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Prenatal Alcohol Exposure Causes Adverse Cardiac Extracellular Matrix Changes and Dysfunction in Neonatal Mice

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Abstract

Fetal alcohol syndrome (FAS) is the most severe condition of fetal alcohol spectrum disorders (FASD) and is associated with congenital heart defects. However, more subtle defects such as ventricular wall thinning and cardiac compliance may be overlooked in FASD. Our studies focus on the role of cardiac fibroblasts in the neonatal heart, and how they are affected by prenatal alcohol exposure (PAE). We hypothesize that PAE affects fibroblast function contributing to dysregulated collagen synthesis, which leads to cardiac dysfunction. To investigate these effects, pregnant C57/BL6 mice were intraperitoneally injected with 2.9 g EtOH/kg dose to achieve a blood alcohol content of approximately 0.35 on gestation days 6.75 and 7.25. Pups were sacrificed on neonatal day 5 following echocardiography measurements of left ventricular (LV) chamber dimension and function. Hearts were used for primary cardiac fibroblast isolation or protein expression analysis. PAE animals had thinner ventricular walls than saline exposed animals, which was associated with increased LV wall stress and decreased ejection fraction. In isolated fibroblasts, PAE decreased collagen I/III ratio and increased gene expression of profibrotic markers, including α-smooth muscle actin and lysyl oxidase. Notch1 signaling was assessed as a possible mechanism for fibroblast activation, and indicated that gene expression of Notch1 receptor and downstream Hey1 transcription factor were increased. Cardiac tissue analysis revealed decreased collagen I/III ratio and increased protein expression of α-smooth muscle actin and lysyl oxidase. However, Notch1 signaling components decreased in whole heart tissue. Our study demonstrates that PAE caused adverse changes in the cardiac collagen profile and a decline in cardiac function in the neonatal heart.

Introduction

Alcohol consumption during pregnancy can lead to a wide range of behavioral and birth defects, collectively known as fetal alcohol syndrome disorders (FASD), with fetal alcohol syndrome (FAS) being the most severe form [1]. Even so, it is reported that 500,000 women a year still drink during pregnancy [2, 3]. As of 2015, it was reported that 10% of pregnant women still report consuming alcohol during pregnancy, and 3% of those women participate in binge drinking. In some states, the alcohol consumption rate for women of child bearing age is as high as 68% as of 2016 [4]. Furthermore, it is estimated that 50% of pregnancies in

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the U.S. are undetected within the first few weeks, increasing the risk of alcohol consumption during early fetal development [5–7]. Given that heavy exposure of alcohol or even light patterns of drinking can cause developmental abnormalities, FASD is clearly an important biomedical issue that requires more attention [8, 9]. Alcohol-induced congenital heart defects are present in 50% of children born with FAS and in 38% of children with FASD [10, 11]. These range from atrial or ventricular septal defects, aortic malformation, and valvular defects [11]. However, less obvious cardiovascular abnormalities may go clinically unnoticed in children born with FASD and may have adverse cardiac implications in adulthood. For example, Serano et. al have shown that prenatal alcohol exposure produces thin ventricular walls in mouse embryos [12]. Further, prenatal alcohol exposure produces elevated blood pressure in adult rats, resulting from aberrant vascular and renal function [13–15]. These alcohol-induced cardiovascular abnormalities may result from changes in the cardiac extracellular matrix, which play a crucial role in proper cardiovascular development [16].

To our knowledge, no studies have examined the effects of fetal alcohol exposure on the composition of the cardiac extracellular matrix or cardiac fibroblasts. The primary function of the fibroblast is to produce the extracellular matrix (ECM), which provides a structural scaffolding for the attachment and organization of cardiomyocytes and other cell types [17, 18]. As such, the fibroblasts play a key role in the development of the heart [18]. The composition and stiffness of the ECM directly regulate developmental processes such as cell proliferation, migration, and differentiation [19, 16]. Additionally, ECM components such as collagen and fibronectin directly affect signaling pathways that regulate cardiac myocyte development and cardiac structure [20]. Despite their importance in development and function of both the fetal and adult heart, fibroblast function is altered in response to noxious stimuli, which can promote further injury [21]. During injury, cardiac fibroblasts become activated and transition to myofibroblasts, which express α-smooth muscle actin and secrete greater amounts of collagen and other ECM components [22]. Our lab and others have shown that alcohol is a noxious stimuli that promotes fibroblast activation through alterations of matrix metalloproteinases, tissue inhibitors of matrix metalloproteinases, and transforming growth factor beta [23, 24]. As such, we investigated the effects of prenatal alcohol exposure on the neonatal heart and how this may impact the cardiac extracellular matrix.

To evaluate potential mechanisms of altered fibroblast function in the neonatal heart, we selected Notch1 signaling for analysis. Notch1 is a transmembrane receptor that when activated, acts as a transcriptional regulator. Upon ligand binding to the extracellular receptor, proteolytic cleavage of the intracellular domain translocates to the nucleus [25]. Important to fetal cardiac development, both perivascular and myocardial fibroblasts have been shown to originate from epithelial-mesenchymal transition (EMT) [26, 27]. This process is regulated by the highly conserved Notch1 cell signaling pathway. Furthermore, Notch1 signaling is necessary for ventricular chamber development, and impairments in Notch1 signaling result in ventricular septal defects [28, 29]. Importantly, Notch1 has a demonstrated role in fibroblast-to-myofibroblast transition in pulmonary fibrosis and other pathological conditions such as cancer [29, 30]. Alcohol has been shown to alter Notch1 signaling in a tissue-specific manner. For example, alcohol feeding decreased Notch1

signaling in mouse colonic cells, but Notch1 signaling increased in skeletal muscle in response to chronic alcohol exposure [31, 32]. We previously reported increased Notch1 signaling in the pressure overloaded heart of adult male rats in response to chronic alcohol administration [33]. For this study, we sought to characterize the cardiac extracellular matrix of the neonatal heart after prenatal alcohol exposure. We hypothesize that prenatal alcohol exposure alters the cardiac collagen profile of the neonatal heart, ultimately producing cardiac dysfunction.

