[Frontiers in Bioscience 11, 3014 -3025, September 1, 2006]

# Connexin 43 gene expression in mice with cardiopulmonary developmental defects

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## 1. ABSTRACT

Gap junctions are vital for cellular integrity, including homeostasis, morphogenesis, differentiation and growth in normal development of organs such as heart. Connexin 43 (Cx43) is a major gap junction protein. Our cDNA microarray analysis of normal and nitrofen-exposed neonatal mice with hypoplastic lungs, associated congenital diaphragmatic hernia (CDH) and heart developmental defects showed up-regulation of Cx43. Our objective was to establish if cardiopulmonary defects in nitrofen-exposed mice may be linked to altered expression of the Cx43 gene. We addressed our objective by performing northern blot analysis, real-time RT-PCR, immunoblotting and immunohistochemistry by localizing Cx43 in hearts and lungs of normal and nitrofen-exposed mice at different gestational stages. The data confirmed up-regulation of Cx43 expression in both hearts and lungs of CDH neonate mice and in lungs at other developmental stages except the pseudoglandular stage. However, Cx43 protein levels were either the same or less in hearts and lungs of nitrofen-exposed mice than in normal tissues except in pseudoglandular lungs. Different expressions of mRNA and protein suggest possible post-transcriptional or translational defects in Cx43. We observed dysmorphic hearts with exaggerated interventricular grooves and deep notches at the apex of the hearts in nitrofen-exposed fetal / neonatal mice; narrowed pulmonary out-flow and various degrees of craniofacial defects in 15-20% of the affected mice. Our data suggest a possible involvement of Cx43 in craniofacial, heart and lung defects in nitrofen-exposed mice. Such cardiopulmonary defects are also observed in human newborns with CDH. Thus, the murine data may help elucidate the pathways of cardiopulmonary defects in the human newborn condition.

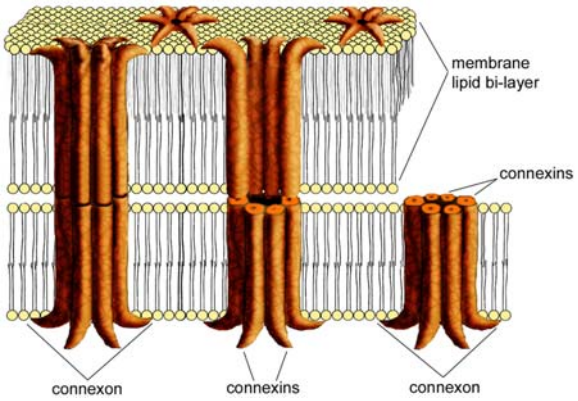


**Figure 1.** Schematic drawing of gap junction channels, where each channel is formed of two hemichannels, called connexons, on adjacent cells. Six connexins (protein subunits) form each connexon.

## 2. INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a common congenital condition in human newborns. The reported incidence of CDH is 1:2500. Pulmonary hypoplasia occurs to some degree in all patients with CDH. In CDH, visceral organs are displaced into the thoracic cavity, usually through a left-sided diaphragmatic defect. Survival rate is only 63% with conventional postnatal treatment (1). Surgery is not the immediate therapy for this condition as associated pulmonary hypoplasia and pulmonary hypertension are lethal factors after birth in affected newborns (2). If all babies are born with the same anatomy, then what is the "hidden mortality" that sentences some to death while others live (3)? Factors contributing to mortality include the degree of pulmonary hypoplasia and the presence of cardiac abnormalities (4).

We used our murine model of pulmonary hypoplasia and CDH (5) to better understand the molecular mechanisms involved in cardiopulmonary defects. Normal heart development necessitates the cellular adhesion and communication that gap junction channels provide. Gap junction channels are formed of two hemichannels called connexons on adjacent cells, each of which is formed by hexameric assembly of connexins, a multigene family of transmembrane protein units (Figure 1). Gap junctions are vital in cellular integrity and differentiation by transmitting ions, amino acids, and electrical activity between cells. In a recent cDNA microarray analysis of normal and hypoplastic murine neonatal lungs, we observed up-regulation of connexin 43 (Cx43) in murine hypoplastic lungs.

Cx43 is a major gap junctional protein, encoded by the gene *Gja1*. Cx43, a member of connexin multigene family has a wide and complex pattern of expression in fetal mouse development including organs originating from all three germ layers, such as epidermis, heart, lung, muscle, kidney and gut (6). Over- or under-expression of this gene results in heart malformations (7,8). In mice with Cx43 targeted mutagenesis, the absence of Cx43 was compatible with the survival of the murine embryos, but they died at birth due to failure of pulmonary gas exchange caused by a swelling and blockage of the right ventricular outflow tract (8).

Gap junction channels coordinate lung and cardiac functions, therefore disruption in its expression or function (ion and metabolite diffusions) might contribute to neonatal lethality of transgenic mice overexpressing Cx43. This lethality was attributed to pulmonary outflow tract obstruction (8,9). Our understanding of the significance of various connexins in vascular integrity (Cx37, Cx40), alveolar epithelial cell functions (Cx26, Cx32, Cx43) prompted us to explore the relationship between Cx43 expression and cardiopulmonary abnormalities found in our murine model of pulmonary hypoplasia.

We hypothesized that alteration in expression of Cx43 (over-expression or under-expression) may be responsible for cardiopulmonary defects observed in CDH mice, where the defects bear similarity to the human newborn condition. Therefore, understanding the significance of Cx43 in murine hearts and lungs may help identify the pathways involved in the cardiopulmonary defects observed in the human newborn condition.

## 3. MATERIALS AND METHODS

### 3.1. Animals

We created pulmonary hypoplasia with CDH in fetal mice by gavaging time-dated pregnant CD-1 mice (Charles River Laboratory, MA), where the effects of nitrofen are dose and day specific (5). We gavaged each pregnant mouse with 25mg nitrofen / 0.5ml olive oil, single dose on the later half of gestational day 8 (Gd8) to create the pulmonary hypoplasia and CDH (5). The dams were euthanized by halothane overdose on Gd13.5, Gd16.5, and Gd19 and the fetuses were delivered via laparotomy. The embryonic membranes were cleared and fetuses were placed in cold Hank's solution. Neonates were vaginally delivered and euthanized with an overdose of halothane. Animal care and usage were in accordance with guidelines of the Institutional Animal Care and Usage Committee International (IACUCI). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). We kept a photographic record of fetal and neonatal craniofacial abnormalities. Following which, a median sternotomy was performed under a dissecting stereomicroscope and the heart-lung units were excised from fetuses and neonates. A comparative morphological analysis of normal and nitrofen-exposed lungs at all stages was performed and documented photographically. Morphologic abnormalities, in the hearts and lungs were recorded.

