MOLECULAR PATHOGENESIS OF GENETIC AND INHERITED DISEASES

***Chd7* Is Critical for Early T-Cell Development and Thymus Organogenesis in Zebrafish**

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Coloboma, heart defect, atresia choanae, retarded growth and development, genital hypoplasia, ear anomalies/deafness (CHARGE) syndrome is a congenital disorder affecting multiple organs and mainly caused by mutations in *CHD7*, a gene encoding a chromatin-remodeling protein. Immunodeficiency and reduced T cells have been noted in CHARGE syndrome. However, the mechanisms underlying T lymphopenia are largely unexplored. Herein, we observed dramatic decrease of T cells in both *chd7* knockdown and knockout zebrafish embryos. Unexpectedly, hematopoietic stem and progenitor cells and, particularly, lymphoid progenitor cells were increased peripherally in nonthymic areas in *chd7*-deficient embryos, unlikely to contribute to the T-cell decrease. Further analysis demonstrated that both the organogenesis and homing function of the thymus were seriously impaired. *Chd7* might regulate thymus organogenesis through modulating the development of both neural crest cell-derived mesenchyme and pharyngeal endoderm-derived thymic epithelial cells. The expression of *foxn1*, a central regulator of thymic epithelium, was remarkably down-regulated in the pharyngeal region in *chd7*-deficient embryos. Moreover, the T-cell reduction in *chd7*-deficient embryos was partially rescued by overexpressing *foxn1*, suggesting that restoring thymic epithelium may be a potential therapeutic strategy for treating immunodeficiency in CHARGE syndrome. Collectively, the results indicated that *chd7* was critical for thymic development and T-lymphopenia in CHARGE syndrome may be mainly attributed to the defects of thymic organogenesis. The current finding may benefit the diagnosis and therapy of T lymphopenia and immunodeficiency in CHARGE syndrome. (*Am J Pathol* 2018; 188: 1043–1058; <https://doi.org/10.1016/j.ajpath.2017.12.005>)

Coloboma, heart defect, atresia choanae, retarded growth and development, genital hypoplasia, ear anomalies/deafness (CHARGE) syndrome (Online Mendelian Inheritance in Man no. 214800) is a complex genetic developmental disorder featuring a unique combination of multiple organ anomalies, including coloboma, heart defects, atresia of the choanae, retarded growth and development, genital hypoplasia, and ear anomalies.^{1,2} Additional features have been recognized in CHARGE syndrome since its first description, such as cranial nerve defects, anosmia, and renal and skeletal abnormalities.^{3–6} Mutations within the

chromodomain helicase DNA binding protein 7 (*CHD7*) gene are the major known cause of CHARGE syndrome, accounting for the disease in at least 60% of affected individuals.^{2,7}

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CHARGE patients usually experience recurrent infections during infancy, and these infections are presumed to result from anatomic anomalies of the craniofacial region and upper airway and defects of the innervating cranial nerves. The contribution of immunodeficiency to these infections has been noted in some case reports, but has received only limited attention.^{8–11} Recent clinical studies suggest that immunodeficiency is more commonly involved than previously thought. For instance, Wong et al¹² reviewed immunodeficiency in CHARGE syndrome and found that 80% of the patients had T lymphopenia. Furthermore, *CHD7* is recognized as a susceptibility gene for severe combined immunodeficiency in newborn screening studies.^{13,14} Thymic aplasia or hypoplasia has been reported in patients with CHARGE syndrome. However, thymus has not been routinely examined in CHARGE patients and the rate of thymic defects varied dramatically in different reports.^{12,15} The current understanding of immunodeficiency, T-cell reduction, and thymus defects in CHARGE syndrome is largely limited to clinical observations and the underlying pathogenic mechanisms are largely unexplored experimentally.

In this study, we investigated the role of *chd7* in T-cell and thymic development using zebrafish as a model organism. Knockdown or deletion of *chd7* decreased hematopoietic stem and progenitor cell (HSPC) numbers in the thymus but increased those numbers in peripheral nonthymic regions of zebrafish embryos. We revealed that *chd7* was critical for thymic organogenesis through regulating both thymic epithelial cells (TECs) and neural crest cells (NCCs). Moreover, T-cell deficiency may be partially rescued by forced expression of *foxn1*. Last, we suggested a molecular pathway regulated by *chd7* that is crucial for thymic organogenesis. These results suggested the involvement of thymic dysplasia and T lymphopenia in immunodeficiency in CHARGE syndrome.

Materials and Methods

Fish Strains and Embryos

Zebrafish strains, including wild type (AB strain), *chd7* mutant, *rag2*:enhanced green fluorescence protein (EGFP), *lyz*:DsRed, and *coro1a*:EGFP transgenic lines, were purchased from the China Zebrafish Resource Center (Wuhan, China). Embryos were obtained by natural spawning and raised in E3 buffer at 28°C. Adult zebrafish were raised in a zebrafish breeding system (HAISHENG Biotech, Shanghai, China) at 28°C. This study was approved by the Ethical Review Committee of Nanchang University (Nanchang, China).

Morpholinos, Primers, mRNA Synthesis, and Microinjection

Standard morpholino (MO) and antisense MO were purchased from GeneTools (Philomath, OR) and prepared as 1 mmol/L stock solutions using double-distilled water (Millipore,

Burlington, MA). The sequences of MOs and the primers used for amplification of RNA probes and coding sequences are listed in Table 1. Capped zebrafish full-length *foxn1* mRNA for injection was synthesized *in vitro* using the mMessage mMachine SP6 transcription kit (Ambion, Carlsbad, CA). MOs (8 ng for *chd7* MO and standard MO) and capped mRNA (100 pg) were injected alone or together into one cell stage zebrafish embryos at the yolk/blastomere boundary.

PCR

All primers used for the preparation of whole mount *in situ* hybridization (WISH) probes, RT-PCR, and real-time quantitative PCR (qPCR) are listed in Table 1. Total RNA was extracted by grinding dechorionated 15 to 20 embryos using RNAiso plus (Takara, Shiga, Japan) and followed by phenol-chloroform extraction. Reverse-transcription reaction was performed by using M-MLV Reverse Transcriptase (Takara). Wild-type and morphant *chd7* transcripts were detected using RT-PCRs. qPCR was performed using the SYBR Premix Ex Taq II (Takara) on the Abi-Step-One plus Real-Time PCR system (Applied Biosystems, Foster City, CA). All of the experiments were repeated three times with triplicates. The data were analyzed using the $\Delta\Delta C_t$ method. Data were represented as means $\pm$ SD, and *t*-test was performed for comparison between control and injected groups. *P* < 0.05 indicates significant difference.

Whole-Mount *in Situ* Hybridization

WISH was performed, as previously described,¹⁶ using probes for *chd7*, *rag1*, *il7r*, *cmyb*, *ikaros*, *mfap4*, *mpo*, *foxn1*, *ccl25a*, and *ccl25b*. Briefly, staged embryos were fixed overnight in 4% paraformaldehyde, and then dehydrated in a methanol gradient. When ready to use, embryos were rehydrated in phosphate-buffered saline containing 0.1% Tween-20. Embryos were permeabilized by proteinase K digestion and then hybridized with digoxin-labeled probes overnight at 70°C. The next day, embryos were washed in a preheated mixture of 50% saline–sodium citrate containing 0.1% Tween-20 and 50% hybridization solution at 70°C. Embryos were washed again at room temperature and incubated in staining solution in the dark until sufficient staining appeared in the embryos. Background staining was cleared by soaking the embryos in a benzylbenzoate-benzylalcohol solution. Embryos were mounted in glycerol and were visualized using a Nikon AZ100 microscope (Nikon, Tokyo, Japan). Images were captured using a Nikon DIGITAL SIGHT DS-Fi1 digital camera (Nikon) and processed with NIS-Elements F 3.0 (Nikon).

