryos staged at 10.5dpc, 11.5dpc or 13.5dpc were selected for analysis. Embryos were injected through their cardiac ventricles with 1:4 India ink diluted with water (World Precisions Instruments, Inc.). After injection was complete, embryos were placed in cardiac relaxation buffer (Williams *et al.*, 2008) for approximately 1 minute. Each embryo was fixed in 95% ethanol, 1% acetic acid, 1% chloroform solution overnight, washed in 70% ethanol, then stored in 100% ethanol. Before analysis using a dissecting microscope (Olympus SZX9), embryos were transferred to clearing solution (benzyl benzoate: methyl salicylate). Only embryos with ink patency along the length of both dorsal aortas were included in analysis of patent thoracic vessels. Images were captured using an Olympus DP10 or Moticam 2300 camera.

Statistical Analysis

Statistical tests were performed using SPSS 16 for Mac (SPSS, Inc.). Chi-squared tests were performed on genotype data for each stage of development and on rates of 5th PAA formation to test the null hypothesis that there was no difference in observed and expected rates. Linear regression was used on data stratified by litter. For 10.5dpc ink injection samples, an ANCOVA was performed to compare genotype, number of arch arteries present, and crown rump length. Residuals were checked to test for assumptions of ANCOVA.

Results

Prenatal Survival in Ts65Dn Embryos

Transmission rates of trisomy were calculated from 54 litters at 10.5dpc, 11.5dpc, 12.5dpc, 13.5dpc, and 14.5dpc to determine if the expected rate of 50% transmission was maintained during development. Although the percent of embryos that were genotyped as trisomic ranged from 37.5 to 51.9%, transmission rates of the T65Dn chromosome were not significantly different from expected at 10.5dpc through 14.5dpc ($\alpha = 0.05$, Table 1). Resorbing embryos that we were unable to genotype were excluded from these analyses. Seven Ts65Dn dams were found not to be pregnant at dissection despite the presence of vaginal plugs indicative of copulation.

Each litter was categorized (blocked) by stage of embryonic development in order to determine if transmission rate of the T65Dn marker chromosome differed significantly between stages as a result of differential mortality. No significant relationship was found between embryonic stage and transmission rate of the T65Dn chromosome at $\alpha = 0.05$ (linear regression: $r^2 = 0.006$, $F_{1,51} = 0.309$, $P = 0.580$). All litters were stratified by litter size in order to determine if the transmission rates of trisomy differed significantly between litter sizes as a result of selective trisomic loss. No significant relationship was found between litter size and transmission rate of the T65Dn chromosome at $\alpha = 0.05$ (linear regression: $r^2 = 0.007$, $F_{1,51} = 0.340$, $P = 0.562$). Furthermore, no significant relationship

was found to exist between embryonic stage of development and litter size (linear regression: $r^2 = 0.007$, $F_{1,51} = 0.376$, $P = 0.543$; Figure 2).

Pharyngeal Arch Arteries

The development of the PAAs in a total of 28 10.5dpc (35.7% Ts65Dn) embryos, 27 11.5dpc (44.4% Ts65Dn) embryos and 43 13.5dpc (48.8% Ts65Dn) embryos were examined. The PAAs were visualized by intracardiac India ink injection to trace the ink-patent arterial vessels. Normal arch artery formation for embryos staged at 10.5dpc is a symmetrical presence of the 3rd, 4th, and 6th arch arteries on both the left and right sides (Figures 1 and 3). Trisomic and euploid sibling embryos at 10.5dpc were scored for the presence or absence of the three arch arteries on each side (Table 2). In some cases, the 6th aortic arch was absent, while other embryos had neither a 4th nor 6th patent arch artery. Of the ten Ts65Dn embryos, eight had at least one PAA absent on the right and/or left side (Figures 3 and 4). Crown rump length (mm) was measured for staging and as a covariate for analyzing the presence and absence of PAAs. A significant difference was found using an ANCOVA in the number of arch arteries present based on the embryo's genotype ($F_{1,24} = 4.821$; $P = 0.038$), as well as the number of arch arteries present on the right and the embryo's crown rump length ($F_{1,24} = 10.330$; $P = 0.004$; $\bar{x} = 4.484$) (Figure 5). The same trend of smaller Ts65Dn embryos lacking arch arteries was seen on the left side (data not shown).

