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Gata4 Interacts With Hedgehog Signaling Pathway In Regulating Outflow Tract Development

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GATA4 INTERACTS WITH HEDGEHOG SIGNALING PATHWAY IN REGULATING OUTFLOW TRACT DEVELOPMENT

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This thesis meets the standards for appearance, conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

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ABSTRACT

Congenital heart diseases (CHDs) are among the most common type of developmental anomaly affecting 8 in 1000 live births. Approximately 30% of CHDs involve cardiac outflow tract (OFT) malformation which leads to significant morbidity and mortality in both children and adults. Development of OFT is regulated by a complex genetic network including Sonic-Hedgehog signaling pathway, TGF-β and BMP signaling pathway. Mutation of a transcription factor, Gata4, has been known to cause OFT defects including double outlet right ventricle (DORV) in both human and mice for decades. Several transcription targets of Gata4 have been identified such as Mef2c, Ccnd2 and Cdk4, however none has been shown to be functionally involved in OFT development. Thus, the important role of Gata4 during OFT development remains unclear.

Here we analyzed the requirement of *Gata4* in several cell progenitors which contribute to the development of OFT and found that knocking down *Gata4* in myocardium, second heart field and cardiac neural crest cells was able to maintain normal OFT development. However, *Gata4* haploinsufficiency in Hedgehog (Hh) receiving cells caused a high penetrance of DORV in embryos at embryonic stage 14.5. Elongation as well as rotation defect were also observed in these mutant embryos compared to wildtype. Through TUNEL apoptosis assay, we found that there was no significant increase in cell apoptosis within the outflow tract region in these mutant embryos compared to wildtype. However, BrdU proliferation assay showed a significant decrease of cell proliferation in

the conal portion of outflow tract in these mutant embryos. By performing real-time PCR, luciferase assay and ChIP-qPCR, we found Gata4 directly binds to Hh signaling effector *Gli1* and regulates its expression. Also, by further blocking Hh signaling pathway in Gata4 mutant embryos or reducing such blockage, we found the occurrence and severity of OFT defect increased or decreased respectively. Together these results suggest that *Gata4* interacts with Hedgehog signaling pathway in regulating outflow tract development.

CHAPTER I

INTRODUCTION

1.1 Introduction to Congenital heart defects

Congenital heart defects (CHDs), also known as congenital heart anomalies or congenital heart diseases, are defects of the heart structure or major blood vessels that are present at birth. In patient with CHD, the normal blood flow through heart and the rest of the body is changed. As a consequence, the patients will experience many kinds of symptoms and complications vary from mild effects including rapid breathing, cyanosis, fatigue to life-threatening effects such as pulmonary hypertension, infective endocarditis, and congestive heart failure.

The worldwide birth prevalence of CHD has increased substantially, from 0.6 per 1,000 live births in 1930 to 9.1 per 1,000 live births after 1995, possibly due to advancement in diagnostic and screening methods such as pulse oximetry screening and echocardiography (van der Linde et al., 2011). The most commonly reported incidence of CHD in the United States is between 4 to 10 per 1,000, clustering around 8 per 1,000 live births (Shuler, Black, & Jerrell, 2013), which translate into a minimum of 40,000 infants who are expected to be affected by CHD each year. Around 25% of these live births require invasive treatment in their first year of life (Mozaffarian et al., 2016). Not only does CHD affect a large population, it also causes a heavy economic burden. In 2004, the total hospital stays for congenital birth defects were 139,000, of which cardiac and circulatory

congenital anomalies accounted for 33.5%, resulting in 1.3 billion hospital costs (Russo & Elixhauser, 2007).

Depending on the size and location that is affected, there are at least 18 distinct types of CHD varying in severity and ranging from a simple hole in the septum such as atrial septal defect (ASD), patent ductus arteriosus (PDA) to combination of simple defects such as tetralogy of Fallot (TOF), Eisenmengenr's syndrome and Holt-Oram syndrome. Among them there are three major types of CHD: atrial septal defect (ASD), ventricular septal defect (VSD) and Outflow tract (OFT) defect, accounting for approximately one third of total CHD prevalence respectively (Mozaffarian et al., 2016).

1.2 Three major types of CHD and the epidemiology

Atrial septal defect (ASD), as indicated by its name, is a congenital heart defect within the interatrial septum. In heart with ASD, the interatrial septum is defective or absent, which allows the oxygen rich blood to flow directly from left atrium and mix with deoxygenated blood in right atrium, leading to a drop in oxygen level in arterial blood that supplies the rest of the body. Depending on the structures involved, ASD can also be divided into many types, including but not limited to ostium primum and secundum atrial septal defect, sinus venosus defect and common atrium, resulting in symptoms ranging from asymptomatic to palpitation and fatigue. The overall incidence of ASD is estimated to be about 1.3 per 1,000 live births (Reller, Strickland, Riehle-Colarusso, Mahle, & Correa, 2008). Most of ASDs are sporadic with no identifiable cause. However, some ASDs are associated with exposure to alcohol and smoking (Alverson, Strickland, Gilboa, & Correa, 2011; Burd et al., 2007), some have been associated with abnormalities in genes, such as

mutations in transcription factors NKX2-5, GATA4 and TBX5 (Baban et al., 2014; Ellesoe et al., 2015; Morine et al., 2015; Sarkozy et al., 2005; Xiang et al., 2014).

Ventricular septal defect (VSD) is the most common type of CHD with a hole in the ventricular septum which separates the ventricle, allowing the heart to pump extra blood leaked from left ventricle to the lung. Since many types of VSD can be asymptomatic at birth and close with age, the precise prevalence of ventricular septal defect varies between studies, depending on the diagnosis techniques and population studied. Commonly the incidence of VSD is estimated to be 2.8 per 1000 live births (Hoffman, Kaplan, & Liberthson, 2004). Like atrial septal defect, VSD have also been associated with some environmental factors such as maternal infection, phenylketonuria and diabetes (Abu-Sulaiman & Subaih, 2004; Rouse et al., 2000). There are also some genetic factors responsible for VSD, including chromosome 18 trisomy (Hyett, Moscoso, & Nicolaides, 1995), chromosome 22q11 deletion (McElhinney et al., 2003), and single gene mutation such as *TBX5*, *NKX2-5*, *HAND2*, *GATA4* and *GATA6* (Garg et al., 2003; Huang et al., 2013; C. X. Liu et al., 2009; Sun et al., 2016; Zheng et al., 2012).

Within the scope of CHD, defects of the cardiac outflow tract (OFT), a transient structure of the developing heart that connects the embryonic ventricles and the aortic sac and that will eventually divide into aorta and pulmonary trunk, are estimated to cause approximately 30% of the CHDs. This include 13.5% valvular pulmonic stenosis, 5.4 % valvular aortic stenosis, 6.1% tetralogy of Fallot (TOF), 2.6% transportation of great artery (TGA), 0.9% double-outlet right ventricle (DORV) and 0.7% persistent truncus arteriosus (PTA) (Mozaffarian et al., 2016).

DORV describes a phenotype in which both the pulmonary trunk and aorta is connected to the right ventricle, instead of the aorta being connected to left ventricle in a normal heart. Most cases of DORV are accompanied by VSD, which can be explained by the need to allow blood to pass from the left ventricle to the aorta and pulmonary artery, allowing the oxygenated blood to mix with deoxygenated blood. However, the majority of blood entering the aorta comes from the right ventricle, which contains deoxygenated blood, so the oxygen level in the blood entering into aorta is reduced. Overriding aorta (OA) is an anomaly similar to DORV in a certain extent, with the main difference being that the aorta is positioned directly over a ventricular septal defect instead of over the left ventricle. Therefore, blood carried to the rest of the body through the aorta is a mixture of oxygenated and deoxygenated blood that comes from both the left and right ventricles. PTA, also known as common truncus, is considered more severe compared to DORV and OA because of the unfavorable prognosis and difficulty in surgical repair. With truncus arteriosus, there is only one single large artery that supplies the pulmonary and systemic circulation instead of a separate aorta and pulmonary artery.

Not only can OFT defect be found in isolated disease as described above, it can also be found as part of human syndromes such as DiGeorge syndrome and Alagille syndrome (Jain, Rentschler, & Epstein, 2010). Unlike ASD and VSD, most of OFT defect requires invasive treatment early in infancy to prevent serious complications such as advanced heart failure and severe pulmonary hypertension, and many other complex forms of OFT defect remain to be surgical challenges.

1.3 Development of outflow tract and etiology of OFT defect

1.3.1 Cell lineages that contribute to OFT development

Formation of outflow tract is a complicated process that requires the contribution of multiple cell lineages. Initially, cells from the primary heart field (PHF) converge and fuse at the midline of the embryo to form the primitive heart tube which will undergo elongation, looping and septation (Figure 1A,B). Starting from mouse embryonic day 8 (E8), additional cardiomyocytes are progressively added into the arterial pole of the heart from a cell population in pharyngeal mesoderm called second heart field (SHF) (Figure 1C,D) (Buckingham, Meilhac, & Zaffran, 2005; Cai et al., 2003; Kelly & Buckingham, 2002; van den Berg et al., 2009). At this stage, the OFT is an unseparated myocardial cylinder lined with endocardial cells. During the rightward looping of the primitive heart tube starting from E9, cardiac neural crest cells (CNCCs), another cell population from the dorsal neural tube, migrates through the pharyngeal arches into the distal (also known as truncal) portion of OFT until approximately E11 (Figure 1F, G) (Hutson & Kirby, 2003). As CNCCs enter the OFT, the endocardial jelly concentrates itself and the endocardial cells undergo endothelial to mesenchymal transition (EMT) to form pairs of OFT cushions between the myocardial and endothelial layers in a spiral conformation throughout the entire OFT (Figure 1G) (Anderson, Spicer, Brown, & Mohun, 2014; Sugishita, Watanabe, & Fisher, 2004). The cushions then undergo solidification, meanwhile cells from SHF have also begun to invade the distal OFT (Buckingham & Relaix, 2007; Franco et al., 2006; Ward, Stadt, Hutson, & Kirby, 2005). After OFT gains a substantial increase in length, the OFT undergoes remodeling, during which the OFT septa converge and separate the OFT into the ascending aorta and pulmonary trunk. The OFT septum further joins

atrioventricular and ventricular septa to align aorta with the left ventricle and pulmonary trunk with the right ventricle in a counterclockwise direction. At E14.5, well-defined chambers are fully separated and correctly aligned to the pulmonary trunk and aorta (Figure 1H).

1.3.2 Signaling pathways that contribute to OFT development

Proper development of OFT requires multiple signaling pathways including fibroblast growth factor (FGF), bone morphogenetic protein (BMP), Wnt, retinoic acid (RA) and Hedgehog (Hh) signaling pathway that are known to play important roles in a wide range of morphogenetic processes and biological functions. Perturbation of any of these pathways results in a spectrum of conotruncal congenital heart defects.

1.3.2.1 Wnt signaling pathway

Wnt signaling has been implicated in OFT development in many aspects. Firstly, knocking out Wnt/ β-catenin signaling specifically in cardiac mesoderm using series of Cre lines including *Isl1-cre*, *MesP1-cre* and *Mef2c-cre* lines causes right ventricular and OFT hypoplasia, while over-activation of β-catenin by LiCl treatment or gain-of-function mutation results in SHF hyperplasia and a greater number of SHF-derived cells in the OFT, suggesting an important role of Wnt signaling in controlling proliferation and differentiation of SHF that contributes to OFT (Cohen et al., 2007; Kwon et al., 2007; Lin et al., 2007). Secondly, Wnt signaling has also been reported to participate in induction, migration and specification of NCC development. Knocking out Wnt signaling member *Dvl2* down-regulates the expression of CNCC markers Pitx2 and Plexin A2, and causes various types of OFT defect such as PTA and DORV (Hamblet et al., 2002). Furthermore, knocking down Wnt1 receptor *Lrp6* results in a reduction of CNCCs in both the neural tube

and pharyngeal arches, as well as high incidences of DORV and VSD in the embryos (Song, Li, Wang, & Zhou, 2010). Thirdly, the EMT process during OFT development is also induced by Wnt signaling. As evidenced by Hurlstone and Liebner, overexpression of Dkk1, an inhibitor of Wnt signaling pathway, and Apc, a component of β-catenin destruction complex, result in hypoplastic endocardial cushion of the OFT (Hurlstone et al., 2003; Liebner et al., 2004).