Whole-Mount Immunostaining

For immunofluorescence microscopy, immunostaining was performed as described previously.¹⁷ Larval zebrafish were fixed overnight with 4% paraformaldehyde in PBS, followed by methanol dehydration and rehydration. After

Table 1 Sequences of the MO and Primers Used in this Study

Name	Sequence	Use
Chd7 MO	5'-TTATTTTCTGGCACTAACCATGTCC-3'	Gene knockdown
Chd7 ATG-MO	5'-TGCAGCCAAGCTTAGAAGCAGGAC-3'	
<i>ikaros</i> -F	5'-TTCCAGTGCAATCAATGTGGTGCTT-3'	qPCR
<i>ikaros</i> -R	5'-GCACACTTATGGGGCTTTCCAACCG-3'	
<i>rag1</i> -F	5'-TAGGAAAGAAGCGGACTAAACCACT-3'	qPCR
<i>rag1</i> -R	5'-GCACCGTGTGATATTCTTTACCCACT-3'	
<i>ccl25a</i> -F	5'-TGGGGACAACTACTTTATCTTGC-3'	qPCR
<i>ccl25a</i> -R	5'-CACCTCTTGTCTTCTGTAGCGGAT-3'	
<i>ccl25b</i> -F	5'-TTGTATGGAAACGCAGAGTTCAACC-3'	qPCR
<i>ccl25b</i> -R	5'-CTCATCTTGAAAACAACAGCGGGAA-3'	
<i>il7r</i> -F	5'-TGCAAGTACACTTAAGAGACGGCAT-3'	qPCR
<i>il7r</i> -R	5'-GCTCATGTTACACCGGATCTCCA-3'	
<i>foxn1</i> -F	5'-TTTAAATAGCGTGAATTCTATGCTG-3'	qPCR
<i>foxn1</i> -R	5'-CTACCGCTTCACATGAACGAG-3'	
<i>rag2</i> -F	5'-AATGAAGATGACCCACCGCAAG-3'	qPCR
<i>rag2</i> -R	5'-CACTCATAATGCCCATTCAATACAGT-3'	
<i>elf1a</i> -F	5'-GATCTACAAATGCGGTGGAATCGAC-3'	qPCR
<i>elf1a</i> -R	5'-GGAGCGGTGAGTTTGAGGCTGGTATCTCC-3'	
<i>ikaros</i> -F	5'-TTGGAAAGCCCCATAAGTGTGCAT-3'	WISH probe
<i>ikaros</i> -R	5'-GTACCCGCATAGGTTGCACTCA-3'	
<i>rag1</i> -F	5'-AGACTGGAAGCCCCACACCTCT-3'	WISH probe
<i>rag1</i> -R	5'-CCTCTGGCACCCTGTGATATTC-3'	
<i>hand2</i> -F	5'-ACCTCATTGATTCCACAACGTGCTC-3'	WISH probe
<i>hand2</i> -R	5'-TAAGCGATATAACTGGTAGCTAAACGA-3'	
<i>Nkx2.3</i> -F	5'-AGTCCCGGCTTCTCAGACAACGA-3'	WISH probe
<i>Nkx2.3</i> -R	5'-TTCAGCCATCATGGTATACTGTTGC-3'	
<i>dlx2a</i> -F	5'-GCCAAAGAAAGTCCGAAAACCTCGT-3'	WISH probe
<i>dlx2a</i> -R	5'-TCAGGTTATTTTCAAGAAGGCACCA-3'	
<i>crestin</i> -F	5'-ACCAACCTGCTGACACAGACCCACT-3'	WISH probe
<i>crestin</i> -R	5'-TGTTGACCACTGCAATATGCCGAT-3'	
<i>ccl25a</i> -F	5'-TGGGGACAACTACTTTATCTTGC-3'	WISH probe
<i>ccl25a</i> -R	5'-GCAGTGTGTGAGAAATGAATATAATCTCT-3'	
<i>ccl25b</i> -F	5'-AGTTTCAAATCTGGCTTTGCTC-3'	WISH probe
<i>ccl25b</i> -R	5'-ACACACAAACATACACGCACAC-3'	

ATG, translation-blocking; Chd, chromodomain helicase DNA binding protein; F, forward; MO, morpholino; qPCR, real-time quantitative PCR; R, reverse; WISH, whole mount *in situ* hybridization.

permeabilization by ice-cold acetone treatment and proteinase K digestion, embryos were incubated with primary and secondary antibodies at the following concentrations: 1:500 mouse anti-alcama (Zirc Co, Buffalo, MN), 1:500 goat anti-GFP (Rockland, Limerick, PA), 1:500 anti-goat IgG Alexa Fluor 488 (Invitrogen, Carlsbad, CA), and 1:500 anti-mouse IgG (Invitrogen). Immunostained embryos were imaged using an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan).

Terminal dUTP Nick End Labeling Assay

Wild-type or *coro1a*:EGFP transgenic embryos were fixed in 4% paraformaldehyde at 4°C overnight and then dehydrated in a methanol gradient and preserved in 100% methanol at -20°C overnight. After rehydration, the embryos were washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20 three times and treated with 5 µg/mL proteinase K for permeabilization. Then, the permeabilized

embryos were refixed in 4% paraformaldehyde for 30 minutes at room temperature with gentle shaking. After being washed three to five times with PBS containing 0.1% Tween-20, the embryos were incubated with a mixture containing 45 µL labeling solution and 5 µL enzyme solution (*In Situ* Cell Death Detection Kit Fluorescein; Roche, Basel, Switzerland) at 4°C overnight. Finally, the stained embryos were washed three times with PBS containing 0.1% Tween-20 and imaged using an Olympus FV1000 confocal microscope.

Confocal Microscopy

Live embryos were anesthetized with tricaine and then mounted onto a coverslip with 1% low-melting agarose. The agar layer was further covered with E3 buffer containing 0.0084 mg/mL tricaine to enable movies to be taken over a long time frame. For live imaging of the thymus, the recordings were started at 60 hours post-fertilization (hpf) and continued for 12 hours. Images were taken every 6 or 5

Figure 1 T-cell development is impaired in *chd7* zebrafish morphants. **A:** The expression pattern of endogenous *chd7* transcripts at different time points. **B:** The expression of T-cell markers, including *rag1*, *ikaros*, and *il7r*, in control and *chd7* morphants. Anterior to the left and dorsal to the top, **red dashed circles** mark the thymus. **C:** Quantitative RT-PCR analysis showing the mRNA level of *rag1*, *ikaros*, and *il7r* in control and *chd7* morphants (*t*-test). **D:** *rag2*:Enhanced green fluorescence protein (EGFP) expression in control and *chd7* morphants at 5 days post-fertilization (dpf). The **red arrowheads** indicate the *rag2*:EGFP expression in thymus. Data are expressed as means $\pm$ SD. *** $P < 0.001$. Hpf, hours post-fertilization; MO, morpholino. Original magnification: $\times 3$ (A, 24 hpf, 48 hpf, and 72 hpf); $\times 6$ (A, 96 hpf); $\times 15$ (B); $\times 20$ (D).

minutes, and single frames were generated as projections of a stack of 46 planes with 2- μ m distance, covering a depth of 90 μ m. For imaging immunostained samples, embryos were mounted onto a coverslip with 1% low-melting agarose. Confocal images were captured by an Olympus FV1000 confocal microscope and three-dimensional projections were generated with the FV100-ASW 4.2 viewer (Olympus). Single frames were generated as projections of a stack of multiple planes. The numbers of immunofluorescence- and terminal dUTP nick end labeling cells were calculated using Imaris7 (Bitplane, Zürich, Switzerland).

Alcian Blue Staining

Zebrafish larvae [5 days post-fertilization (dpf)] were fixed in 4% paraformaldehyde at 4°C overnight. Then, the fixed

larvae were digested in 10% trypsin for 1 hour. After washing in PBS three times, the larvae were stained in 0.1% (w/v) Alcian blue (Sigma, Darmstadt, Germany) for 3 hours. Then, the staining reaction was terminated with PBS, and the larvae were washed again in PBS. The stained larvae were mounted in 90% glycerol and visualized using a Nikon AZ100 microscope. Images were captured using a Nikon DIGITAL SIGHT DS-Fil1 digital camera and processed with NIS-Elements F 3.0 software (Nikon).

Statistical Analysis

Values are presented as the means (SD). *t*-Test was used to compare means of different groups, and $P < 0.05$ was considered significant.