Embryos analyzed at 11.5dpc were scored based on the presence of the 3rd, 4th, and 6th PAAs, thickening of the 4th and 6th PAAs on the left side (left side dominance), and beginning formation of the brachiocephalic artery, with parallel right and left dorsal aorta branches (Figure 3); at this stage all of these characteristics are expected (Bard and Kaufman, 1999). In both euploid and trisomic embryos with cardiovascular development of 11.5dpc, variability in external morphological appearance was found. In one of the 11.5dpc trisomic embryos (8.3%), the right 6th arch artery was absent. Another trisomic embryo exhibited right side dominance (thickening of the right aortic arch and right-sided dorsal aorta, Figure 4), indicating the potential formation of right aortic arch. The left 6th arch artery was found to be hypoplastic in one trisomic embryo, and the right 6th arch artery hypoplastic in another. Of the fifteen euploid embryos scored, three had notable hypoplastic 6th arch arteries (2 left, 1 right), indicating that variability in the size of the 6th PAA does not correlate with genotype. In both euploid and trisomic embryos with cardiovascular development at 11.5dpc, 17 embryos (63%) exhibited an ink-patent 5th arch artery or vascular ring formed by the potential 5th arch artery found between the dorsal aorta, the 4th arch artery, and the 6th arch artery (Figures 3 and 4). Eight 11.5dpc euploid embryos (53%) and nine Ts65Dn embryos (75%) exhibited a 5th arch artery phenotype on the right or left side, an occurrence that is expected to be found in only 50% of embryos in normal mammalian development (Khan and Nihill, 2006). Incidence of the vascular ring formed from a prospective 5th arch artery was significantly higher than expected in trisomic mice when analyzed at $\alpha = 0.10$ ($n=12$, $\chi^2 = 3.00$, $P = 0.083$).

Embryos staged at 13.5dpc were also injected with India ink. The embryos analyzed were scored based on the presence and location of the right and left subclavian arteries, right and left carotid arteries, a right side dorsal aortic trunk with left side dominance and dorsal aorta on the left side (Figure 3). Presence of the ductus arteriosus and the right and left pulmonary arteries were scored if visible. These characteristics are expected at this stage in normal embryonic development (Bard and Kaufman, 1999). Delayed migration of the RSC was identified in two trisomic (9.5%) and two euploid (9.1%) embryos at 13.5dpc (Figure 4). During cardiovascular development, the distal segment of the RSC arises from the right-sided dorsal aorta as it thins and regresses from the thickened, dominant left-sided dorsal aorta. This typically occurs at 12dpc - 12.5dpc, with complete migration by 13.5dpc. In all

four cases, migration of the right-sided dorsal aorta was not yet complete. Of the 21 Ts65Dn embryos observed, one (4.8%) exhibited a right aortic arch with Kommerell's diverticulum (Figure 3). This anomaly is characterized by a right aortic arch with a retroesophageal vascular ring encircling the trachea and esophagus. No cardiovascular anomalies were noted in the euploid embryos ($n = 22$).

Discussion

Transmission Rates

Transmission rate of the T65Dn marker chromosome was investigated to confirm normal chromosomal segregation during oogenesis and identify any selective trisomic loss. We tested the null hypothesis that the expected 1:1 Mendelian ratio would be followed by equal survival at five different points in gestation. At 10.5dpc, 11.5dpc, 12.5dpc, 13.5dpc, and 14.5dpc, no statistically significant difference from the expected ratio was found (Table 1). These results indicate that the significantly reduced rates of trisomy occurring at birth (Williams *et al.*, 2008) in the Ts65Dn mouse model are a result of differential mortality during later stages (>14.5 dpc) of embryonic development.

If selective fetal loss occurs as a result of trisomy, both transmission rate and litter size should be reduced at later stages of development. To identify any low levels of selective loss, embryos were grouped by litter to test litter size versus transmission rate, embryonic stage versus transmission rate, and embryonic stage versus litter size. No significant relationship was found between embryonic stage and transmission rate of trisomy between litters, indicating that between 10.5dpc and 14.5dpc, differential mortality does not have an effect on transmission rate in litters. In addition, each litter was stratified by litter size to determine if transmission rate of the T65Dn marker chromosome differed significantly between litter sizes as a result of differential mortality; no significant relationship was found in this study. Therefore, smaller litters are not primarily euploid and premature death of trisomic embryos is not a causative factor of reduced litter size at these stages. Linear regression was used to test the potential effects of embryonic stage of development on litter size (Figure 2). Although there was a slight negative slope representing the relationship between embryonic stage of development and litter size, no statistically significant relationship was found to exist, indicating that litters produced between 10.5dpc and 14.5dpc do not vary significantly in size.