Normal and nitrofen-exposed fetal/neonatal mice were selected from each developmental stage to assess pulmonary arterial outflow. Some hearts and lungs were excised, lightly blotted off to remove excess Hank's solution and processed for various morphologic and biochemical analyses, others were placed within

appropriately labeled microfuge tubes, snap frozen in nitrogen, and stored in -80$^{o}$C until later use in RNA extraction or protein determination.

### 3.2. Visualization of pulmonary arterial outflow

To visualize the pulmonary-arterial outflow in of normal and nitrofen-exposed mice, a sternotomy was performed and micro-quantities of India ink (black) were injected via a 30 gauge needle and tuberculin syringe into a few hearts '*in situ*' at Gd16.5, Gd19 and at neonatal stage. No specific criteria were used to select mice from any given group. Injections were placed slightly left of the apex at the basal tip of the right ventricle with the needle inserted superiorly. The pulmonary outflow tract was visualized and a photographic log was kept.

### 3.3. Light Microscopy

Normal and nitrofen-exposed Gd16.5 hearts, both with and without ink perfusion, and lungs were fixed in 10% buffered formaldehyde, and processed for routine histology. Five-micrometer thick serial coronal sections were cut and stained with Harris hematoxylin and counterstained with eosin.

### 3.4. cDNA microarray

The Mouse 1.2 arrays (CLONTECH Laboratories, Inc., Palo Alto, CA) were used to compare the expression of the altered genes in the neonatal lungs from normal and nitrofen-exposed mice. Two identically spotted membranes with array profiles about 1,200 genes with crucial cellular pathways and functions were used. Total RNA extracted from lungs were used for cDNA expression arrays, where cDNAs were labeled with $^{32}$P-dCTP to maximize the sensitivity and allow detection of the low abundance transcripts. Both identically prepared membranes were hybridized with labeled probe prepared either with normal or hypoplastic lung cDNA.

The data were analyzed using AtlasImage™ Software. A differential gene expression ratio of 1.5 times was considered significant, when observed in more than one experiment. The difference in expression of genes greater than the threshold, but less than 1.5 times the cutoff value was taken as a weak signal or low trust data and were not pursued for further analysis. The data array analyses were obtained as excel sheets, phosphoimager data showing the actual differences in the signals, pie charts and pseudocolor image of differential expression data.

### 3.5. Extraction of Total RNA and Northern Blotting

Isolation and purification of total RNA from lung and heart tissues was done (Qiagen RNeasy Midi Kit, QIAGEN Inc., Valencia, CA). Each RNA sample was prepared by pooling normal or nitrofen-exposed tissues at specific time-points: Gd13.5 – 8-12 lungs; 9 hearts; Gd16.5 – 2 lungs; 3 hearts; Gd19 – 1 lung; 2 hearts; neonate – 1 lung; 2 hearts, where pooling was necessary to extract appropriate yield of RNA. A total of n=5 samples were used for Gd13.5 lung tissue; whereas a total of n=10 samples were used for Gd16.5, Gd19, and neonate lung tissue. At each time point, a total n=5 samples were used for heart tissue. The RNA was quantified and twenty micrograms of total RNA from normal and nitrofen-exposed lungs were electrophoresed and transferred onto nitrocellulose membrane, hybridized with $^{32}$P dCTP-labeled probes for Cx43 cDNA (6), phosphorImaged and the relative abundance of mRNA signals was quantified. Each membrane with heart or lung RNAs was respectively hybridized to GAPDH cDNA. The intensity of signals from hybridization to Cx43 cDNA were normalized to those obtained by hybridization to GAPDH cDNA.

### 3.6. Preparation of $^{32}$p probes

Radiolabeled cDNA probes for 1.3-kb fragment of Cx43 and 1.4-kb PstI fragment of rat glyceraldehydes 3-phosphate dehydrogenase (GAPDH) were prepared using multiprime DNA labeling systems (Amersham, NJ), for each probe, the specific activity was calculated and greater than 1 x $10^6$ cpm/mL was used for hybridizations as described previously (10).

### 3.7. Quantitative Real Time RT-PCR

To confirm our microarray results for Cx43 and northern blot analysis, we performed quantitative real-time RT-PCR in hearts and lungs at different developmental stages. Aliquots of 10 µg of total RNA were taken from each heart and lung sample and processed for RT-PCR using methods described earlier (11). Tissues were obtained from two different experimental set ups (n=2) and the samples were run in triplicates. Using the sequence data from Fishman *et al.* (12) (GenBank no. M65188) and Primer Express (version 1.0, Perkin-Elmer), a real-time RT-PCR probe/primer design software that optimizes sequences used in real-time RT-PCR sequences for human Cx43 cDNA probes and primers were designed (11): 5'-TCT CAC CTA TGT CTC CTC CTG GGT ACA A-3' for the probe, 5'-GCT CCT CAC CAA CCG CT-3' for the forward primer, and 5'-TTG CGG CAG GAG GAA TTG-3' for the reverse primer. The real-time RT-PCR data were obtained from n=3 and normalized to 18S mRNA as described earlier (11). Steady state Cx43 mRNA levels were measured by real time RT-PCR using a Perkin-Elmer ABI Prism 7700 sequence detection system.

### 3.8. Western blotting

Lung and heart tissues of normal and nitrofen-exposed mice at each time point were homogenized individually on ice in PBS (pH 7.5) with protease inhibitors (aprotinin, 1 mg/mL; antipain, 2mg/mL; and leupeptin, 2 mg/mL) (13). Total protein concentrations in the tissues were assessed by using a modification of the Bradford protein micro-assay (13-15). Using a 12% SDS polyacrylamide gel, 30 micrograms of total protein from lungs and heart from each developmental time-point were separated electrophoretically, electroblotted to PVDF membrane (Millipore, Bedford, MA) overnight and blocked with 5% milk for one hour. Rabbit anti-Connexin 43 primary antibody diluted 1:125 (Zymed Laboratories, Inc., San Francisco, CA) and horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Amersham, NJ) diluted 1:2000 were used. The antigen-antibody complexes were detected on the membranes, using an enhanced chemiluminescence (ECL) kit for western blot

