Chapter 8
Environmental Sensitivity to Trichloroethylene (TCE) in the Developing Heart

Ornella I. Selmin, Om Makwana, and Raymond B. Runyan

Abstract Epidemiological reports first suggested a specific teratogenicity of trichloroethylene (TCE) in drinking water but due to other potential contaminants, uncertain exposure levels and selection of controls, these reports were controversial. Early animal studies explored the effects of high doses of TCE on heart development and a drinking water model was established that showed the ability of TCE to produce cardiac malformations. However, these doses were well above typical levels of environmental exposure and the significance of TCE as a cardiac teratogen remained controversial. Using molecular and functional measures of cardiac development, several laboratories began to examine the effects of TCE in animal models at relevant exposure levels. This work demonstrated a non-monotonic dose response where significantly more effects on gene expression and cardiac function were seen just above the current maximum contamination level (5 parts per billion) than at much higher dose levels. An examination of early heart valve formation showed that formation of valve progenitors was impaired. Molecular studies pointed towards changes in expression of several muscle genes

O.I. Selmin, PhD (✉)
Department of Nutritional Sciences, The University of Arizona Cancer Center, The University of Arizona, Tucson, AZ, USA

The University of Arizona Cancer Center, Tucson, AZ, USA

The Sarver Heart Center, The University of Arizona, Tucson, AZ, USA
e-mail: selmin@email.arizona.edu

O. Makwana
Department of Medicine, University of California, San Francisco, CA, USA

Veterans Affairs Medical Center, San Francisco, CA, USA

R.B. Runyan
Department of Cellular and Molecular Medicine, The University of Arizona Cancer Center, Tucson, AZ, USA

The Sarver Heart Center, The University of Arizona, Tucson, AZ, USA

K.M. Gilbert, S.J. Blossom (eds.), *Trichloroethylene: Toxicity and Health Risks*, Molecular and Integrative Toxicology, DOI 10.1007/978-1-4471-6311-4_8,

involved in calcium homeostasis and myocardial contraction. Further studies showed that calcium-mediated contraction in the heart was impaired and that this corresponded to changes in intracellular calcium flux and cardiac output. To explore the non-monotonic dose curve, expression of phase I metabolic enzymes were examined. The early heart was found to be a specific site of cytochrome p450 expression prior to the development of the liver and CYP2C expression was elevated with TCE exposure. Surprisingly, the CYP expression pattern replicated the non-monotonic dose response and did not explain it. This suggests that a metabolite of TCE is likely to be the teratogen. To explore the teratogenicity of TCE, microarray data from exposed chick hearts were analyzed by utilization of interactome analysis. This analysis identified a subset of highly-linked genes that were perturbed by TCE exposure. The most centrally linked gene in the interactome was the transcription factor HNF4a. Though not previously known in the heart, HNF4a expression was confirmed. Its level of expression was unchanged with TCE exposure but its level of phosphorylation was altered. Ongoing studies are investigating the hypothesis that HNF4a is a proximal target of TCE exposure and that misregulation of gene expression by this transcription factor is a significant mediator of congenital heart defects produced by TCE.

Keywords Embryonic heart • Epithelial mesenchymal transition • Gene expression • Cytochrome P450 • Hepatocyte nuclear factor 4 alpha

8.1 Introduction

Recent epidemiological studies reinforced earlier controversial findings indicating that TCE exposure during pregnancy increased the frequency of congenital heart disease (CHD) in newborns. Every year, approximately 1 % of all children are born with heart defects around the world. This number includes 0.6 % of newborns with severe and moderate defects with additional measures of 0.3–6.9 % of newborns with lesser defects as determined by various protocols (Hoffman and Kaplan 2002). It is estimated by the Children's Heart Foundation that inpatient surgery to repair heart defects in the US exceeds $2.2 billion/year. Estimates in TCE-exposed populations show an odds ratio of 2.5–3.0 compared to control groups (The Toxicological Review of TCE; Goldberg et al. 1990; Bove et al. 1995). That only a small proportion of these CHDs can be attributed to specific genetic defects, highlights the apparent importance of environmental factors in embryonic heart maldevelopment. The most convincing data attesting to TCE cardiotoxicity derive from mechanistic studies, conducted in chick embryos, using a wide range of doses during different phases of heart differentiation. In vitro studies unraveled likely mechanisms of action by which TCE may interfere with normal heart development. As the heart is the first functional organ in the embryo, and must continue to develop and remodel throughout embryonic and fetal life, it utilizes a number of specific cellular and molecular processes that are sensitive to TCE. Endothelial-mesenchymal transition

is a cellular process that produces the progenitors of the valves and septa while a regulated calcium flux is required to mediate muscle contraction and produce blood flow. As described below, both processes are altered by TCE exposure. Although independent groups have confirmed similar findings in different species, outstanding questions to be addressed remain the bi-modal behavior of TCE dose–response, the role of P450 cytochromes in TCE metabolism in the embryonic heart, and the role of TCE metabolites in determining cardiac toxicity in mammals and avians. In this chapter we review data from epidemiologic, animal and in vitro studies supporting the notion that sensitivity of the embryonic heart to TCE exposure may be responsible for the increased number of congenital heart defects observed in contaminated areas. Lastly, we present a new hypothesis suggesting the role of a nuclear transcription factor in mediating, at least in part, the negative effects of TCE in the developing heart.

8.2 Epidemiological Studies

Very few epidemiological studies analyzed the effects of maternal TCE exposure on the incidence of CHD in children and these show mixed results (The Toxicological Review of TCE). Reports of cardio-teratogenicity first came from human studies (Goldberg et al. 1990) that were later verified by Bove et al. (1995). The spectrum of defects seen in children exposed to up to 270 parts per billion (ppb) TCE in drinking water *in utero* included aortic stenosis, valvular, septal and muscular defects and the odds ratio (OR) for all heart defects was between 3.0 (Goldberg et al. 1990) and 2.5 (Bove et al. 1995). In a retrospective study, looking at ambient air exposure to TCE, Yauck et al. (2004) reported an increase in heart defects among mothers ≥38 years of age living within 1.33 miles of a TCE-emitting site (OR = 3.2; 95 %). More recently, Forand et al. (2012) reported that both birth and cardiac birth defects approximately doubled in populated areas of Endicott, New York that were found to be contaminated with TCE and perchloroethylene (PCE). When the analysis was limited to the conotruncal defects, these, although rare, were nearly five times more prevalent in both the PCE and TCE exposed areas. A few community-based studies have looked at the association between TCE exposure and incidence of birth defects other than cardiac malformations. Lagakos et al. (1986), using a health survey of ~5,000 residents of Walburn (MA), observed an increased rate of eye anomalies (OR = 14.9) and perinatal deaths (OR = 10) associated with exposure to TCE-contaminated water wells. Other studies using the same populations were not conclusive, although a retrospective analysis looking at hospital records reported increased OR for many birth defects including congenital heart diseases (NAS Report 2006).