Figure 2 Hematopoietic stem and progenitor cell numbers are reduced in the thymus but increased in the caudal hematopoietic tissue (CHT) region in *chd7* morphants. **A:** The expression of hematopoietic progenitor markers, *ikaros* and *cmyb*, in control and *chd7* morphants at 48 hours post-fertilization (hpf). Red arrows denote hematopoietic progenitors expressing *ikaros* or *cmyb* in the aorta-gonad-mesonephros region. **B:** Quantitative RT-PCR analysis showing the mRNAs of *cmyb*, *ikaros*, *mpo*, and *maf4* in control and *chd7* morphant at 48 hpf (t-test). **C:** The expression of T-lymphoid primed progenitor marker, *irf4a*, in control and *chd7* morphant at 48 hpf. Red arrows indicate the T-lymphoid primed cells expressing *irf4a* in the CHT region. **D:** Double transgenic [*coro1a*:enhanced green fluorescence protein (EGFP)/*lyz*:DsRed] wild-type and *chd7* morpholino (MO)-injected embryos showing the numbers of lymphoid progenitors, macrophages, and neutrophils in the CHT region of control and *chd7* morphants at 60 hpf. White dashed rectangles indicate the posterior part of the CHT region. **E:** Enumeration of *coro1a*:EGFP-positive and *lyz*:DsRed-negative cells or *lyz*:DsRed cells shown in **D**. **F:** The expression of *mpo* and *mfap4*, which mark neutrophils and macrophages, respectively, in the CHT region of control and *chd7* morphants at 3 dpf. Red arrows denote neutrophils expressing *mpo* or macrophages expressing *mfap4* in the CHT region. **G:** Neutrophils marked by *lyz*:DsRed expression in the CHT region of control and *chd7* morphants at 3 days post-fertilization (dpf). White arrows indicate the DsRed-positive neutrophils. **H:** The expression of *cmyb* in control and *chd7* morphants at 4 dpf. Red dashed rectangles denote the pharyngeal regions; blue dashed rectangles, the CHT regions; red dashed circles, the region of thymus; and red arrows, the hematopoietic stem progenitor cells and myeloid lineage cells in the CHT region. Data are expressed as means $\pm$ SD. * $P < 0.05$. Original magnification: $\times 9$ (A, C, and F); $\times 10$ (D and G); $\times 1.8$ (H).

Results

T-Cell Development Is Impaired in *chd7*-Deficient Embryos

Embryonic T lymphopoiesis consists of several successive steps, and the process is evolutionarily conserved between zebrafish and higher vertebrates.¹⁸ In zebrafish, at around 48 hpf, the endodermal progenitors in the third pharyngeal pouch begin to differentiate into TECs, marked by the expression of tissue-specific transcription factors, *foxn1*. Subsequently, by interacting with postmigratory neural crest cells derived from the dorsal ectoderm, the thymic anlage detaches from the pharynx and moves into a location underneath the otic vesicles. Meanwhile, lymphoid progenitors in the aorta-gonad-mesonephros and caudal hematopoietic tissue (CHT) regions migrate into the

thymic rudiment and become established for subsequent differentiation and maturation, and then T lymphopoiesis is initiated after the expression of *rag1* at 78 hpf.¹⁹

WISH was performed in zebrafish embryos, and it was found that *chd7* was mainly expressed in central nervous system and gut (Figure 1A). It could be detected in rhombomeres at 24 hpf, from which some neural crest cells are derived (Figure 1A). In the developing pharynx from 48 to 96 hpf, *chd7* was also strongly expressed (Figure 1A). These WISH data indicated that *chd7* might participate in multiple aspects of embryonic development, including neural crest development and pharyngeal segmentation, which are related to thymic development.

An antisense oligonucleotide MO targeting the junction between exon 13 and intron 13 of *chd7* to block splicing²⁰

Figure 3 Thymic epithelium development is severely impaired in *chd7* morphants. **A:** The expression of a thymic marker, *foxn1*, in the thymus of control and *chd7* morphants. **Red dashed circles** mark the thymus. **B:** Quantitative RT-PCR showing the mRNA level of *foxn1* in control and *chd7* morphants at 4 days post-fertilization (dpf; *t*-test). **C:** The morphology of endodermal pouches shown by expression of *nkx2.3*, in control and *chd7* morphants at 48 hours post-fertilization (hpf). Numbers indicate the pharyngeal pouches, and **red asterisks** and **red line** indicate the moderate and severe defects, respectively, in endodermal pouches 3, 4, and 5. **D:** Immunohistochemistry with *zn5* showing the morphology of endodermal pouches in *chd7* morphants at 48 hpf. Numbers denote the pharyngeal pouches, and **red asterisk** indicates the absence of fifth pharyngeal pouch. Data are expressed as means $\pm$ SD. *******P* < 0.01. MO, morpholino. Original magnification: $\times 12$ (A); $\times 15$ (C); $\times 20$ (D).

(Table 1) was injected into zebrafish zygotes. Sequencing the RT-PCR product revealed that intron 13 was retained in the transcript, introducing a premature stop codon at the 1175th codon and presumably resulting in a truncated protein missing SNF2, helicase, and BRK domains (Supplemental Figure S1). Several CHARGE-like phenotypes were observed among the *chd7* morphants, including coloboma, abnormal otoliths, cartilage defects, and reversed heart looping (Supplemental Figure S2), which were consistent with a previous report.²⁰

To explore the role of *chd7* in T lymphopoiesis, WISH was performed to check the expression of several T-cell markers, including *rag1*, *ikaros*, and *il7r*. Compared with control, the expression of all these markers was dramatically reduced in the thymus at 4 dpf (Figure 1B). Real-time quantitative PCR further confirmed the WISH observations that T-cell markers were significantly down-regulated in 4-dpf morphants (Figure 1C). Moreover, when *chd7* MO was injected into a *rag2:EGFP* transgenic fish line at the single cell stage, the number of GFP-positive T-cell precursors was significantly decreased at 5 dpf (Figure 1D). Taken together, these results indicated that knockdown of *chd7* may model the T lymphopenia found in CHARGE patients.

Hematopoietic Progenitor and T-Lymphoid Primed Progenitor Numbers Are Increased in the Nonthymic Region of *chd7* Morphants

The reduction of T cells may be attributable to the decrease of HSPCs, the defects of thymus organogenesis, or abnormal proliferation or apoptosis of thymocytes. The development of HSPCs was first studied. Surprisingly, HSPCs were increased in the aorta-gonad-mesonephros and CHT regions in *chd7* morphants at 48 hpf, as indicated by the expression of *cmyb* and *ikaros*, two HSPC markers at the prethymic stage (Figure 2A). qPCR data further confirmed the WISH findings (Figure 2B). To further address the development of lymphoid progenitors, the expression of *irf4a*, a recently identified marker of T-lymphoid primed progenitors,²¹ was examined, and it was also found to be increased in the CHT region at 50 hpf in *chd7* morphants (Figure 2C). Because *irf4a* is only expressed in a small population of T-lymphoid-primed progenitors, we used *coro1a:EGFP/lyz:DsRed* double transgenic embryos to mark the lymphoid progenitors and macrophages (*coro1a:EGFP* expression marks both myeloid and lymphoid lineages, and *lyz:DsRed* expression

Figure 4 The development of post-migratory neural crest cells is impaired in *chd7* morphants. **A:** The expression of post-migratory neural crest cells markers, *dlx2a* and *hand2*, in pharyngeal arches in control and *chd7* morphants at 30 hours post-fertilization (hpf). **Red arrows** indicate the reduced post-migratory neural crest cells. **B:** The expression of post-migratory neural crest cells markers, *dlx2a* and *hand2*, in pharyngeal arches in control and *chd7* morphants at 48 hpf. Numbers denote the pharyngeal arches; **red asterisks**, the defective pharyngeal arches; **red line**, the obscure pharyngeal arches. MO, morpholino. Original magnification: $\times 9$ (**A**, lateral views); $\times 12$ (**A**, dorsal views, and **B**).

marks neutrophils).²² The GFP-positive but DsRed-negative cells (lymphoid progenitors and macrophages) were modestly increased in the CHT region on *chd7* knockdown (Figure 2, D and E). These results implied that the T-cell deficiency in *chd7* morphants was unlikely to be attributed to a lack of T-lymphoid-primed progenitors at prethymic stage. It was further tested whether the HSPCs and myeloid lineages were changed when thymus rudiment was formed. The expression of *mpo* and *mfap4*, which mark neutrophils and macrophages, respectively, was first examined. Both WISH and qPCR data showed that *mpo* and *mfap4* were increased in the CHT region at 3 dpf (Figure 2, B and F). Moreover, when the *chd7* MO was injected into *Lyz:DsRed* transgenic embryos, the DsRed-positive neutrophils were increased at 3 dpf (Figure 2G). Furthermore, to overview the distribution of hematopoietic cells in *chd7* morphants, the expression of *cmyb*, which was expressed in HSPCs and myeloid lineage cells, was examined. The expression of *cmyb* was specifically reduced in the thymus, whereas it was moderately increased in the CHT region of *chd7* morphants at 4 dpf (Figure 2H). Analogously, when the *chd7* MO was injected into *coro1a:EGFP* transgenic embryos, a specific

reduction of EGFP-positive T cells was detected in the thymus at 4 dpf (Supplemental Figure S3). Collectively, our data demonstrated that HSPCs were specifically reduced in the thymic region but increased in the nonthymic region, suggesting that the T-cell defect in *chd7* morphants seemed unlikely to be explained by the increase of T-lymphoid progenitors. Alternatively, it may be caused by the defects of other T-cell development processes (eg, thymic organogenesis).