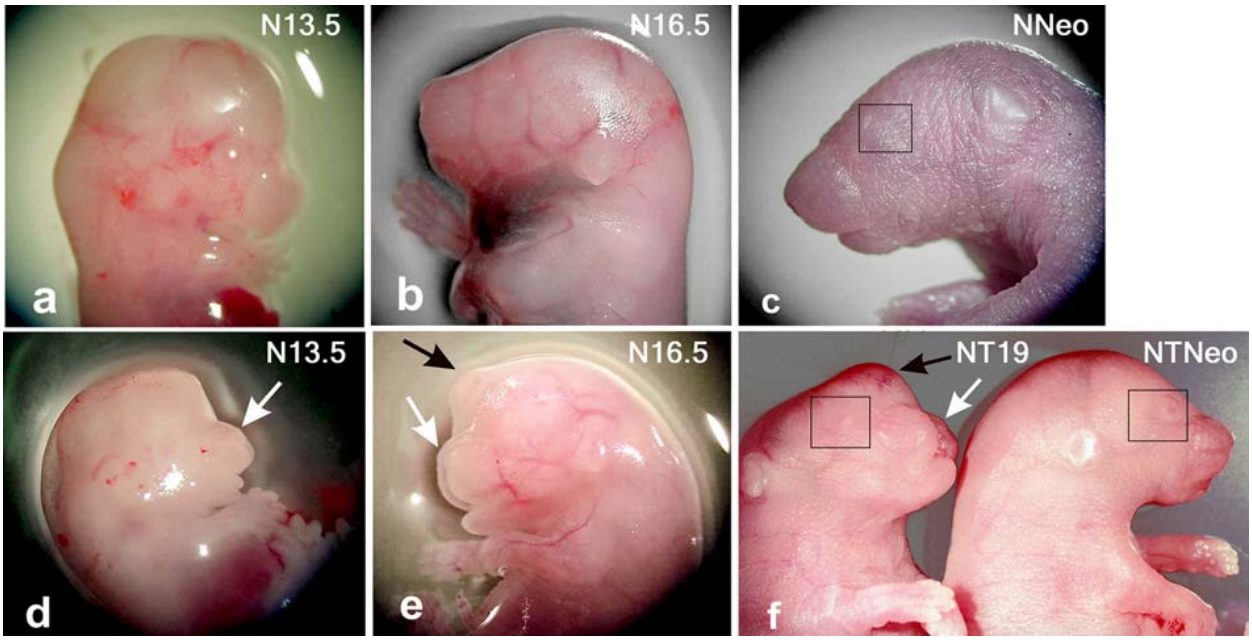


**Figure 2.** Representative photographs of N and NT fetuses and neonates (a) N Gd13.5 (x11.25); (b) N Gd16.5 (x7.5); (c) N neonate (x9.375); (d) NT Gd13.5 (x11.25); (e) NT Gd16.5 (x7.5); (f) NT Gd19 (left) and NT neonate (right) (x7.5, each). NT fetuses and neonates [(d), (e), (f)] demonstrated a smaller head to body size ratio and a reduced snout to ear distance compared to N counterparts [(a), (b), (c)]. Craniofacial abnormalities found in NT animals included mid-facial cleft [white arrows in (d), (e), and (f)], absence of eyes [presence and absence of eyes are indicated by the squares outlining the areas in (c) and (f)], and occasional cranial bulging [black arrows in (e) and (f)]. We related the craniofacial defects of affected mice with severely hypoplastic lungs and coexistent diaphragmatic hernia. N=normal and NT= nitrofen-exposed.

detection (Amersham, NJ), and exposed to Kodak film (X-OMAT). The relative abundance of Cx43 protein signals was quantified on a 100A Molecular Dynamics densitometer using the Protein Data Basis Information Software for obtaining mean optical density.

### 3.9. Immunohistochemistry

Immunohistochemical staining for Cx43 was performed in neonatal hearts and lungs from normal and nitrofen-exposed tissues with Cx43 primary antibody (1:100 dilution; Zymed Laboratories, Inc., San Francisco, CA) and biotinylated anti-rabbit secondary antibody (1:500 dilution; Vector Laboratories, Burlingame, CA) using methods published by us earlier (16-17).

### 3.10. Statistical analysis

A total of six pregnant dams were used for each normal and nitrofen-exposed time point, i.e., four time-points each in normal and nitrofen-exposed animals x ten different experiments = 80 pregnant animals. Each dam had a litter of 10 to 14 fetuses/pups. A morphometric record of craniofacial defects was kept from each litter and the pulmonary arterial flow was checked randomly in at least two hearts at each time-point from each litter. RNA extraction and northern blot analysis were n=5 for heart tissue normal and nitrofen-exposed mice at the four gestational time points; for Gd13.5 lung tissues n=5 and for other developmental stages n=10. RNA extraction and northern blot analysis were n=10; real-time RT-PCR n=3 and immunoblotting as well as immunohistochemistry n=4. ANOVA was used to compare the data among the normal and nitrofen-exposed hypoplastic lungs. When a difference was found, Dunnett's procedure for multiple comparisons was applied with statistical significance set at $p<0.05$.

## 4. RESULTS

### 4.1. Mice with craniofacial defects

Nitrofen-exposed (NT) fetal / neonatal mice were smaller in stature with decreased weight and spongy bodies (5) compared to the normal (N) mice of equivalent age. At birth, most nitrofen-exposed animals were cyanotic and visibly in respiratory distress, had fragile, pale, wrinkled skin. Fifteen to 20% of these animals demonstrated a smaller head to body size ratio than untreated normal animals (Figure 2). Nitrofen-exposed animals had craniofacial defects including mid-facial cleft, reduced snout to ear distance, absence of eyes and occasional cranial bulging. The craniofacial defects were observed in affected mice with severely hypoplastic lungs and coexistent diaphragmatic hernia, and were not observed in the mice with less severe pulmonary hypoplasia and no diaphragmatic hernia.

### 4.2. Lung Morphology

All lungs from nitrofen-exposed mice at different developmental stages were hypoplastic with more than 70% showing severe hypoplasia of the left lung. When

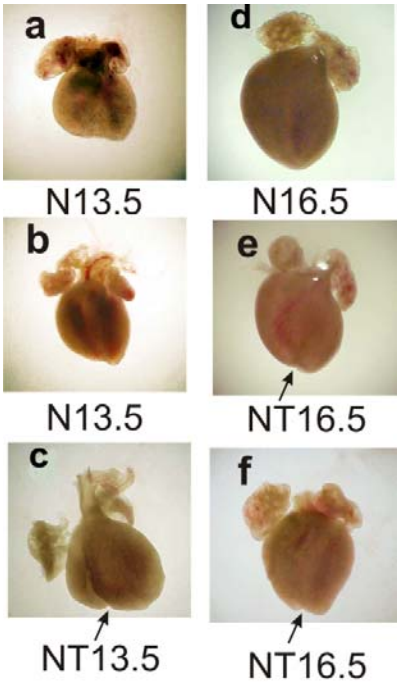


**Figure 3.** Representative pictures of fetal hearts: (a) N Gd13.5 (x18.75); (b) N Gd13.5 (x18.75); (c) NT Gd13.5 (x18.75); (d) N Gd16.5 (x15); (e) and (f) NT Gd16.5 (x15, each). Overall, NT hearts, as compared to N hearts, had greater width and baggier appearance. NT hearts demonstrate an exaggerated interventricular groove overlaying the ventricular septum that extends to the apex of the heart as a deep notch (indicated by arrow), possibly indicating incomplete formation or delayed development of the heart. N=normal and NT= nitrofen-exposed.