Studies performed at the US Marine Corps Base at Camp Lejeune, NC, where residents had been exposed to TCE, DCE and PCE for as long as 30 years, found that TCE exposure was associated to smaller male, but not female infants (Agency

for Toxic Substances and Disease Registry 1998; Sonnenfeld et al. 2001). Risk was higher for women older than 35 and those with a history of fetal loss. Limitations of these studies included misclassification of the groups (i.e. exposed vs nonexposed, and long term vs short term exposure) lack of information about water consumption, dermal and inhalation exposure, tobacco and alcohol usage. Taken altogether, data collected from available studies provide robust indication that environmentally relevant doses of TCE exposure through drinking water increased the risk of intrauterine growth reduction. These findings are similar to those reported for maternal alcohol consumption (alcohol has a metabolism similar to TCE), and are confirmed by observations gathered from animal studies (Johnson et al. 1998a, b; Fisher et al. 2001; Smith et al. 1992, 1989). In summary, epidemiological studies were complicated by several factors including chemical co-exposure, difficulty in establishing the doses and duration of TCE exposure, small number of cases, aggregation of a wide range of malformations, and the unknown role of genetic variations in determining sensitivity to TCE. However, considered as a whole, these studies indicate a relatively consistent elevation for cardiac defects in the range of 2.5–3.0-fold (Chiu et al. 2013), and other birth defects including eye malformations and low birth weight.

8.3 In Vivo Studies

While the timing varies between species, the developmental processes of heart development are consistent. The embryonic heart develops as a hollow tube consistent of two cellular layers, the inner endothelium and the exterior myocardium, separated by extracellular matrix (Fig. 8.1a–c). The heart tube then loops as part of a developmental process to bring the inflow and outflow portions of the heart to their adult orientation. During the looping process, there is a signal derived from the myocardium that initiates valve formation by endothelial cells lining the atrioventricular canal (Runyan and Markwald 1983). A subset of endothelial cells in this region loses cell-cell adhesions and migrates into the adjacent extracellular matrix, becoming mesenchymal cells. This process, called epithelial-mesenchymal transition (EMT) produces the progenitors of the valves and membranous septa of the mature heart. Factors altering this process of valve and septa formation would affect the size and shape of the mitral and tricuspid valves and/or produce septal defects between the right and left sides of the heart. Valve or septal defects would, in turn, impair normal hemodynamics and produce muscular malformations. Additional developmental processes include muscular protrusions that divide the atria and ventricles into right and left sides. Fusion between the intraventricular muscle tissue and the fused cardiac cushions normally closes the septum between the two ventricles and separates the function of the right and left chambers. The atrial muscular septum is comprised of two layers with offset openings that permit blood to pass from the right to the left side of the heart during fetal life. Changes in blood pressure

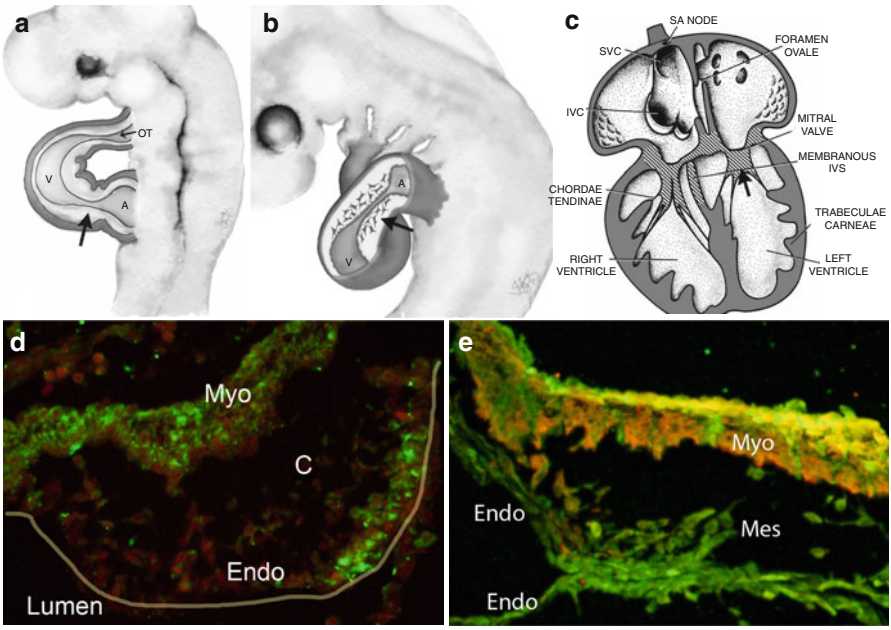

Fig. 8.1 Heart Development and Gene Expression. (**a**) The early embryonic heart is seen at this stage as a single tube E2 (HH14)- chick, E9.25-mouse, and E20- human. The outer layer is the myocardium and the inner layer is endothelium. The intervening space is filled with extracellular matrix and protrudes into the lumen in the canal between the single atrium and the single ventricle (*arrow*) as paired cardiac cushions. (**b**) By HH17 in the chick and E9.5 in the mouse the atrium has continued to rotate and shift towards the head. Endothelial cells lining the AV canal have begun to undergo an epithelial-mesenchymal transition to form the progenitors of the valve fibroblasts within the cushions (*arrow*). (**c**) Later in development, the four-chambered heart has developed from the heart tube. The striped area of the valves and septa in this cross section are derived from the cells that invaded the cushions in (**b**). (**d**) An embryonic chick heart stained for CYP2C after 8 ppb exposure to TCE. A single cardiac cushion is outlined by a *light yellow line*. Staining (*green*) is seen in the myocardium and in a subset of endothelial cells proximal to the atrium. (**e**) A segment of the heart stained for HNF4a (*green*) and cardiac myosin (*orange/red*). HNF4a is seen throughout the embryonic heart. Abbreviations- *A* atrium, *V* ventricle, *OT* outflow tract, *Myo* myocardium, *Endo* endothelium, *Mes* mesenchyme, *C* cushion

at birth cause the two layers to fuse and functionally separate the pulmonary and corporeal blood flows. By the end of the embryonic period of development, the heart is largely in its final form. In the rodent model, the heart starts contracting around 8.5 days, begins looping to bring the inflow and outflow of the heart together around day 9 and the valvular primordial begin to develop around day 9.5. Much of the early morphogenesis of the heart is complete by day 12. In the human, the heartbeat begins about day 18 and looping and early valve formation take place between day 21 and 30. The equivalent period in the chick embryo is between days 2 and 3.5. Studies showing cardio-teratogenic effects of TCE in each of these species appear to consistently target this early organogenic period of heart development.

