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Effects of constitutively active IKKβ on cardiac development

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ABSTRACT

NF-κB is a major transcription factor regulating cell survival, organ development and inflammation, but its role in cardiac development has been inadequately explored. To examine this function, we generated mice in which IKKβ, an essential kinase for NF-κB activation, was constitutively activated in embryonic cardiomyocytes. For this purpose, we used smooth muscle-22α (SM22α)-Cre mice, which are frequently used for gene recombination in embryonic cardiomyocytes. Embryonic hearts of SM22αCre-CA (constitutively active) IKKβ$^{flox/flox}$ mice revealed remarkably thin, spongy and hypoplastic myocardium. In exploring the mechanism, we found that the expression of bone morphogenetic protein 10 (BMP10) and T-box transcription factor 20 (Tbx20), major regulators of cardiac development, was significantly downregulated and upregulated, respectively, in the SM22αCre-CAIKKβ$^{flox/flox}$ mice. We also generated NK2 homeobox 5 (Nkx2.5) Cre-CAIKKβ$^{flox/wt}$ mice since Nkx2.5 is also expressed in embryonic cardiomyocytes and confirmed that the changes in these genes were also observed. These results implicated that the activation of NF-κB affects cardiac development.

1. Introduction

NF-κB is a major family of transcription factors playing essential roles in cell survival [1], organ development [2] and inflammation [3,4], but its role in cardiac development has not been adequately explored. In unstimulated condition, NF-κB exists in cytoplasm with binding to inhibitor of κB (IκB). When cell is stimulated with wide variety of stimuli like inflammatory cytokines, IκB is phosphorylated and degraded by ubiquitination, then NF-κB is translocated to nucleus and cytokine transcription is activated [3–7]. The IκB is phosphorylated by a multiunit complex containing two catalytic subunits, IκB kinase alpha and IKKβ, and the regulatory subunit IKKγ (NEMO). Among them, IKKβ is the central kinase in mediating of IκB phosphorylation [6].

To determine the effects of NF-κB activation on cardiac development, we generated two lines of mice in which constitutively active IKKβ (CAIKKβ) is expressed in embryonic cardiomyocytes.

2. Materials and methods

2.1. Mice

All animal experiments were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (ARC-MedKyotoUniv, permission number: Med Kyo 21002-3) and performed in accordance with the institutional guidelines of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University (Kyoto, Japan). Constitutively active IKKβ^{flox} (R26Stop-^{FL}IKK2CA, Stock # 008242 [8]), SM22αCre (Tg (Tagln-cre)1Her/J, Stock # 004746 [9]), and mT/mG mice (B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato, EGFP)Luo/J, Stock# 007676 [10]) were obtained from The Jackson Laboratory. Nkx2.5Cre mice were transferred from Prof. Kyoko Imanaka-Yoshida (Mie University Graduate School of Medicine, Japan) with the permission of Prof. Robert J. Schwartz (University of Houston, TX).

2.2. Histology and immunohistochemistry

Embryos were dissected after intraperitoneal administration of a mixture of three drugs (medetomidine: 0.3 mg/kg body weight, midazolam: 4 mg/kg body weight, and butorphanol: 5 mg/kg body weight) [11] to pregnant mice at selected time points, fixed in 4% paraformaldehyde solution (PFA) for two days, and then dehydrated in 70% ethanol for paraffin embedding.

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Nonstandard abbreviations and acronyms

NF-κB	nuclear factor kappa B
Myh6	myosin heavy chain 6
Sm22α	smooth muscle 22α
Nkx2.5	NK2 homeobox 5
IκB	inhibitor of kappa B
IKKβ	IκB kinase subunit beta
EGFP	enhanced green fluorescent protein
mT/mG	membrane-localized tdTomato/membrane-localized EGFP
Tbx20	T-box transcription factor 20
BMP10	bone morphogenetic protein 10

Paraffin-embedded samples were cut into 4 μm sections, deparaffinized and dehydrated, and then used for haematoxylin-eosin staining (HE) to observe embryonic heart morphology. The thickness of the compact and noncompact layers was measured in images acquired by an Axio Observer (Carl Zeiss) microscope. Six measurements were taken for each heart section using Zen Lite (Carl Zeiss) and averaged individually. The NCC ratio was analysed by dividing the thickness of the compact layer by the thickness of the trabecular layer.