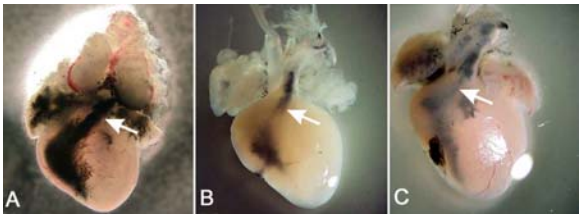


**Figure 4.** Representative pictures of normal (a) and nitrofen-exposed [(b) and (c)] Gd16.5 fetal hearts. Significant narrowing of the pulmonary outflow tracts visualized by India ink injection in (b) and (c) compared to (a) (white arrows). (x15 for all).

compared to normal, unexposed lungs. Nitrofen-exposure results in time and dose dependent laterality of the defects (5). The right lung showed slight general hypoplasia and in mice with less severe left hypoplasia, the size of the right lung was comparable to the normal, unexposed lungs. [Published earlier, (5,17)].

**4.3. Heart Morphology**

Hearts from nitrofen-exposed mice demonstrated characteristic phenotypic abnormalities similar to those observed by others in Cx43 over-expressing transgenic mice. As shown in Figure 3, hearts from nitrofen-exposed mice had an exaggerated interventricular groove overlaying the ventricular septum that extended to the apex of the heart as a deep notch, possibly indicating incomplete formation or delayed development of the heart.

Overall, nitrofen-exposed hearts were dysmorphic with a baggier appearance and increased width relative to normal hearts. India ink injection enabled us to visualize the pulmonary outflow tracts. Figure 4 shows, the narrowing of these tracts in hearts of nitrofen-exposed mice compared to normals. All affected mice had the narrowed outflow tracts, with variability in severity of defect in different mice from same litter or different litters.

**4.4. Heart Histology**

Untreated, normal hearts were larger in size. Histologically, the normal hearts had more cellularity compared to the hearts from nitrofen-exposed mice. More red blood cells were visible within the tissue of normal hearts, between the myocardial cells, most evident in the ventricular septum area. The left chambers of the hearts from nitrofen-exposed mice were smaller and the septum not as thick or heavily vascularized as in normal hearts (pictures not shown). We have previously demonstrated the histological differences in lungs from normal and nitrofen-exposed mice (16-18).

**4.5. Identification of altered gene expressions by cDNA microarray**

The Clontech membrane microarray-mouse 1.2 array analysis of the neonatal lungs from normal and nitrofen-exposed showed altered expression of several genes (Figures 5 and 6). Among these genes, differences in the expressions of members of the connexin family were noted. Cx43, gap junction alpha protein gene (*Gja; Genbank access #M63801*) was up-regulated in hypoplastic lungs by 1.5 to 2 fold in two different experiments (arrows in the second row in Figure 5). Cx32 (*Gjb1, Genbank access #M63802*), Cx26 (*Gjb2, Genbank access #M63803*), Cx46 (*Gja3; Genbank access #U44955*), Cx50 (*Gja8; Genbank access #M91243*) had weak signals and therefore were considered low trust and not pursued. Cx37gene expression (*Gja4; Genbank access #X5797;* the circles in Figure 5) and Cx40 gene expression (*Gja5; Genbank access #X61675*), which are markers for endothelial cells (although Cx37 is also expressed in oocytes and neural / glial cells and Cx40 is highly expressed in arterial myocytes), were down-regulated by about 1.5 to 2.0 fold in hypoplastic lungs compared to the normal lungs suggesting presence of immature endothelial cells or fewer endothelial cells. (This work is discussed elsewhere in a separate manuscript). Because over- or under-expression of Cx43 is related to the presence of heart defects, we decided to focus on Cx43 in our study.

**4.6. Cx43 mRNA expression**

Analysis of Cx43 mRNA demonstrated similar levels in hearts of nitrofen-exposed mice compared to normal hearts at all prenatal stages of development; however, Cx43 up-regulation was only significant at the neonatal stage ($p<0.05$), as shown in Figure 7. Cx43

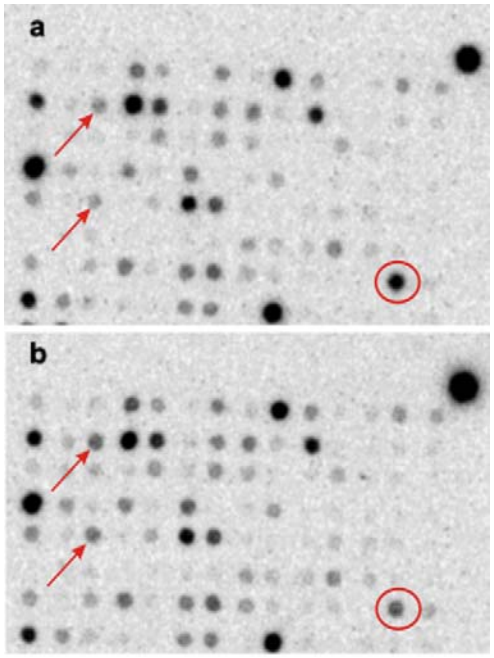


**Figure 5.** A representative Mouse 1.2 array (Clontech) analysis: up-regulation of Cx43 gene expression in neonatal hypoplastic lungs and a down-regulation of Cx37 gene expression. Blots: (a) Normal lung; (b) Nitrofen-exposed hypoplastic lung.

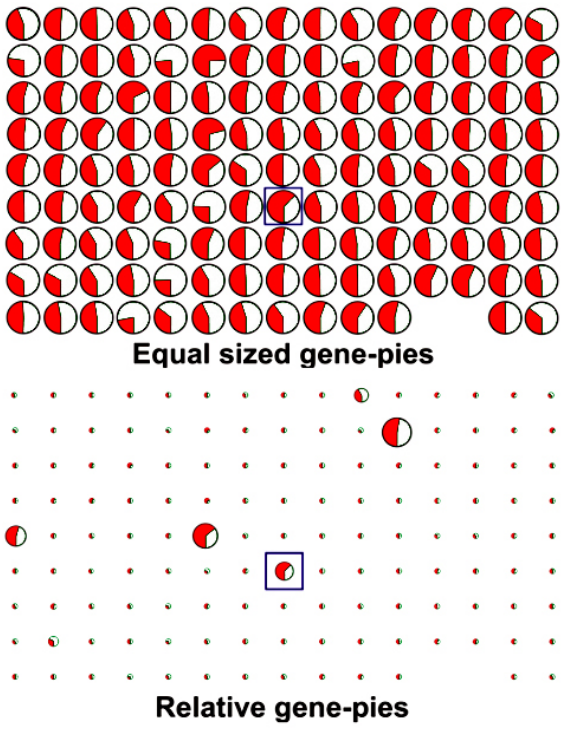


**Figure 6.** The differential gene expression in normal (white) and nitrofen-exposed (red) neonatal lungs shown through the equal-sized gene pie charts. The one boxed in represents the expression of Cx43.

mRNA at different stages of development of lung showed a gradual increase of Cx43 in both normal and nitrofen-exposed lungs (Figure 8). Where, Cx43 expression in lung tissue was significantly up-regulated by nitrofen-exposure compared to normal lungs of equivalent age, at all stages of development tested in this study ($P<0.05$), except at day 13.5 pseudoglandular stage. The data were normalized to GAPDH expression and the bar graphs present mean ratios of Cx43/GAPDH in the respective tissues.