1.3.2.2 FGF signaling pathway

FGF signaling affects the contribution of cell progenitors for OFT development. Conditional ablation of *Fgf8, Fgfr1* and *Fgfr2* in the SHF using Cre-loxP technique results in failure of OFT alignment and decreased cell proliferation within the SHF, implicating a role of FGF signaling during SHF proliferation and OFT development (Ilagan et al., 2006; E. J. Park et al., 2006; Eon Joo Park et al., 2008). FGF signaling has also been reported to play important roles in both the EMT process of OFT development and the contribution of CNCC to OFT. Ablation of FGF signaling adaptor *Frs2α* not only results in expansion of SHF cells into the OFT, but also leads to reduced cellularity in OFT cushions and defective CNCCs migration (J. Zhang et al., 2008). Deletion of *Ext1*, a gene encoding heparin sulfate biosynthesis enzyme necessary for cardiac morphogenesis, is reported to impair contribution of the SHF and CNCCs to OFT via down-regulation of FGF signaling. Moreover, exposure of both the OFT explants and pharyngeal explants to Fgf8 rescues the defects induced by *Ext1* deletion (R. Zhang et al., 2015).

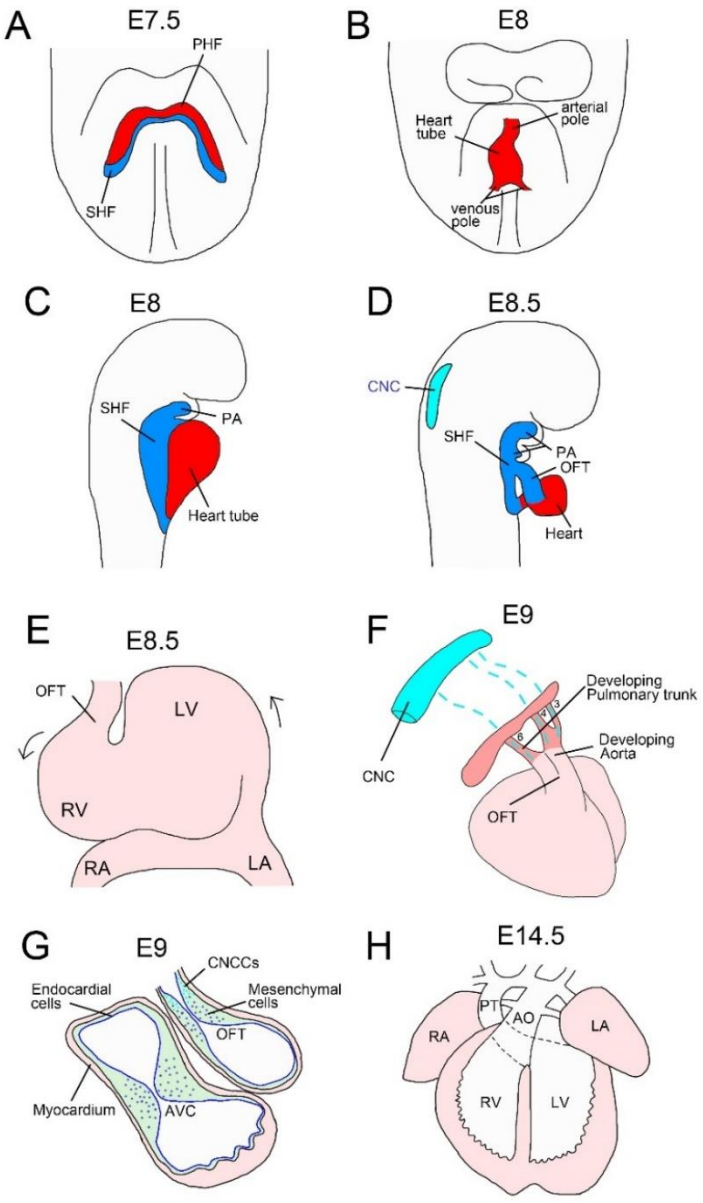


Figure 1. Development of outflow tract

(A) Two heart forming regions. (B) At E8, cells from the FHF converge at the midline of the embryo to form a heart tube. (C, D) Cells from the SHF are added to the arterial pole of the development heart. (E) Contribution of SHF cells results in elongation of OFT and looping of the heart. (F) Cells from CNC migrate into the distal OFT starting from around E9. (G) Endocardial cells receive signals from multiple sources and undergo EMT, resulting in the separation of OFT. (H) At E14.5, a heart with well-defined chambers is formed. FHF, first heart field; SHF, second heart field; PA, pharyngeal arches; CNC, cardiac neural crest; RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium; OFT, outflow tract; AVC, atrioventricular canal. (adapted from (Fung & Aikawa, 2013; Gessert & Kühl, 2010; Zaffran, Robrini, & Bertrand, 2014))

1.3.2.3 BMP signaling pathway

BMP signaling has been shown to be required by SHF and CNCC for OFT formation. Knocking out BMP type II receptor (*Bmpr2*) causes absence of conotruncus septation and PTA, suggesting the involvement of BMP signaling in OFT seperation (Délot, Bahamonde, Zhao, & Lyons, 2003). BMP signaling is required in SHF for OFT formation, as evidenced by the abnormal OFT morphology and SHF specification found in mutant embryos with overexpression or conditional loss of *Bmp4*, *Bmp7* and *Bmpr1* (Bmp type1 receptor) (W. Liu et al., 2004; L. Yang et al., 2006). Further investigation of the requirement of BMP signaling in SHF shows that BMP signaling regulates the EMT process of the OFT via *Vegfa* (Bai et al., 2013). BMP signaling has also been reported to be required by CNCCs for OFT formation, as specific ablation of *Bmpr1* in neural crest causes shortened OFT with defective septation. Moreover, BMP signaling can also interact with other signaling pathways including FGF signaling, which is supported by the finding that FGF signaling in the OFT myocardium enhances differentiation of NCCs in OFT cushion through up-regulation of *Bmp4* (J. Zhang et al., 2010). BMP signaling pathway also interacts with Hedgehog signaling, which is evidenced by the finding that SAG (Hedgehog signaling agonist)-induced proliferation of SHF explants is inhibited by Bmp2 (L. A. Dyer et al., 2010).

1.3.2.4 Retinoic acid signaling pathway

Retinoic acid (RA) is a vitamin A-derived metabolite and acts as a ligand for nuclear RA receptors (RARs). Upon binding to ligand RA, RAR alters its conformation and changes the binding affinity of RAR/RXR heterodimer to the retinoic acid response elements (RAREs) on DNA, thus regulating the transcription activity of nearby genes. RA

signaling has been reported to participate in OFT development two decades ago through gene knockout study, in which the knockout embryos shows abnormal ventricular trabeculation and defective OFT septation (Kastner et al., 1994; Mendelsohn et al., 1994). Further study by Li group identifies an essential role of RA signaling in regulating SHF differentiation and patterning TGFβ expression (P. Li, Pashmforoush, & Sucov, 2010). This interaction between RA and TGFβ signaling is recently confirmed by specifically knocking down TGFβ ligand gene *Tgfb2* in endocardium cells of RAR knockout embryos, which rescued the OFT septation defect in RAR mutants by 50% and demonstrated that CAT in RAR knockout mutants results from dysregulated TGFβ signaling in endocardium (M. C. Ma et al., 2016). RA signaling is also found to regulate the orientation and positioning of CNCCs as well as the coordination between CNCCs and endocardium in OFT septation (El Robrini et al., 2016).

1.3.2.5 Hedgehog signaling pathway

Hedgehog signaling pathway is a major regulator of embryonic development and plays critical roles in maintaining homeostasis of adult tissues. The name of the pathway originates from an intercellular signaling molecule called Hedgehog (Hh) which can cause a stubby and spiked phenotype in Hh mutant Drosophila larvae. Hh is involved in establishing the differences between the anterior and posterior parts of Drosophila body segments. In both vertebrate and invertebrates, Hh first binds to the Patched (PTCH) receptor, then PTCH activates SMO, a downstream protein of the pathway. SMO then further activate zinc-finger transcription factors GLI. Activated GLI then accumulates in the nucleus and regulates the transcription of specific target genes (Jacob & Lum, 2007; Varjosalo & Taipale, 2007).

Hedgehog (*Hh*) null mouse embryos shows defects in development of conotruncal and pharyngeal arch, and also a defect resembling tetralogy of Fallot (Smoak et al., 2005). Further analysis of the requirement in CNCC by crossing *Wnt1-cre* line with *Smo flox* line reveals that all mutant embryos displayed OFT defect, ranging from hypoplastic pulmonary artery to single OFT. Fate mapping of the CNCCs within the OFT shows a reduction in CNCCs within OFT, suggesting that *Hh* is required by CNCCs to survive and populate into OFT cushions (Goddeeris, Schwartz, Klingensmith, & Meyers, 2007). By specifically knocking out Hh signaling in SHF, the same group also found that Hh signaling is required in SHF for OFT septation and elongation (Goddeeris et al., 2007). Further study by Dyer LA found that *Hh* is required for SHF cell proliferation and that this *Hh*-induced proliferation may be regulated by BMP signaling (Laura A Dyer & Kirby, 2009; L. A. Dyer et al., 2010). Not only does Hh signaling regulates OFT development through CNCCs and SHF, it also acts via Hh-receiving cells, as showed in Hoffman's finding that Hh-receiving cells marked by Gli1-cre expression is found to migrate between E9.5 and E11.5 from SHF into the atrial septum and pulmonary artery (Andrew D Hoffmann, Peterson, Friedland-Little, Anderson, & Moskowitz, 2009). Several transcription factors have been identified to interact with Hh signaling in heart development. For example, Isl1 null mice has downregulated Shh in foregut endoderm and exhibits defects in aortic arch artery and outflow tract, suggesting an interaction between *Isl1* and Hh signaling pathway for cardiogenesis (Lin, Bu, Cai, Zhang, & Evans, 2006). Besides, constitutive activation of Hh signaling using *SmoM2* line recuses the ASD in *Tbx5* SHF haploinsufficiency mice, suggesting *Tbx5* acts upstream or in parallel to Hh signaling in atrial septation (L. Xie et al., 2012). Also, analysis of atrioventricular septal defect (AVSD) caused by compound

haploinsufficiency for *Foxf1and Foxf2* identifies a *Foxf1a* cis-regulatory element that is bound by Gli1 and Tbx5, suggesting that *Foxf* genes act downstream of *Tbx5* and Hh signaling for atrial septation (A. D. Hoffmann et al., 2014).

1.4 Transcription factors

With the morphogenesis of OFT being such a complex process, mutations of numerous genes including *Nkx2.5, Notch1,* T-box family and Gata family have been identified to be responsible for inherited and sporadic OFT defects. Mutations in the homeobox protein Nkx2.5, which plays a critical role in regulating tissue-specific gene expression for determining the temporospatial patterns of development, have been implicated as a cause of tetralogy of Fallot (Goldmuntz, 2001). Loss or mutation of T-box transcription factors including Tbx1 and Tbx3, have also been reported to be responsible for DORV, PTA and TOF (Liao et al., 2004; Karim Mesbah, Harrelson, Théveniau-Ruissy, Papaioannou, & Kelly, 2008).

1.4.1 T-box family and its relation to OFT defect

Tbx1, a member of the T-box family, is a transcription factor identified by analysis of the chromosome 22q11.2 deletion induced DiGeorge syndrome (Jerome & Papaioannou, 2001; Lindsay et al., 2001; Yagi et al., 2003). By specifically knocking out *Tbx1* using multiple Cre mouse lines, Xu et al. found that *Tbx1* is required in the pharyngeal endoderm for OFT septation and in SHF for OFT alignment (Xu et al., 2004). It is also found that *Tbx1* is required in the mesoderm for regulating the *Fgf8* in SHF, suggesting a relationship between *Tbx1* and FGF signaling in cardiac OFT development (Z. Zhang, Huynh, & Baldini, 2006). Tbx2 is another member of the T-box family which has been implicated in development of both the atrioventricular canal and septation of outflow tract (Harrelson et

al., 2004). It is found that the expression pattern of Tbx2 is complementary to that of chamber myocardium-specific genes including *Nppa*, *Cx40* and *Cx43*, suggesting a role of *Tbx2* in differentiating the formation of OFT and cardiac chambers (Christoffels et al., 2004). T-box family members also interact with each other in governing pharyngeal and OFT development, as showed by Mesbah that knocking out one T-box family member expression causes expression pattern changes of other T-box family member and loss of two of three genes (Tbx1/ Tbx2/ Tbx3) results in severe pharyngeal hypoplasia and heart tube extension defects (K. Mesbah et al., 2012).

1.4.2 Transcription factor Gata4 and its relation to OFT defect

Gata4 is a zinc finger transcription factor that recognizes the GATA motif and regulating gene transcription essential for embryogenesis. Many known mutations of GATA4 such as K319E, T280M, G21V and G296S have been associated with atrial septal defects (ASD) or ventricular septal defects (VSD) in both human and mouse studies (Y. Chen et al., 2010; Garg et al., 2003; Hirayama-Yamada et al., 2005; Rajagopal et al., 2007; Xiang et al., 2014). Other GATA4 mutations such as A9P, L51V and E216D or Gata4 haploinsufficiency were found to associate with OFT defects such as TOF and DORV (Nemer et al., 2006; Y. Q. Yang et al., 2013).