Materials and Methods

Prenatal Alcohol Exposure (PAE) Model

All experimental procedures were performed in the mouse strain C57Bl/6 from Envigo (Indianapolis, IN). All studies and animal handling conformed to the principles of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals 8th edition, and were approved by LSU Health Sciences Center's Institutional Animal Care and Use Committee. Adult male and female mice approximately 8 weeks of age were mated overnight. The breeding pairs were separated the next morning, and the presence of a mucosal vaginal plug was determined as gestation day 0.5. Pregnant mice were administered two intraperitoneal injections of 2.9 grams ethanol/kg maternal body weight of a 30% (volume/volume) ethanol solution in sterile saline on gestation days 6.75 and 7.25 (at 11:00 AM and 5 PM). Control mice were administered an equal volume of vehicle saline based upon body weight. The gestational period continued uninterrupted. After birth, cardiac function was analyzed in 5 day old pups using echocardiography. Whole hearts were then collected for fibroblast isolation or for tissue analysis.

Echocardiography

Echocardiography was performed in sedated 5 day old neonates (2% isoflurane) to assess left ventricular (LV) chamber dimension and function (VEVO 770, VisualSonics; Toronto, CA). LV short axis-view was used to obtain B-mode two-dimensional images and M-mode tracings. Using this method, LV chamber diameter, and posterior and anterior wall thickness were measured at both systole and diastole. These parameters were used to calculate ejection fraction, fractional shortening, and LV volumes using the VisualSonics software. There were no significant differences in heart rate between groups (average rate of 417±15 BPM).

Neonatal Cardiac Fibroblast Isolation

Whole hearts were obtained from saline control or PAE 5 day old neonates. Hearts were pooled from one litter (~ 6-8 per litter). Each litter of pups was designated as n of 1, and each experimental group had a sample size of n = 4-6. The atria were removed, and ventricles minced in a physiologic saline solution at 37°C. Ventricular tissue was then placed in 0.1% collagenase digestion buffer (Worthington, Type 4 LS004186) and stirred gently for 20 min. The solution containing the cells was removed, and a stop solution comprised of 20% FBS in Hanks Balanced Salt Solution was administered to deactivate enzymatic activity. Cells were pelleted using centrifugation at 2000 rpm for 8 minutes. The digestion/pelleting process was repeated 3 times per each pool of hearts. The cell pellets were resuspended using Dulbecco's Modified Eagle Medium with 10% FBS and 5% NBS

(Sigma, D5030). Resuspended cells were plated onto a T25 flask and placed into a 5% CO_2 incubator and grown to 90% confluence.

Quantitative Real-time PCR

Buffer RLT lysis buffer was used to lyse isolated cardiac fibroblasts (Qiagen #79216; Hilden, Germany). RNeasy Fibrous Tissue Kit was used to extract RNA from lysed fibroblasts. 1 μg of RNA from each sample was reverse transcribed into CDNA using iScript from Bio-Rad. qPCR was performed using SYBR green master mix (Bio-Rad #172-5121) with primers listed in Table 1(Integrated DNA Technologies, Inc.; Coralville, IA). Thermocyclying was performed using CFX96 Therymocycler (Bio-RAD), and data were analyzed using the ΔΔCT method. Gene expression for all genes was normalized to RPS13 values.

Western Blot Analysis

One randomly selected male neonate was chosen from each litter for Western blot analyses, which were performed as previously described[33]. Each male from a litter counted as sample size of 1. Western Blot analyses had sample sizes ranging from n = 3-6 for each group. Briefly, 15 mg of ventricular tissue for each sample was homogenized in RIPA buffer (#899900; Pierce Antibody Products; ThermoFisher Scientific; Waltham, MA) with HALT protease and phosphatase inhibitor cocktail (#78440; ThermoFisher Scientific). Protein concentration of homogenized tissue was assessed using Bradford assay. Proteins (50 μg) were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes (PVDF). Indicated dilutions of primary antibody were incubated with PVDF membranes overnight at 4° against collagen I (1:1000, Product #34710; Abcam; Cambridge, UK), collagen III (1:5000, Abcam #7778), LOX (1:1000, Abcam #31238), αsmooth muscle actin (1:1000, Abcam #5694), Aph1α (1:50, Abcam #12104), Hes1 (1:2000, Abcam #71559), Hey1 (1:100, Abcam #22614), and Notch1 (1:100, Abcam #52627). Histone H3 (1:5000, Abcam #1791) was used as a loading control. The membranes were incubated with secondary antibody (1:1000, Abcam #97051) for 1 hour at room temperature. Protein bands were visualized by exposure to Western ECL substrate kit (Clarity, Biorad; Hercules, CA) and images collected with ImageQuant LAS 4000 imager (GE Healthcare Life Sciences; Malborough, MA). Densitometry was performed using CareStream software. All densitized bands were normalized to histone H3 expression.

Statistics

Data acquisition and processing were performed for echocardiography (Vevo 770), Western Blot analyses (ImageQuant LAS 4000 and Carestream software), and real-time PCR (BioRad C1000 Thermocycler and CFX96 Real Time System). The data in this study are expressed as mean ± SEM. Comparisons in data between Saline animals and Prenatal Alcohol Exposed animals were compared by unpaired Student's *t*-test. P values of <0.05 were considered statistically significant. All data were processed and analyzed using Graphpad 5.0 (Prism, San Diego, CA).

Results

Morphological Measurements

Body weight was measured prior to sacrifice on neonatal day 5. Ventricular heart weight was also measured and then normalized to body weight (Fig 1). At neonatal day 5, body weight decreased significantly in the PAE group compared to Saline control (Fig 1A). Heart weight in the neonates decreased compared to Saline with a p value of 0.058 (Fig 1B). Heart weight was normalized to body weight for each animal, and the ratio decreased significantly in the PAE neonates (Fig 1C). Representative images of the ventricles from Saline and PAE animals are shown in Fig 1D. These images were taken from Saline and PAE animals that were approximately 3.3 grams in body weight. The ventricular tissue of the Saline and PAE neonates weighed approximately 18 and 13 milligrams, respectively.