### 4.7. Quantitative Real time RT-PCR for Cx43

Real-time RT-PCR indicating steady state Cx43 mRNA levels further confirmed the results of northern blot analysis of Cx43 mRNA expression. Nitrofen-exposed hearts and lungs at Gd16.5, Gd19 and neonate showed up-regulation in Cx43 steady state levels, which were more predominant than those seen in northern blot analysis. However, only n=2 (run in triplicates) was performed for real-time RT-PCR. Therefore, the data were confirmatory, but statistically not significant. No changes were noted at Gd13.5.

### 4.8. Cx43 protein levels

Since up-regulation of Cx43 mRNA expression/steady state levels were confirmed by cDNA microarrays, northern blot analysis and real-time RT-PCR, we further determined Cx43 protein levels by immunoblotting lung and heart homogenates of normal and nitrofen-exposed mice. Despite the higher levels of Cx43 mRNA in hearts of nitrofen-exposed mice, the phosphorylated as well as the non-phosphorylated (NP) forms of Cx43 protein showed similar pattern of signal densities in normal and nitrofen-exposed hearts and lungs. We present signal densities of the phosphorylated form of Cx43, as these signals were stronger than the NP form. Cx43 protein was similar in hearts of normal and nitrofen-exposed mice at equivalent developmental stages, except in neonatal hearts, where Cx43 protein was significantly lower in the hearts of nitrofen-exposed mice ($p<0.05$) (Figure 9). Furthermore, in early developmental stages of lung, Cx43 protein was significantly higher in hypoplastic lungs than normal lungs ($p<0.05$) (Figure 10); it was down-regulated in hypoplastic lungs during canalicular and saccular stages ($p<0.05$) and was comparable in both at birth (Figure 10).

### 4.9. Immunohistochemical localization of Cx43

Immunohistochemical localization of Cx43 protein in neonatal lungs (Figure 11a, b) and hearts (Figure 11c, d) from normal and nitrofen-exposed mice revealed its distinct localization in normal lungs and hearts (dark brown staining). It was localized in type II cells with no significant difference in the intensity or localization between normal and hypoplastic lungs. However, abnormal distal structure of hypoplastic lungs was visible. In normal heart, Cx43 localization was strong and punctate, whereas in nitrofen-exposed heart it was diffuse. The localization intensity of Cx43 protein in normal and nitrofen-exposed neonatal lungs and hearts was supportive of Cx43 protein levels observed through immunoblotting.

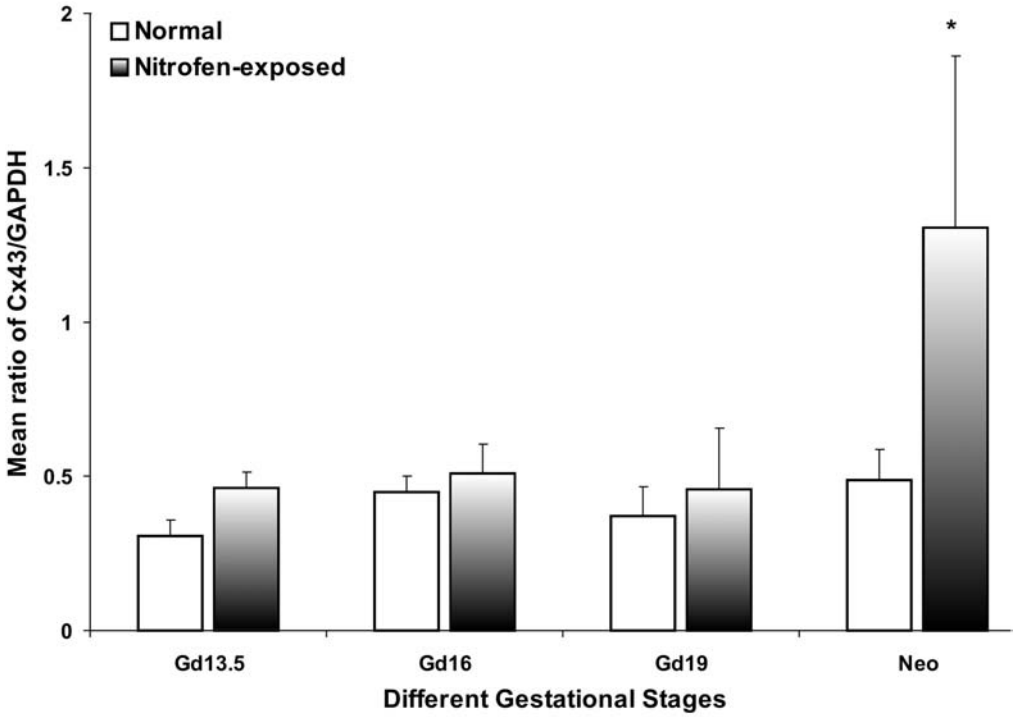


**Figure 7.** Cx43 mRNA in heart tissue of normal and nitrofen-exposed mice normalized to GAPDH expression, presented as mean ratio of Cx43/GAPDH, show up-regulation of Cx43 in hearts of nitrofen-exposed mice compared to normal hearts at all stages of development; however, it was only significant in the neonatal hearts.