For frozen sections, embryonic murine hearts were washed with cold phosphate-buffered saline (PBS), fixed with 4% PFA for 2 h at 4°, and then dehydrated in 10%, 20%, and 30% sucrose in PBS at 4° for a few hours overnight according to the size of the tissue. Tissue samples were snap-frozen in liquid nitrogen following Optimal Cutting Temperature compound (Sakura) embedding, sectioned at 8 μm thickness and air-dried for 30 min at room temperature. Tissue slides were washed with PBS 2 times and counterstained with mounting medium containing DAPI (Vector Laboratories) before coverslipping.

2.3. Real-time PCR

Total RNA was isolated from embryonic hearts using TRIzol (Thermo). cDNA synthesis was performed with the extracted RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo) according to the manufacturer's instructions. Gene expression levels were measured by quantitative PCR using Thunderbird SYBR qPCR Mix (Toyobo) and a real-time PCR system (StepOnePlus; Thermo). The relative mRNA levels of each gene were normalized to the 18S ribosomal RNA levels in the same sample. The following primer sequences were used: BMP10: forward: 5′-CCTGAAACCATTCTCCCTCA-3′, reverse: 5′-CTTGAGTGTGTTCGCTTGGA-3'; Tbx20: forward: 5′-GCAGCAGAGAACACCATCAA-3′, reverse: 5′-GTGAGCATCCAGACTCGTCA-3'; Nkx2.5: forward: 5′-CAAGTGCTCTCCTGCTTTCC-3′, reverse: 5′-GGCTTTGTCCAGCTCCACT-3'; myocardin: forward: 5′-ACTGAGGTGAGCCTCTCCAA-3′, reverse: 5′-CTGAGCCAGGAGTGAGATCC-3'.

2.4. Statistical analysis

Differences between two groups were compared by the Wilcoxon rank sum test as a nonparametric comparison test. The experimental results were analysed using JMP, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. SM22αCre-induced constitutively active IKKβ in hearts results in a noncompaction phenotype

For the purpose of this study, we chose SM22αCre mice to induce Cre-LoxP recombination in embryonic cardiomyocytes. SM22α is expressed in cardiomyocytes at E8.75–12.5 [12,13], and SM22αCre has been frequently used for analysing cardiac development [14–16].

First, we mated SM22αCre mice with mT/mG mice to confirm recombination in embryonic cardiomyocytes. In those mice, EGFP is expressed on the membrane of cells in which Cre-LoxP recombination takes place [10]. We found that all embryonic cardiomyocytes at E17.5 expressed EGFP (Fig. 1a). This result indicated that SM22αCre mice can be used to induce recombination in embryonic cardiomyocytes.

Then, we generated mice with CAIKKβ in cardiomyocytes by crossing SM22αCre mice with CAIKKβ^{flox} mice and found that homozygous SM22αCre-CAIKKβ$^{flox/flox}$ (IKK^{SM}) mice died just after birth. To investigate the causes of perinatal death in IKK^{SM} mice, we examined embryonic hearts at E17.5. Interestingly, we found that IKK^{SM} mice were characterized by severe hypoplasia with spongy myocardium (Fig. 1b). We also analysed the earlier embryonic hearts at E15.5 to obtain a more evident phenotype, which revealed a remarkably thin compact layer in the ventricular wall (Fig. 1c). We quantified the thickness of the compact and trabecular layers and found that the NC (non-compact)/C (compact) ratio was significantly increased in IKK^{SM} hearts. Since high NC/C ratio has been used as sign of noncompaction [17–19], the results indicated that IKK^{SM} mice have a phenotype of noncompaction (Fig. 1d).