8.4 Rodents

After the original epidemiological studies in humans, Goldberg and his group (Johnson et al. 1998a, b, 2003; Dawson et al. 1990, 1993) reported on TCE cardiac teratogenicity in rat embryo studies that were controversial largely because of very high exposures (1,100 parts per million, ppm, which approximates the maximum solubility of TCE in water) and atypical dose response curves (Dawson et al. 1993; Johnson et al. 2003). These authors found a correlation between TCE exposure in rat embryos through maternal drinking water and congenital heart defects, but it was statistically significant only for the highest doses and appeared to be protective at the lowest dose tested of 2.5 ppb. Conversely, in an independent study similar high doses of TCE (500 mg/kg) provided by gavage to pregnant rats from day 6 to 15 of pregnancy, produced no defects in embryos (Fisher et al. 2001). The contrasting results were attributed to differences in modality and time of exposure that included maternal drinking water vs. gavage in soybean oil, and exposure starting at gestational day (GD) 0 vs 6. A few studies investigated the effects of TCE metabolites on cardiac malformations in exposed embryos. In humans and other mammals TCE is partially excreted by expiration. The ingested TCE is converted into trichloroethanol (TCEtOH) through an unstable chloral compound by CYP2E1. Conversely, ALDH1 mediates the conversion of the intermediate chloral into trichloroacetic acid (TCA), which is eliminated through the urine, unaltered or after dechlorination in form of di- or mono-chloroacetic acid (DCA and MCA, respectively). TCEEtOH is excreted either as a free compound or as a glucoronide conjugate (Davidson and Beliles 1991). TCE can cross the placenta and CYP2E1 has been detected in rat placenta and embryos.

Smith et al. (1992, 1989) reported that both TCA and DCA administered by gavage to Long-Evans dams between GD 6 and 15, produced significant increase in levocardia at doses of TCA equal or >330 mg/kg-day, and intraventricular septal defects at >800 mg/kg-day. The same types of defects were observed at doses of DCA equal or >900 mg/kg-day for levocardia and equal or >1,400 mg/kg-day for intraventricular septal defects. Epstein et al. (1992) reported that GD between 9 and 12 was particularly sensitive to TCA exposure (one dose of 2,400 mg/kg-day) in Long-Evans rats and produced intraventricular septal defects. The authors explained their findings as a failure of the proliferating intraventricular septal tissue to fuse with the tight tubercle of the atrioventricular (AV) cushion tissue. Subsequent studies from Johnson et al. (1998a, b) found that TCA induced significant increases in cardiac defects at the highest dose (2,730 ppm = 291 mg/kg-day) when administered by maternal drinking water during GD 1–22. Of all the other TCE metabolites tested, only dichloroethylene (DCE) produced cardiac defects in a similar model of exposure at either 0.15 or 110 ppm (= .015 and 10.64 mg/kg-day, respectively), when administered pre- and during pregnancy. No particular type or grouping of cardiac anomaly was observed in these studies.

The same animal model of TCE exposure described by Johnson et al. (1998a), was used to identify proteins whose expression is affected by TCE in embryonic hearts. Using a differential display approach, Collier et al. (2003) reported that

moderate doses (100 ppm) of TCE in maternal drinking water altered the expression of genes critical for heart development, including vimentin, a marker of mesenchymal differentiation, and the calcium ATP-ase Serca2a.

8.5 Chicks

Original observations regarding TCE cardiac teratogenicity in avians were made by Loeber et al. (1988) who reported that in ovo exposure of chick embryos led to several cardiac malformations including conotruncal, AV cushion and myocardium abnormalities. Although this study suffered from lack of accurate information concerning time and concentration of TCE used, it provided a solid rationale for using chick embryos as a sensitive model for TCE cardiac sensitivity.

Mishima et al. (2006) provided additional data using a chick whole-embryo culture system in which they were able to determine the TCE concentration in the medium, and pin-point the stage of embryonic development most sensitive to TCE cardiotoxicity. In particular, they observed that when cultures were prepared containing initial concentrations between 25 and 250 ppm of TCE, after equilibration (1 h), about 70 % of the added TCE was lost and none was detected after 24 h. Under these conditions, the authors estimated the chick embryos were exposed to TCE for an average of 6 h and the most sensitive stages were at 2 days when the heart began to loop. After 24 h, more than 80 % of 3 day embryos exposed to 80 ppm TCE were apparently normal. However, a closer examination at the cellular level revealed a significant reduction in the number and altered distribution of mesenchymal cells in the AV cushion.

These results were independently confirmed by Drake et al. (2006a), using low doses of TCE (4 nM = 8 ppb) injected into chick eggs between HH stages 13 and 20 (2–3.5 days), corresponding to the time of development of cardiac cushions. The authors reported a significant reduction in mesenchymal cells and cardiac function, using pulsed-Doppler ultrasound, when embryos were exposed to 8 ppb TCE and similar concentrations of metabolites TCA and trichloroethanol (TCOH). Interestingly, these effects were not observed when embryos at earlier and later stages of cardiac morphogenesis (HH 3+ and HH20) were exposed to TCE (Drake et al. 2006b). In contrast, 4nM TCE or TCA increased myocyte proliferation in these embryos exposed at an early developmental time. The conclusion from these studies is that cardiac sensitivity to TCE in chick is dependent on specific stages of heart development.

8.6 Mechanisms of Action

The notion that TCE can disrupt the early events of cardiac differentiation emerged in an earlier in vitro study (Boyer et al. 2000) looking at the effects of TCE on EMT in chick AV explants by collagen gel assays. Results from Boyer et al. (2000)

indicated that the EMT process was impaired by doses of TCE between 50 and 250 ppm in vitro. As these doses reflected starting levels in plastic dishes with some airspace above each culture, the actual effective dose was likely far lower than reported. In the dose range tested, TCE inhibited the expression of Mox-1 and fibrillin2, which are components of the EMT process during the AV canal formation.