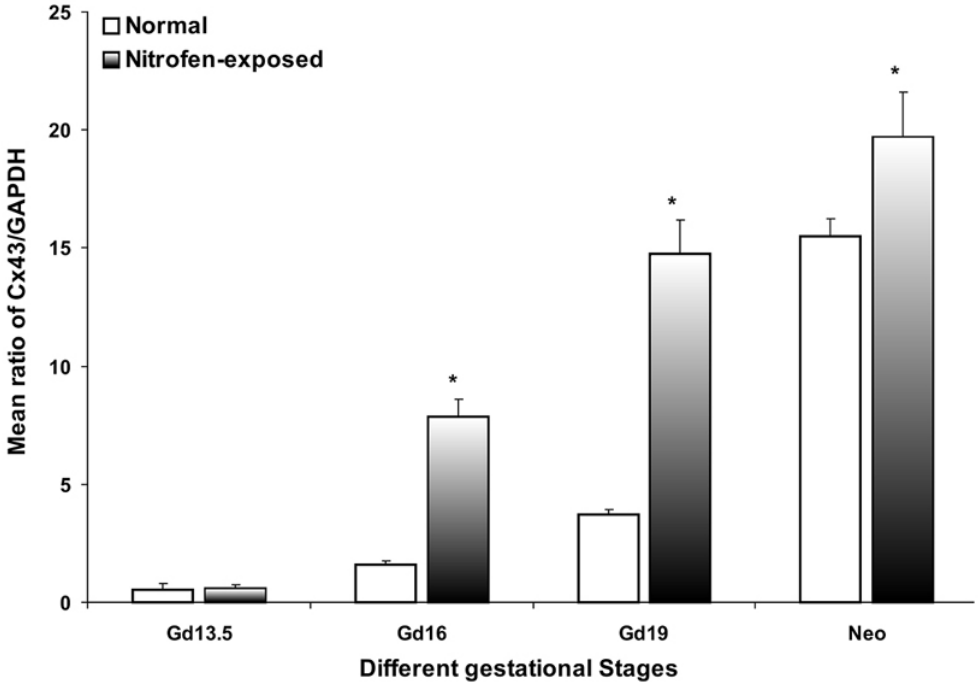


**Figure 8.** Cx43 mRNA in lung tissue of normal and nitrofen-exposed mice normalized to GAPDH expression, presented as mean ratio of Cx43/GAPDH, show significant Cx43 up-regulation in nitrofen-exposed lungs compared to normal lungs at Gd16, Gd19 and neonatal stage.

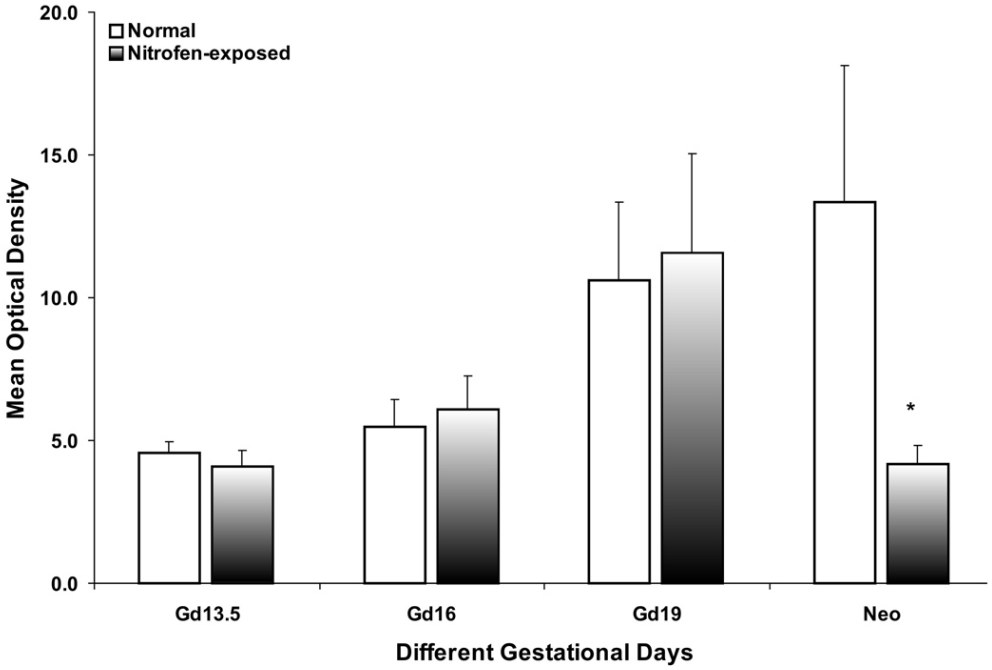


**Figure 9.** The graph represents Cx43 protein levels in hearts of normal and nitrofen-exposed mice. Unlike Cx43 mRNA, Cx43 protein was not different in normal and exposed hearts at equivalent developmental stages, except in neonatal hearts, where Cx43 was significantly lower in the hearts of nitrofen-exposed mice.

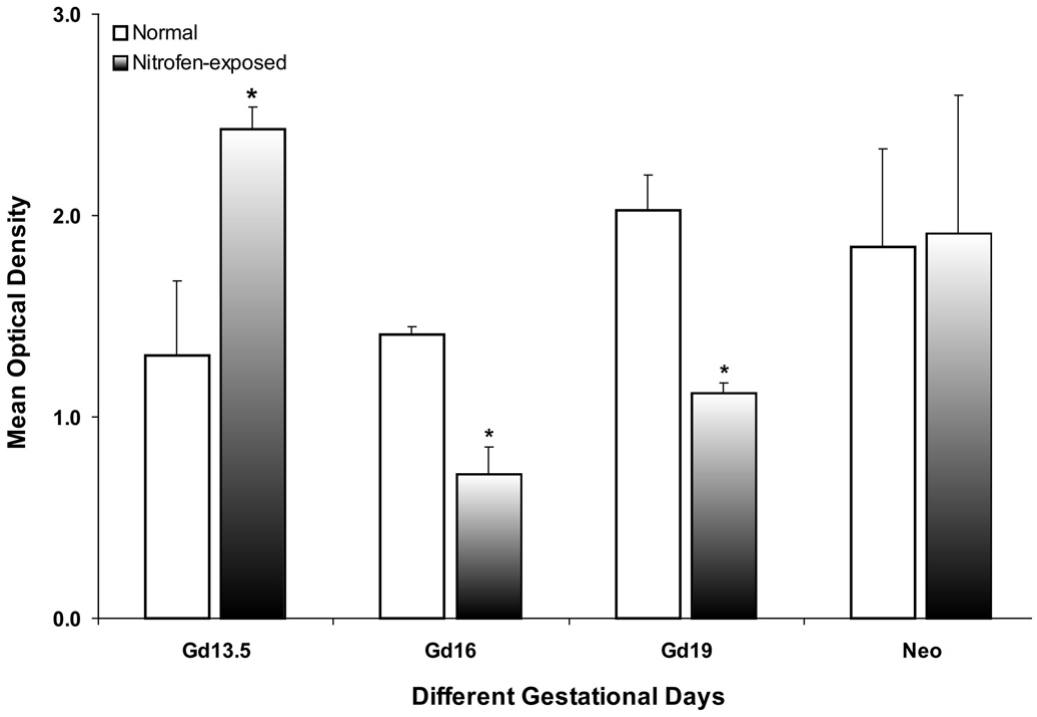


**Figure 10.** The graph represents Cx43 protein levels in normal and nitrofen-exposed lungs. Cx43 protein was significantly higher in early developmental stages in hypoplastic lungs than normal lungs and it then reduced during canalicular and saccular stages. At birth the Cx43 protein was comparable in normal and hypoplastic lungs.