A complication that arose during this study was the presence of resorbing embryos that we were unable to accurately genotype and which were excluded from analyses (Table 1). Resorption occurs as a result of developmental failure; however, no relationship between trisomy and embryonic degeneration could be tested. In addition, despite the presence of a vaginal plug indicative of copulation, seven dams were found not to be pregnant at the time of dissection. At later stages, dams were monitored for appropriate weight gain to prevent sacrifice on non-pregnant trisomic dams. We found female infertility and failed progression through pregnancy presented obstacles in collecting a larger sample size in this trisomic DS model.

Genetic insults affecting heart development cause an increased rate of severe cardiac abnormalities resulting in significantly increased fetal loss between 10.5dpc and 14.5dpc of murine development (Ranger *et al.*, 1998; Wu *et al.*, 1999). Our multiple analyses of both litters and populations at these stages indicate that in our DS model, differential mortality is not occurring during the critical stages of heart development. Since transmission rates were not found to differ from expected at these stages, cardiac defects that occur during this time period were not severe enough to cause death during septation of the heart (Conway *et al.*, 2003). This period of murine development parallels the first seven weeks of human

development. Although researchers have detected trisomy and studied rates of fetal loss in human embryos in the second trimester (Hook *et al.*, 1995; Morris *et al.*, 1999), evidence of embryonic mortality in the first trimester has been variable. We have found that triplication of half of the *Hsa21* genes that are found on T65Dn do not cause cardiac anomalies that impede survival of trisomic embryos during the stages studied. These findings are similar to other DS mouse models in that cardiovascular abnormalities, but not embryonic lethality during cardiovascular development, are associated with *Hsa21* (O'Doherty *et al.*, 2005) or *Mmu16* (Li *et al.*, 2007) dosage imbalance in mice.

Analysis of Pharyngeal Arch Arteries

India ink injections into the PAAs were performed to elucidate pathogenic mechanisms responsible for malformations present in Ts65Dn neonates. The tracing of ink patent vessels in arch artery system at 10.5dpc has revealed abnormalities in Ts65Dn ranging from the absence of the 6th arch artery to the absence of both the 4th and 6th arch arteries (Table 2, Figures 3 and 4). The crown rump length (mm) was measured in all 10.5dpc samples as a covariate to investigate if size, in addition to or instead of embryonic genotype, caused arch artery malformations. A significant difference was found in the number of arch arteries present based on the embryo's genotype as well as the number of arch arteries present and the embryo's crown rump length. These results indicate that the Ts65Dn lack more PAAs than euploid siblings (Figure 5). The impact of embryo size on arch artery presence could be that the smaller crown rump length indicates developmental delay and the Ts65Dn will develop the missing arch arteries later in development. It is important to note that while some small euploid embryos demonstrated the presence of all three arteries, similarly sized trisomic embryos did not (Figure 5).

The tracing of the arch arteries at 11.5dpc and 13.5dpc revealed both developmental delay and abnormality in the Ts65Dn embryos. At 11.5dpc, a 6th PAA was hypoplastic in two trisomic and three euploid embryos. This suggests a delayed formation of the 6th arch artery in these embryos, but this potential delay was not limited to trisomic embryos. However, the right 6th arch artery was found to be absent in one trisomic embryo. Another Ts65Dn embryo exhibited thickening of the right-sided dorsal aorta and arch arteries (Figure 4), suggesting the potential formation of an abnormal RAA at later stages. Our findings with 11.5dpc embryos show variability in mild phenotypes such as hypoplastic vessels; but more severe phenotypes, like missing vessels and incorrect maintenance and regression, are limited to trisomic embryos.

In both trisomic and euploid embryos, a potential 5th PAA contributing to formation of a vascular ring was identified between the junction of the dorsal aorta, 4th arch artery, and 6th arch artery on either the right or left side (Figures 3 and 4). In our study, the incidence of 5th PAA was 53% of euploid embryos scored and 75% of trisomic embryos scored; a significantly higher rate than expected correlating with trisomy. Many of the human cases identified have been associated with other cardiovascular defects in the patient, including right aortic arch with a retroesophageal ring, aberrant left subclavian artery, patent ductus arteriosus, atrioventricular septal defects, and tetralogy of Fallot (Donti *et al.*, 1997; Lee *et al.*, 1999; Stevenson and Hall, 2006). Although no cases have linked persistent 5th arch artery to DS, patients with this anomaly exhibit many cardiovascular phenotypes also found in DS and Ts65Dn mice (Williams *et al.*, 2008). The increased presence of a 5th PAA in our trisomic embryos suggests a basis for abnormal development in later stages, including cardiovascular defects associated with persistent 5th PAA.