PAE Increased Gene Expression of Profibrotic Markers in Isolated Cardiac Fibroblasts

Gene expression of profibrotic markers was assessed using qPCR in cardiac fibroblasts isolated from saline and PAE neonates (Fig 2). Collagen I mRNA (Col1a1) decreased in fibroblasts from PAE hearts vs. Saline (Fig 2A), whereas collagen III mRNA (Col3a1) increased (Fig 2B). These changes in collagen expression resulted in a significant reduction of Col1a1-to-Col3a1 ratio (Fig 2C). Collagen 1 confers more tensile strength to the ECM providing stiffness, whereas collagen III provides more compliance. A decrease in this ratio could be linked to a dilated phenotype in neonatal hearts [34]. Further, Acta2 (mRNA for α-smooth muscle actin) was used as a profibrotic marker of fibroblast-to-myofibroblast transition. PAE caused an increase in cardiac Acta2 compared to Saline (Fig 2D). Lastly, LOX mRNA was significantly increased in PAE hearts versus Saline (Fig 2E). LOX is a collagen crosslinking enzyme necessary for the maturation and deposition of collagen into the ECM and is associated with fibrosis.

PAE Increased Notch1 Signaling in Cardiac Fibroblasts

Notch1 signaling is crucial for cardiovascular development. Notch1 receptor and downstream transcription factor, Hey1, were assessed in isolated cardiac fibroblasts from PAE and Saline neonatal hearts (Fig 3). Both Notch1 receptor (Fig 3A) and HEY1 (Fig 3B) gene expression were significantly elevated in PAE cardiac fibroblasts. This increase in Notch1 signaling may indicate an increase in fibroblast-myofibroblast transition.

PAE Increased Profibrotic Markers in Whole Heart

Protein expression of profibrotic markers were assessed in ventricular heart tissue of Saline and PAE neonates (Fig 4). Similar to results using the isolated cardiac fibroblasts, collagen I protein expression decreased in PAE hearts compared to Saline controls (Fig 4A). Also, collagen III expression increased in response to PAE (Fig 4B). As in the isolated fibroblasts, these changes in protein expression produced a dramatic reduction in collagen I/III ratio in the PAE hearts versus Saline (Fig 4C). A decrease in the collagen I/III ratio indicates a more compliant phenotype in the PAE neonatal hearts. Alpha smooth muscle actin was significantly increased in PAE hearts potentially indicating fibroblasts transitioning into

myofibroblasts (Fig 4D). As in the isolated fibroblasts, LOX expression was increased in the PAE hearts (Fig. 4E).

PAE Caused Decreased Notch1 Signaling Components in Whole Heart

Notch1 receptor and its downstream effectors were assessed in whole heart tissue (Fig 5). Notch1 receptor protein expression was not significantly different between Saline and PAE (Fig 5A). Gamma secretase cleaves and activates the Notch intracellular component once the Notch receptor is activated. Aph1a, the active component in gamma secretase, was decreased in PAE hearts versus Saline (Fig 5B). In contrast to the increase found in isolated fibroblasts, the downstream transcription factor Hey1 was not significantly altered, but there was a decreasing trend ($p = 0.067$; Fig 5C). However, Hes1 transcription factor that is downstream of Notch1 activation was significantly decreased by PAE (Fig 5D). Although Notch1 activity was not determined, a decrease in the Notch1 signaling components would likely be associated with a decrease in Notch1 activity in the whole ventricular tissue. This is in contrast with our results on Notch1 signaling components in isolated cardiac fibroblasts, and may indicate a more complicated effect of PAE on Notch1 signaling in other cardiac cell types.

PAE caused Cardiac Dysfunction in Neonates

Diastolic function in 5 day old neonates was assessed using echocardiography; representative M-mode images are provided from Saline and PAE neonates (Fig 6). LV end diastolic diameter at diastole (LVIDd) was not different between Saline and PAE neonates (Fig 6A). LV posterior wall thickness at diastole (LVPWd) was reduced in response to PAE, indicating ventricular wall thinning in those pups (Fig 6B). Using these two parameters, eccentric index was calculated using the equation LVID/(2*LVPW). Eccentric index was significantly increased in PAE hearts versus Saline, which indicates a potential increase in wall stress in these hearts (Fig 6C). Systolic function in day 5 neonates was assessed using echocardiography. Ejection fraction (Fig 6D) and fractional shortening (Fig 6E) were significantly decreased in PAE neonates versus Saline.

Discussion

Fetal alcohol spectrum disorders (FASD) encompass the entire range of behavioral and physiological deficits, from mild to severe, that result from prenatal alcohol exposure. Fetal alcohol syndrome (FAS) describes the most severe condition on the spectrum [35]. Alcohol-induced congenital heart defects are present in half of children born with FAS [10]. Though many of these defects can be surgically repaired, the mechanisms by which prenatal alcohol exposure causes cardiac abnormalities remain unclear. Additionally, 80% of children born with FASD may have no overt physical symptoms and remain undiagnosed [36]. Although Hoyme et al. proposed a clinical approach to diagnosing the entire spectrum of FASD, practitioners only give a clinical diagnosis of FAS based on official guidelines [35]. The Journal of American Medical Association recently published a study with weighted prevalence of FASD affecting as many as 9% of school aged children in some U.S. communities [37]. Standard screening of newborns may not detect subtle cardiac changes such as ventricular wall thinning, changes in ECM composition, and ventricular compliance

[38, 39]. These more subtle cardiac phenotypes may lead to adverse cardiovascular outcomes later in life [13]. Here, we focused on how prenatal alcohol exposure changes the neonatal cardiac extracellular matrix (ECM) and how this may impact cardiac function.