3.2. BMP10 and Tbx20 expression is modified in SM22αCre-CAIKKβ^{flox} hearts

To investigate the mechanism for the defects in cardiac development of IKK^{SM} hearts, we analysed the mRNA expression in IKK^{SM} hearts. First, we quantified the IKKβ mRNA expression, and it was significantly higher in the IKK^{SM} hearts than in the control hearts, as expected (Fig. 2a).

Next, we analysed the representative genes related to cardiac development, including noncompaction, such as BMP10, Tbx20, Nkx2.5 and myocardin [18,20–23]. We found no change in Nkx2.5 or myocardin, but the levels of BMP10 and Tbx20 were significantly downregulated and upregulated, respectively, in the IKK^{SM} embryonic hearts (Fig. 2b–e). These results indicated that transcriptional regulation in cardiac development was disorganized in the IKK^{SM} hearts.

3.3. The expression levels of BMP10 and Tbx20 are also modified in Nkx2.5Cre-CAIKKβ^{flox} hearts

Given that SM22α is expressed in embryonic cardiomyocytes relatively late at E8.75, we generated other mice by using Nkx2.5Cre mice since Nkx2.5 is expressed in cardiomyocytes as early as E7.5 [24–26].

Before mating the Nkx2.5Cre mice with CAIKKβ^{flox} mice, we mated them with mT/mG mice to ensure that recombination occurred in embryonic cardiomyocytes. The results indicated that all cardiomyocytes expressed EGFP (Fig. 3a).

Next, we mated Nkx2.5Cre mice with CAIKKβ^{flox} mice to generate mice with constitutively active IKKβ in embryonic cardiomyocytes and found that all of the heterozygous Nkx2.5Cre-CAIKKβ$^{flox/wt}$ (IKK$^{Nkx2.5}$) mice died within a few days after birth. The qPCR results indicated that the expression of IKKβ in embryonic