Also noted during the collection of the 11.5dpc embryos was increased variability between embryo morphology and stage within litters. Roper *et al.* (2008) report variability in embryonic stage in 19 litters collected at 9dpc from Ts65Dn dams. During dissections, we

found notable variability in two of nine litters collected at 11.5dpc. In both litters, embryos varied from 9dpc to 12dpc in morphological appearance. We observed some Ts65Dn dams that failed to become pregnant after multiple matings, plugged dams with no embryos present upon dissection, and variability of developmental stage within litters that suggest a reduced ability to sustain pregnancies in Ts65Dn dams. Underdeveloped embryos were genotyped as both Ts65Dn and euploid, suggesting that survival during pregnancy is nondiscriminatory in trisomic dams.

To determine if the developmental anomalies identified at 10.5 and 11.5dpc had an effect on cardiovascular development in later stages of embryonic maturity, 13.5dpc embryos were also injected with India ink for cardiovascular tracing. In two trisomic and two euploid embryos, delayed regression of the RSC was found at 13.5dpc. Chaoui *et al.* (2005) reports 36% incidence of aberrant RSC in association with DS, an anomaly characterized by the development of the RSC arising as a fourth vessel from the dorsal aorta. This abnormality has also been found in Ts65Dn neonates and adults (Williams *et al.*, 2008). It is possible that developmental delay may play a role in formation of this anomaly. Despite previous findings and occurrence of delayed regression of the right dorsal aorta in both euploid and trisomic embryos used in our study, unambiguous aberrant RSC was not found at the latest stage analyzed. However, a clear vascular defect previously seen in Ts65Dn neonates, right aortic arch with Kommerell's diverticulum, was found in one trisomic 13.5dpc embryo (Figure 3).

Together, the results of PAA analysis in 10.5dpc, 11.5dpc, and 13.5dpc Ts65Dn embryos provide evidence that trisomic embryos form at a retarded rate in entirety when compared to their euploid sibs. Developmental delays in some trisomic embryos at these critical phases of development, such as small body size at 10.5dpc and late formation of pharyngeal arch arteries (lack of PAAs at 10.5 and 11.5dpc, hypoplastic at 11.5dpc) may ultimately result in significant abnormalities in the adult morphology. Some of the anomalies found at 11.5dpc, such as vascular rings and right-sided dominance, could contribute to cardiovascular anomalies like the formation of the RAA found at 13.5dpc and defects previously identified in Ts65Dn neonates. It is possible that blood flow in embryonic trisomic mice may have been disrupted through either malformation in the umbilical vessels or anomalies in septation and valve development. Previous studies have shown that altered hemodynamics can drastically effect the formation of the PAAs, causing anomalies such as persistent 4th PAA and a reversed aortic arch (Hogers *et al.*, 1997). Alternatively persistent 5th PAA and vascular rings can alter blood flow and are associated with gross cardiovascular abnormalities during development (Lee *et al.*, 1999; Stevenson and Hall, 2006; Donti *et al.*, 1997). Our study confirms that delayed development and cardiovascular abnormalities are present in Ts65Dn embryonic mice at 10.5dpc, 11.5dpc, and 13.5dpc. Hemodynamic analysis of the embryonic circulatory system may elucidate the mechanisms for the formation of cardiovascular anomalies in trisomy.

Although the findings of our study show a basis for developmental abnormalities such as the RAA found in 8.3% of neonatal Ts65Dn mice by Moore (2006), other cardiovascular abnormalities, such as aberrant RSC, were not observed in the embryonic samples analyzed. Survival of trisomic embryos was not reduced during the critical stages of heart septation studied. Because trisomy is strongly associated with cardiovascular abnormalities, delays found during development could be a precursor for the anomalies we see at birth. Based on our observed recovery of "missing arches" by 11.5dpc and 13.5dpc in most trisomic embryos, our study suggests that cardiovascular anomalies found in Ts65Dn are the result of early developmental delays due to trisomy. With further insight into the factors that influence the formation of the great thoracic arterial system, we might better understand cardiovascular anomalies found in DS patients.

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Changes in pharyngeal arch artery pattern during cardiovascular development. Each number represents the respective arch artery. The dotted lines indicate transient structures that will be lost throughout development. Color-coding indicates contributions to mature adult structures in the arterial system. This schematic shows the symmetrical development of each arch artery pair before loss of symmetry that occurs at approximately 11.5dpc.