Using a mouse model of PAE, ethanol was administered on gestation days 6.75 and 7.25. This is comparable to gestation day 18 in human development on the Carnegie scale for cardiac organogenesis [40–43]. Although no cardiac structure exists at this stage, progenitor cells comprising the cardiac heart fields have begun migration and differentiation [40]. Several studies have illustrated that the developing heart is most vulnerable to ethanol exposure during this period [12, 8, 44]. Further, at this early stage of pregnancy, a woman may be unaware that she is pregnant, which may increase her likelihood to inadvertently expose the fetus to alcohol, including heavy binge levels [4, 7]. The NIAAA defines binge alcohol levels as achieving a blood alcohol content (BAC) above the legal limit of 0.08 g/dL within 2 hours, or about 4 standard drinks for women within this time frame [45]. In our study, 2.9 g ethanol/kg of body weight was administered to achieve BAC of about 0.35. This represents a high binge level and previous studies indicate that this level of ethanol produces the most adverse cardiac changes while being still compatible with survival [12]. We found that PAE produced a decrease in both neonatal heart weight and body weight. Pups exposed to PAE had an overall decrease in heart weights even after normalization with their significantly lower body weights. Decreased body weight is a major characteristic of fetal alcohol exposure in humans. Decreased heart weight could indicate cardiac atrophy and thinning ventricular walls [46–48]. In particular, studies in adult rats subjected to chronic ethanol ingestion show a decrease in heart weight that was accompanied by thinner ventricular walls [49, 50]. Using a similar model of PAE, Serrano et. al found that PAE embryos had thinner ventricular walls and valve defects [12]. Our PAE neonates exhibited thinner ventricular walls than controls, which may result in increased wall stress. According to LaPlace's Law, wall stress is directly related to chamber diameter and inversely related to wall thickness [51, 52]. As such, a decrease in the thickness of the ventricular wall with no corresponding change in chamber diameter would indicate an increase in diastolic eccentric index and, thus, increased wall stress in these hearts. Although thinner ventricular walls are generally associated with a dilated phenotype, we did not find an increase in ventricular diameter by echocardiography [34]. Likewise, our previous study examining the effects of chronic ethanol exposure in adult rats with pressure overload indicate a similar decrease in relative wall thickness with no apparent ventricular dilation [33].

Wall thinning may be due in part to a change in the organization of the ECM. Cardiac fibroblasts are the primary regulators of the ECM, and play a key role in tissue architecture and homeostatic function [18]. As such, we assessed their gene expression of profibrotic markers in response to PAE. To verify whether the changes observed in the fibroblasts had physiological effects in the whole heart, cardiac tissue was also analyzed for protein expression of profibrotic markers. We observed that PAE decreased collagen I and increased collagen III mRNA levels in isolated cardiac fibroblasts. Consistent with these data, we found decreased protein expression of collagen Iand increased collagen III in whole heart tissue. Collagen I confers tensile strength, whereas collagen III gives the heart a more compliant phenotype. A decrease in the ratio of collagen I/III in the PAE hearts is consistent with the wall thinning that was observed, and suggests a more compliant ventricle in the

PAE neonates [34]. This change in cardiac tissue property may increase end diastolic volume, and impact the ability of the heart to adequately contract and eject volume. The changes in the collagen expression profile coincided with fibroblast transition into the active myofibroblast phenotype, which was marked by an increase α-smooth muscle actin gene (Acta2) and protein expression. Further, collagen must be crosslinked by the enzyme lysyl oxidase (LOX) in order to produce mature, insoluble interstitial fibers and be stabilized in the ECM. LOX is primarily secreted by fibroblasts, and we found concomitant increases in LOX expression in fibroblasts and whole heart tissue from PAE neonates. These collagen profile changes caused by PAE were associated with significant cardiac dysfunction.

To our knowledge, no studies have analyzed cardiac function in neonates after PAE. We found that PAE increased LV wall stress during diastole and decreased systolic function in 5-day-old neonates. The decreases in ejection fraction and fractional shortening found in PAE neonates are reflective of myocardial contractile dysfunction. In line with our findings, Wold et al. found decreased contractile function in ventricular papillary muscles isolated from rats aged to 10-12 weeks that had been subjected to PAE [53, 54]. Further, it has been observed that the mechanisms of alcoholic cardiomyopathy from chronic ethanol consumption in adults are similar to the mechanisms of cardiac dysfunction from prenatal alcohol exposure. For example, Ren et al. found that ethanol caused apoptosis in cardiac myocytes via induction of caspase-3 signaling [54]. Likewise, Capasso et. al also found that chronic ethanol ingestion decreased cardiac contraction in adult rats due to damage of myocytes as well as remodeling of the ventricular wall [49, 50]. Similarly, our own studies in adult rats exposed chronically to ethanol during pressure overload determined that relative wall thickness of the ventricles decreased along with a decrease in collagen I/III ratio [33]. Collectively, we interpret our data as PAE creating an altered collagen phenotype in neonatal mouse hearts ultimately resulting in cardiac dysfunction, as measured by eccentric index, ejection fraction, and fractional shortening. Although outside the scope of this study, the effects of PAE on cardiac myocyte contractility is worthy of investigation. These data would determine whether the depression in systolic function is caused by altered mechanotransduction due to a change in collagen phenotype or a reduction of cardiomyocyte contractility.

We next sought to assess potential dysregulation in cell signaling that may be responsible for fibroblast-to-myofibroblast transition due to PAE, which contributed to this cardiac phenotype. During development, Notch1 signals epithelial-to-mesenchymal transition (EMT) to produce the population of fibroblasts in the developing heart [30]. In adult models of disease, Notch1 has been shown to induce fibrosis in various tissues, including fibrotic repair in the heart after myocardial infarction [55, 56]. Additionally, in a model of bleomycin-induced lung injury, Notch1 signaling stimulated pulmonary fibrosis by inducing myofibroblast differentiation and promoting EMT [57]. Indeed, Zeisburg et al. demonstrated that endothelial to mesenchymal transition plays a role in cardiac fibrosis [58]. Further, Notch1 signaling is differentially regulated by ethanol exposure in various tissues. For example, ethanol has been shown to downregulate Notch1 receptors in human coronary smooth muscle cells, whereas it stimulates Notch1 signaling in human umbilical vein endothelial cells [59, 60]. Importantly, Sarmah et al. found that PAE zebrafish have increased Notch1 signaling throughout the entire ventricle [61]. Notch1 and its ligands are

both transmembrane proteins, so Notch1 is activated by cell-to-cell contact with its ligands, Jagged and Delta-like protein [62]. Upon activation, the Notch1 intracellular domain (NICD) is cleaved by gamma secretase. The NICD then translocates to the nucleus and binds to the CSL promotor to transcribe Fles and Fley transcription factors [63]. PAE resulted in upregulation of Notch1 receptor expression and the downstream Fleyl in neonatal fibroblasts. Notch/CSL interaction directly targets gene transcription of α-SMA [64]. The increases we found in Notch1 gene transcription in the isolated fibroblasts could indicate a mechanism by which PAE activates fibroblasts via transcription of α-SMA.