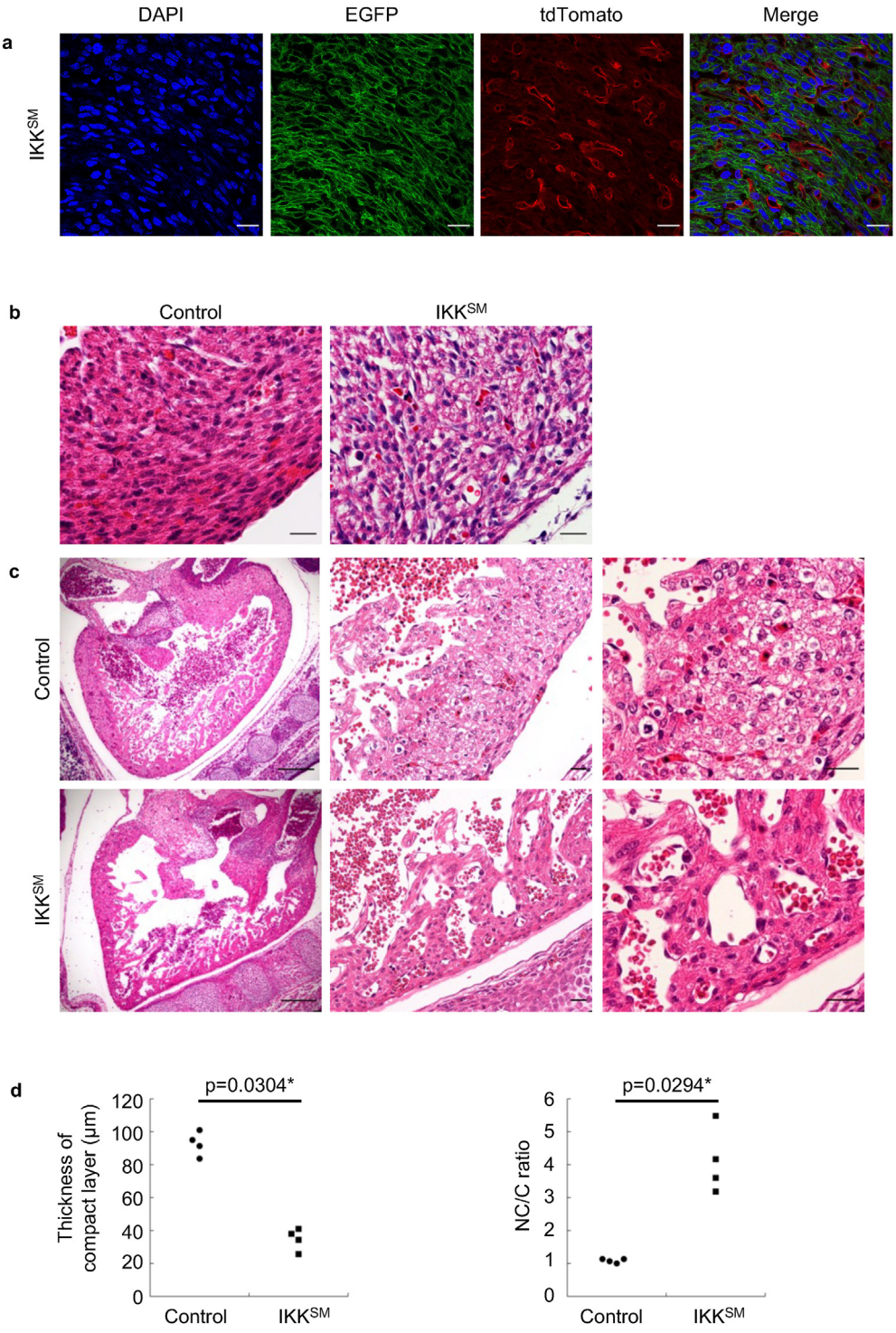

Fig. 1. IKK^{SM} mice displayed a noncompaction phenotype.
a, SM22αCre-mediated recombination was indicated by EGFP expression on the cell membrane. Fluorescence microscope images of SM22aCre-mTmG$^{flox/wt}$ heart cryosections at E17.5 (n = 1). Scale bars: 20 μm **b and c**, Haematoxylin and eosin staining in coronal sections of the littermate control and IKKˢᴹ hearts at E17.5 (b) and E15.5 (c) ((b) control, n = 3; (b) IKKˢᴹ, n = 4; (c) control, n = 4; (c) IKKˢᴹ, n = 4). The right panels of (c) show magnified views of the left panel. Scale bars: 200 μm for the whole hearts, 20 μm for the magnified views. **d**, Quantification of the thickness of the compact layer and NC (noncompaction)/C (compaction) ratio in the littermate control and IKKˢᴹ hearts at E15.5 (n = 4). *p < 0.05, evaluated by the Wilcoxon rank sum test.