Lots of efforts have been placed on elucidating the role of Gata4 in heart development. Pu et al. found that cardiac morphogenesis including OFT development is dependent on the dosage of Gata4 (Pu, Ishiwata, Juraszek, Ma, & Izumo, 2004). Gata4 is known to work in combination with other essential cardiac transcription factors such as Nkx2.5 and Tbx5 to regulate proper cardiac development (Garg et al., 2003; Riazi et al., 2009; Sepulveda, Vlahopoulos, Iyer, Belaguli, & Schwartz, 2002). Gata4 also interacts

with other Gata family members including Gata5 and Gata6 in regulating OFT development. Mice with compound haploinsufficiency for *Gata4* and *Gata5* exhibited OFT defect with increased incidence and severity (Laforest & Nemer, 2011). By specifically knocking out *Gata4* in SHF using *Mef2c-cre* and *Nkx2.5-cre* line, Rojas found that *Gata4* is required in the derivatives of the SHF for cardiomyocyte proliferation. Further analysis of the heart mRNA reveals that Gata4 regulates a sets of cell cycle genes including *Cyclin D2* and *Cdk4*, both *in vivo* and *in vitro* (Rojas et al., 2008). By using a *Tie2-cre* transgenic mouse line, Rivera-Feliciano et al. identified Gata4 as a positive regulator of EMT of the atrioventricular cushion (AVC) by acting upstream of *Erbb3-Erk* pathway, both of which are essential factors for EMT (Rivera-Feliciano et al., 2006). Gata4 has also been reported to interact with Hh signaling. By eliminating Gata4 in the midgut endoderm using *Shh-cre* line, Kohlnhofer found that *Gata4* is required in Hh expressing cells for intestinal epithelial cell proliferation (Kohlnhofer, Thompson, Walker, & Battle, 2016). Daoud found that BMP-mediated induction of Gata factors blocks the induction of Hh dependent gene expression in presomitic mesoderm, suggesting that Gata factors act as repressors of Hh signaling (Daoud et al., 2014). The inhibition effect is supported by the finding of Xuan that knocking out *Gata4* and *Gata6* upregulates Hh signaling in mutant pancreata (Xuan & Sussel, 2016).

Recently, our lab found *Gata4* haploinsufficiency in posterior part of SHF (pSHF) caused abnormal atrial septation, suggesting the requirement of *Gata4* by SHF for heart morphogenesis (paper in publishing). Specifically, Gata4 is found to regulate the expression of cell cycle genes in SHF and interact with transcription factor Tbx5 in AV septation. Moreover, the finding that mice with compound haploinsufficiency of *Gata4*

and *Smo* in SHF shows severe AVSDs compared to control suggested *Gata4* interacts with Hh signaling in AV septation.

1.5 Gap in knowledge and contribution of this study

Despite ample studies of Gata4 in heart development, its role in OFT development is rarely studied and remains unclear. Specifically, although it is already known that improper deployment of SHF (Goddeeris et al., 2007), loss or reduction of the CNC (Hutson & Kirby, 2003), defects in the OFT endocardial cell (Y. Zhang et al., 2009) all contribute to OFT defects, whether Gata4 is required in these processes remains unknown. Whether Gata4 interacts with BMP, FGF and especially Hh signaling pathways or acts on its own for OFT development is also a question to be answered.

In the present study, we first analyzed the requirement of *Gata4* in the cell progenitors that contribute to OFT development by crossing several cell type-specific Cre mouse lines with *Gata4 flox* mouse line. We found that ablation of one allele of *Gata4* in myocardium, anterior second heart field and cardiac neural crest cells did not induce OFT defects in mutant embryos. Ablation of one allele of *Gata4* in Hh-receiving cells, however, induced a high occurrence of DORV in mutant embryos at embryonic stage 14.5 (E14.5) as well as elongation and rotation defects in mutant embryos at E10.5 and E11.5. TUNEL apoptosis assay showed no difference in cell apoptosis between Gata4 mutants and wildtype. BrdU proliferation assay showed a significant decrease of cell proliferation in conal OFT in mutants compared with that of wildtype. We then performed a real-time PCR to compare the expression of Hh signaling molecules *Gli* and *Smo* in SHF, and we found that the transcription level of *Gli1* and *Smo* was significantly decreased in mutants. By further performing luciferase assay and ChIP-qPCR, we showed that Gata4 can directly

bind to promoter regions of *Gli*. Last, by blocking or over activating Hh signaling pathway from Gata4 mutant embryos, we found the incidence and severity of OFT defect was increased or decreased respectively. Thus, these results suggest that *Gata4* interacts with Hedgehog signaling pathway in regulating OFT development.

1.6 Principle of major techniques used

1.6.1 Cre-loxP system

Cre-loxP recombination is a site specific recombinase technique able to mediate deletion, insertion, translocation and inversion at specific locations on DNA. Cre recombinase is a 38 kDa protein originally found in bacteriophage P1 that can recognize and bind to specific DNA sequences called loxP and result in homologous recombination. For the past decade, the Cre-loxP system has been widely used to overcome embryonic and early postnatal lethality in germline knockout studies (Le & Sauer, 2001; Nagy & Mar, 2001). By crossing mice which has a gene flanked by two loxP sites (resulting in an allele named GeneB^{flox} or GeneB^{fl}) and mice (GeneA^{Cre}) which carries Cre that is controlled by a tissue specific promoter, targeted gene knockdown and knockout can be achieved (Figure 2A,B). If the Cre recombinase is modified, by fusing the Cre to a mutated hormone-binding domains of estrogen receptor (Ert2) which can be activated by tamoxifen, the recombination can be regulated both spatially and temporally (Figure 2C).

Another application of the Cre-loxP system is cell fate mapping. By crossing mice (R26R^{flox} or R26R^{fl}) which has a loxP flanked STOP sequence followed by β-galactosidase (lacZ) gene with mice which expresses Cre under a tissue specific promoter, tissues with Cre expression will express lacZ, which encodes β-galactosidase. Upon addition of the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), β-galactosidase will

cleave X-gal and result in a dark blue precipitate, marking the Cre-expressing cell population (Figure 2D).

1.6.2 Luciferase Assay and ChIP Assay

Luciferase assay is a technique widely used to study the promoter activity *in vitro* by measuring luminescence from interaction of luciferase expressed under the promoter of interest and a substrate. In order to study transcriptional regulation, an expression vector that expresses transcription factor of interest and a reporter vector that contains the putative regulatory region upstream of the reporter gene are constructed. By transfecting cells with both the expression vector and reporter vector, luciferase enzyme will be produced only if the regulatory region is activated.

Chromatin immunoprecipitation (ChIP) is a method used to investigate the transcriptional regulation through transcription factor-DNA binding interactions *in vivo*. After DNA and the transcription factor protein are crosslinked to each other, antibody that specifically targets the protein of interest is used to pull down the DNA-protein complex. DNA fragments bound by transcription factor is then purified and recovered. By comparing to negative control loci, enrichment of transcription factor binding at regions of interest can be determined by quantitative PCR (qPCR).

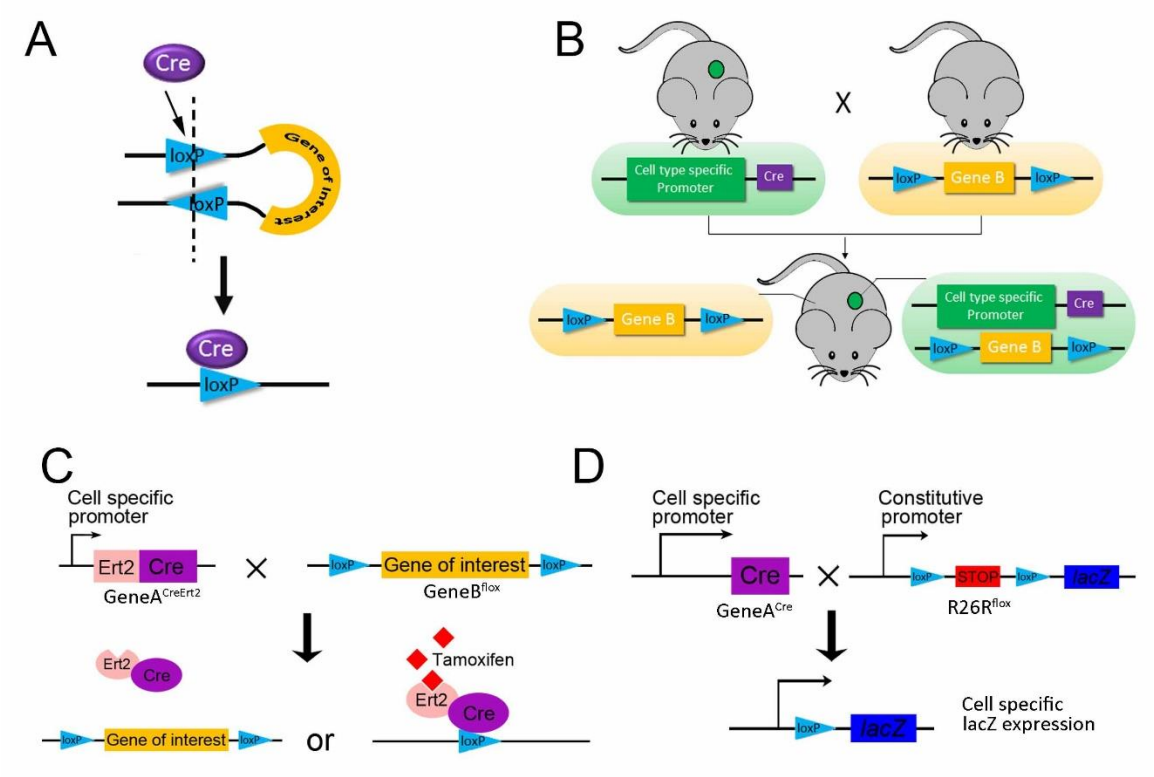


Figure 2. Cre-loxP techniques

(A) Cre recombinase recognizes loxP sites and delete the gene of interest through homologous recombination. (B) Transgenic mice (GeneA^{Cre}) carries Cre under cell/tissue specific promoter are mated to mice (GeneB^{flox}) with a gene flanked by loxP sites, resulting in cell/tissue specific deletion of the gene in the offspring. (C) Cre-loxP can be modified to enable temporal regulation. Cre is fused with mutated hormone-binding domains of estrogen receptor (Ert2) which can be activated by tamoxifen. Cre mediated excision will only occur when tamoxifen is administrated (lower right). (D) Mice (GeneA^{Cre}) which has cell specific Cre expression are crossed with mice (R26R^{fl}) that has stop sequence flanked by loxP sites and followed by a β-galactosidase (lacZ) gene, resulting in cell specific expression of β-galactosidase which can be used for mapping the cell fate

CHAPTER II

METHOD

2.1 Mouse Strains

All mice were maintained in a mixed B6/129/SvEv background. The *Gata4*$^{fl/fl}$ *Gli1*$^{CreERT2/+}$, *Mef2c*$^{AHF\text{-}Cre/+}$, *Tie2*$^{Cre/+}$, *Smo*$^{fl/fl}$ and *SmoM2*$^{fl/fl}$ mouse lines were obtained from Dr. Ivan Moskowitz lab (University of Chicago, Chicago). *EIIa*$^{Cre/+}$ and *Wnt1*$^{cre/+}$ mouse line was purchased from the Jackson Laboratory. *Tnt*$^{Cre/+}$ mouse line was from Dr. Yiping Chen lab (Tulane University, New Orleans). Generation of the *Gata4*$^{fl/fl}$, *Smo*$^{fl/fl}$, *SmoM2*$^{fl/fl}$, *Eiia*$^{Cre/+}$, *Gli1*$^{CreERT2/+}$, *Mef2c*$^{AHF\text{-}Cre/+}$, *Tie2*$^{Cre/+}$, *Tnt*$^{Cre/+}$, *Wnt1*$^{CreERT2/+}$ mouse lines have been reported previously (Ahn & Joyner, 2004; Jeong, Mao, Tenzen, Kottmann, & McMahon, 2004; Jiao et al., 2003; Kisanuki et al., 2001; Lakso et al., 1996; Long, Zhang, Karp, Yang, & McMahon, 2001; Pu et al., 2004; Verzi, McCulley, De Val, Dodou, & Black, 2005; Zervas, Millet, Ahn, & Joyner, 2004). For genotyping, yolk sacs or tail biopsies were first isolated and treated with 50 mM NaOH for 30 min to extract DNA, PCR and gel electrophoresis were then performed to determine the genotypes of the mice and embryos.

Mouse experiments were completed according to a protocol reviewed and approved by the Institutional Animal Care and Use Committee of the University of North Dakota, in compliance with the USA Public Health Service Policy on Humane Care and Use of Laboratory Animals.