The Development of Thymic Epithelial Cells Is Severely Impaired in *chd7* Morphants

TECs are the primary functional cells that form the micro-environment to support T-cell differentiation. FOXP1 is a marker of TECs and a pivotal regulator of TEC differentiation, proliferation, and survival²³; therefore, its expression was examined. Both WISH and qPCR data showed a dramatic reduction of *foxp1* expression in the thymus of *chd7* morphants at 4 dpf (Figure 3, A and B). Because both the cortical and medullary TECs might arise from the single endodermal layer in the third pharyngeal pouch,²⁴ it was

Figure 5 The thymic homing is disrupted and chemokine ligands are down-regulated in *chd7* morphants. **A:** Whole mount *in situ* hybridization for *ccl25a* and *ccl25b* in the control and *chd7* morphants at 54 hours post-fertilization (hpf). Red dashed circles and rectangles indicate the thymic regions. **B:** Real-time quantitative PCR analysis of mRNA level of *ccl25a* and *ccl25b* in the control and *chd7* morphants at 54 hpf (*t*-test). **C:** Number of *coro1a*:enhanced green fluorescence protein (EGFP) cells entering the thymus every 60 minutes in the control and *chd7* morphants during the homing process from 60 to 72 hpf. **D:** Confocal imaging of *coro1a*:EGFP/*lyz*:DsRed embryos showing the numbers of *coro1a*:EGFP cells in the thymus in control and *chd7* morphants. White circles indicate the thymus. Data are expressed as means $\pm$ SD. *n* = 3 (**C**). $**P < 0.01$. Original magnification: $\times 9$ (**A**); $\times 60$ (**B**). MO, morpholino.

tested whether the development of pharyngeal pouches was impaired in *chd7* morphants. To address this, the expression of *nkx2.3*, a homeobox transcription factor that is expressed in the endoderm of developing pharyngeal pouches in vertebrates, was examined.²⁵ The expression of *nkx2.3* in *chd7* morphants was greatly decreased in pouches 3, 4, and 5, and the whole pharynx appeared shrunken (Figure 3C). To further corroborate this result, immunohistochemistry was performed with antibody zn5, which recognizes activated leukocyte cell adhesion molecule expressed in pharyngeal endoderm.^{26,27} The immunofluorescence in all five pharyngeal pouches was decreased, indicating that the development of these pouches was disrupted in *chd7* morphants (Figure 3D). Taken together, these results indicated that both morphogenesis of pharyngeal endoderm and TEC differentiation were disrupted in *chd7* morphants.

The Development of Post-Migratory Neural Crest Cells Is Impaired in Pharyngeal Arches in *chd7* Morphants

NCCs are a transient population of pluripotent cells arising from the dorsal ectoderm and are required for thymus

development.^{28–30} CHD7 has been characterized as a critical regulator in migratory and multipotent neural crest cells.³¹ Therefore, it is reasonable to speculate that the impaired thymus organogenesis in *chd7* morphants is partially attributed to impaired development of NCCs. The expression of migratory neural crest marker, *crestin*, was almost absent in *chd7* morphant rhombomeres at 24 hpf (Supplemental Figure S4). To further check the development of post-migratory NCCs, the expression of two post-migratory NCC markers was examined, *hand2* and *dlx2a*. Reduced expression of *hand2* was observed in the developing pharynx at 30 hpf, and the *dlx2a* expression was specifically reduced in the population underneath the otic vesicle at 30 hpf, which would be segmented into pharyngeal arch 5 (Figure 4A). As pharyngeal segmentation continued, a distinct reduction in *hand2* expression may be observed in pharyngeal arches 4 and 5 and a prominent reduction may also be detected in the expression of *dlx2a* in pharyngeal arch 4 in *chd7* morphants at 48 hpf (Figure 4B). These results demonstrated that not only the development of the third endodermal pouch but also the surrounding post-migratory neural crest cells in the fourth pharyngeal arch were severely impaired in *chd7* morphants.

Figure 6 Reduced proliferation and increased apoptosis are present in *chd7* morphants. **A:** Immunohistochemistry of anti-phospho-histone H3 (PH3) antibody in control and *chd7* morphants at 48 and 5 days post-fertilization (dpf). **White dashed lines** indicate the pharynx and otic vesicle; **white ellipses**, the thymus; and **insets**, higher magnification of the thymus. **B:** Terminal dUTP nick end labeling (TUNEL) assay of the control and *chd7* morphants at 48 and 3 dpf. **White dashed lines** indicate the regions of the pharynx and otic vesicle; **white ellipses**, the thymus; and **insets**, higher magnification of the thymus. **C:** TUNEL assay and immunostaining for *coro1a*:enhanced green fluorescence protein (EGFP) transgenic wild-type and *chd7* morphants showing the apoptotic T-cell precursors in control and *chd7* morphants at 5 dpf. **White dashed rectangles** denote the thymic regions. **Insets** denote higher magnification of the thymus. Original magnification: $\times 14$ (**A** and **B**); $\times 20$ (**C**). Hpf, hours post-fertilization; MO, morpholino; OV, otic vesicle.

Homing of Lymphoid Progenitors to the Thymic Rudiment Is Impaired in *chd7* Morphants

It has been demonstrated that the ability of thymus to attract the migratory lymphoid progenitors is crucial for commitment of lymphoid progenitors to early T-cell progenitors and subsequent differentiation, survival, and proliferation. In this study, T-cell and thymic development were severely impaired in *chd7* morphants. It was examined whether the thymic homing was affected in *chd7* morphants. The expression of a chemokine ligand, *ccl25*, which was demonstrated to be indispensable for thymic homing and might be regulated by *foxn1* in mice and medaka, was first examined.³² Both qPCR and WISH data showed that *ccl25a* and *ccl25b* were down-regulated in the thymus of *chd7* morphants (Figure 5, A and B, and Supplemental Figure S5), indicating that the ability of the thymus to attract lymphoid progenitors might be impaired. To further analyze dynamic trafficking of T-cell progenitors to the thymus in *chd7* morphants, time-lapse imaging was used from 60 to 72 hpf. To distinguish lymphoid cells from myeloid cells, *coro1a*:EGFP/*lyz*:DsRed double transgenic embryos were used. The *coro1a*:EGFP⁺ and *lyz*:DsRed⁻ cells with round shape (lymphoid progenitors) were observed to migrate into the thymus of control embryos. However, a significantly

reduced number of these cells migrated into the thymus of *chd7* morphants (Figure 5, C and D, and Supplemental Video S1, S2, S3, and S4). These results indicated that the compromised ability of thymus to attract hematopoietic progenitors may also account for the reduced T cells in *chd7* morphants.