Taken together, prenatal alcohol exposure may increase Notch1 signaling in cardiac fibroblasts in the developing myocardium, and these increases persist into the neonatal period. The increase in Notch1 signaling suggests a mechanism by which α-SMA is transcribed and expressed in cardiac fibroblasts, creating a profibrotic myofibroblast phenotype in the neonatal heart. These alterations in the ECM contribute to a reduction in cardiac function. It is worth noting, however, that increases in Notch1 gene transcription were only found in the isolated cardiac fibroblasts. In contrast, Notch1 protein analysis in whole ventricular tissue did not change. Protein expression of downstream transcription markers and the gamma secretase decreased in PAE hearts, which contrasts with the upregulation in gene expression of Notch1 signaling components that was found in the isolated fibroblasts. This contrary finding represents a more complicated role of Notch1 signaling in the cardiac effects of prenatal alcohol exposure. Notch1 signaling proteins are expressed in multiple cardiac cell types aside from fibroblasts. Endothelial, myocardial, and endocardial cells also express Notch1, which may regulate cell growth and maturation [65]. Indeed, our study is limited by the lack of thorough investigation into the role of Notch1 signaling in other cardiac cell populations as well as assessing Notch1 ligands such as Jagged1 and Delta-like ligand. It is possible that prenatal alcohol exposure may increase Notch1 signaling in fibroblasts to produce a profibrotic phenotype, whereas Notch1 signaling in cardiomyocytes may be downregulated by alcohol and limit cell growth and maturation. Further, investigation into whether inhibition of Notch1 signaling in cardiac fibroblasts can prevent the adverse effects of prenatal alcohol exposure is needed.

To our knowledge, this is the first study to characterize the changes in cardiac fibroblasts and ECM after prenatal alcohol exposure, and how these changes impact viable neonates. Future studies are warranted to further examine the cellular mechanisms responsible and whether these changes in the neonatal heart contribute to susceptibility to cardiovascular diseases in adulthood.

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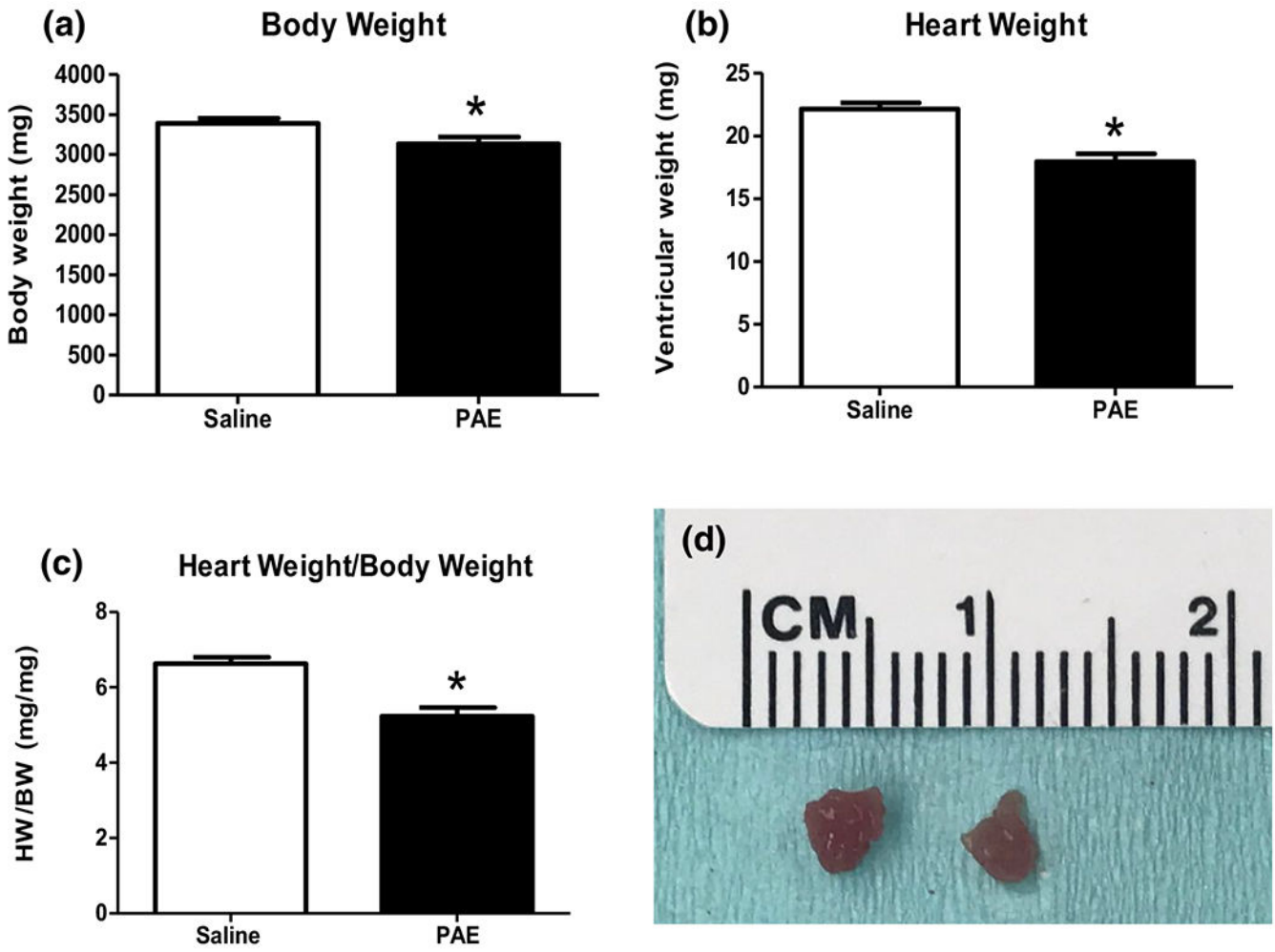

Figure 1:

Morphological measurements at neonatal day 5. A) Body and B) heart weight were significantly decreased in prenatal alcohol exposure (PAE) mice. C) Despite the decrease in body weight, PAE heart weight-to-body weight ratio was less than saline controls. D) Representative images of ventricles from Saline and PAE neonates. (*$p<0.05$ versus saline control; HW=heart weight; BW=body weight). Each litter of pups was designated as n of 1. N = 7-10 litters.