2.2 Mouse Treatment

Superovulation technique (Luo et al., 2011) was used to obtain a greater number of eggs than normal in female mice. Female mice of 5-8 weeks old are injected intraperitoneally (IP) with 5 IU pregnant mare serum (PMS, Sigma) between 1:00 PM and 4:00 PM on day 1 to stimulate the development of the ovarian follicle. On day 3, forty-two to fifty hours after the PMS injection, the mice received an IP injection of 5 IU human chorionic gonadotropin (HCG, EMD Millipore) to promote the maintenance of the corpus luteum during the beginning of pregnancy. Female mice were then placed into appropriate male cage immediately following HCG injection. Female with vaginal plugs on day 4 was considered to be pregnant, and the embryos were considered as at embryonic stage 0.5 (E0.5). Tamoxifen-induced activation of CreERT2 was accomplished by oral gavage with two doses of 75mg/kg (Zhou et al., 2015) tamoxifen (TMX, Sigma) dissolved in corn oil on day10 and day11 (E7.5 and E8.5).

2.3 Histology Study

Embryos at E13.5 or E14.5 were first collected and fixed in 10% buffered formalin phosphate overnight at 4 °C. Dehydration by a graded ethanol series from water through 50%-70%-80%-90%-95%-100% ethanol, clearing by two changes of 100% xylene and infiltration by three changes of paraffin wax were then performed to process the embryos for embedding. For embryos at E13.5 and E14.5, embryos were cut across the body between the heart and liver, and the upper part was placed on embedding cassette with the transection facing against the cassette to ensure proper transverse orientation. Tissue blocks were then sectioned transversely and serially at 5 μm, and stained by hematoxylin and eosin (HE) staining. Incidences of defects in both mutants and control embryos were

recorded and compared with Fisher's exact test. Embryos at E10.5 and E11.5, with an R26R allele from a parent of the transgenic line which carries a floxed LacZ gene that encodes β-galactosidase, were first harvested and stained by lacZ staining, which uses 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, RPI) as substrate to develop color in β-galactosidase-expressing cells. Embryos were then fixed in 4% paraformaldehyde overnight at 4 °C, processed, embedded, sectioned and counterstained with 50% Eosin. Looping and elongation defects were then examined in each embryo. For embryos at E10.5 and E11.5, the limbs were removed first, then embryos were placed on embedding cassette with the left side facing against the cassette to ensure proper sagittal orientation.

In order to compare the elongation of OFT, three embryos from both wildtype and mutant were first selected randomly. Then section with the longest OFT length from each embryo was selected and measured using ImageJ. Results from both genotypes were compared using Student's t-test.

For BrdU proliferation assay, two doses of 100 mg/kg (Zhou et al., 2015) of bromodeoxyuridine (BrdU, RPI) were first given to female mice by IP injection 3 h and 6 h before sacrifice. Embryos were collected at E10.5, processed normally without lacZ staining and sectioned at 5 μm. Cell proliferation was then analyzed on sections with a BrdU kit (EMD Millipore). Briefly, paraffin embedded tissues were then deparaffinized in xylene followed by a graded ethanol series. After deparaffinization, the slides were immersed in 3% hydrogen peroxide to suppress endogenous peroxidase activity. 0.2% trypsin solution was applied to the slides to retrieve the antigen. Slides were then incubated with detector antibody at room temperature for 60 min, followed by 10 min incubation in

streptavidin-horseradish peroxidase conjugate at room temperature. 3,3 ′ -Diaminobenzidine (DAB) was applied on each slide for 5-10 min to stain the BrdU labelled cell. Slides were finally counterstained by hematoxylin. TUNEL apoptosis assay was performed with a peroxidase in-situ apoptosis detection kit (EMD Millipore). Briefly, slides were first treated with 20 µg/ml proteinase K to retrieve the antigen. After quenching with 3% hydrogen peroxide for 5 min, slides were then incubated with terminal deoxynucleotidyl transferase (TdT) for 1 h at 37 °C. TdT recognizes the 3'-OH ends of DNA and catalyzes the addition of digoxigenin-conjugated nucleotide to the ends. Anti-digoxigenin conjugate was then applied to each slide and incubated for 30 min to label the apoptotic cells that have significant numbers of DNA 3'-OH ends. DAB staining was then performed to stain the apoptotic cells followed by hematoxylin counterstaining.

For both BrdU assay and TUNEL assay, three embryos from both mutant and wildtype were selected for staining. Percent of labeled apoptotic or proliferative cells (brown) were counted in truncal OFT or conal OFT on 2-3 sections of each embryo from both control and mutant group. Measuring and counting work was done by well-trained students blinded to the study design. Results were analyzed and compared using Student's t-test.

2.4 RNA Extraction, RNA-Seq and qRT-PCR

E9.5 embryos were micro-dissected as previously described (L. Xie et al., 2012). Briefly, thoracic region of the embryo was obtained by isolating the part between the upper and lower limb buds. The neural tube was then removed by cutting through the foregut, then the anterior SHF (aSHF) and posterior SHF (pSHF) was separated by cutting between

the outflow and inflow tracts. The heart, aSHF and pSHF were collected in RNAlater (Sigma) and stored at -80 °C until RNA extraction. Total RNA was then extracted from each tissue type using RNeasy Mini Kit (Qiagen) per instructions.

For RNA-Seq, an aliquot of the extracted RNA from each sample was sent to University of Chicago for Next Generation Sequencing on Illumina HiSeq 2500 system. Heatmap of genes with the significant variations across the samples and sample grouping using hierarchical clustering were first done to determine the quality and consistency of sample collection. Cuffdiff and gene set enrichment analysis (GSEA) was then used for differentially expressed genes (DEG) analysis and gene set analysis (GSA).

For qRT-PCR, an aliquot of the extracted RNA was first reverse transcribed into cDNA using ReadyScript cDNA synthesis Kit (Sigma). Quantitative real-time PCR was then performed with All-in-One qPCR mix (GeneCopoeia) on iQ5 thermal cycler (Bio-Rad). Results were analyzed by delta-delta Ct method with Gapdh as normalization control (Livak & Schmittgen, 2001). Primers tested are listed in Table 1.

2.5 Luciferase Assay and Site-directed Mutagenesis

Gata4 expression vector was obtained from Dr. Ivan Moskowitz lab (University of Chicago, Chicago). Regulatory regions of Gli1 were amplified and cloned into pGL3 Basic vector (Promega) to make *firefly* reporter vectors. pRL-TK *Renilla* reporter vector (Promega) was used as internal control to remove experimental variability caused by transfection efficiency and cell lysis efficiency. Reporter vector and pRL-TK vector were co-transfected into 2×10^4 HEK293T cells, with or without Gata4 expression vector at a ratio of 50:1 or 50:50:1, using FuGene HD transfection Reagent (Promega). Cells were

then cultured for 24 h, lysed and assayed using Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. Briefly, the growth media was first removed and cells were washed using PBS. Then 20 µl passive lysis buffer was applied to each well for 15 min on orbital shaker to lyse the cells. Lysate was then transferred to new 96 well culture plate for luciferase assay. For each well, 100 µl Luciferase Assay Reagent II was first added to measure the *firefly* luciferase activity using multi-detection microplate plate reader (BioTek). 100 µl Stop&Glo was then added to stop the *firefly* activity and stimulate the *Renilla* activity. Results were represented as *firefly*/*Renilla* ratio.

Mutant reporter vectors were generated by deleting Gata4 binding sites on the reporter vector using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Briefly, mutant vectors were first synthesized by PCR using primers for mutagenesis and the original reporter vector as template. After treating with *Dpn* I enzyme to digest the original vector, the remaining mutant vector was then used for transformation following a stand transformation protocol. Primers used for mutagenesis are listed in Table1. Detailed information for genomic regions of Gli1 and mutation points are listed in Table 3.

2.6 ChIP-qPCR

E9.5 embryos were micro-dissected in PBS containing Protease Inhibitor Cocktail (Roche) on ice to isolate the heart region. Approximately 20 tissues were pooled for each sample. Tissues were treated with 1% formaldehyde for 15 min at room temperature to cross-link DNA and protein, and terminated with 0.2 mol/L glycine. Then tissues were washed twice with PBS and dissociated in Collagenase, Type II (Gibco) solution by shaking at 37 °C for 1~2 h at 100 rpm. Sonication was performed using Covaris S220

sonicator to generate fragments with an average size of 600 bp. After reserving an aliquot as Input control, samples were incubated with Gata4 antibody (Santa Cruz, sc-1237) overnight at 4 °C for immunoprecipitation. Samples were then incubated with Dynabead Protein G (Life Technologies), washed and reverse cross-linked.

qPCR was then performed using DNA recovered by phenol-chloroform extraction to compare the enrichment of Gata4 at different loci. qRT-PCR data of the regulatory region were expressed as fold enrichment relative to negative control region. Primers used to amplify the interested genomic regions (see Table 3) are listed in Table 1.

Table 1. Primers information

RT-PCR primers		
Gata4	CAACCCTGGAAGACACCCC	CTTAATGAGGGGCCGGTTGA
Gapdh	GACCTCATGGCCTACATGGC	GTTGGGATAGGGCCTCTCTTG
Shh	CAATCTGCAACGGAAGCGAG	TCCCCAAGGGATGCATGGTC
Ptch1	GACAGCTGGGAGGAAATGCT	ACAAGGGCCACATCAAGAGG
Smo	CAGCAAGAT CAACGAGACCA	GCTGAAGGTGATGAGCACAA
Gli1	GCCTTGAAAACCTCAAGACG	ATGGCTTCTCATTGGAGTGG
Gli3	GCAACCTCACTCTGCAACAA	ACCCTGCTGCTCTGACTCAT
Foxf1a	GCTCAACGAGTGCTTCATCA	CGGATCGATGGTCCAGTAGT
Gas1	ATGTTCGGCCCTCTTCTGTG	CTTGAAAGACCCCCACCGTT
Primers for Luciferase Assay		
Gli1-Fr (HindIII/SmaI)	AGTCAAGCTTTGAACATGGCGTCTCAGG	GTCACCCGGGTTCTCTTCTGGC CCTACC
Primers for Site-directed Mutagenesis		
Gli1-Fr-M1	GGAACGAAACAGAGAATGACAGTTTCAGGC	GCCTGAAACTGTCATTCTCTGTT TCGTTCC
Gli1-Fr-M2	CCTCGTTTCAGTCCACTGGTAGGGCCAG	CTGGCCCTACCAGTGGACTGAA ACGAGG
Primers for ChIP-qPCR		
Gli1-Fr1	TGGCGTCTCAGGGAAGGATG	CTGAGATGAGGGTTAGAGGC
Gli1-Fr2	GTGATGGTGGAACACACGG	TTCTCTTCTGGCCCTACC
Neg-ctrl	GAGGGATACTTAGGCGGC	GTTGCAGCAAGGCCTTTAGC

CHAPTER III

RESULTS

3.1 Gata4 is involved in OFT development

Mutations of Gata4 have been shown to cause a wide spectrum of congenital heart abnormalities in human including ASD, VSD, DORV and TGA (J. Chen et al., 2016; Mohan et al., 2014; Nemer et al., 2006; W. Zhang et al., 2008). Previous studies have demonstrated that Gata4 is required for multiple aspects of embryonic cardiac morphogenesis in mice such as heart tube formation and cardiomyocyte proliferation (Kuo et al., 1997; Pu et al., 2004). To confirm *Gata4* is required for OFT development, we analyzed mouse haploinsufficient for *Gata4* in the germ line by crossing $Gata4^{fl/fl}$ mouse line, which has loxP sites flanking the second exon of *Gata4*, with the $EIIa^{Cre/+}$ mouse line which is widely used to delete loxP-flanked genes in germ line (Krag & Vissing, 2015; Lakso et al., 1996; Paschos et al., 2012; Qu et al., 2006). Whereas $Gata4^{fl/+}$ (n=13) embryos had normal atrial septum and outflow tract alignment, 39% of $Gata4^{fl/+}$,$EIIa^{Cre/+}$ embryos showed ASD (7/18, Figure 3D vs. 3A) and 61% of mutant embryos showed DORV (11/18, p=0.0004, Figure 3E,3F vs. 3B,3C; Table 2). These results confirmed that Gata4 is involved in OFT development.