Figure 2.

The effects of embryonic stage of development on litter size. Embryos staged between 10.5dpc and 14.5dpc were genotyped by interphase FISH analysis and stratified by litter size. No significant relationship was found between embryonic stage and litter size at these stages in embryonic development (linear regression, $P = 0.543$).

Figure 3.

Vessel tracings of PAAs at 10.5dpc, 11.5dpc, and 13.5dpc. Embryos from trisomic dams were injected with Indian ink for visualization of the arterial system. A: Euploid embryo staged at 10.5dpc with all three PAAs (3rd, 4th, and 6th) present. B: Trisomic embryo staged at 10.5dpc missing the 4th and 6th PAA on both the right and left sides. C: Right side 3rd, 4th, and 6th PAAs of a euploid embryo staged at 11.5dpc. D: Right side PAAs of a trisomic embryo staged at 11.5dpc. The 5th PAA is present at the junction of the right DA, 4th, and 6th PAAs forming a vascular ring (arrow). E: Dorsal view of euploid 13.5dpc embryonic vasculature. F: Dorsal view of a trisomic 13.5dpc embryo exhibiting a right aortic arch. [DA, dorsal aorta; LDA, left dorsal aorta; AoA, normal aortic arch; LCC, left common carotid; BA, brachiocephalic artery; RCC, right common carotid; RSC, right subclavian artery; PA, pulmonary artery (with arrows); R AoA, right aortic arch.] Scale bar = 1 mm (A, B); Scale bar = 0.5 mm (C, D, E, F).

Figure 4.

Vessel tracings of pharyngeal arch arteries at 10.5dpc, 11.5dpc, and 13.5dpc. Embryos from trisomic dams were injected with Indian ink for visualization of the arterial system. A: Trisomic embryo staged at 10.5dpc with only one PAA (3rd PAA) present on each side. B: Trisomic embryo staged at 10.5dpc missing the 6th PAA on the left side. C: Right side 3rd, 4th, and 6th PAAs of a euploid embryo staged at 11.5dpc with the prospective 5th PAA present at the junction of the right DA, 4th, and 6th PAAs forming a vascular ring. D: Right side PAAs of a trisomic 11.5dpc embryo with a 5th PAA forming a vascular ring. E: Dorsal view of trisomic 11.5dpc embryonic vasculature with a dominant right aortic arch and right dorsal aorta with smaller left dorsal aorta: the potential origin of a right aortic arch. F: Dorsal view of a 13.5dpc embryo exhibiting a delay in regression of the right aorta, accompanying delay in cephalic migration of the left and right subclavian arteries. [DA, dorsal aorta; LDA, left dorsal aorta; LCC, left common carotid; BA, brachiocephalic artery; RCC, right common carotid; RSC, right subclavian artery; R AoA, right aortic arch.] Scale bar = 0.5 mm.

Figure 5.

Relationship of genotype, crown – rump length and the number of PAAs. Each embryo was staged as 10.5dpc, imaged for crown-rump length, genotyped as trisomic (n=10) or euploid (n=18), and injected with ink. Cleared embryos were scored for the presence of the three PAAs on the right and left side. Presence of only the 3rd PAA is denoted by “1” on the y-axis. Presence of only the 3rd and 4th PAAs is indicated by “2” on the y-axis. Presence of all PAAs (3rd, 4th and 6th) is represented by a “3” on the y-axis. Right-sided PAAs are shown; the left side showed a similar pattern with fewer PAAs formed in trisomic embryos by 10.5dpc.

Table 1

Chi-squared analysis of transmission rate of Ts65Dn during murine cardiovascular development.

Stage in dpc (n)	Ts65Dn Transmission (%)	χ^2	P value	Litters	Resorbing embryos
10.5 (84)	40.5	3.048	0.081	14	7
11.5 (54)	51.9	0.160	0.689	9	3
12.5 (44)	50	0.0	1.0	7	8
13.5 (99)	47.5	0.248	0.619	16	10
14.5 (32)	37.5	2.0	0.157	7	9

Table 2

The percentage of 10.5dpc embryos with one, two, or all three of the pharyngeal arch arteries typically identifiable after India ink injection at this stage of development.

PAA present	Euploid (n= 18)		Ts65Dn (n=10)	
	Right	Left	Right	Left
3 rd	33%	17%	70%	60%
3 rd & 4 th	-	22%	10%	10%
3 rd 4 th & 6 th	67%	61%	20%	30%