Ou et al. (2003) reported that, in differentiated bovine coronary endothelial cells (BCEC), TCE altered the function of endothelial nitric oxide synthase (eNOS) by inhibiting its interaction with the heat shock protein hsp90, and inducing an increased production of O_2^- in a dose–response fashion. The reduced synthesis of NO in favor of O_2^- may have a negative impact on endothelial cell proliferation and lead to defects in valve and septa development. Consistent with this analysis, Feng et al. (2002) observed that eNOS-KO mice had increased incidence of congenital heart defects. In a microarray analysis looking at changes in gene expression caused by TCE exposure, our group found that molecules involved in regulation of calcium signaling (e.g. CamKII and Ryr2) were affected by TCE exposure as low as 1 and 10 ppb, in mouse embryonal carcinoma cell line P19. These cells were used as a model of undifferentiated cardiac myocytes and provided important clues regarding possible molecular mechanisms mediating TCE action (Selmin et al. 2008). In particular, we observed that many transcripts affected either by TCE or its metabolite, TCA, encoded for calcium-responsive proteins or are dependent on cellular DNA methylation for their function (e.g. rhodopsin-G-proteins).

The importance of molecules involved with regulation of calcium flux during early cardiogenesis was confirmed by subsequent studies looking at the effects of low (10–1,000 ppb) and moderate (10–100 ppm) doses of TCE on vasopressin-induced calcium flux in rat myoblasts, using FURA2 measurements. Using the H9c2 cell line, Caldwell et al. (2008) observed a dose-dependent response in which doses of TCE between 10 and 100 ppb inhibited calcium flux, whereas higher doses (10 ppm TCE) did not alter either calcium flux or expression of proteins involved in calcium regulation (e.g. Serca2a and Ryr2). This bimodal behavior of cell response to low versus high doses of TCE has been confirmed in numerous studies using different systems (Mishima et al. 2006; Drake et al. 2006a) but the reasons have not been elucidated. Makwana et al. (2013) recently proposed a role for p450 cytochrome oxygenases in TCE metabolism as essential to explain this phenomenon, and their findings will be discussed below.

Numerous lines of evidence from research groups examining TCE carcinogenicity in liver and kidney (Tao et al. 1999, 2000; Dow and Green 2000) suggested that TCE might exert its negative action by disrupting the folate/homocysteine pathway and ultimately altering the normal methylation status of proteins and nucleic acids. To test this hypothesis Caldwell et al. (2010) analyzed the transcriptome of mouse embryonic hearts isolated from dams that had been exposed to 10 ppb TCE through maternal drinking water and a diet containing 0, 2, or 8 mg/kg folate. The goal of this study was to identify cellular pathways altered in the developing heart following exposure to low, environmentally significant doses of TCE and to determine whether folic acid supplementation might counteract the effects of TCE. Standard chow for pregnant rats usually contains 5–8 mg of folate

per kg of pellet and provides ~200 ug/day of folate. The recommended daily allowance (RDA) for pregnant women is 600 ug/day. A gross examination of the embryos collected from the three folate groups indicated that both high (8 mg/kg) and low (0 mg/kg) folate in the maternal diet lead to similar morphological outcomes. In fact, in both groups we observed an increased rate of resorbed and developmentally delayed embryos, accompanied by a reduced number of normally developed embryos. However, when compared with the TCE exposed groups, we observed an almost perfect inversion of phenotypic outcome, suggesting that (i) any alteration from the optimal level of folate in the pregnant rats may alter fetal development, and (ii) low levels of TCE may facilitate developmental progress through otherwise restrictive developmental checkpoints. In fact, we observed no change in percentage of embryos resorbed or delayed in dams exposed to TCE and low folate supplementation (0 mg/kg) compared with those not exposed to TCE and receiving a normal (2 mg/kg) folate level in their diet (Dow and Green 2000). In addition, results from these studies showed that exposure to the low dose of TCE caused extensive alterations in transcripts encoding proteins involved in transport, ion channel, transcription, differentiation, cytoskeleton, cell cycle, and apoptosis. Exogenous folate did not offset the effects of TCE exposure on normal gene expression, and both high and low levels of folate produced additional significant changes in gene expression. We concluded that a mechanism in which TCE induced a folate deficiency did not explain altered gene expression patterns in the embryonic mouse heart. The data further suggested that use of folate supplementation, in the presence of TCE, might be detrimental and not protective of the developing embryo.

A possible mechanism behind these effects is suggested by recent findings reported by our laboratory (Palbykin et al. 2011). We found that TCE affects DNA methylation of the Serca2 promoter region both in rat myoblasts H9c2 cells, and in murine embryonic hearts exposed to TCE via maternal drinking water. The Serca2 gene encodes for a calcium ATPase that is essential for regulation of the calcium flux in myocyte. In particular, we observed a modest increase in CpG methylation across the Serca2 proximal promoter, accompanied by a dramatic hypermethylation of a CpG dimer adjacent to a SP1 binding site, which had been previously described as involved in Serca2 transcriptional activity (Brady et al. 2003). These changes were paralleled by reduced levels of Serca2a protein both in TCE exposed H9c2 cells and embryonic hearts. Methylation of CpG dinucleotides is catalyzed by DNA methyltransferases and uses S-adenosyl-methionine (SAM) as the methyl-donor group. The reduced amounts of SAM observed in this study were explained by the fact that TCE-induced hypermethylation of the Serca2 promoter, and likely other actively transcribed genes, may deplete the cellular content of SAM, which in turn may lead to delayed embryonic development and possible congenital birth defects, including heart defects (Ifergan and Assaraf 2008). Although these findings reported a cellular depletion of SAM, the methylation status of each promoter is regulated by dynamic and complex processes that determine gene activation or repression during embryonic development. Therefore, in order to better understand the mechanisms of action of TCE, it is necessary to investigate the nature of the interactions between

TCE and crucial transcription factors that mediate gene expression during critical phases of embryonic development.

The hypothesis that TCE exposure may cause a reduction in calcium homeostasis in myocytes and directly impact cardiac morphology, was tested by Makwana et al. (2010) using chick embryos exposed to TCE in ovo. Cardiac myocytes were isolated from E18 embryos exposed to either 8 or 800 ppb TCE at HH stage 13 (E2) by in ovo injection into the yolk. Sarcomeric function was assessed by measuring the rate of contraction after electrical stimulus. A reduced half-width of contraction by the sarcomere was observed in myocytes exposed to 8 ppb, whereas sarcomere length itself was not affected, suggesting the change was due to an alteration in Ca++ handling and not to changes in sarcomere structure. Despite the comparatively early exposure of these myocytes in development, sarcomere function remained altered in these cells 16 days later. The functional loss of sarcomeric contraction was also seen by reduced expression of markers of shear stress, NOS-3 and KLF2 in the heart. These data are consistent with observations that altered Ca++ homeostasis can reduce blood flow and the loss of flow can lead to congenital heart defects (Hogers et al. 1997). Independent observations from Rufer et al. (2010) confirmed these ideas with further experimental data. In their study, chick embryos exposed to 8 ppb TCE in ovo, between HH15 and 17, displayed high embryonic mortality and functional dysmorphologies. In particular, a significant high frequency of ventricular septal defects (VSDs) was observed. These defects occurred in 37.5 % of the exposed embryos, and in none of the controls, exposed to vehicle only. The authors concluded that since cardiac hemodynamics are a major contributor to VSDs, their findings support a mechanism of TCE cardiac teratogenesis based on altered calcium handling and blood flow.