3.2 Gata4 knockdown in myocardium, second heart field cells and cardiac neural crest cells does not affect OFT development

Table 2. Incidence of OFT defects in embryos

Genotype	OFT defect	Total	P value (χ^2 test)	
Gata4$^{fl/+}$,*Eiia*$^{Cre/+}$	11	18	vs. *Gata4*$^{fl/+}$ (0/13)	p=0.0004
Gata4$^{fl/+}$,*Tnt*$^{Cre/+}$	0	12	vs. *Gata4*$^{fl/+}$ (0/12)	p=1.0000
Gata4$^{fl/+}$,*Mef2c*$^{AHF\text{-}Cre/+}$	0	22	vs. *Gata4*$^{fl/+}$ (0/24)	p=1.0000
Gata4$^{fl/+}$,*Wnt1*$^{CreERT2/+}$	0	24	vs. *Gata4*$^{fl/+}$ (0/27)	p=1.0000
Gata4$^{fl/+}$,*Gli1*$^{CreERT2/+}$	11	18	vs. *Gata4*$^{fl/+}$ (0/15)	p=0.0002
Gata4$^{fl/+}$,*Smo*$^{fl/+}$,*Gli1*$^{CreERT2/+}$	5*	9	vs. *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ (2/6) vs. *Smo*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ (0/7)	p=0.6084 p=0.0337
Gata4$^{fl/+}$,*SmoM2*$^{fl/+}$,*Gli1*$^{CreERT2/+}$	0	9	vs. *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ (3/6) vs. *SmoM2*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ (2/7)	p=0.0440 p=0.1750

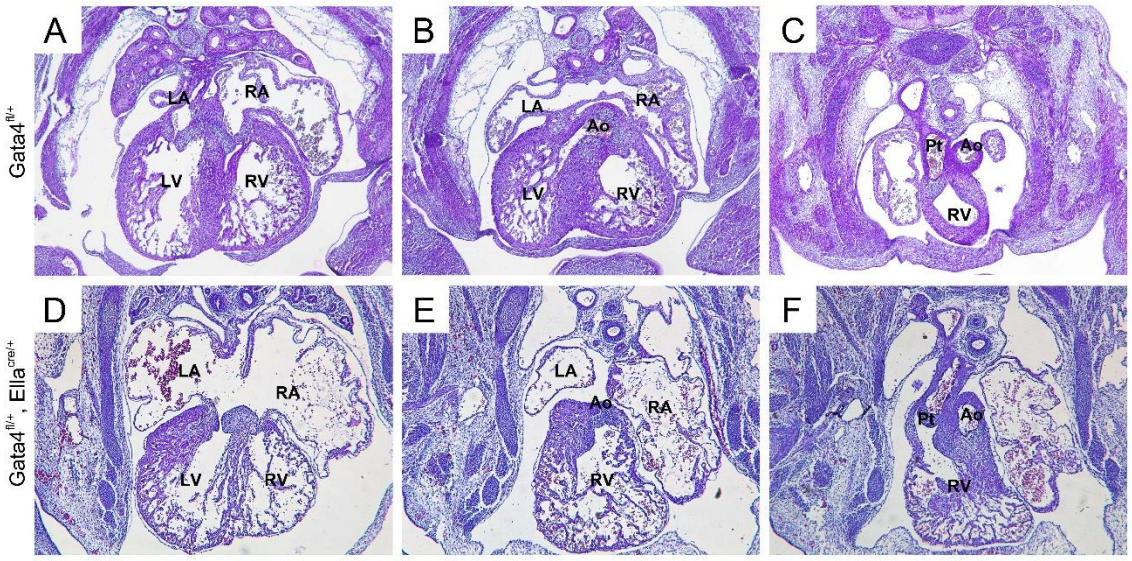


Figure 3. *Gata4* is required for OFT development

Histology of *Gata4*$^{fl/+}$ and *Gata4*$^{fl/+}$,*EIIa*$^{Cre/+}$ mouse embryos at E13.5. (A) Normal septation between left atrium (LA) and right atrium (RA). (B and C) Normal structure of OFT, with left ventricle and right ventricle being connected to aorta and pulmonary trunk respectively. (D) *Gata4*$^{fl/+}$,*EIIa*$^{Cre/+}$ embryos had ASD, as shown by the hole in atrial septum. (E and F) *Gata4*$^{fl/+}$,*EIIa*$^{Cre/+}$ embryos also had DORV, as both the aorta and pulmonary trunk was connected to right ventricle. RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium; Ao, aorta; Pt, pulmonary trunk;

Considering the multiple cell progenitors that contribute to the formation of OFT as described in the Introduction, we then wanted to know in which cell lineage Gata4 is required for OFT development. To achieve our goal, we knocked down Gata4 in myocardium, second heart field cells and cardiac neural crest cells individually by breeding *Gata4*$^{fl/fl}$ mice with *Tnt*$^{Cre/+}$, *Mef2c*$^{AHF\text{-}Cre/+}$ and *Wnt1*$^{CreERT2/+}$ mice, respectively. *Tnt*$^{Cre/+}$ mouse has *Cre* expression under the control of rat cardiac TroponinT (cTnT) promoter and has been shown to induce Cre recombinase activity in the cardiomyocyte lineage at early stage (Jiao et al., 2003). It is widely used as a marker for cardiomyocytes (J. W. Chen et al., 2006; D. Li et al., 2016; Sah, Bates-Withers, Jin, & Clapham, 2010; von Gise & Pu, 2012; L. Xie et al., 2012). *Mef2c*$^{AHF\text{-}Cre/+}$ mouse has *Cre* expression under the control of *Mef2c* anterior heart field enhancer and has been shown to be expressed only in the anterior SHF and its descendants (Verzi et al., 2005). *Wnt1*$^{CreERT2/+}$ mouse expresses Cre under the control of wingless-related MMTV integration site 1 (*Wnt1*) promoter and enhancer. It is widely used in studying the development of neural crest and its derivatives including OFT (Etheridge et al., 2008; Goddeeris et al., 2007; Olaopa et al., 2011; Paul, Harvey, Wegner, & Sock, 2014; Plein et al., 2015).

As shown in Figure 4, normal OFT alignment was observed in the following*Gata4* mutant embryos (*Gata4*$^{fl/+}$,*Tnt*$^{Cre/+}$ 12/12; *Gata4*$^{fl/+}$,*Mef2c*$^{AHF\text{-}Cre/+}$ 22/22; *Gata4*$^{fl/+}$,*Wnt1*$^{CreERT2/+}$ 24/24; Figure4B-D, 4F-H vs. 4A,E; Table 2) at E14.5, although ASD was noted in *Gata4*$^{fl/+}$,*Mef2c*$^{AHF\text{-}Cre/+}$ embryos which may indicates that *Gata4* is required in SHF for atrial septation (data not shown). These results suggested that *Gata4* knockdown in myocardium, anterior SHF cells and cardiac neural crest cells individually does not affect normal OFT development.

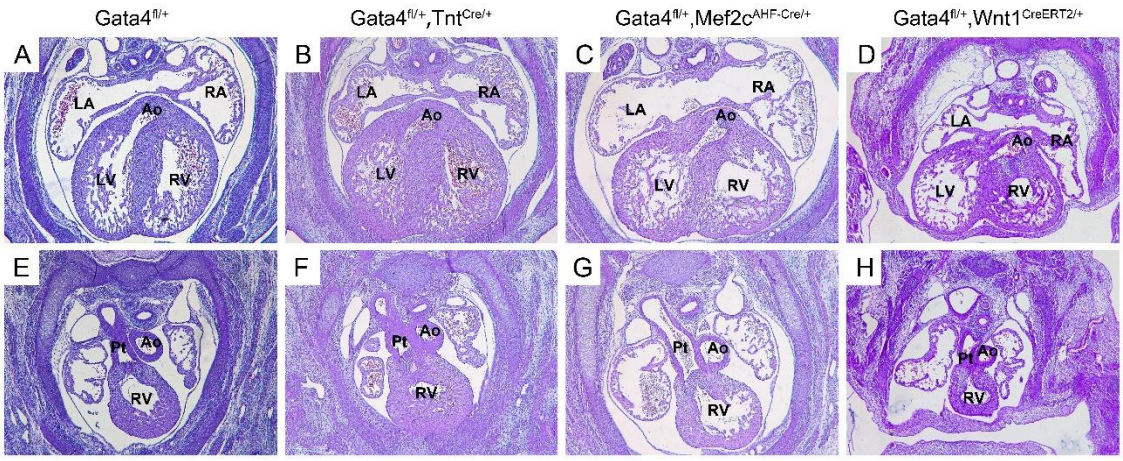


Figure 4. *Gata4* knockdown in myocardium, SHF cells and CNCCs retains normal OFT development

Histology of mouse embryos heart at E14.5. All *Gata4* mutant embryos showed normal OFT alignment compared to wildtype (B and F, C and G, D and H vs. A and E)

3.3 Gata4 haploinsufficiency in Hh-receiving cells impairs normal OFT development

Previous studies have shown that Hedgehog signaling is directly required by both the SHF and CNCC fields for OFT morphogenesis (Goddeeris et al., 2007; Smoak et al., 2005). Therefore, we wondered if *Gata4* interacts with Hedgehog signaling for OFT development. We tested this hypothesis by specifically ablating one copy of *Gata4* in Hh-receiving cells with the help of the $Gli1^{CreERT2/+}$ mouse line, which expresses Cre under the promoter of an Hh signaling effector, *Gli1*. *CreERT2* was activated by tamoxifen (TMX) administration at E7.5 and E8.5 in $Gata4^{Gli1Cre\text{-}ERT2/+}$ embryos, a regimen previously used to implicate SHF Hh signaling in AV septation. ASD was observed in 73.3% (11/15) of TMX treated $Gata4^{fl/+}$,$Gli1^{CreERT2/+}$ embryos, but not in control $Gata4^{fl/+}$ embryos (0/15) or $Gli1^{CreERT2/+}$ embryos (0/15) at E14.5. DORV was also observed in 61% (11/18, p=0.0002; Figure 5E,F) of $Gata4^{fl/+}$,$Gli1^{CreERT2/+}$ embryos at E14.5, but not in control $Gata4^{fl/+}$ embryos (0/15, Table 2; Figure 5B,C).

It is well known that OFT elongation is required for correct cardiac looping and proper alignment of the OFT (Karim Mesbah et al., 2008; Ramsbottom et al., 2014; Roux, Laforest, Capecchi, Bertrand, & Zaffran, 2015). Thus, we tested if the OFT length is affected in $Gata4^{fl/+}$,$Gli1^{CreERT2/+}$ embryos. We measured the OFT length in both mutant and wildtype embryos at E10.5 and found that the OFT length of $Gata4^{fl/+}$,$Gli1^{CreERT2/+}$ embryo was significantly shorter than that of wildtype (0.8759±0.0150 vs. 1.0000±0.0420, p=0.0320, Figure 6B,D vs. 6A,C). Moreover, serially sagittal sections of the embryonic hearts showed that cardiac looping in $Gata4^{fl/+}$,$Gli1^{CreERT2/+}$ embryos was abnormal compared to wildtype, as indicated by the relative orientation of the OFT and AVC (Figure

6F vs. 6E, 6H vs 6G). Together these results suggested that Gata4 is required in Hh-receiving cells for OFT development.

3.4 Gata4 haploinsufficiency in Hh-receiving cells affects cell proliferation within conal OFT cushion

Besides the abnormal cardiac looping, we also noticed there seemed to be less cushion cells within the conal OFT cushion in *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos at E11.5 (Figure 6H vs. 6G). It was possible that the reduced cushion cell number was due to an increase of cell apoptosis or a decrease of cell proliferation, two commonly reported events involved in OFT development (Laforest & Nemer, 2011; Leung et al., 2016; H. Y. Ma, Xu, Eng, Gross, & Kioussi, 2013; Plein et al., 2015). To analyze cell apoptosis of the OFT, TUNEL apoptosis assay was performed on serially sagittal sections of E10.5 embryonic hearts. We observed very few apoptotic cells within the OFT, with no significant changes in the amount of apoptotic cells in OFT of *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos compared to control embryos.

We then wondered if defects in proliferation was involved. Cell proliferation was evaluated by BrdU incorporation assay at E10.5. Consistent with the observations in E11.5 embryos (Figure 6), the proliferation rate of the truncal OFT cushion showed no difference between *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos and control embryos (Figure 7B vs. 7A; Figure 7C). Cell proliferation of the conal OFT cushion in *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos, however, was significantly reduced compared with controls (Figure 7E vs. 7D; Figure 7F). These results implied that *Gata4* is required in Hh-receiving cells for regulating cell proliferation within the conal OFT cushion.