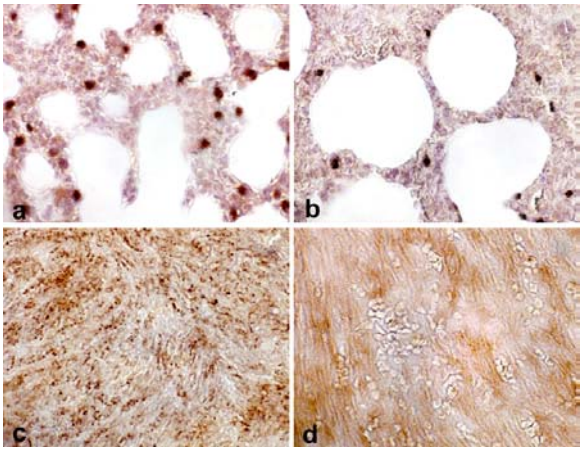


**Figure 11.** Immunohistochemical localization of Cx43 in normal and nitrofen-exposed lungs (a,b) and hearts (c,d) reveals that the normal tissues (a,c) have distinct localization of Cx43. In lungs it is localized in the type II cells and the normal lungs (a) have significantly greater localization compared to hypoplastic lungs (b). The normal hearts (c) had punctate staining of Cx43 compared to the diffused staining seen in hearts of nitrofen-exposed mice (d).

## 5. DISCUSSION

### 5.1. Connexins: Cell Growth and Differentiation

Connexins are a multigene family with more than 20 different homologous sequences identified in the mouse genome. Seven of which lack introns in the coding sequence (19).

Throughout embryogenesis gap junctions are expressed and they are vital to cellular functions, including homeostasis, patterning, cell differentiation, morphogenesis and growth control (20-23). Junctional permeability regulates cellular growth, and growth limitation, via a negative feed-back loop. Modulation of gap junctional communication can be achieved by alterations in transcription, translation, stability, post-translational processing (especially phosphorylation), gating, and insertion or removal from the plasma membrane (24), where reduction and alterations in the levels or types of connexins may be correlated with tumor progression and metastasis (24-26).

### 5.2. Craniofacial Developmental Defects

We have observed that about 15 to 20% of mice with CDH and pulmonary hypoplasia expressed various degrees of craniofacial defects, including blunted snouts and absence of eyes. Lacanda *et al.* (27) has shown that in Cx43-null mice genetic deficiency of Cx43 affects skeletal development *in vivo*. They have retarded ossification of the clavicles, ribs, vertebrae and limbs, thus demonstrating that the observed skeletal defects were not restricted to neural crest defects. They suggested that lack of Cx43 led to delayed mineralization and skull abnormalities, indicating the significance of Cx43 mediated cell-to-cell signaling in craniofacial development, osteogenesis and osteoblastic functions.

Literature indicates that craniofacial defects may be due to altered expression of the glucocorticoid receptors (GR) and/or vitamin A deficiency. Cx43 is downstream of steroid-thyroid-retinoid nuclear receptor superfamily. We have recently shown that this superfamily is down-regulated in murine hypoplastic lungs (15) leading to reduced number of alveoli and abnormal alveolar structure in hypoplastic lungs, which may be attributed to down-regulated retinoic acid receptors (RARs) (15-18). About 25% of human newborns have craniofacial defects, which include cleft palate, cleft lip, choanal atresia, micrognathia and microstomia.

### 5.3. Cardiac Developmental Defects

Cardiac defects have been observed in human newborns with CDH and also in rat model of CDH. We found similarities between our murine studies and one of the rare reported studies of eight infants with left sided CDH: a decreased cardiac mass due to hypoplasia of the left atrium and ventricle and interventricular septum may be due to the compression of mediastinal structures by herniated abdominal viscera during prenatal life (28). Interrupted aortic arch, tetralogy of Fallot, transposition of great vessels, and double outlet right ventricle have been demonstrated by Benjamin *et al.* (29). In rats, cardiac maldevelopment was recently identified as a contributor to the high mortality rate in babies with CDH. Guarino *et al.*, (30) tested the hypothesis that alteration of the extracellular matrix (ECM) may contribute to the cardiac abnormalities seen. They found that the myocardium in rats with CDH was structurally immature (reduced cardiac tropoelastin and procollagen gene expression, and alpha elastin content.) Previously, we have demonstrated altered collagen content in the body-wall of nitrofen-exposed murine fetuses (5), although we did not assess collagen content in hearts or lungs of these affected mice. Left to right ventricular diameter ratio and aortic to pulmonary root diameter ratio were significantly decreased in rat fetuses with CDH in comparison with controls (31).

### 5.4 Cx43 modulations and Cardiopulmonary Abnormalities

We have focused on Cx43, because both, over- and under-expression of Cx43 result in cardiac malformations. We observed that connexins such as Cx37 and Cx40, known markers of vascular endothelial cells, were down-regulated in the hypoplastic lungs. Reaume *et al.* (8), discovered that the embryonic mice with targeted mutagenesis of Cx43 gene survived to term in the absence of Cx43, however they died at birth because of blockage of the right ventricular outflow tracts and subsequent failure in pulmonary gas exchange. In all mice with CDH and pulmonary hypoplasia, we observed narrowed pulmonary outflow tract, smaller left ventricle as compared to the right ventricle and exaggeration of the interventricular sulcus. Although, the degree of defects varied in different mice.

In addition to these morphological differences, we observed that there were higher levels of Cx43 mRNA in hearts and lungs of nitrofen-exposed mice with developmental abnormalities. However, levels of

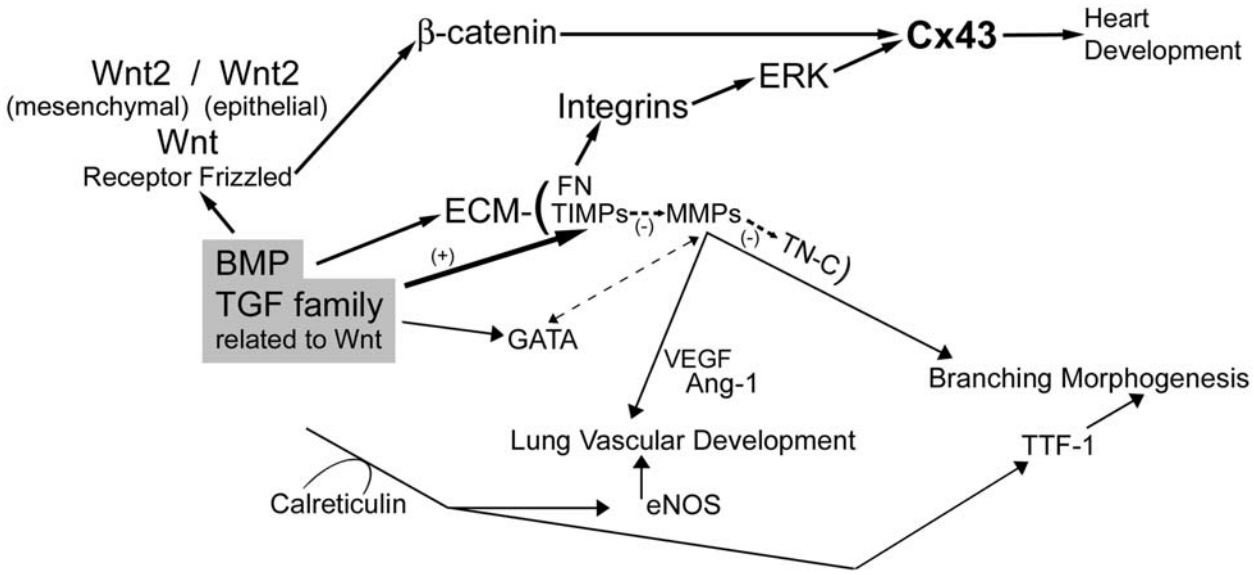


**Figure 12.** Schematic of pathways downstream of TGF / BMP superfamily. This schematic shows that ECM molecules are the common downstream molecules in regulation of heart, vascular and lung development. Most interlinks shown in this schematic are based on the work published by our laboratory.