In a recent study, Makwana et al. (2013) explored the possibility that the non-monotonic dose–response curve could reflect issues on cardiac metabolism of TCE. Previously, Lash et al. (2000) had identified specificity of the cytochrome P450 CYP2 family for TCE metabolism in human liver microsomes and in murine systems. Makwana et al. (2013) showed the presence of two members of the CYP2 family in embryonic chick hearts (2H1and 2C45), and more importantly, exposure to 8 ppb TCE, but not 800 ppb, induced the expression of CYP2H1 in particular in myocardial cells and in AV canal endothelial cells most proximal to the atrium. No detectable expression of cytochrome p450s was observed in extracardiac tissues (Fig. 8.1d). These results indicate that the earliest embryonic expression of phase I detoxification enzymes are in the developing heart, which may explain its distinct sensitivity to TCE. Later, cytochrome p450 expression in the heart drops and develops in the newly-formed liver. This roughly coincides with the period of TCE sensitivity observed by Rufer et al. (2010). While the bi-modal response curve remains poorly understood, Makwana et al. (2013) suggest that low doses of TCE are metabolized, causing toxic effects, whereas at higher TCE doses, CYPs are degraded faster than can be synthesized. The question whether in the latter case, TCE metabolites cause any cardiac toxicity in the embryonic heart, is still unanswered.

8.7 Current Studies

Altering the expression of proteins that regulate myocyte calcium flux may be a significant mechanism by which TCE causes heart defects. Consistent with the notion of altered gene regulation is the finding that numerous transcription factors involved in early embryonic cardiac differentiation (e.g. brachyuryT, NFATc1, Hoxa1, Foxa1) were down-regulated in mouse embryonic hearts exposed to TCE (Caldwell et al. 2010). The central question remains: what are the proximal signals induced or inhibited by TCE exposure and how do they regulate the expression of these structural and regulatory proteins? In an attempt to address this question, our group returned to the chick system to explore microarray analysis of embryos treated with 8 ppb TCE. The microarray data were subjected to analysis using a chick interactome database (Konieczka et al. 2009). The chick interactome is a compendium of seven databases derived from biological data across many species. The nodes in the database (Fig. 8.2) are molecules converted to a consistent species nomenclature (avian) and the edges are connections derived from public databases on transcriptional regulation, yeast two hybrid screens, protein interaction immunoprecipitation data and other evidence of biological interaction. Of the approximately

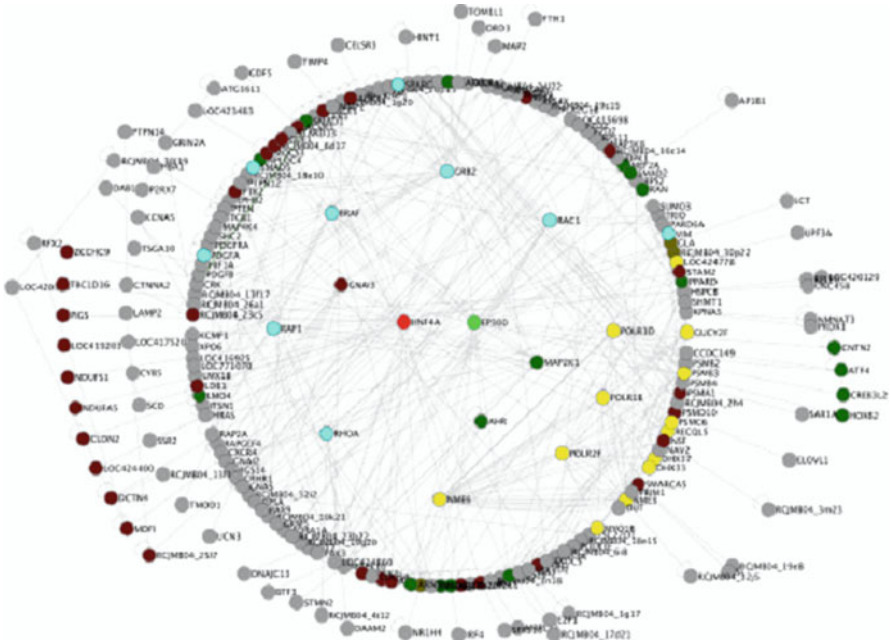

Fig. 8.2 TCE interactome. Circles represent nodes (molecules) and connecting lines are edges showing an identified interaction in one or more of the core databases. The data are displayed in a circular pattern by Cytoscape and the most highly linked nodes were moved to the center of the circle. HNF4a is shown in *red* and directly linked nodes are shown in *maroon*

Table 8.1 Mostly highly linked nodes in interactome analysis of TCE

Name	1′ Neighbors	2′ Neighbors	1′ Sig DN	1′ Sig UP
HNF4A	159	866	35	55
TRAF6	116	778	12	43
IKBKE	100	621	11	28
14-3-3z	89	670	9	25
14-3-3 g	80	555	14	20
EIF1B	63	431	5	16
PRKAB1	56	518	8	13
MAP3K3	54	646	6	17
HSPA8	54	540	4	16

HNF4a shows 159 directly linked (1′ Neighbors) nodes in the TCE dataset and indirect linkage to a further 866 nodes (2′ Neighbors). The combined 1′ and 2′ linkage represents approximately 76 % of the total 1,345 nodes found in the TCE interactome. Of the 159 directly linked nodes, 35 were significantly down-regulated and 55 were up-regulated by 8 ppb TCE. Thus HNF4a is the most highly linked node and is central to the greatest number of regulated targets in the dataset. Most of the regulated primary targets of HNF4a were also found in the mouse dataset. Cytoscape analysis is heuristic and begins randomly in the dataset. Linkage was determined after 5 reiterations. After 20 repeats, EP300 moved to 12th in rank and is not shown in table despite a retained involvement

4,000 genes significantly altered in the chick arrays by TCE exposure, 1,345 genes were found in the interactome dataset that linked to each other. This subset was identified as the TCE interactome. As shown in Table 8.1, we ranked, within the TCE interactome, the set of genes with the highest levels of interconnections in the dataset. The most highly linked molecule was the hepatocyte nuclear factor 4 alpha, HNF4a. In fact, 76 % of the 1,345 molecules included in the TCE interactome are directly or indirectly linked to HNF4a through 1 partner. The transcription factor HNF4a (bright red) and the transcriptional co-activator EP300 (bright green) are shown as highly linked genes. Interestingly, although the HNF4a transcript, itself, is not altered by TCE, in both mouse and chick, the expression of the coactivator EP300, which has acetyl transferase activity, is reduced by TCE, along with other cofactors including HDAC11, HNF1b, and Ppargc1a and 1b (Caldwell et al. 2010). This is consistent with a hypothesis that HNF4a protein is a proximal target of a TCE or its metabolite and that alteration of HNF4a activity would perturb expression of molecules normally regulated by HNF4a.