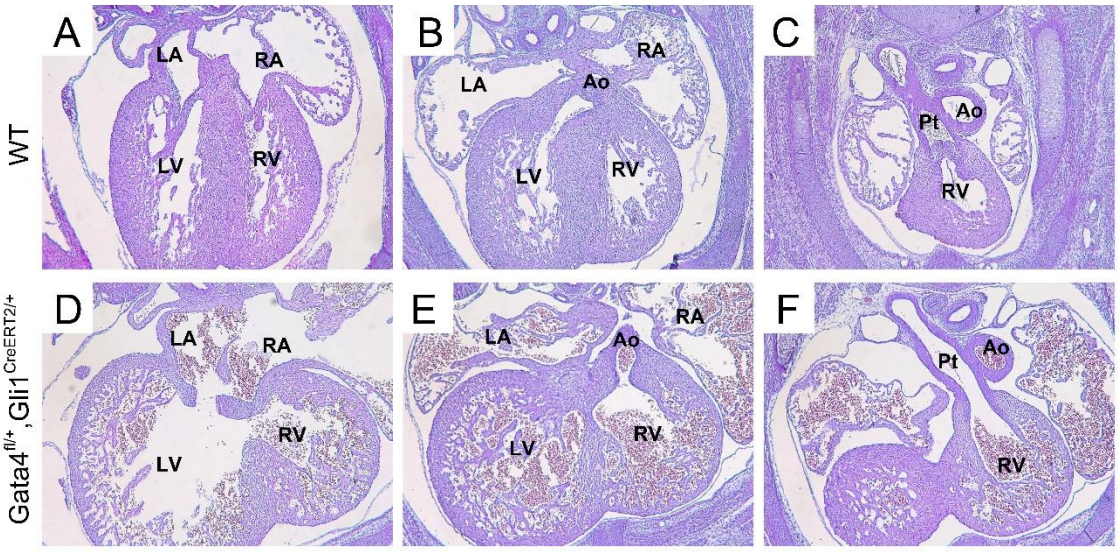


Figure 5. *Gata4* haploinsufficiency in Hh-receiving cells affects OFT development

Histology of both wildtype and *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos heart at E14.5. (A-C) Normal atrial septation and OFT alignment in wildtype embryos. (D-F) Atrial septal defect (ASD) and double outlet right ventricle (DORV) was observed in *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos.

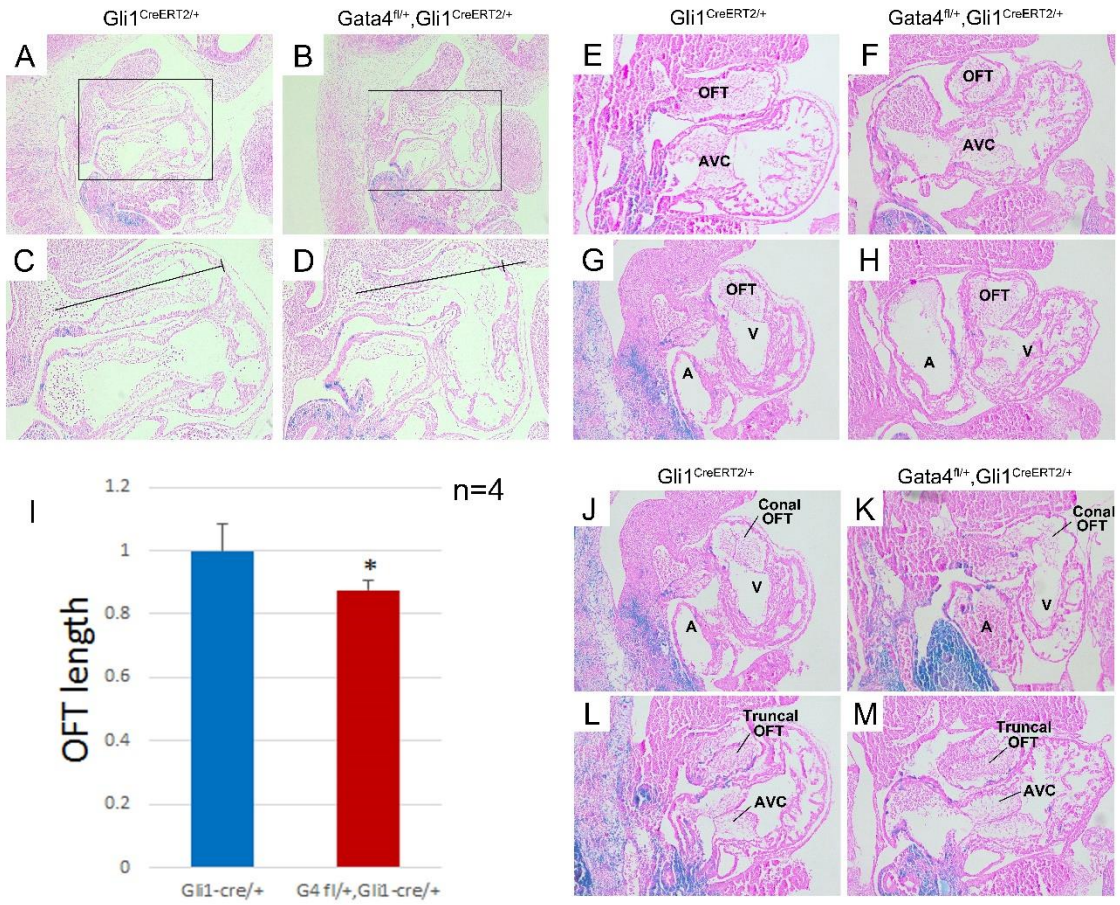


Figure 6. *Gata4* haploinsufficiency in Hh-receiving cells affects OFT lengthening, cardiac looping and cell proliferation of conal OFT

(A-D) Histology of wildtype and *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos heart at E10.5. *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos showed a relatively shorter OFT length compared to that of wildtype, as indicated by the black line (C vs. D). Abnormal OFT orientation was observed in *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos with atrioventricular canal as reference (E vs. F, G vs. H). Mutant embryos also seemed to have less cells within the conal OFT cushion compared to that of control (J vs. K), and there was no apprent difference within truncal OFT cushion and AV cushion. (I) Statistical analysis of embryos of each genotype showed a significant decrease in OFT length in mutant embryos. Results were presented as mean±SD; n=4, $P<0.05$. Magnification in panels A and B is 50X; Magnification in C-D, E-H and J-M is 100X. OFT, outflow tract; AVC, atrioventricular cushion; A, atrium; V, ventricle.

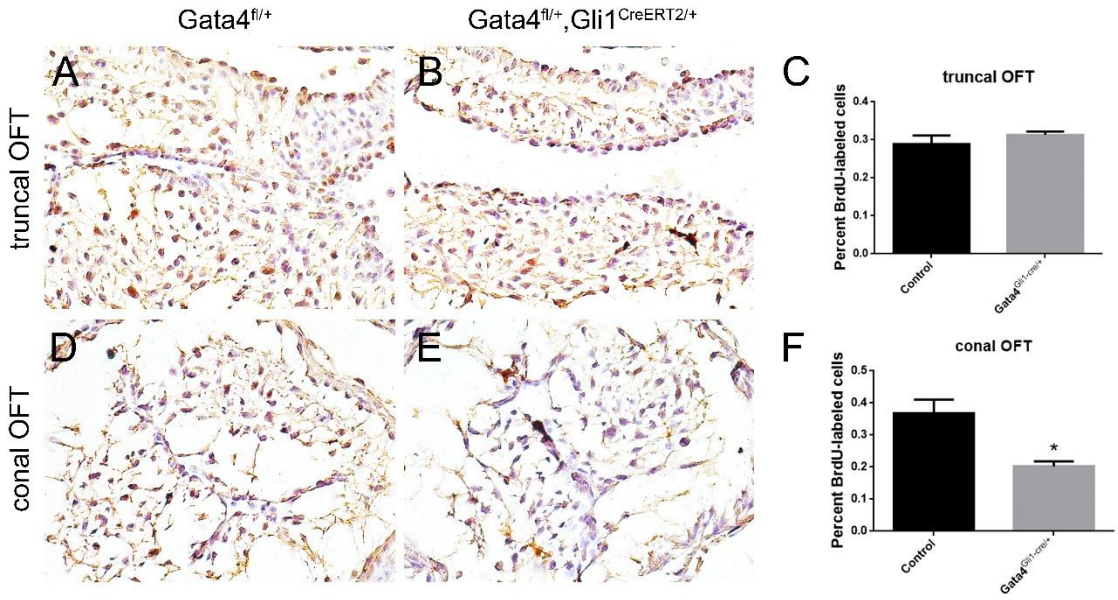


Figure 7. *Gata4* haploinsufficiency in Hh-receiving cells affects cell proliferation of conal OFT

(A-B, D-E) Histological analysis of cell proliferation within truncal OFT and conal OFT respectively in both wildtype and *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos. Proliferating cells were labelled in brown, other cells were labelled in blue. (C and F) Statistical analysis of percent of proliferating cells within truncal OFT and conal OFT respectively. Results were presented as mean±SD; n=3, $P<0.05$. A siginificant decrease of the ratio of proliferating cells was observed in conal OFT cushion compared to that of control (D vs. E; F).

3.5 Gata4 dose-dependent transcription profiles identified Hh signaling pathway as regulator of OFT development

To identify the potential Gata4 dependent molecular networks required for OFT development, we compared the SHF transcriptomes of *Gata4*$^{fl/+}$ and *Gata4*$^{fl/fl}$,*Gli1*$^{CreERT2/+}$ embryos at E9.5. As shown in the heatmap (Figure 8A), the biological replicates of the same genotype were clustered together, showing good consistency in sample collection. And *Gata4*$^{fl/+}$ and *Gata4*$^{fl/fl}$,*Gli1*$^{CreERT2/+}$ samples were clustered into different groups, suggesting that they have distinct transcription profiles. Alteration of 12 signaling pathways including Hh signaling pathway were identified by gene set enrichment analysis (GSEA) with p value less than 0.05 as cutoff (Figure 8B). The involvement of Hh signaling was further tested and confirmed by real-time PCR analysis of several Hh signaling genes, including *Gli1*, transcription activator, *Gli3*, both transcription activator and repressor, *Smo*, a component of Hh pathway, *Foxf1a*, a target of Hh signaling and *Shh*, the sonic hedgehog ligand. As shown in Figure 8C, mRNA expression of *Shh*, *Gli1* and *Foxf1a* were significantly changed in SHF of *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos compared to control. These results suggest that the Hh-signaling integrity is dependent on the dose of Gata4 in the SHF.

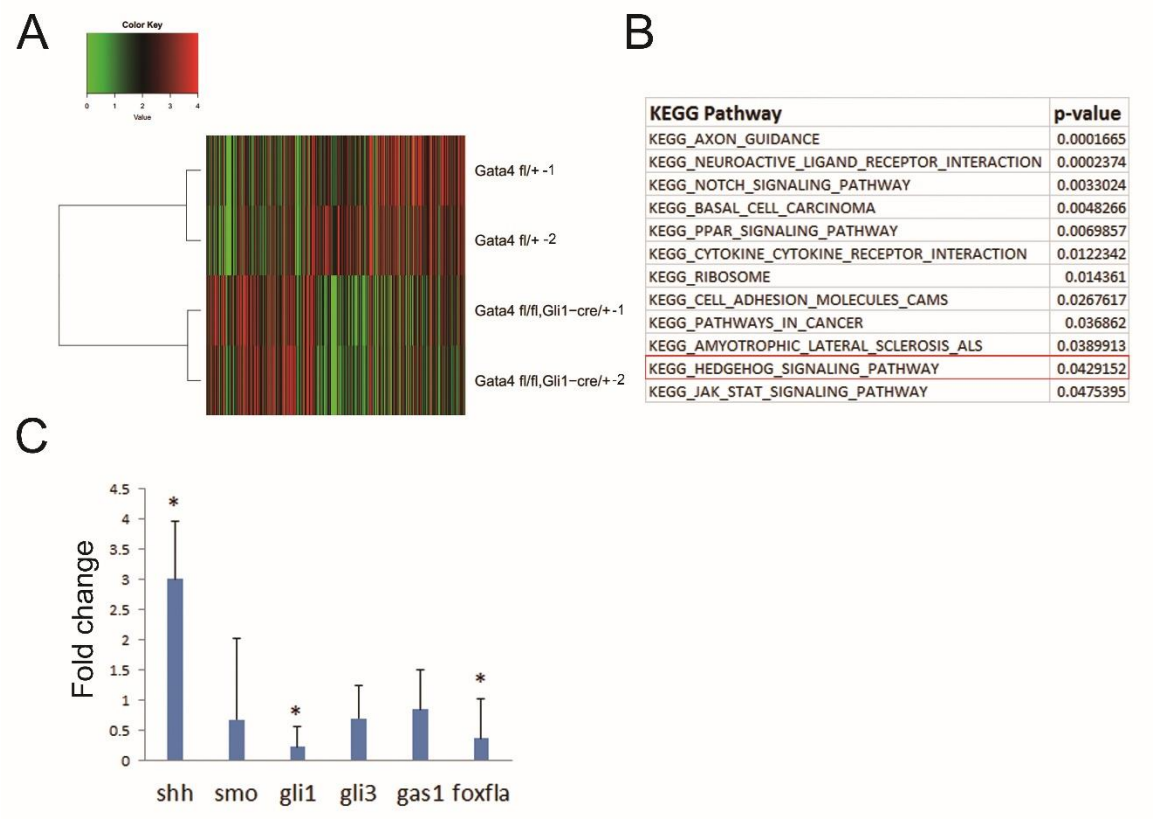
3.6 Gata4 directly regulates expression of Hh signaling effector Gli1

We wondered if the regulation of *Gli1* by Gata4 was direct or indirect. To answer this question, we amplified a 0.8 kb fragment of *Gli1* (Gli1-Fr, Figure 9A) containing conserved Gata4 binding motifs, (A|T)GATA(A|G). The amplified region is in close proximity to Gata4 binding sites reported in ChIP-sequencing data of the developing murine heart (He et al., 2014). We then tested cis-regulatory function of this region by

luciferase assay. Gata4 significantly transactivated this region in HEK293T cells, as indicated by luciferase reporter expression (Figure 9B and Table 3). We further mutated the *Gata4* binding sites in the fragment and found that the mutant constructs (Gli1-Fr-M1 and Gli1-Fr-M2) failed to activate luciferase expression. To confirm our finding, ChIP-qPCR was performed using microdissected SHF tissues of wildtype mouse embryos at E9.5. Two *Gli1* genomic regions (Gli1-Fr1, Gli1-Fr2) containing Gata4 binding motifs as well as a negative control genomic region (Gli1-Neg-ctrl) contains no Gata4 binding motif were tested for Gata4 binding activity. ChIP-qPCR demonstrated significant Gata4-dependent enrichment for those two Gli1 fragments containing Gata4 binding motif, but not for the control fragments (Figure 9C and Table 3). Together these results showed that Gata4 directly binds to regulatory genomic regions of Hh signaling effector Gli1.