phosphorylated and non-phosphorylated (NP) Cx43 protein levels were either the same as or lesser than those seen in the normal tissues. These data suggest possible post-transcriptional / translational defects in Cx43 in nitrofen-exposed mice, which may be responsible for specific developmental defects in hearts and lungs. The literature describes mRNA profiling as a means to define cell phenotypes, however it is also criticizes that this may not correctly represent the proteome. There are a limited number of reports on comparison of proteins and their corresponding mRNA, all of which suggest that mRNA abundance is a poor indicator of the amount of the corresponding protein. Thus, the discrepancy observed in the mRNA and proteins levels may indicate the relevance of additional mechanisms besides transcription in the control of gene expression, which include nuclear export, mRNA localization and stability, translational regulation and protein degradation.

Decreased Cx43 phosphorylation is associated with decreased gap junctional conductance (24). Reduction in phosphorylated Cx43 decreases gap junctional communication, i.e., it reduces the adhesion and results in leakage through increased permeability. We have observed down-regulation of phosphorylated and NP Cx43 at all stages of development in hypoplastic lungs except at the pseudoglandular stage and in neonatal hearts.

**5.5. Cx43 and ECM molecules**

Figure 12, is a schematic of the possible upstream regulation of Cx43. Transcription factors and extracellular matrix (ECM) molecules that regulate are common regulators of heart development, pulmonary branching morphogenesis and vascular development are shown. Through microarray analysis, we have found multiple genes in these pathways with altered expressions, such as hepatocyte nuclear growth factors (HNFs), bone morphogenic proteins (BMPs), SMADs, GATAs, connexins, MyoD and myogenin, matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), signal transducers and activators of transcription (STATs) and epidermal growth factor receptor (EGFR). Several of these genes are transcriptional regulators with significant roles during development of an embryo (32). Our research related to MMPs / TIMPs, vascular endothelial growth factor (VEGF) / Ang-1, have been discussed in separate publications (16,32). We propose that early embryonic signal transduction pathway involving TGF and BMP superfamily is down-regulated in nitrofen-exposed mice, which in turn affects the downstream pathways leading to abnormal heart, lung and vascular development (schematic interlinks of regulatory molecules are shown in Figure 12). We have also demonstrated the altered expressions of eNOS and TTF-1 (15,32). Taken together, ECM molecules play a key role in organogenesis and cytodifferentiation related pathways and are upstream of Cx43, which is one of the key regulators of heart development.

**5.6. Significance of Cx43**

Cx43 is located at the intercalated discs of the cardiac myocyte, suggesting a critical role in synchronization of myocyte contraction (33). Cx43 is not known to be expressed in the outflow tract and in knockouts no significant changes in the conduction velocity activation pattern or other conduction parameters were reported (34). Histological review of Cx43 transgenic mouse myocardium revealed a spongy appearance of the right ventricular apical myocardium (due to absence of compact layer and disorganization of trabeculare), right- and inter-ventricular septum hypertrophy, and enlargement of the proximal portion of the atrioventricular conduction system (7). We observed cellularity in the myocardium of normal, untreated animals unlike the myocardium of nitrofen-exposed mice.

Reaume *et al.* (7) and Ewart *et al.* (8) suggest that the embryos lacking Cx43 might survive the embryonic stages, but die upon birth. The pups become cyanotic, suggesting a failure of gas exchange. Abnormal heart morphogenesis and pulmonary outflow obstruction was observed. However, it was also suggested that this was not a direct effect of Cx43 deficiency, but may be due to the abnormal neural crest migration during the embryogenesis. Too little or too much of Cx43 can be disturbing for the gap junctional communication. Histologically, other tissue types such as brain, gut, skin, lungs, kidney and limbs revealed no overt abnormalities, however the subtle cell type specific and / or physiological effects cannot be excluded. Heart morphogenesis was disrupted in both Cx43 transgenics and mice with a deletion of the Cx43 gene. In both, defects in the right ventricle and pulmonary outflow tract were observed (7). However, the malformations were not identical, which is a significant finding.

India ink injection into the right ventricles of normal and nitrofen-exposed mice revealed a narrowed pulmonary outflow in mice with CDH and pulmonary hypoplasia. The pulmonary outflow defects and the ventricular abnormalities were similar to those shown by Ewart *et al.* (7) in Cx43 over-expressing transgenic mice. We propose that the lung defects, in part, can be attributed to the pulmonary vascular defects caused by altered expression of Cx43. Due to close interactions of the pulmonary vasculature with the alveolar development, the latter is affected. Thus, even if the lung defects are not clearly observed histologically in the Cx43 deficient mice, they may exist.

## 6. CONCLUSIONS

Based on our studies, we suggest that up-regulation of Cx43 mRNA expression may alter the permeability of gap junctions and thus result in cardiopulmonary and vascular defects observed in nitrofen-exposed mice. The differences in mRNA expression and levels of protein in heart and lung suggested possible post-transcriptional/translational defects in Cx43 in nitrofen-exposed mice resulting in developmental defects. Future studies on upstream regulators of Cx43, such as ECM proteins and RAR signal transduction pathways will enable us to provide evidence to further support our previous work on involvement of nuclear receptor pathways in regulation of lung airway branching morphogenesis as well as cardio-vascular development.

## 7. ACKNOWLEDGMENTS

This work was supported in part by Grant-in-Aid #0050341N from the American Heart Association (National) and a Career Award from American Lung Association of Central Pennsylvania to MRC, Grant-in-Aid #0151545U from the AHA (Penn-Delaware Affiliate) to REC and ML was supported in part by the Medical Student Summer Fellowship. The authors thank Shane Miller for his technical assistance at the various stages of this study, and Lisa McCully for her assistance in the preparation of this manuscript.

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**Key Words:** Developmental Biology, Gene Expression, Gap Junctions, Connexins, Matrix Metalloproteinase (MMP)

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