We used PCR and Western analysis to investigate the expression of HNF4a in mouse heart (embryonic and maternal) and in rat cardiomyoblasts, H9c2 cells. The results illustrated in Fig. 8.3 indicate that HNF4a protein is expressed in both embryonic and maternal heart, and that TCE exposure in H9c2 cells inhibits HNF4a phosphorylation but does not affect its protein level. We had previously shown that Serca2a expression is altered by TCE. Examination of the promoter region of Serca2a showed a candidate HNF4a binding site. After an acute low dose exposure of TCE on H9c2 rat cardiomyocytes at two intervals a CHIP analysis was carried

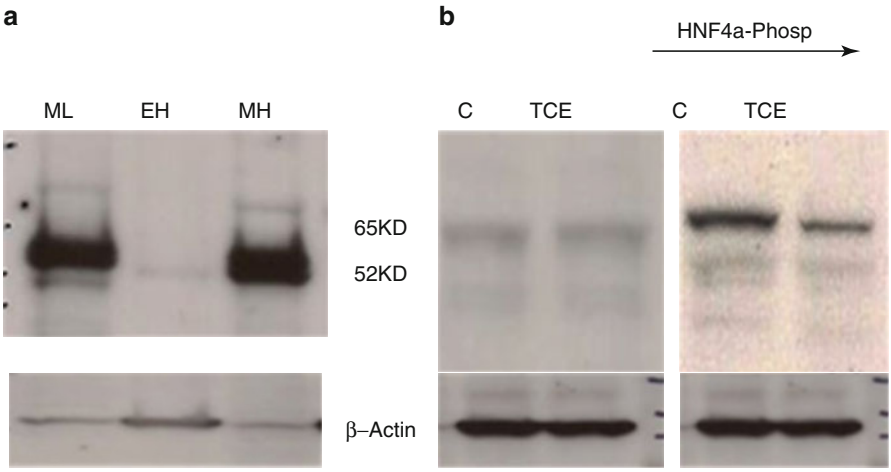

Fig. 8.3 Expression of HNF4a. (**a**) Tissue homogenate from mouse maternal liver (*ML*), Day 18 embryonic heart (*EH*), and maternal heart (*MH*), was analyzed by Western blot using anti HNF4a antibodies which recognized in all tissues a major band of ~52 kD, and a minor band of ~65 kD band in maternal liver and heart only. Lower levels of HNF4a are present in EH, as expected. (**b**) Equal amounts of H9c2 cell lysates were analyzed by Western blot using the same HNF4a antibody in panel **a** (*left panel* in **b**) or antibodies against the phosphorylated form of HNF4a (*right panel* in **b**). H9c2 cells exposed to 10 ppb TCE for 24 h (TCE) show reduced levels of phosphorylated HNF4a compared to control, not exposed cells

out to verify an increased HNF4a expression after TCE exposure. H9C2 cells were fixed, the genomic DNA was sheared and an immunoprecipitation was performed with anti-HNF4a antibodies. The eluted DNA was then measured by quantitative real time PCR to measure changes in HNF4a bound Serca2 promoter region compared to untreated control cells. In Fig. 8.4, we show that 10 ppb TCE exposure after 30 min and 1 h results in an increase in HNF4a transcription factor binding to this region. These data confirm that HNF4a is found in cardiomyocytes and confirm an alteration of activity consistent with a proximal role for HNF4a in mediating some of the effects of TCE on cardiomyocytes. We followed this up by collecting stage embryonic chick heart material from the early, looped heart through a stage nearing completed septation into the 4-chambered heart. These stages are all before a functional liver has developed in the embryo. Quantitative PCR showed a variable pattern of expression during development but a loss of signal about the time that the liver begins to form (data not shown). Immunostaining with the HNF4a antibody (stage 18 hearts) showed that endothelia lining the heart are positive for HNF4a and that there is a slight loss during the transition into valvular mesenchyme in the region where the mitral and tricuspid valves will form. There is also expression in the myocardial cell layer that is heaviest in the outermost (epicardial) layer of muscle and in clusters of myocardial cells that stain poorly for myosin (Fig. 8.1e). These cells that are positive for HNF4a staining likely represent undifferentiated myoblasts. Thus, HNF4a is found in the heart early in development and could be the target of TCE exposure.

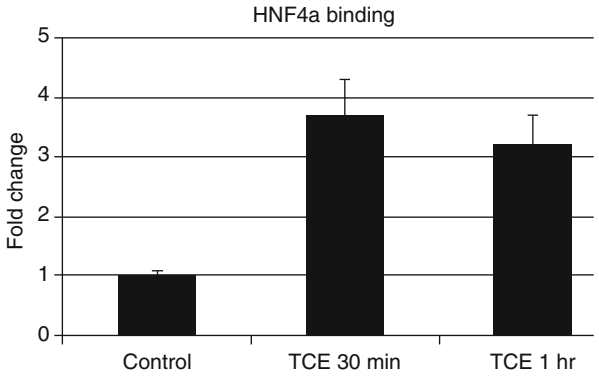

Fig. 8.4 ChIP analysis of HNF4a binding to the Serca2 promoter. H9c2 cells were exposed to 10 ppb TCE for 30 min or 1 h before chromatin was isolated for immunoprecipitation using HNF4a specific antibodies, and primers specific for Serca2 proximal promoter region flanking a HNF4a putative binding site for real time PCR analysis. The graph represents the average of three PCR runs in which each sample was run in quadruplicate. HNF4a binding was normalized by subtracting non specific IgG binding from each sample and value were expressed as average fold change compared with control, non exposed cells. The bars represent standard error