Reduced Proliferation and Increased Apoptosis Are Present in *chd7* Morphants

The defects of T lymphopoiesis and thymus organogenesis in *chd7* morphants may be caused by impaired cell proliferation or abnormal apoptosis. Immunostaining with an anti-phospho-histone H3 antibody was first performed to detect proliferating cells in control and *chd7* morphants. Phospho-histone H3-positive cells were significantly decreased in the region of the pharynx in *chd7* morphants at 48 hpf, when thymus organogenesis was initiated, indicating that reduced proliferation might account for the impaired thymus organogenesis (Figure 6A). Moreover, at 5 dpf, when the thymus rudiment was formed and lymphoid progenitors were settled in it for subsequent development, the proliferating cells were decreased in the thymus region of *chd7* morphants (Figure 6A). To evaluate apoptosis in *chd7* morphants, the terminal dUTP nick end labeling assay was

Figure 7 The expression of *rag1* and *rag2* can be rescued by *foxn1* overexpression in *chd7* morphants. **A:** Whole mount *in situ* hybridization (WISH) analysis for the expression of *rag1* in control, *chd7* morphant, *chd7* morphants coinjected with *foxn1* mRNA, and wild type injected with *foxn1* mRNA at 4 days post-fertilization (dpf) and WISH analysis for the expression of *rag2* at 5 dpf. The dashed red circles indicate the regions of thymus. **B:** Quantitative RT-PCR analysis of the expression of *rag1* and *rag2* in control, *chd7* morphants, *chd7* morphants coinjected with *zfoxn1* mRNA, and wild type injected with *zfoxn1* mRNA at 4 dpf. Data are expressed as means $\pm$ SD (**B**). * $P < 0.05$, *** $P < 0.001$. Original magnification: $\times 15$ (**A**, WISH results); $\times 20$ (**A**, ISH results). GFP, green fluorescence protein; MO, morpholino.

used. Apoptosis was not changed in the developing pharynx of *chd7* morphants at 48 hpf, when thymus organogenesis was initiated and the neural crest cells had finished their migration and settled in the developing pharynx, indicating that the impaired organogenesis of the thymus might not be attributed to apoptosis but at least partially to compromised proliferation in *chd7* morphants (Figure 6B). However, high numbers of apoptotic cells were detected in thymus area in *chd7* morphants at 3 dpf, when thymus rudiment was formed and lymphoid progenitors were settled in it (Figure 6C). Furthermore, to find out whether these intrathymic apoptotic cells included T-cell precursors, a half dose of *chd7* MO was injected into *coro1a:EGFP* embryos because a full dose of the MO severely compromised the homing of the lymphoid progenitors to thymus, and examined the apoptosis of intrathymic T-cell precursors. The cells dual labelled by terminal dUTP nick end labeling and GFP were much more than in the control group, indicating that increased apoptosis accounted for the reduction of *coro1a*-positive T cells in the thymus of *chd7* morphants. Moreover, examination of apoptosis in the CHT region showed no change in apoptosis (data not shown) (Figure 6C). These results suggested that the reduction of T cells in *chd7* morphants may be attributed to the impaired proliferation at early stage of thymus organogenesis and to the aberrant apoptosis in thymus at a later stage, particularly

the apoptosis of T-cell precursors. In combination with the HSPC results, these data implied that the decrease of T cells in *chd7* morphants may be mainly caused by the defects of thymus organogenesis.

T-Cell Decrease in *chd7* Morphants Can Be Partially Rescued by Forced Expression of *foxn1*

Our results indicated that the down-regulated *foxn1* might contribute to the impaired development of thymus in *chd7* morphants. Many studies have documented that *foxn1* is crucial for TEC differentiation and maintenance.^{23,33,34} Furthermore, TEC regeneration can be achieved by up-regulating *foxn1* expression.^{35–37} These findings prompted us to speculate whether overexpression of *foxn1* may ameliorate the defects of thymus organogenesis and restore the reduced T cells in *chd7* morphants. To test this idea, *foxn1* mRNA and *chd7* MO were coinjected into zebrafish embryos at the single-cell stage. More than half of the embryos survived in the *foxn1* overexpressing group (Supplemental Figure S6A). As anticipated, the expression of *rag1* was moderately restored at 4 dpf (Figure 7A). Similarly, when the coinjection was performed in *rag2:EGFP* transgenic embryos, the number of GFP-positive T-cell precursors was also moderately restored (Figure 7A). The qPCR results of T-cell markers further

Figure 8 *Chd7* regulates *foxn1* expression in thymus organogenesis, probably mediated by *bmp* signaling. **A:** The expression of *bmp4* and *bmp2b* in control and *chd7* morphants at 50 hours post-fertilization (hpf). Numbers denote the pharyngeal arches. **B:** The expression of *foxn1* in control and Dorsomorphin treatment group at 72 hpf. Dorsomorphin treatment was performed from 44 to 54 hpf. Red dashed circles indicate the thymus. Original magnification: $\times 12$ (A); $\times 15$ (B). MO, morpholino; OV, otic vesicle.

verified the rescue effects (Figure 7B and Supplemental Figure 6B). Moreover, when *foxn1* was injected into wild-type embryos, the expression of *ccl25a*, a chemokine ligand important for the homing function of TECs, was largely restricted to the thymus area. Interestingly, ectopic expression was occasionally detected (Supplemental Figure S7), further demonstrating that *foxn1* was critical for TEC differentiation and maintenance.

Chd7 Regulates *foxn1* Expression in Thymus Organogenesis, Which Is Probably Mediated by *bmp* Signaling

It has been well established that *foxn1* is crucial for thymus organogenesis; however, there is limited information regarding its upstream transcriptional regulation mechanism. *Bmp4* has been found to be required for the establishment of thymic fate and *foxn1* expression.^{38–40} *Bmp2* can be up-regulated after deletion of *Bmp4* to provide compensatory function in thymic organogenesis.⁴¹ It has been found that *Chd7* might directly regulate *Bmp4* expression in mouse.⁴²

From these findings, it was conjectured that *Chd7* may regulate the process of thymus organogenesis through modulating the expression of *Bmp4* and *foxn1* in zebrafish. To verify this hypothesis, the expression of *bmp4* and *bmp2b* was examined in *chd7* morphants; the expression of *bmp4* was almost absent, whereas *bmp2b* is reduced, in the pharynx at 50 and 48 hpf, respectively (Figure 8A), indicating that *chd7* might regulate thymus organogenesis by modulating the expression of *bmps*. Moreover, Dorsomorphin was used to block the *bmp* signals from 44 to 54 hpf, a window of time during thymic organogenesis. *foxn1* Transcript was strikingly reduced in the pharyngeal region at 60 hpf (Figure 8B), indicating that the *bmp* signaling might regulate *foxn1* expression. Taken together, these results suggested that *chd7* might regulate *foxn1* expression through *bmp* signaling in thymus organogenesis.

It has been demonstrated recently that *foxn4*, a metazoan ancestor of *foxn1*, can promote thymic epithelium formation in teleost fish.⁴³ It was tested whether *foxn4* was involved in the thymic defects in *chd7* morphants. The expression pattern of *foxn4* was examined. *foxn4* Was detected in the pharyngeal region at 4 dpf but not 48 and 72 hpf, indicating that *foxn4* might not participate in the early thymic organogenesis (Supplemental Figure S8A). It was next checked whether the expression of *foxn4* changes in *chd7* morphants at 4 dpf. Unexpectedly, little change of *foxn4* was detected in the pharyngeal region (Supplemental Figure S8B), indicating that *foxn4* might not be involved in the thymic defects in *chd7* morphants. These results further suggested that the *chd7-foxn1* molecular axis might be conserved between zebrafish and mammals.

Chd7 Mutants and *chd7* Morphants Display Similar Phenotypes

To validate some of the phenotypes observed in splice-blockling MO treated embryos, a translation-blocking MO against *chd7* (*chd7* ATG-MO) was injected into the embryos at the single cell stage and the T-cell and TEC markers were also reduced in the thymus region at 4 dpf (Supplemental Figure S9). Moreover, a *chd7* mutation with a 23-bp deletion in the second exon was generated using zinc-finger nucleases (Figure 9A). The 23-bp deletion resulted in a deductive loss of all functional domains of *Chd7* (Figure 9B) and was verified by genomic PCR (Figure 9C) and direct sequencing analysis (data not shown). The mutants displayed pericardial edema, cranial cartilage malformation, and swim bladder defects (Supplemental Figure S10), similar to the defects found in the morphants. Then, the expression of T-cell and thymic markers was examined in the mutants using WISH, and it was found that *ikaros*, *il7r*, *foxn1*, *rag1*, and *ccl25a* were all down-regulated in the mutants at 4 dpf, indicating that T-cell and thymic development were impaired in the absence of *Chd7* (Figure 9D and Supplemental Figure S11), consistent with the observations in *chd7* morphants.

Figure 9 *Chd7* mutants and *chd7* morphants display similar phenotypes. **A:** Location and sequence of the zinc-finger nuclease (ZFN) target sites for the *chd7* gene. **B:** Schematic of the zebrafish Chd7 protein in siblings and the mutants. Yellow, sapphire, brown, and yellow rectangles represent the chromodomain, SNF2, helicase, and BRK domains, respectively. **C:** Agarose gel electrophoresis analysis of wild-type siblings, *chd7* heterozygote PCR products, and *chd7* homozygote PCR products. M denotes the DNA marker. **D:** The expression of T-cell and thymic markers, *ikaros*, *il7r*, and *foxn1*, in the thymus in siblings and *chd7* mutants at 4 days post-fertilization (dpf). Red arrows indicate the expression of T-cell and thymic markers in the thymus. **E:** The morphology of pharyngeal arches marked by expression of *hand2* in siblings and *chd7* mutants at 48 hours post-fertilization (hpf). Numbers denote the arches. Asterisks mark the defective arches. **F:** The morphology of pharyngeal endodermal pouches marked by expression of *nkx2.3* in siblings and *chd7* mutant at 48 hpf. Numbers denote the endodermal pouches. Original magnification: $\times 15$ (D); $\times 9$ (E); $\times 12$ (F). M0, morpholino.