3.7 Further blocking Hh signaling pathway leads to higher occurrence and more severe form of OFT defect

Both Gli1 and Smo are required for Hh signal transduction, therefore we hypothesized further disrupting Hh signaling can validate our results that *Gata4* interacts with Hh signaling in regulating OFT development. We tested our hypothesis by ablating both a copy of *Gata4* and a copy of *Smo* in Gli1-expressing cells by crossing the $Gata4^{fl/+}$,$Smo^{fl/+}$ mouse line with $Gli1^{CreERT2/+}$ mouse line. Surprisingly, not only did we observe DORV in $Gata4^{fl/+}$,$Smo^{fl/+}$,$Gli1^{CreERT2/+}$ embryos (2/9; Figure 10H,K), but we also observed OA (1/9; Figure 10I,L) as well as PTA (2/9; Figure 10J,M). Although the total incidences of OFT defect between $Gata4^{fl/+}$,$Gli1^{CreERT2/+}$ embryos and $Gata4^{fl/+}$,$Smo^{fl/+}$,$Gli1^{CreERT2/+}$ embryos were not significantly different from each other (2/6 vs. 5/9, p=0.6084, Table 2), considering that PTA is a more severe form of OFT defect, we confirmed that *Gata4* interacts with Hh signaling in regulating OFT development.



KEGG Pathway	p-value
KEGG_AXON_GUIDANCE	0.0001665
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	0.0002374
KEGG_NOTCH_SIGNALING_PATHWAY	0.0033024
KEGG_BASAL_CELL_CARCINOMA	0.0048266
KEGG_PPAR_SIGNALING_PATHWAY	0.0069857
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	0.0122342
KEGG_RIBOSOME	0.014361
KEGG_CELL_ADHESION_MOLECULES_CAMS	0.0267617
KEGG_PATHWAYS_IN_CANCER	0.036862
KEGG_AMYOTROPHIC_LATERAL_SCLEROSIS_ALS	0.0389913
KEGG_HEDGEHOG_SIGNALING_PATHWAY	0.0429152
KEGG_JAK_STAT_SIGNALING_PATHWAY	0.0475395

Figure 8. *Gata4*-dependent transcript profile identified Hh signaling pathway in OFT development

(A) Heatmap of 1213 genes with the largest variations in E9.5 SHF of $Gata4^{fl/fl}$,$Gli1^{CreERT2/+}$ vs. $Gata4^{fl/+}$ embryos. (B) 12 pathways identified by GSEA with $p<0.05$ as cutoff. (C) Transcription level changes of Hh-signaling pathway genes in E9.5 SHF of mutant embryos compared to wildtype.

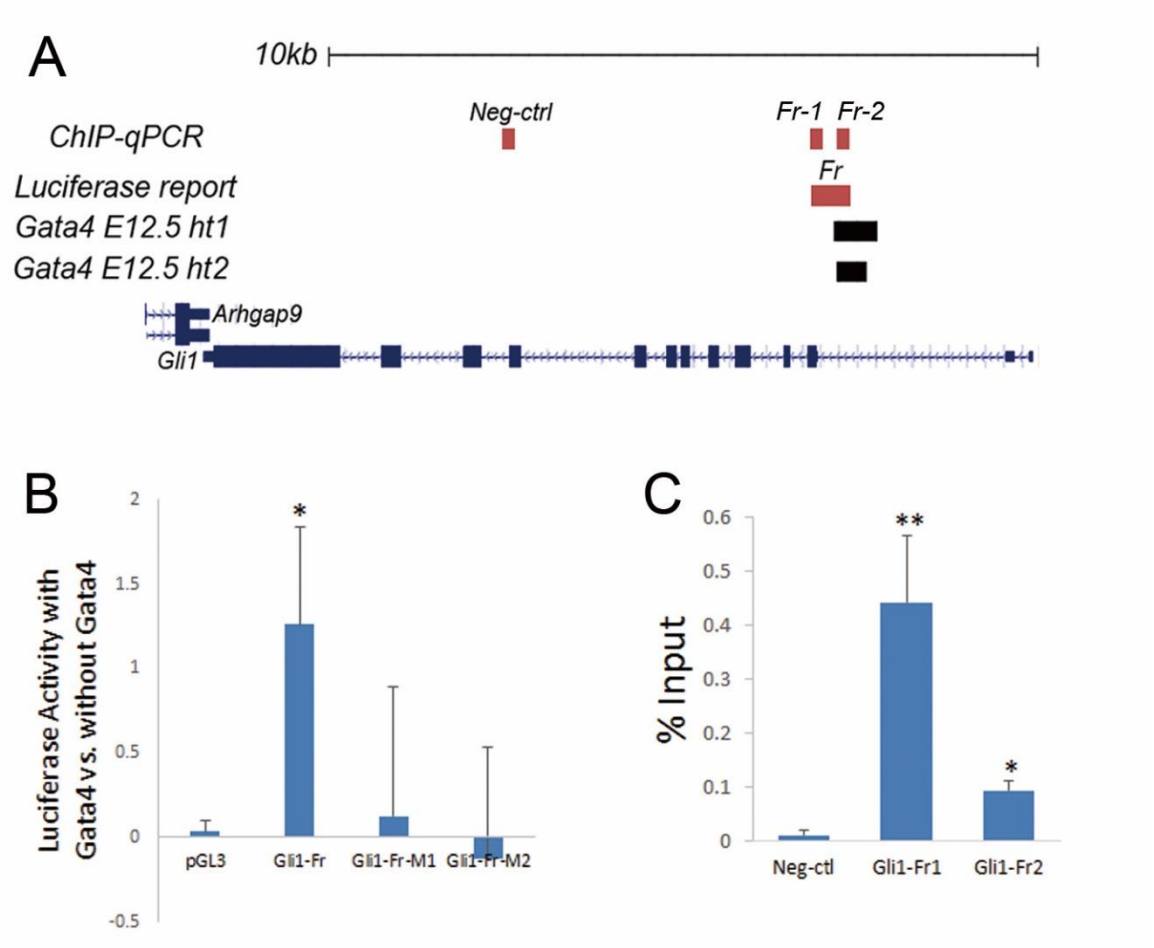


Figure 9. Gata4 directly regulates expression of Hh signaling effector *Gli1*

(A) Schematic figure showing positions of amplified *Gli1* genomic regions for ChIP-qPCR and luciferase assay. Black blocks indicate Gata4 binding from published data (He 2014). Red blocks are regions tested by ChIP-qPCR (Fr1, Fr12 and Neg-ctrl) and luciferase assay analysis (Fr). (B) Gata4 stimulated firefly luciferase expression in wildtype *Gli1*-Fr, but not in mutated *Gli1*-Fr-M1 and *Gli1*-Fr-M2 fragments. Results are presented as mean±SEM; *P<0.05. (C) *Gli1* fragments containing Gata4 binding motifs were enriched compared to negative control genomic region. ** P<0.01, * P<0.05.

Table 3. Genomic regions assessed in luciferase reporter assay and ChIP-qPCR

GENE NAME	LUCIFERASE ASSAY				CHIP		
	Genomic fragment	Locus	Luciferase results	Gata4 binding sites in subcloned fragments	Genomic fragment	Locus	ChIP results
Gli1	Gli1-Fr	chr10:126775570-126776129	1.26±0.58 p=0.0478	chr10:126775655-126775660 chr10:126776103-126776108	Gli1-Fr1	chr10:126775576-126775736	0.44±0.12 p=0.0039
	Gli-Fr-M1	chr10:126775570-126776129	0.13±0.77 p=0.4711	chr10:126776103-126776108	Gli1-Fr2	chr10:126775984-126776129	0.09±0.01 p=0.0163
	Gli1-Fr-M2	chr10:126775570-126776129	-0.13±0.67 p=0.4270	chr10:126775655-126775660	Neg ctrl	chr10:126771190-126771322	0.01±0.01

*All genomic coordinates are shown in mouse genome build mm9.

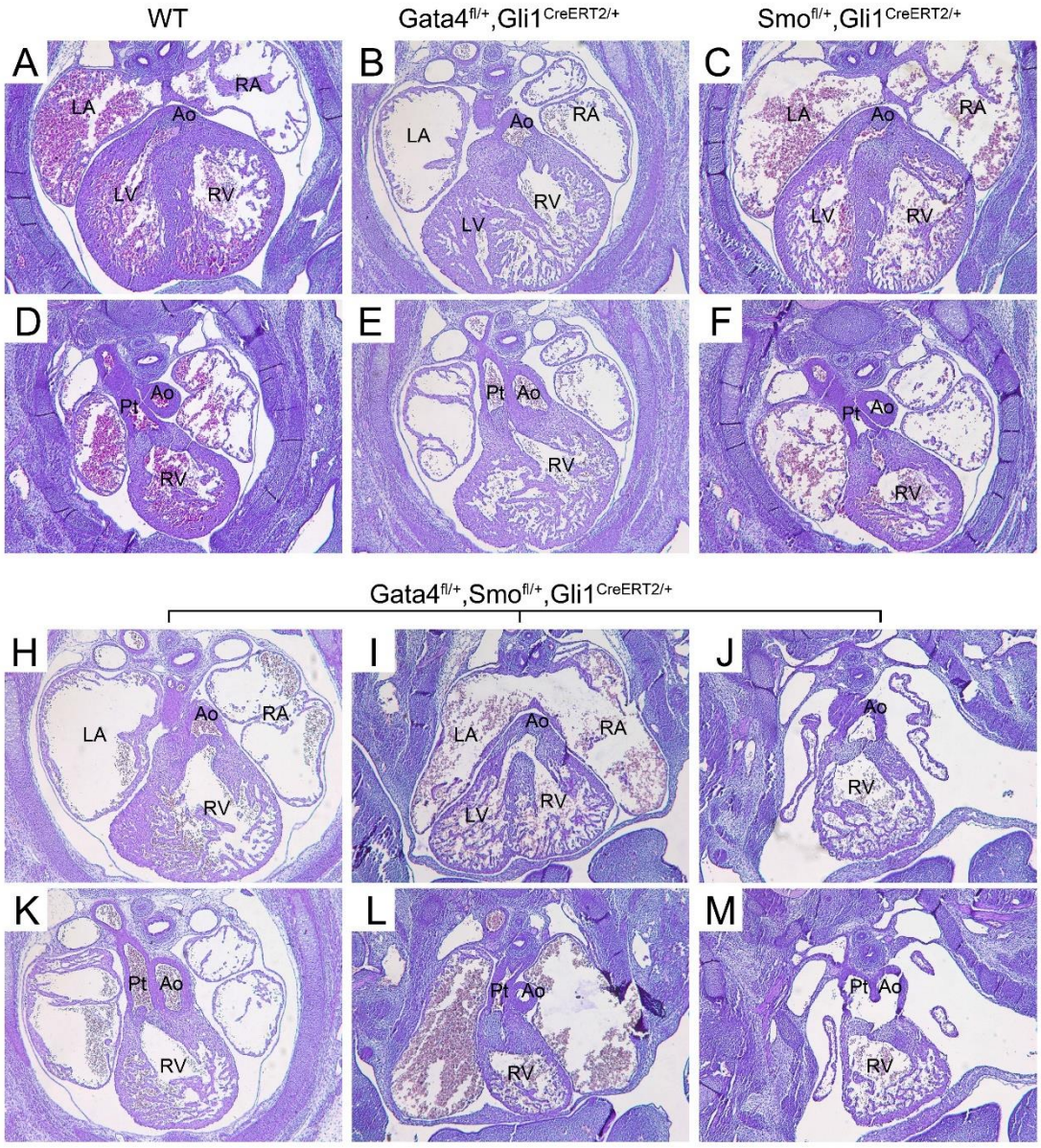


Figure 10. Further blocking Hh signaling leads to increased severity of OFT defect