8.8 Conclusions

Earlier studies were controversial, in large part, due to a poorly understood non-monotonic dose curve. In the last 10 years findings from chick and murine models of TCE exposure unequivocally support the embryonic heart sensitivity to low, environmentally relevant doses of TCE. In addition, the embryonic heart is particularly susceptible to TCE toxicity during the phase of transition between a looped heart divided in two compartments separated by the AV cushion and a 4-chambered functional heart. The most current hypothesis corroborated by several independent groups is that TCE affects the ability of the cardiac tissue to regulate calcium flux, thus altering myocyte contraction and maintenance of normal blood flow. Altered hemodynamics likely lead or contribute to development of the variety of congenital heart malformations observed in humans and animal models. Although the molecular mechanisms are still unclear, our current studies suggest a nuclear transcription factor, HNF4alpha, as a most proximal target of TCE action. Future studies will focus on elucidating the mechanisms by which TCE may disrupt transcriptional regulation of HNF4a target genes involved in heart differentiation and metabolism, including CYP2 members found in the embryonic heart (Table 8.2). While the bimodal or non-monotonic behavior of TCE dose–response is not unique and has been observed for other compounds, including serotonin, opiates, alcohol, and formaldehyde (Calabrese 2001; Calabrese and Baldwin 2003; Gaylor and Aylward 2004; Sari and Zhou 2003), the mechanism underlying this effect is still unclear. However, the finding that low doses of TCE can be metabolized in the embryonic chick heart by members of the CYP2 family, highlight the possibility that cardiac toxicity from TCE and other teratogens may be influenced by a transitory localized metabolizing ability.

Table 8.2 TCE effects on cytochrome P450 expression

Gene	Effect	Tissue/cell	Compound	Mouse homolog
Cyp1b1	UP	Mouse e. h.	10ppbTCE	
Cyp39a1	UP	Mouse e.h.	10ppbTCE	
Cyp26a1	UP	Mouse e.h.	10ppbTCE	
Cyp7b1	UP	Mouse e. h.	10ppbTCE	
Cyp2c66	UP	Mouse e.h.	10ppbTCE	
Cyp2d11	DOWN	Mouse e.h.	10ppbTCE	
Cyp24a1	DOWN	Mouse e.h.	10ppbTCE	
Cyp4f13	DOWN	Mouse e.h.	10ppbTCE	
Cyp4f16	DOWN	Mouse e.h.	10ppbTCE	
Cyp2c37	DOWN	Mouse e.h.	10ppbTCE	
Cyp2g1	DOWN	Mouse e.h.	10ppbTCE	
Cyp2a12	UP	P19 cells	1ppmTCE	
Cyp2r1	UP	P19 cells	1ppmTCA	
Cyp2h1	UP	Chick e. h.	8ppbTCE	Cyp2e1
Cyp1a4	UP	Chick e.h.	8ppbTCE	Cyp1a1
Cyp2c45	UP	Chick e.h.	8ppbTCE	Cyp2c6

Data were extracted from Caldwell et al. (2010), and Makwana et al. (2010)

Level of expression of Cyp transcripts in mouse and chick embryonic heart (e.h.) were determined by microarray or PCR analyses

References

Agency for Toxic Substances and Disease Registry (1998) Volatile organic compounds in drinking water and adverse pregnancy outcomes: U.S. Marine Corps Camp Lejeune, North Carolina. Atlanta: US Department of Health and Human Services

Bove FJ, Fulcomer MC, Klotz JB, Esmart J, Dufficy EM, Savrin JE (1995) Public drinking water contamination and birth outcomes. Am J Epidemiol 141(9):850–862

Boyer AS, Finch WT, Runyan RB (2000) Trichloroethylene inhibits development of embryonic heart valve precursors in vitro. Toxicol Sci 53(1):109–117

Brady M, Koban MU, Dellow KA, Yacoub M, Boheler KR, Fuller SJ (2003) Sp1 and Sp3 transcription factors are required for trans-activation of the human SERCA2 promoter in cardiomyocytes. Cardiovasc Res 60(2):347–354

Calabrese EJ (2001) The future of hormesis: where do we go from here? Crit Rev Toxicol 31(4–5): 637–648

Calabrese EJ, Baldwin LA (2003) Ethanol and hormesis. Crit Rev Toxicol 33(3–4):407–424. Review

Caldwell PT, Thorne PA, Johnson PD, Boitano S, Runyan RB, Selmin O (2008) Trichloroethylene disrupts cardiac gene expression and calcium homeostasis in rat myocytes. Toxicol Sci 104(1):135–143

Caldwell PT, Manziello A, Howard J, Palbykin B, Runyan RB, Selmin O (2010) Gene expression profiling in the fetal cardiac tissue after folate and low-dose trichloroethylene exposure. Birth Defects Res A Clin Mol Teratol 88(2):111–127

Chiu WA, Jinot J, Scott CS, Makris SL, Cooper GS, Dzubow RC, Bale AS, Evans MV, Guyton KZ, Keshava N, Lipscomb JC, Barone S Jr, Fox JF, Gwinn MR, Schaum J, Caldwell JC (2013) Human health effects of trichloroethylene: key findings and scientific issues. Environ Health Perspect 121:303–311

Collier JM, Selmin O, Johnson PD, Runyan RB (2003) Trichloroethylene effects on gene expression during cardiac development. Birth Defects Res A Clin Mol Teratol 67(7):488–495

Davidson IW, Beliles RP (1991) Consideration of the target organ trichloroethylene in terms of metabolite toxicity and pharmacokinetics. Drug Metab Rev 23(5–6):493–599. Review. PubMed PMID: 1802654

Dawson BV, Johnson PD, Goldberg SJ, Ulreich JB (1990) Cardiac teratogenesis of trichloroethylene and dichloroethylene in a mammalian model. J Am Coll Cardiol 16(5):1304–1309

Dawson BV, Johnson PD, Goldberg SJ, Ulreich JB (1993) Cardiac teratogenesis of halogenated hydrocarbon-contaminated drinking water. J Am Coll Cardiol 21(6):1466–1472

Dow JL, Green T (2000) Trichloroethylene induced vitamin B(12) and folate deficiency leads to increased formic acid excretion in the rat. Toxicology 146(2–3):123–136

Drake VJ, Koprowski SL, Lough J, Hu N, Smith SM (2006a) Trichloroethylene exposure during cardiac valvuloseptal morphogenesis alters cushion formation and cardiac hemodynamics in the avian embryo. Environ Health Perspect 114(6):842–847

Drake VJ, Koprowski SL, Hu N, Smith SM, Lough J (2006b) Cardiogenic effects of trichloroethylene and trichloroacetic acid following exposure during heart specification of avian development. Toxicol Sci 94(1):153–162

Epstein DL, Nolen GA, Randall JL, Christ SA, Read EJ, Stober JA, Smith MK (1992) Cardiopathic effects of dichloroacetate in the fetal Long-Evans rat. Teratology 46(3):225–235

Feng Q, Song W, Lu X, Hamilton JA, Lei M, Peng T, Yee SP (2002) Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. Circulation 106(7):873–879