Moreover, to determine whether the reduced expression of T-cell and thymic markers resulted from impaired thymic organogenesis, the development of the pharynx in the *chd7* mutants was examined. The expression of endodermal marker, *nkx2.3*, was down-regulated in all five pouches, and the expression of post-migratory marker, *hand2*, was dramatically down-regulated in arches 2, 3, 4, and 5 at 48 hpf, which was consistent with the results in the *chd7* morphants (Figure 9, E and F). These data demonstrated that interfering with *chd7* expression at either genomic or transcriptional level resulted in similar T-cell and thymic developmental phenotypes.

Discussion

In this study, we demonstrate that the T-cell reduction in CHARGE syndrome can be phenocopied in zebrafish by

knocking down or knocking out *chd7*, the main causative gene for the congenital disorder. T cells play a central role in cell-mediated immunity, and T lymphopenia usually leads to immunodeficiency. The T-cell numbers range from low to absent, and the severity of immunodeficiency varies from mild to severe among the reported individuals of CHARGE patients. In some cases, CHD7 is associated with severe combined immunodeficiency.^{8–11,13,14,44} The spectrum of immunodeficiency may be at least partially attributed to the different mutations found in CHD7, ranging from benign and missense mutations to truncating mutations. Although truncating mutations seem to be associated with a more severe phenotype than missense variants,⁴⁵ further studies are required to find out the correlation between genotype and phenotype of CHD7 mutations.

The defects of thymic organogenesis observed in *chd7* morphants are consistent with the clinical reports of thymus hypoplasia or aplasia in CHARGE patients. It seems likely

that the T lymphopenia and immunodeficiency in CHARGE syndrome mainly contributed to the defects of thymus organogenesis. However, the rate of reported thymus defects varies between different studies. Thymus hypoplasia or aplasia is rare in postnatal CHARGE syndrome cases,⁴⁶ whereas it is observed in 7 of 10 CHARGE fetuses.¹⁵ This may be interpreted by, for example, the bias of the selected cohort of patients, the severity of various *CHD7* mutations, and different imaging techniques used to detect the thymus. Otherwise, CHARGE patients with thymic defects are more prone to die from immunodeficiency or other accompanying anomalies, such as heart defects. It is also possible that the athymic patients are diagnosed as having DiGeorge syndrome rather than CHARGE syndrome. Furthermore, relatively normal morphology of the thymus might not necessarily mean normal function of the thymus or T cells in CHARGE patients because the homing function of the thymus might also be disrupted, as observed in our animal model.

The patients with proved variants in *CHD7* are occasionally diagnosed as having complete DiGeorge syndrome, a typical athymic disorder that is mainly caused by defects in pharyngeal arches and pouches.^{47,48} To treat complete DiGeorge syndrome patients, transplantation of allogenic cultured thymic tissue was adopted as a therapy and obtained satisfying effects.^{49,50} Adoptive transfer of mature T cells from human leukocyte antigen identical siblings through bone marrow transplantation has been proposed.⁵¹ Nevertheless, no definite guidelines have been proposed for the clinical management of immunodeficiency in CHARGE syndrome. Various types of hematopoietic stem cells, including T-lymphocyte-depleted haploidentical paternal hematopoietic stem cells, have been used clinically but seem unsuccessful,¹⁰ which is probably attributable to a lack of understanding the underlying mechanisms. Our data highlight that the immunodeficiency in CHARGE syndrome is mainly attributed to thymic defects and indicate that restoring the morphology and function of thymus by, for example, thymic cell transplantation may be a potential strategy for treating immunologic abnormalities. However, the present study mainly focuses on the early development of T-cell and thymus organogenesis. Further studies are needed to clarify whether the thymic defects can recover with development and aging and whether the development of T cells is still compromised at later stages.

It is well established that *Foxn1* plays a central role in thymus organogenesis and TEC differentiation.^{23,52} However, the upstream network that regulates the expression of *Foxn1* still remains largely a mystery.⁵² In the present study, it was found that *chd7* can regulate the expression of both *foxn1* and *bmp4*. Several studies have demonstrated that *Bmp4* regulates the expression of *Foxn1* during the early development of thymus. And this role of *Bmp4* is evolutionarily conserved between zebrafish and mammals.⁵³ It has been demonstrated that *Chd7* might directly regulate the expression of *Bmp4* *in vitro*, and *Bmp4* is down-regulated in

the forebrain of *Chd7*-mutant mice.⁴² It has also been shown that *Bmp4* participates in maintaining the expression of *Foxn1* in mature thymus⁴⁰ and *Bmp4* is a critical component of a set of factors that direct the differentiation of human embryonic stem cells into functional thymic epithelial cells to support T-cell development.⁵⁴ These studies are consistent with our finding that *chd7* might regulate the expression of *foxn1* in thymus organogenesis through modulating the *bmp4* signaling. Besides participating in thymus morphogenesis, *Bmp4* is also revealed to directly regulate the development of thymocytes by signaling through *Bmp* receptor expressed on the thymocytes.⁵⁵ Further studies are required to clarify whether *CHD7* regulates the expression of *BMP4* in adults and whether *BMP4* contributes to the T-cell lymphopenia in CHARGE syndrome by regulating the differentiation of thymic epithelial cells or the maturation of thymocytes into T cells or both.

A widely accepted pathogenetic theory of CHARGE syndrome is that it is caused by maldevelopment of the neural crest cells.^{31,56,57} However, the theory has often faced challenges because it could not explain all aspects of the disorder. Several other theories have been proposed [eg, a disruption of the mesenchymal-epithelial interaction (mesenchymal includes neural crest mesenchyme and mesoderm, and epithelial includes ectoderm and endoderm)].⁵⁶ Restoring *Chd7* expression specifically in neural crest cells fails to rescue the defects of pharyngeal arch artery in *Chd7* mutant mice, indicating that *Chd7* may also function in other tissues besides neural crest cells.⁵⁸ Our study illustrates that *chd7* is critical for the development of not only migratory (marked by *crestin*) and post-migratory neural crest cells (marked by *crestin*, *dlx2a*, and *hand2*) but also the pharyngeal endoderm (marked by *nkx2.3* and *zn5*) and differentiated thymic epithelial cells (marked by *foxn1* and *ccl25*) during thymus organogenesis. These data suggest that *chd7* may participate in the interaction between epithelial and neural crest-derived mesenchymal cells in thymus morphogenesis. The down-regulation of *bmp4* in the pharynx of *chd7* morphants further supports the idea because *Bmp4* has been shown to mediate the mesenchymal-epithelial interaction during thymus development.^{39,59} The rescue of thymus defects in *chd7* morphants by overexpressing *foxn1* also suggests the importance of endoderm-derived thymic epithelial cells in thymus development and T lymphopoiesis. Both our data and other studies support the mesenchymal-epithelial interaction theory in CHARGE pathogenesis.⁵⁶

The increase of HSPCs peripherally in *chd7*-deficient embryos observed in this study is consistent with a previous report.⁶⁰ Particularly, the increase of lymphoid precursors makes it difficult to explain the decrease of T cells in *chd7* morphants; however, it can be easily explained by thymus defects. However, the contribution of the abnormal HSPCs cannot be excluded. For most patients, T lymphopenia is concomitant with T-cell dysfunction. Further investigation is required to examine whether the increased early HSPCs

can develop normally into functional T cells and whether *chd7* is involved in HSPCs at later stages. The developing thymocytes may cross talk with thymic stromal cells, and whether the abnormal HSPCs influence the development of thymus also needs further study. Although the report of T-cell decrease in CHARGE syndrome is usually accompanied with relatively normal B and natural killer cells, there are occasional reports of low or high numbers of these cells.¹² A recent study demonstrates that *Chd7* deficiency might suppress leukemogenesis through inhibiting cellular proliferation.⁶¹ These reports suggest that the role of *Chd7* in hematopoiesis might be complex, and more study is needed to clarify whether abnormal HSPCs contribute to the rare abnormal B and natural killer cells and other potential hematopoietic defects. In addition, because CHARGE syndrome is occasionally diagnosed as DiGeorge syndrome because of their overlapping features, including immunodeficiency and T-cell decrease, the participation of CHD7 in HSPC development might be helpful for differential diagnosis and treatment of the two types of disorders.