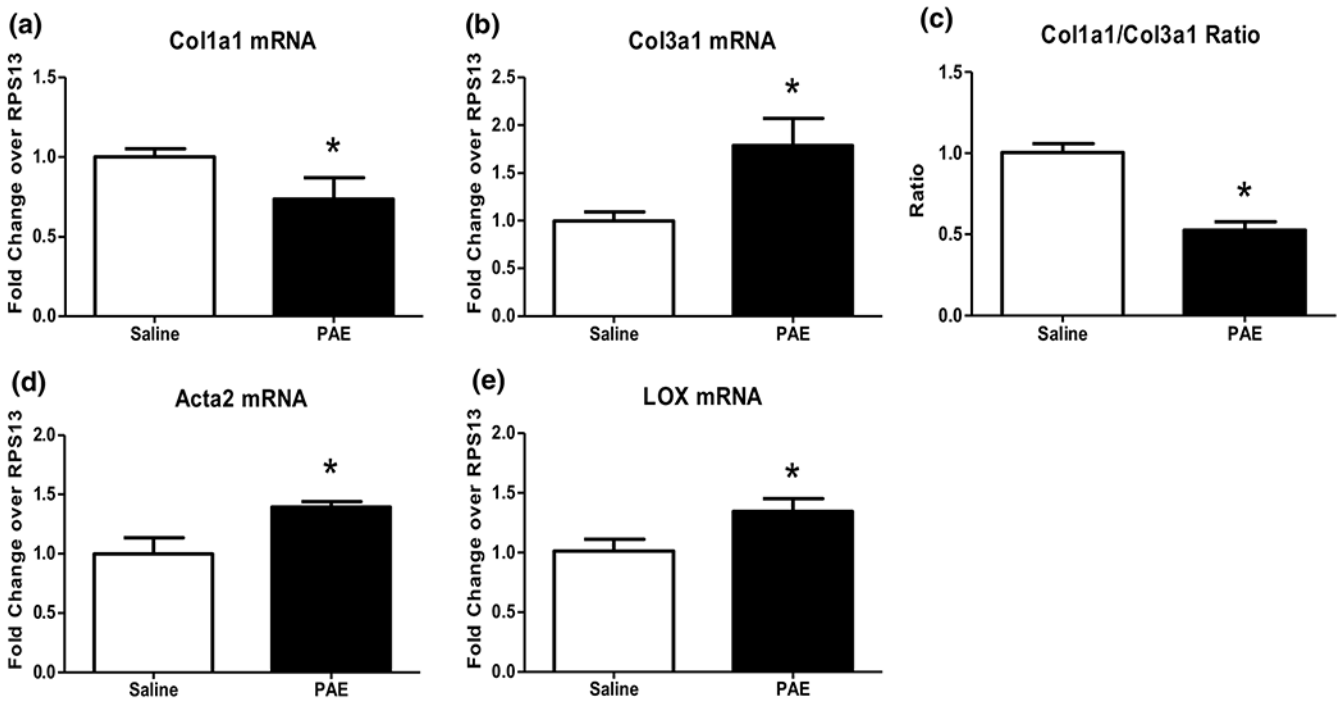

Figure 2:

Profibrotic markers in isolated primary neonatal cardiac fibroblasts. A) PAE decreased collagen I (Col1a1) gene expression, B) increased expression of collagen III (Col3a1), C) decreased the collagen I/III ratio, D) increased expression of α-smooth muscle actin (Acta2), and E) increased lysyl oxidase (LOX) mRNA expression (*$p<0.05$ versus saline control). Each litter of pups was designated as n of 1. N = 4-7.

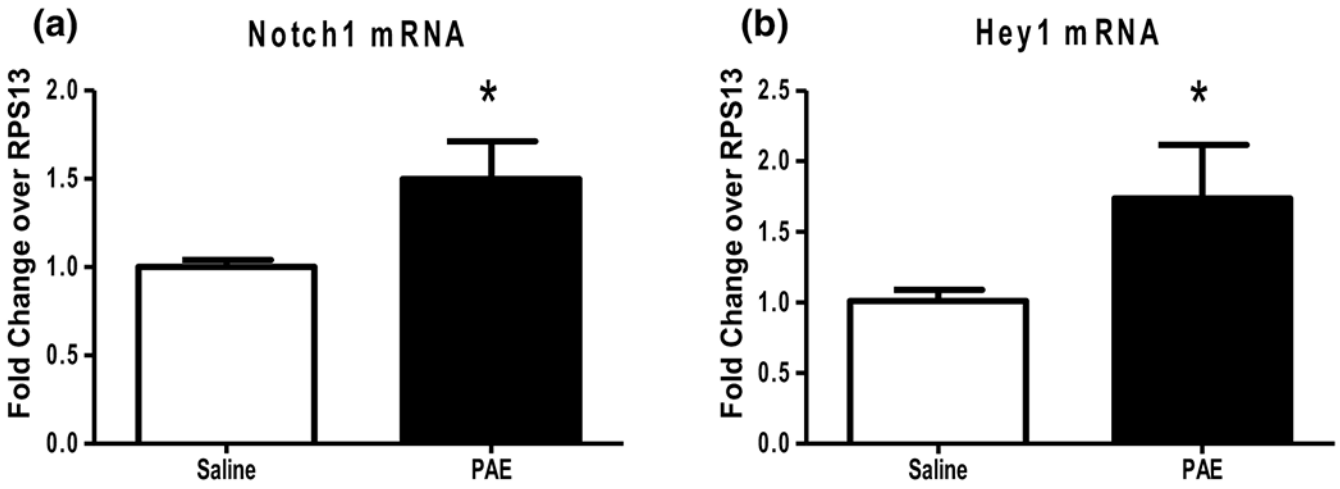

Figure 3:

Gene expression of Notch1 signaling in isolated fibroblasts. A) PAE increased Notch1 receptor (Notch1) gene expression and b) downstream Hey1 transcription factor. (*$p<0.05$ versus saline control). Each litter of pups was designated as n of 1. N = 6-7 litters.