Histology of embryos at E14.5 generated from crossing *Gata4*$^{fl/+}$ mice with *Smo*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ mice. Normal OFT structure was observed in both wildtype and *Smo*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos (A and D, C and F). DORV was observed in *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos (B and E). Three types of OFT of different severity were observed in *Gata4*$^{fl/+}$,*Smo*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos, including DORV (H and K), OA (I and L) and PTA (J and M)

3.8 OFT defect in embryos haploinsufficient for Gata4 in Hh receiving cells is rescued by overactivation of Hh signaling

SmoM2 is a mutated form of Smoothened which contains a point mutation W529L and renders it constitutively active (J. Xie et al., 1998). When *SmoM2* flox mouse line is crossed with a Cre line, Cre recombinase is expressed and cut off the stop sequence that inhibits the expression of *SmoM2*, resulting in enhanced Hh signaling in Cre expressing cells (Jeong et al., 2004). By crossing the $Gata4^{fl/+}$,$SmoM2^{fl/+}$ mouse line with $Gli1^{CreERT2/+}$ mouse line, we were able to study the relation between *Gata4* and Hh signaling when Hh signaling is enhanced. As shown in Figure 11, DORV was observed in $Gata4^{fl/+}$,$Gli1^{CreERT2/+}$ embryos (Figure 11B,F) as expected. Surprisingly, DORV was also observed in $SmoM2^{fl/+}$,$Gli1^{CreERT2/+}$ embryos (Figure 11C,G), suggesting the proper dosage of Hh-signaling is required for OFT alignment. However, we did not observe any type of OFT defects in $Gata4^{fl/+}$,$SmoM2^{fl/+}$,$Gli1^{CreERT2/+}$ embryos, which was significantly different than that of $Gata4^{fl/+}$,$Gli1^{CreERT2/+}$ embryos (0/9 vs. 3/6, p=0.044, Table 2; Figure 11D,H vs. 11B,F). These results further confirmed the crosstalk between *Gata4* and Hh signaling.

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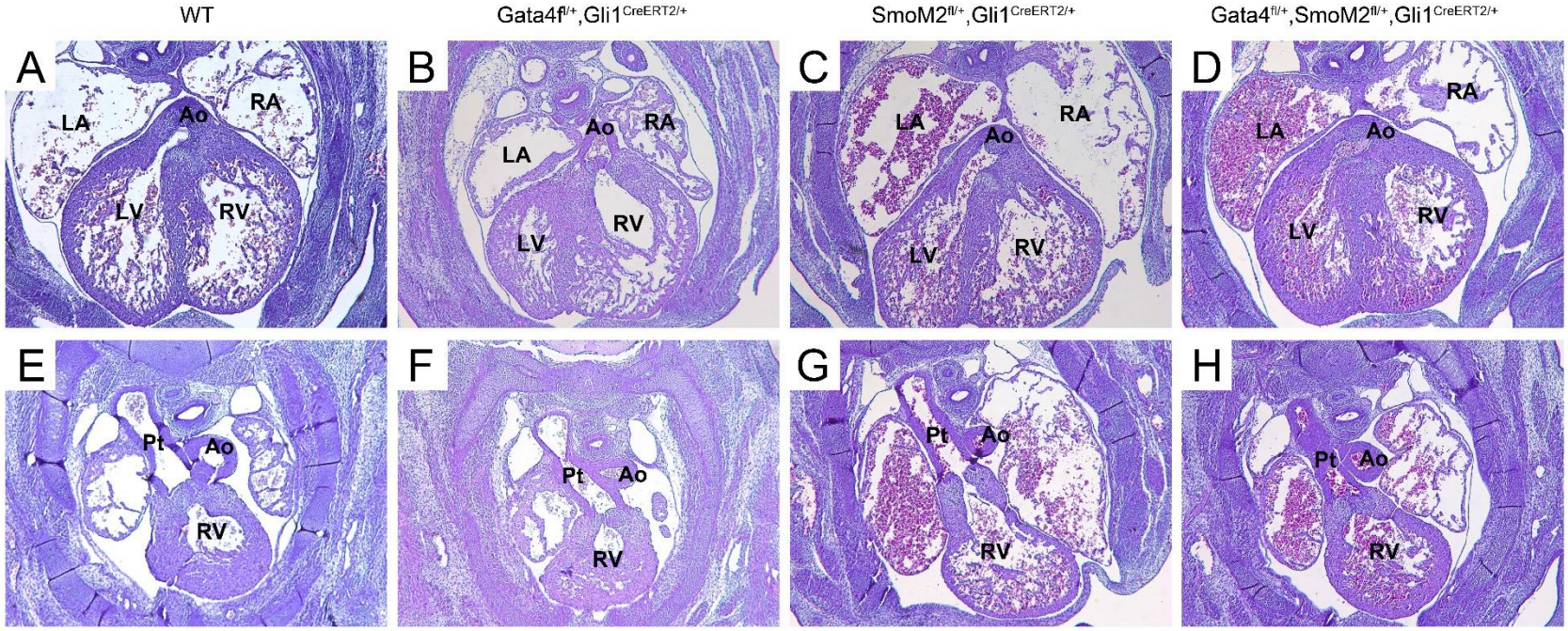

Figure 11. Enhanced Hh signaling rescues OFT defects

Histology of embryos at E14.5 from crossing Gata4$^{fl/+}$ mice with *SmoM2$^{fl/+}$,Gli1$^{CreERT2/+}$* mice. (A and E, C and G) Normal OFT morphology was observed in all wildtype and most *SmoM2$^{fl/+}$,Gli1$^{CreERT2/+}$* embryos (Table 2). DORV was observed in *Gata4$^{fl/+}$,Gli1$^{CreERT2/+}$* embryos (B,F). All *Gata4$^{fl/+}$,SmoM2$^{fl/+}$,Gli1$^{CreERT2/+}$* showed normal OFT morphology (D,H).

CHAPTER IV

DISCUSSION

OFT development is a complicated process that requires intricate spatiotemporal orchestration of several different cell progenitor populations both from the heart itself and from multiple extra-cardiac regions. Gata4 is a key cardiac transcription factor and has been associated with OFT defects for decades. However, rarely has it been studied the cell lineages in which Gata4 is required for OFT development and the pathways in which Gata4 plays a role. In the present study, we demonstrate that *Gata4* is required in the Hh-receiving cells for OFT lengthening and cardiac looping. Moreover, our work demonstrated that Gata4 interacts with Hh signaling pathway in regulating OFT development.

Previous study showed that *Gata4* is a dosage-sensitive regulator of cardiac morphogenesis (Laforest & Nemer, 2011; Maitra et al., 2009; Pu et al., 2004). Specifically, a 70% reduction of the normal expression level of Gata4 caused common atrioventricular canal (CAVC), DORV, myocardial hypoplasia and embryonic lethality in mice (Pu et al., 2004). In our study, germline deletion of one copy of Gata4 caused 39% ASD and 61% DORV in mutant embryos (Table 2), confirming the involvement of *Gata4* in guiding OFT development. Our data showed that mice with *Gata4* knockdown in myocardium, SHF cells and CNCCs exhibited normal OFT alignment at E14.5 (Figure 4), suggesting ablating one copy of Gata4 in these cell lineages does not affect OFT development

However, we cannot rule out the possibility that *Gata4* is still required in those cell lineages. It is possible that OFT development is not sensitive to one copy deletion of Gata4 in these cells lineage. For example, conditional knockdown of *Tbx5* in SHF which contains a cell population necessary for atrial septum development did not cause ASD, however *Tbx5* knockout in the same region resulted in ASD with a 100% penetrance (L. Xie et al., 2012). Therefore, in order to find out whether *Gata4* is required in SHF for OFT development, the next step is to investigate into the effect of *Gata4* KO in SHF on OFT development. It is possible the same situation applies to the results of *Gata4* knockdown in CNCCs, despite that our data is consistent with Rivera Feliciano's finding (Rivera-Feliciano et al., 2006). Further experiments detecting the transcription level of *Gata4* is necessary in order to test this possibility.

Hh-receiving cells marked by $Gli1^{Cre}$ expression is known to migrate from SHF into atrial septum and pulmonary trunk between E9.5 and E11.5 (Andrew D Hoffmann et al., 2009). Informatively, our data shows *Gata4* haploinsufficiency in Hh-receiving cells between E8 to E10.5 not only caused high incidence of ASD and DORV (Table 2), but also lead to a hypoplastic conal OFT cushion which was caused by decreased cell proliferation (Figure 6 and 7), as well as a significant OFT lengthening defect (Figure 6). These data suggested to us that OFT development is very sensitive to reduced level of Gata4 in Hh-receiving cells during E8 to E10.5.

However, considering that Hh-receiving cells do not mark the conal portion of the OFT cushion by the GIFM (Andrew D Hoffmann et al., 2009), and that Shh is required for SHF cells and CNCCs to survive and populate into OFT ((Laura A Dyer & Kirby, 2009; Goddeeris et al., 2007)), it is possible that the OFT defects observed in

Gata4$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos is caused by abnormal cell apoptosis, proliferation, differentiation or migration of the SHF and CNC cells. Future study is needed to find out: 1. are cell survival and proliferation in the SHF and CNC changed? 2. are migration and differentiation of the SHF and CNC cells affected in *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos?

It is interesting that a hypoplastic conal OFT was observed in *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos, which is possible due to less mesenchymal cells via EMT in the *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ embryos. Therefore, future study remains to answer: 1. Does EMT defect present in conal OFT? 2. Is the initial signaling for EMT affected in the endocardium or myocardium? 3. Is the defect in EMT caused by Gata4 knocking down in the Hh-receiving cells?

The mechanism whereby transcription factor haploinsufficiency causes developmental phenotypes remains largely unanswered. Our RNA-Seq data and GSEA result shows that the SHF transcriptomes of the wildtype and *Gata4*$^{fl/+}$,*Gli1*$^{CreERT2/+}$ mutant were distinct from each other and did identify Hh signaling as one of the pathways that contribute to the distinction. Our qPCR data confirmed the involvement of Hh signaling pathway, suggesting that Gatat4 regulates the development of OFT through activation of Hh signaling. In our study, Gata4 upregulates Hh pathway activity through activation of one of its components, Gli1. Consistent with previous report that Gata factor bind to *Gli1* by both protein-DNA interaction and protein-protein interaction (Daoud et al., 2014), our data from luciferase assay and ChIP-qPCR further demonstrated that Gata4 directly binds to two Gata4-responsive cis-regulatory elements of Gli1 (Figure 9), providing clear evidence for the interaction between Gata4 and Hh signaling. Remarkably, SHF-specific

constitutive Hh signaling rescued DORV/OA in *Gata4* mutant embryos, indicating Gata4 functionally plays upstream of Hh-signaling for OFT development.

In summary, we found *Gata4* is required in Hh receiving cells for OFT development. Further analysis of the requirement leads us to propose a novel mechanistic model in which Gata4 interacts with Hh signaling in regulating OFT development. Together these observations suggest a model in which deficiency of discrete pathways of Gata4 downstream targets are required for abnormal OFT alignment and CHD causation.

APPENDIX

List of abbreviations

ASD	atrial septal defect
AVC	atrioventricular cushion
AVSD	atrioventricular septal defect
BMP	bone morphogenetic protein
BrdU	bromodeoxyuridine
CHD	congenital heart disease
ChIP	chromatin immunoprecipitation
CNCC	cardiac neural crest cell
DAB	3,3′-Diaminobenzidine
DORV	double outlet right ventricle
EMT	endothelial to mesenchymal transition
FGF	fibroblast growth factor
GSEA	Gene set enrichment analysis
HCG	human chorionic gonadotropin
Hh	hedgehog
lacZ	β-galactosidase
OA	overriding aorta
OFT	outflow tract
PDA	paten ductus arteriosus
PHF	primary heart field
PMS	pregnant mare serum
PTA	persistent truncus arteriosus

RARE	retinoic acid response element
SAM	significance analysis of microarray
SHF	second heart field
TdT	terminal deoxynucleotidyl transferase
TGA	transportation of great artery
TMX	tamoxifen
TOF	tetralogy of Fallot
VSD	ventricular septal defect
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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