In summary, we have illustrated the role of *chd7* in early T-cell development and thymus organogenesis and the underlying molecular and cellular mechanisms. Our data indicate that, similar to complete DiGeorge syndrome, pharyngeal defects in arches and pouches, resulting in impaired thymic organogenesis, might be the major cause of thymic defects and T-cell decrease in CHARGE syndrome. From this, we propose that transplantation of allogeneic cultured thymic tissue or adoptive transfer of mature T cells from human leukocyte antigen identical siblings through bone marrow transplantation should be used to treat severe combined immunodeficiency patients with CHARGE syndrome. Zebrafish will serve as an ideal model organism for studying immunologic deficiency in CHARGE syndrome.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.12.005>.

References

- Sanlaville D, Verloes A: CHARGE syndrome: an update. *Eur J Hum Genet* 2007, 15:389–399
- Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, de Vries BB, Janssen IM, van der Vliet WA, Huys EH, de Jong PJ, Hamel BC, Schoenmakers EF, Brunner HG, Veltman JA, van Kessel AG: Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004, 36:955–957
- Blake KD, Hartshorne TS, Lawand C, Dailor AN, Thelin JW: Cranial nerve manifestations in CHARGE syndrome. *Am J Med Genet A* 2008, 146A:585–592
- Chalouhi C, Faulcon P, Le Bihan C, Hertz-Pannier L, Bonfils P, Abadie V: Olfactory evaluation in children: application to the CHARGE syndrome. *Pediatrics* 2005, 116:e81–e88
- Wang RY, Earl DL, Ruder RO, Graham JM Jr: Syndromic ear anomalies and renal ultrasounds. *Pediatrics* 2001, 108:E32
- Zentner GE, Layman WS, Martin DM, Scacheri PC: Molecular and phenotypic aspects of CHD7 mutation in CHARGE syndrome. *Am J Med Genet A* 2010, 152A:674–686
- Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, van Hagen JM, Donnai D, de Ravel TJ, Veltman JA, Geurts van Kessel A, De Vries BB, Brunner HG, Hoefsloot LH, van Ravenswaaij CM: CHARGE syndrome: the phenotypic spectrum of mutations in the CHD7 gene. *J Med Genet* 2006, 43:306–314
- Chopra C, Baretto R, Duddridge M, Browning MJ: T-cell immunodeficiency in CHARGE syndrome. *Acta Paediatr* 2009, 98:408–410
- Jyonouchi S, McDonald-McGinn DM, Bale S, Zackai EH, Sullivan KE: CHARGE (coloboma, heart defect, atresia choanae, retarded growth and development, genital hypoplasia, ear anomalies/deafness) syndrome and chromosome 22q11.2 deletion syndrome: a comparison of immunologic and nonimmunologic phenotypic features. *Pediatrics* 2009, 123:e871–e877
- Gennery AR, Slatter MA, Rice J, Hoefsloot LH, Barge D, McLean-Tooke A, Montgomery T, Goodship JA, Burt AD, Flood TJ, Abinun M, Cant AJ, Johnson D: Mutations in CHD7 in patients with CHARGE syndrome cause T-B + natural killer cell + severe combined immune deficiency and may cause Omenn-like syndrome. *Clin Exp Immunol* 2008, 153:75–80
- Assing K, Nielsen C, Kirchhoff M, Madsen HO, Ryder LP, Fisker N: CD4+ CD31+ recent thymic emigrants in CHD7 haploinsufficiency (CHARGE syndrome): a case. *Hum Immunol* 2013, 74:1047–1050
- Wong MT, Scholvinck EH, Lambeck AJ, van Ravenswaaij-Arts CM: CHARGE syndrome: a review of the immunological aspects. *Eur J Hum Genet* 2015, 23:1451–1459
- Yu H, Zhang VW, Stray-Pedersen A, Hanson IC, Forbes LR, de la Morena MT, Chinn IK, Gorman E, Mendelsohn NJ, Pozos T, Wiszniewski W, Nicholas SK, Yates AB, Moore LE, Berge KE, Sorte H, Bayer DK, ALZahrani D, Geha RS, Feng Y, Wang G, Orange JS, Lupski JR, Wang J, Wong LJ: Rapid molecular diagnostics of severe primary immunodeficiency determined by using targeted next-generation sequencing. *J Allergy Clin Immunol* 2016, 138:1142–1151.e2
- Chinn IK, Shearer WT: Severe combined immunodeficiency disorders. *Immunol Allergy Clin North Am* 2015, 35:671–694
- Sanlaville D, Etchevers HC, Gonzales M, Martinovic J, Clement-Ziza M, Delezoide AL, Aubry MC, Pelet A, Chemouny S, Cruaud C, Audollent S, Esculpavit C, Goudefroye G, Ozilou C, Fredouille C, Joye N, Morichon-Delvallez N, Dumez Y, Weissenbach J, Munnich A, Amiel J, Encha-Razavi F, Lyonnet S, Vekemans M, Attie-Bitach T: Phenotypic spectrum of CHARGE syndrome in fetuses with CHD7 truncating mutations correlates with expression during human development. *J Med Genet* 2006, 43:211–217
- Thisse C, Thisse B: High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc* 2008, 3:59–69
- Chalasani SH, Sabol A, Xu H, Gyda MA, Rasband K, Granato M, Chien CB, Raper JA: Stromal cell-derived factor-1 antagonizes slitr-1 signaling in vivo. *J Neurosci* 2007, 27:973–980
- Langenau DM, Zon LI: The zebrafish: a new model of T-cell and thymic development. *Nat Rev Immunol* 2005, 5:307–317
- Ma D, Wei Y, Liu F: Regulatory mechanisms of thymus and T cell development. *Dev Comp Immunol* 2013, 39:91–102