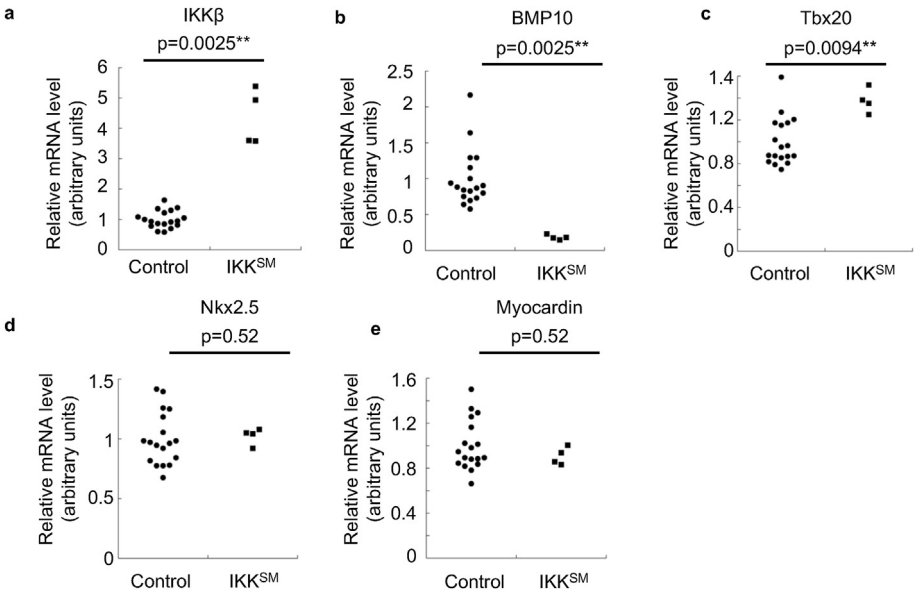

Fig. 2. The expression of key genes regulating cardiac development.
The expression of mRNA for each gene from embryonic hearts at E17.5 is shown (control: n = 18; IKKˢᴹ: n = 4). **p < 0.01, evaluated by Wilcoxon rank sum test.