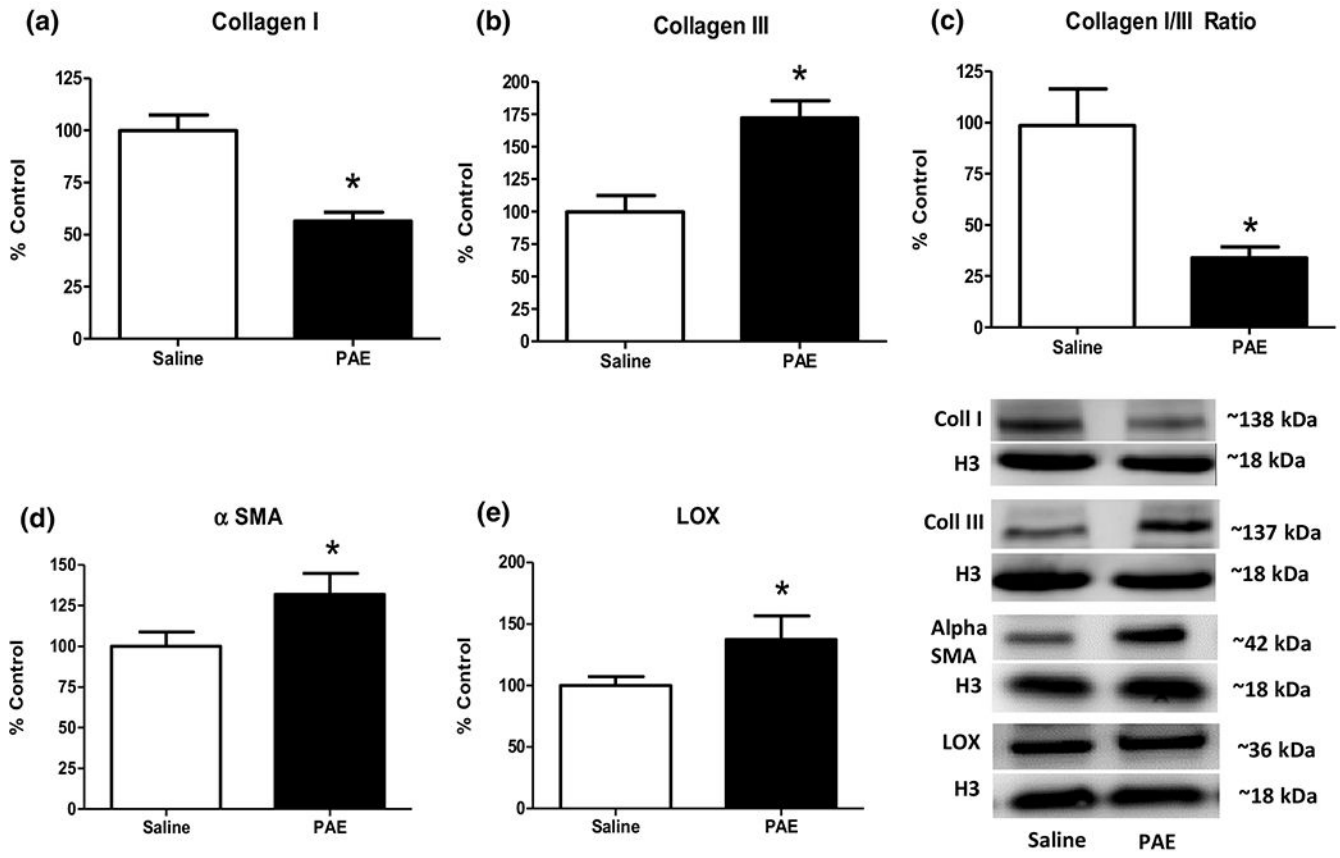

Figure 4:

Profibrotic markers in whole heart tissue. A) PAE decreased collagen I protein expression, B) increased collagen III expression, C) decreased collagen I/III ratio, D) increased α-smooth muscle actin (α SMA), and E) increased protein expression of lysyl oxidase (LOX) in whole heart tissue. (*$p<0.05$ versus saline control; H3 = Histone3 used as a loading control). One randomly selected male from each litter of mouse pups was designated as n of 1. N = 3-5.

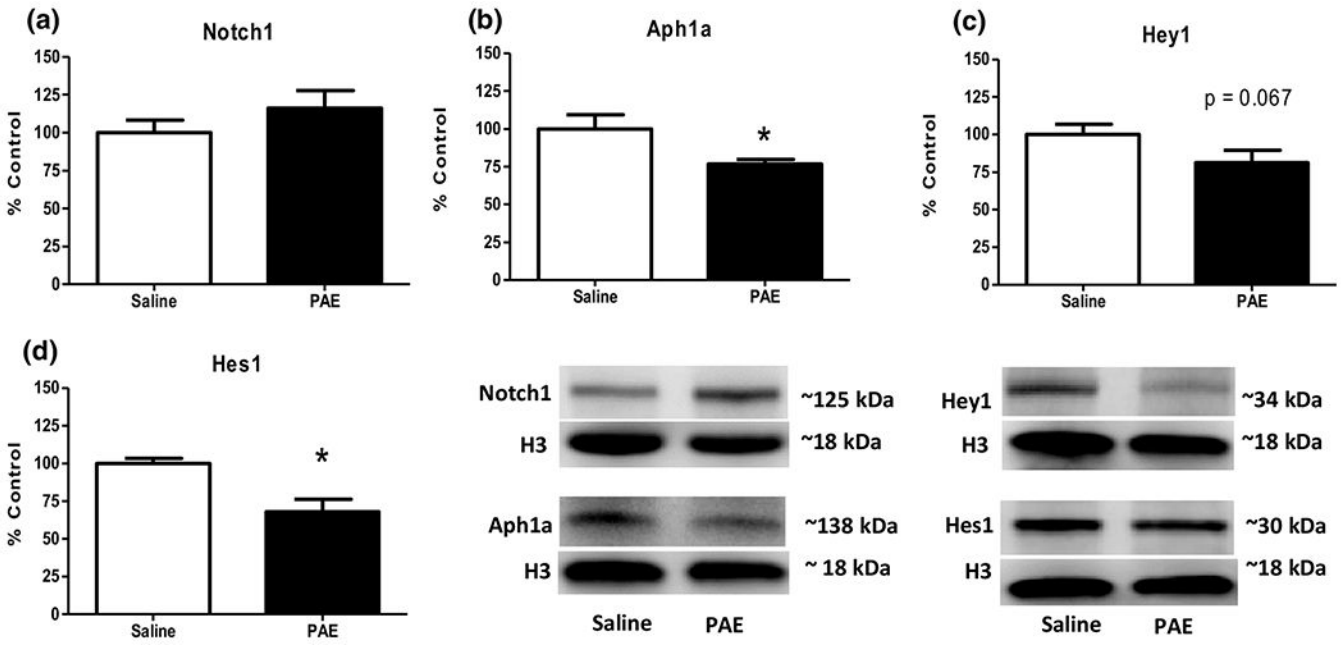

Figure 5:

Protein expression of Notch1 signaling components in whole heart tissue. A) There were no changes in Notch1 receptor expression between Saline and PAE whole heart tissue. B) PAE decreased protein expression of Aph1a, the active subunit of λ-secretase. λ-secretase cleaves the Notch1 intracellular domain upon activation. C) PAE decreased the downstream transcription factors, Hey1 and d) Hes1 . (*$p<0.05$ versus saline control). One randomly selected male from each litter of mouse pups was designated as n of 1. N = 3-4.

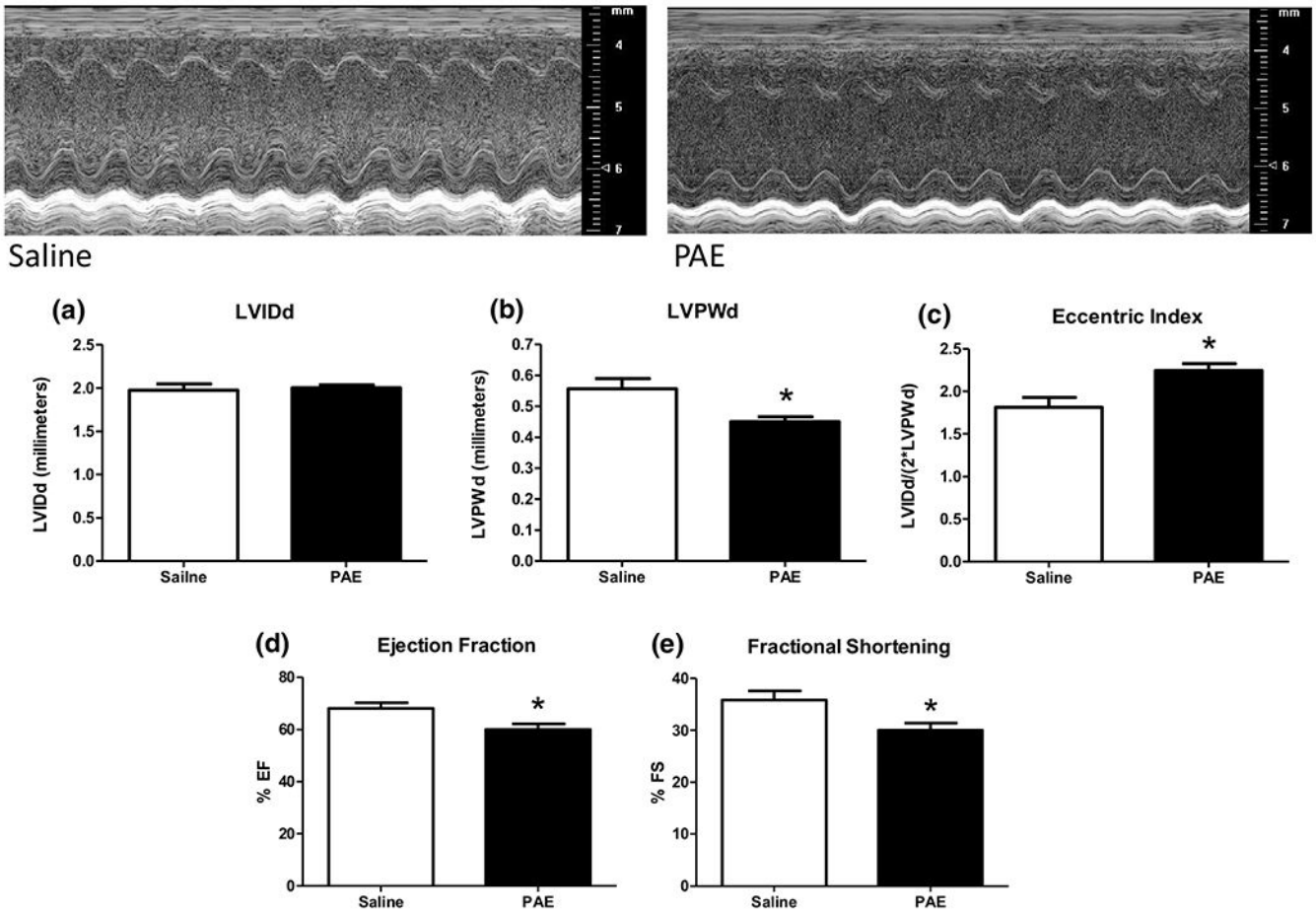

Figure 6:

Representative M-mode echocardiography images and cardiac function of Saline and PAE neonates. A) There were no changes in left ventricular interior diameter at diastole (LVIDd) between PAE and Saline controls. B) PAE decreased left ventricular posterior wall thickness at diastole (LVPWd), C) increased eccentric wall index, D) decreased ejection fraction, and E) decreased fractional shortening. (*$p<0.05$ versus saline control). One randomly selected male from each litter of mouse pups was designated as n of 1. N = 8-9.

Table 1.

Primers used in qPCR

Gene	Accession #	Forward (5′-3′)	Reverse (5′-3′)
COL1a1	NM_007742(1)	CATTGTGTATGCAGCTGACTTC	CGCAAAGAGTCTACATGTCTAGG
COL3a1	NM_009930(1)	TCTCTAGACTCATAGGACTGACC	TTCTTCTCACCCTTCTTCATCC
Acta2	NM_007392(1)	CTGTTATAGGTGGTTTCGTGGA	GAGCTACGAACTGCCTGAC
LOX	NM_010728(1)	CCAGGTACGGCTTTATCCAC	GACATTCGCTACACAGGACA
NOTCH1	NM_008714(1)	AGGATCAGTGGAGTTGTGC	CGTTACATGCAGCAGTTTCTG
Hey1	NM_010423(1)	ACTCCGATAGTCCATAGCCA	GTACCCAGTGCCTTTGAGAA