Fisher JW, Channel SR, Eggers JS, Johnson PD, MacMahon KL, Goodyear CD, Sudberry GL, Warren DA, Latendresse JR, Graeter LJ (2001) Trichloroethylene, trichloroacetic acid, and dichloroacetic acid: do they affect fetal rat heart development? Int J Toxicol 20(5):257–267

Forand SP, Lewis-Michl EL, Gomez MI (2012) Adverse birth outcomes and maternal exposure to trichloroethylene and tetrachloroethylene through soil vapor intrusion in New York State. Environ Health Perspect 120(4):616–621. Epub 2011 Dec 5

Gaylor DW, Aylward LL (2004) An evaluation of benchmark dose methodology for non-cancer continuous-data health effects in animals due to exposures to dioxin (TCDD). Regul Toxicol Pharmacol 40(1):9–17

Goldberg SJ, Lebowitz MD, Graver EJ, Hicks S (1990) An association of human congenital cardiac malformations and drinking water contaminants. J Am Coll Cardiol 16(1):155–164

Hoffman JL, Kaplan S (2002) The incidence of congenital heart disease. J Am Coll Cardiol 39(12):1890–1900

Hogers B, DeRuiter MC, Gittenberger-de Groot AC, Poelmann RE (1997) Unilateral vitelline vein ligation alters intracardiac blood flow patterns and morphogenesis in the chick embryo. Circ Res 80(4):473–481

Ifergan I, Assaraf YG (2008) Molecular mechanisms of adaptation to folate deficiency. Vitam Horm 79:99–143

Johnson PD, Dawson BV, Goldberg SJ (1998a) Cardiac teratogenicity of trichloroethylene metabolites. J Am Coll Cardiol 32(2):540–545

Johnson PD, Dawson BV, Goldberg SJ (1998b) A review: trichloroethylene metabolites: potential cardiac teratogens. Environ Health Perspect 106(Suppl 4):995–999. Review

Johnson PD, Goldberg SJ, Mays MZ, Dawson BV (2003) Threshold of trichloroethylene contamination in maternal drinking waters affecting fetal heart development in the rat. Environ Health Perspect 111(3):289–292

Konieczka JH, Drew K, Pine A, Belasco K, Davey S, Yatskievych TA, Bonneau R, Antin PB (2009) BioNetBuilder2.0: bringing systems biology to chicken and other model organisms. BMC Genomics 10(Suppl 2):S6

Lagakos SW, Wessen BJ, Zelen M (1986) An analysis of contaminated well water and health effects in Woburn, Massachusetts. J Am Stat Assoc 81:583–596

Lash LH, Fisher JW, Lipscomb JC, Parker JC (2000) Metabolism of trichloroethylene. Environ Health Perspect 108(Suppl 2):177–200. Review

Loeber CP, Hendrix MJ, Diez De Pinos S, Goldberg SJ (1988) Trichloroethylene: a cardiac teratogen in developing chick embryos. Pediatr Res 24(6):740–744

Makwana O, King NM, Ahles L, Selmin O, Granzier HL, Runyan RB (2010) Exposure to low-dose trichloroethylene alters shear stress gene expression and function in the developing chick heart. Cardiovasc Toxicol 10(2):100–107

Makwana O, Ahles L, Lencinas A, Selmin OI, Runyan RB (2013) Low-dose trichloroethylene alters cytochrome P450-2C subfamily expression in the developing chick heart. Cardiovasc Toxicol 13(1):77–84

Mishima N, Hoffman S, Hill EG, Krug EL (2006) Chick embryos exposed to trichloroethylene in an ex ovo culture model show selective defects in early endocardial cushion tissue formation. Birth Defects Res A Clin Mol Teratol 76(7):517–527

NAS Report (2006) Toxicological Review of Trichloroethylene (TCE). http://cfpub.epa.gov/ncea

Ou J, Ou Z, McCarver DG, Hines RN, Oldham KT, Ackerman AW, Pritchard KA Jr (2003) Trichloroethylene decreases heat shock protein 90 interactions with endothelial nitric oxide synthase: implications for endothelial cell proliferation. Toxicol Sci 73(1):90–97

Palbykin B, Borg J, Caldwell PT, Rowles J, Papoutsis AJ, Romagnolo DF, Selmin OI (2011) Trichloroethylene induces methylation of the Serca2 promoter in H9c2 cells and embryonic heart. Cardiovasc Toxicol 11(3):204–214

Rufer ES, Hacker TA, Flentke GR, Drake VJ, Brody MJ, Lough J, Smith SM (2010) Altered cardiac function and ventricular septal defect in avian embryos exposed to low-dose trichloroethylene. Toxicol Sci 113(2):444–452

Runyan RB, Markwald RR (1983) Invasion of mesenchyme into three-dimensional collagen gels: a regional and temporal analysis of interaction in embryonic heart tissue. Dev Biol 95(1):108–114

Sari Y, Zhou FC (2003) Serotonin and its transporter on proliferation of fetal heart cells. Int J Dev Neurosci 21(8):417–424

Selmin OI, Thorne PA, Caldwell PT, Taylor MR (2008) Trichloroethylene and trichloroacetic acid regulate calcium signaling pathways in murine embryonal carcinoma cells p19. Cardiovasc Toxicol 8(2):47–56

Smith MK, Randall JL, Read EJ, Stober JA (1989) Teratogenic activity of trichloroacetic acid in the rat. Teratology 40(5):445–451

Smith MK, Randall JL, Read EJ, Stober JA (1992) Developmental toxicity of dichloroacetate in the rat. Teratology 46(3):217–223

Sonnenfeld N, Hertz-Picciotto I, Kaye WE (2001) Tetrachloroethylene in drinking water and birth outcomes at the US Marine Corps Base at Camp Lejeune, North Carolina. Am J Epidemiol 154(10):902–908

Tao L, Ge R, Xie M, Kramer PM, Pereira MA (1999) Effect of trichloroethylene on DNA methylation and expression of early-intermediate protooncogenes in the liver of B6C3F1 mice. J Biochem Mol Toxicol 13(5):231–237

Tao L, Yang S, Xie M, Kramer PM, Pereira MA (2000) Effect of trichloroethylene and its metabolites, dichloroacetic acid and trichloroacetic acid, on the methylation and expression of c-Jun and c-Myc protooncogenes in mouse liver: prevention by methionine. Toxicol Sci 54(2):399–407

The Toxicological Review of TCE, Chapter 4, CAS-No79-01-6, EPA/635/r-09/011F at www.epa.gov/iris

Yauck JS, Malloy ME, Blair K, Simpson PM, McCarver DG (2004) Proximity of residence to trichloroethylene-emitting sites and increased risk of offspring congenital heart defects among older women. Birth Defects Res A Clin Mol Teratol 70(10):808–814