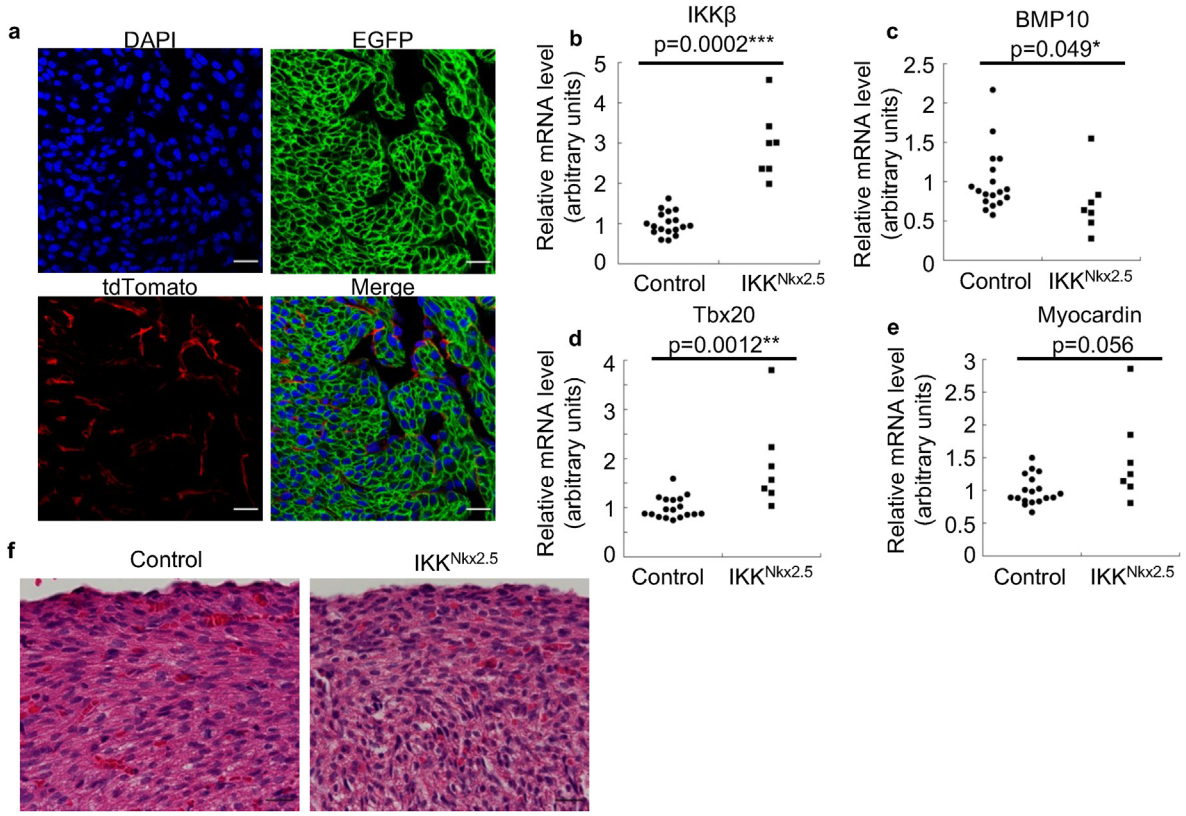

Fig. 3. The expression of BMP10 and Tbx20 was also modified in heterozygous IKK$^{Nkx2.5}$ mice.
a, Nkx2.5Cre-mediated recombination was indicated by EGFP expression on the cell membrane. Fluorescence microscope images of Nkx2.5Cre-mTmG $^{flox/wt}$ heart cryosections at E17.5 (n = 3). Scale bars: 20 μm **b**, The expression of IKKβ mRNA from embryonic hearts at E17.5 is shown (control: n = 18; IKK$^{Nkx2.5}$: n = 7). ***p < 0.001, evaluated by Wilcoxon rank sum test. **c-e**, The expression of mRNA for each gene from embryonic hearts at E17.5 is shown (control: n = 18; IKK$^{Nkx2.5}$: n = 7). *p < 0.05; **p < 0.01, evaluated by the Wilcoxon rank sum test. **f**, IKK$^{Nkx2.5}$ mice did not display a noncompaction phenotype. Haematoxylin and eosin staining in coronal sections of the littermate controls and IKK$^{Nkx2.5}$ ^{mice} at E17.5 (n = 3). Scale bars: 20 μm.

hearts of IKK$^{Nkx2.5}$ mice was significantly increased, but the change was mild compared to that of IKKˢᴹ mice due to heterozygosity (Fig. 3b). Interestingly, the expression levels of BMP10 and Tbx20, but not myocardin, were significantly down- and upregulated, respectively, compared with the controls, similar to IKKˢᴹ (Fig. 3c–e). These results confirmed that constitutive activation of

IKKβ in cardiomyocytes modified the expression of BMP10 and Tbx20. Meanwhile, histological analysis indicated that $IKK^{Nkx2.5}$ mice did not show an apparent noncompaction phenotype (Fig. 3f), which was supposedly due to the heterozygosity. Indeed, the change in BMP10 between the control and $IKK^{Nkx2.5}$ mice was much milder than that between the control and IKK^{sm} mice, as shown in Figs. 2b and 3c.

4. Discussion

NF-κB is a transcription factor regulating cell survival, organ development and inflammation. In terms of organ development, it has been reported that manipulation of NF-κB results in severe developmental defects during embryogenesis in multiple organs, including limb, liver, skin, lung, neural, notochord, muscle, skeletal, and haematopoietic cells [2]. However, its roles in cardiac development have not been adequately examined. In this study, we generated two lines of mice with constitutively active IKKβ in embryonic cardiomyocytes and found that IKK^{sm} mice showed a noncompaction phenotype. Furthermore, we found that constitutive activation of IKKβ in cardiomyocytes modified the expression of BMP10 and Tbx20, major genes critical for cardiac development.

Kraut et al. showed that constitutively active IKKβ in cardiomyocytes under the αMHC (=Myh6)-tTA system induces abnormalities in heart development, resulting in embryonic lethality [27]. This was a careful and detailed report; however, previous studies revealed that expression of modified activator protein caused cardiomyopathy. Indeed, McCloskey et al. raised important caution in using this αMHC-tTA system [28]. They examined whether cardiac expression of the tTA is associated with cardiac function, and found that αMHC-tTA mice had ventricular dilatation compared with Wt littermate, which is similar phenotype seen in Kraut's study. Furthermore, the expression levels of 153 genes were significantly changed between αMHC-tTA and Wt. The report revealed that αMHC-tTA construct itself causes significant change on myocardial gene expression and major functional abnormality in vivo and in vitro, which made us cautious in interpreting the results in the Kraut's report. Therefore, we examined the function of NF-κB on cardiac development with other modality, the Cre-LoxP system, in this study. We considered to use Myh6-Cre mice in this study, but previous report indicated that Myh6-Cre-mediated gene manipulation does not typically induce morphogenic defects in cardiac development, likely due to the relatively late expression of Cre-recombinase in embryo [29]. Therefore, we used SM22α-Cre and Nkx2.5-Cre mice. Consequently, it was meaningful that we observed noncompaction phenotype in IKK^{sm} mice, and found that critical genes for cardiac development were modified by constitutive activation of IKKβ in cardiomyocytes, which were not detected in Kraut's report.