20. Patten SA, Jacobs-McDaniels NL, Zaouter C, Drapeau P, Albertson RC, Moldovan F: Role of Chd7 in zebrafish: a model for CHARGE syndrome. *PLoS One* 2012, 7:e31650
21. Wang S, He Q, Ma D, Xue Y, Liu F: Irf4 regulates the choice between T lymphoid-primed progenitor and myeloid lineage fates during embryogenesis. *Dev Cell* 2015, 34:621–631
22. Li L, Yan B, Shi YQ, Zhang WQ, Wen ZL: Live imaging reveals differing roles of macrophages and neutrophils during zebrafish tail fin regeneration. *J Biol Chem* 2012, 287:25353–25360
23. Vaidya HJ, Briones Leon A, Blackburn CC: FOXP1 in thymus organogenesis and development. *Eur J Immunol* 2016, 46:1826–1837
24. Gordon J, Wilson VA, Blair NF, Sheridan J, Farley A, Wilson L, Manley NR, Blackburn CC: Functional evidence for a single endodermal origin for the thymic epithelium. *Nat Immunol* 2004, 5:546–553
25. Lee KH, Xu Q, Breitbart RE: A new tinman-related gene, nkx2.7, anticipates the expression of nkx2.5 and nkx2.3 in zebrafish heart and pharyngeal endoderm. *Dev Biol* 1996, 180:722–731
26. Crump JG, Swartz ME, Kimmel CB: An integrin-dependent role of pouch endoderm in hyoid cartilage development. *PLoS Biol* 2004, 2:E244
27. Piotrowski T, Nusslein-Volhard C: The endoderm plays an important role in patterning the segmented pharyngeal region in zebrafish (*Danio rerio*). *Dev Biol* 2000, 225:339–356
28. Griffith AV, Cardenas K, Carter C, Gordon J, Iberg A, Engleka K, Epstein JA, Manley NR, Richie ER: Increased thymus- and decreased parathyroid-fated organ domains in *Spotch* mutant embryos. *Dev Biol* 2009, 327:216–227
29. Jenkinson WE, Bacon A, White AJ, Anderson G, Jenkinson EJ: An epithelial progenitor pool regulates thymus growth. *J Immunol* 2008, 181:6101–6108
30. Chen L, Zhao P, Wells L, Amemiya CT, Condie BG, Manley NR: Mouse and zebrafish *Hoxa3* orthologues have nonequivalent in vivo protein function. *Proc Natl Acad Sci U S A* 2010, 107:10555–10560
31. Bajpai R, Chen DA, Rada-Iglesias A, Zhang J, Xiong Y, Helms J, Chang CP, Zhao Y, Swigut T, Wysocka J: CHD7 cooperates with PBAF to control multipotent neural crest formation. *Nature* 2010, 463:958–962
32. Bajoghli B, Aghaallaei N, Hess I, Rode I, Netuschil N, Tay BH, Venkatesh B, Yu JK, Kaltenbach SL, Holland ND, Diekhoff D, Happe C, Schorpp M, Boehm T: Evolution of genetic networks underlying the emergence of thymopoiesis in vertebrates. *Cell* 2009, 138:186–197
33. Nowell CS, Bredenkamp N, Tetelin S, Jin X, Tischner C, Vaidya H, Sheridan JM, Stenhouse FH, Heussen R, Smith AJ, Blackburn CC: Foxn1 regulates lineage progression in cortical and medullary thymic epithelial cells but is dispensable for medullary sublineage divergence. *PLoS Genet* 2011, 7:e1002348
34. Romano R, Palamaro L, Fusco A, Giardino G, Gallo V, Del Vecchio L, Pignata C: FOXP1: a master regulator gene of thymic epithelial development program. *Front Immunol* 2013, 4:187
35. Bredenkamp N, Nowell CS, Blackburn CC: Regeneration of the aged thymus by a single transcription factor. *Development* 2014, 141:1627–1637
36. Zook EC, Krishack PA, Zhang S, Zeleznik-Le NJ, Firulli AB, Witte PL, Le PT: Overexpression of Foxn1 attenuates age-associated thymic involution and prevents the expansion of peripheral CD4 memory T cells. *Blood* 2011, 118:5723–5731
37. Bredenkamp N, Ulyanchenko S, O'Neill KE, Manley NR, Vaidya HJ, Blackburn CC: An organized and functional thymus generated from FOXP1-reprogrammed fibroblasts. *Nat Cell Biol* 2014, 16:902–908
38. Patel SR, Gordon J, Mahbub F, Blackburn CC, Manley NR: Bmp4 and Noggin expression during early thymus and parathyroid organogenesis. *Gene Expr Patterns* 2006, 6:794–799
39. Neves H, Dupin E, Parreira L, Le Douarin NM: Modulation of Bmp4 signalling in the epithelial-mesenchymal interactions that take place in early thymus and parathyroid development in avian embryos. *Dev Biol* 2012, 361:208–219
40. Barsanti M, Lim JM, Hun ML, Lister N, Wong K, Hammett MV, Lepletier A, Boyd RL, Giudice A, Chidgey AP: A novel Foxn1eGFP/+ mouse model identifies Bmp4-induced maintenance of Foxn1 expression and thymic epithelial progenitor populations. *Eur J Immunol* 2017, 47:291–304
41. Bleul CC, Boehm T: BMP signaling is required for normal thymus development. *J Immunol* 2005, 175:5213–5221
42. Jiang X, Zhou Y, Xian L, Chen W, Wu H, Gao X: The mutation in Chd7 causes misexpression of Bmp4 and developmental defects in telencephalic midline. *Am J Pathol* 2012, 181:626–641
43. Swann JB, Weyn A, Nagakubo D, Bleul CC, Toyoda A, Happe C, Netuschil N, Hess I, Haas-Assebaum A, Taniguchi Y, Schorpp M, Boehm T: Conversion of the thymus into a bipotent lymphoid organ by replacement of FOXP1 with its paralog, FOXP4. *Cell Rep* 2014, 8:1184–1197
44. Boudny P, Kurrer MO, Stamm B, Laeng RH: Malakoplakia of the colon in an infant with severe combined immunodeficiency (SCID) and charge association. *Pathol Res Pract* 2000, 196:577–582
45. Bergman JE, Janssen N, van der Sloot AM, de Walle HE, Schoots J, Rendtorff ND, Tranenjaerg L, Hoefsloot LH, van Ravenswaaij-Arts CM, Hofstra RM: A novel classification system to predict the pathogenic effects of CHD7 missense variants in CHARGE syndrome. *Hum Mutat* 2012, 33:1251–1260
46. Blake KD, Davenport SLH, Hall BD, Hefner MA, Pagon RA, Williams MS, Lin AE, Graham JM: CHARGE association: an update and review for the primary pediatrician. *Clin Pediatr* 1998, 37:159–173
47. Markert ML, Devlin BH, Chinn IK, McCarthy EA: Thymus transplantation in complete DiGeorge anomaly. *Immunol Res* 2009, 44:61–70
48. Janda A, Sedlacek P, Mejstrikova E, Zdrahalova K, Hrusak O, Kalina T, Sieglova Z, Zizkova H, Formankova R, Keslova P, Hubacek P, Sediva A, Bartunkova J, Dlsak K, Sary J: Unrelated partially matched lymphocyte infusions in a patient with complete DiGeorge/CHARGE syndrome. *Pediatr Transplant* 2007, 11:441–447
49. Rice HE, Skinner MA, Mahaffey SM, Oldham KT, Ing RJ, Hale LP, Markert ML: Thymic transplantation for complete DiGeorge syndrome: medical and surgical considerations. *J Pediatr Surg* 2004, 39:1607–1615
50. Markert ML, Boeck A, Hale LP, Kloster AL, McLaughlin TM, Batchvarova MN, Douek DC, Koup RA, Kosty DD, Ward FE, Rice HE, Mahaffey SM, Schiff SE, Buckley RH, Haynes BF: Transplantation of thymus tissue in complete DiGeorge syndrome. *N Engl J Med* 1999, 341:1180–1189
51. McGhee SA, Lloret MG, Stiehm ER: Immunologic reconstitution in 22q deletion (DiGeorge) syndrome. *Immunol Res* 2009, 45:37–45
52. Gordon J, Manley NR: Mechanisms of thymus organogenesis and morphogenesis. *Development* 2011, 138:3865–3878
53. Soza-Ried C, Bleul CC, Schorpp M, Boehm T: Maintenance of thymic epithelial phenotype requires extrinsic signals in mouse and zebrafish. *J Immunol* 2008, 181:5272–5277
54. Parent AV, Russ HA, Khan IS, LaFlam TN, Metzger TC, Anderson MS, Hebrok M: Generation of functional thymic epithelium from human embryonic stem cells that supports host T cell development. *Cell Stem Cell* 2013, 13:219–229
55. Hager-Theodorides AL, Ross SE, Sahni H, Mishina Y, Furmanski AL, Crompton T: Direct BMP2/4 signaling through BMP receptor 1A regulates fetal thymocyte progenitor homeostasis and differentiation to CD4+CD8+ double-positive cell. *Cell Cycle* 2014, 13:324–333
56. Williams MS: Speculations on the pathogenesis of CHARGE syndrome. *Am J Med Genet A* 2005, 133A:318–325
57. Siebert JR, Graham JM Jr, MacDonald C: Pathologic features of the CHARGE association: support for involvement of the neural crest. *Teratology* 1985, 31:331–336
58. Randall V, McCue K, Roberts C, Kyriakopoulou V, Beddow S, Barrett AN, Vitelli F, Prescott K, Shaw-Smith C, Devriendt K,

- Bosman E, Steffes G, Steel KP, Simrick S, Basson MA, Illingworth E, Scambler PJ: Great vessel development requires biallelic expression of *Chd7* and *Tbx1* in pharyngeal ectoderm in mice. *J Clin Invest* 2009, 119:3301–3310
59. Gordon J, Patel SR, Mishina Y, Manley NR: Evidence for an early role for BMP4 signaling in thymus and parathyroid morphogenesis. *Dev Biol* 2010, 339:141–154
60. Huang HT, Kathrein KL, Barton A, Gitlin Z, Huang YH, Ward TP, Hofmann O, Dibiase A, Song A, Tyekucheva S, Hide W, Zhou Y, Zon LI: A network of epigenetic regulators guides developmental haematopoiesis in vivo. *Nat Cell Biol* 2013, 15:1516–1525
61. Zhen T, Kwon E, Zhao L, Hsu J, Hyde RK, Lu Y, Alemu L, Speck NA, Liu PP: CHD7 deficiency delays leukemogenesis in mice induced by *Cbfb-MYH11*. *Blood* 2017, 130:2431–2442