BMP10 belongs to the TGF-β superfamily and is expressed exclusively in cardiomyocytes during myocardium development [23,30]. BMP10 is a key regulator of cardiac morphogenesis, and BMP10-deficient mice show a thinned myocardium, which leads to embryonic lethality at midgestation [23]. Recently, high-resolution melt screening (HRM) for patients with left-ventricle noncompaction (LVNC) identified a missense variant in BMP10 [31]. Regardless of its importance in cardiac development, the regulation of BMP10 is poorly understood.

Tbx20 is a member of the Tbx1 subfamily and plays an essential role in embryonic heart development, including chamber differentiation and cardiac looping [22,32,33]. Indeed, Nkx2.5Cre-mediated Tbx20 overexpression in embryonic cardiomyocytes led to embryonic lethality and resulted in a thin ventricular wall [34]. It was reported to be downstream of BMP10 [35]; therefore, it was somewhat unexpected that the change in expression levels of BMP10 and Tbx20 were not in similar direction. However, Bouvard et al. reported that Tbx20 expression was not significantly downregulated with double knockout of BMP9 and BMP10 [36], which indicated that the relationship between BMP10 and Tbx20 is not one-to-one. Constitutively active IKKβ in cardiomyocytes may have independently modified Tbx20 and BMP10.

The reason why heterozygous $IKK^{Nkx2.5}$ mice showed perinatal death without apparent histological phenotypes is unclear, but it should be noted that Nkx2.5 is heterozygously knocked out in Nkx2.5Cre mice [26]. A previous report showed embryonic lethality due to arrhythmia in Nkx2.5-ablated hearts [21]. It is possible that such electrophysiological changes induced by modification of Nkx2.5 affected the survival of $IKK^{Nkx2.5}$ mice.

Our study has limitations. First, it is possible that constitutively active IKKβ in noncardiomyocytes affected the noncompaction phenotype in IKK^{sm} heart since SM22α-Cre-induced recombination was reported to occur in multiple kinds of cells other than cardiomyocytes [37]. As SM22α-Cre has been frequently used for analysing cardiac development as mentioned above, the possibility would not be high; however, it should not be ignored. To verify it, SM22α-Cre-induced recombination at noncardiomyocytes in heart should be evaluated precisely. Shen et al. examined SM22α-Cre-induced recombination in detail [37], but they did not focus on cells in the heart. We have been studying this issue as an independent project. Second, changes in BMP10 or Tbx20 induced by constitutive activation of IKKβ in cardiomyocytes were interesting, but we could not explain the mechanism of the noncompaction phenotype in IKK^{sm} hearts. Heterozygous Nkx2.5Cre-$CAIKK\beta^{flox/wt}$ mice ($IKK^{Nkx2.5}$) did not show a noncompaction phenotype despite changes in the expression of BMP10 and Tbx20. This finding might be because the change was modest due to heterozygosity, but it could not be proven. Further experiments to examine whether modification of these genes can rescue the noncompaction phenotype in IKK^{sm} heart would be needed for direct explanation.

It is widely known that viral or bacterial infection during pregnancy can cause congenital heart diseases (CHDs) [38,39]. However, the mechanism of how infection during pregnancy can cause CHD has not been adequately analysed. Given that virus or bacterial infection activates NF-κB and that NF-κB was reported to be activated in CHD [40], the results of this study add new information on how infection during pregnancy induces CHD.

Data availability

All data generated or analysed during this study are included in this published article.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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