Peptides 136 (2021) 170459

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Cholecystokinin peptide signaling is regulated by a TBX5-MEF2 axis in the heart

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ARTICLE INFO

Keywords:
Heart peptide
Heart development
TBX5
MEF2C
Cholecystokinin
Heart failure

ABSTRACT

The procholecystokinin (proCCK) gene encodes a secreted peptide known to regulate the digestive, endocrine, and nervous systems. Though recently proposed as a biomarker for heart dysfunction, its physiological role in both the embryonic and adult heart is poorly understood, and there are no reports of tissue-specific regulators of cholecystokinin signaling in the heart or other tissues. In the present study, mRNA of proCCK was observed in cardiac tissues during mouse embryonic development, establishing proCCK as an early marker of differentiated cardiomyocytes which is later restricted to anatomical subdomains of the neonatal heart. Three-dimensional analysis of the expression of proCCK and CCKAR/CCKBR receptors was performed using in situ hybridization and optical projection tomography, illustrating chamber-specific expression patterns in the postnatal heart. Transcription factor motif analyses indicated developmental cardiac transcription factors TBX5 and MEF2C as upstream regulators of proCCK, and this regulatory activity was confirmed in reporter gene assays. proCCK mRNA levels were also measured in the infarcted heart and in response to cyclic mechanical stretch and endothelin-1, indicating dynamic transcriptional regulation which might be leveraged for improved biomarker development. Functional analyses of exogenous cholecystokinin octapeptide (CCK-8) administration were performed in differentiating mouse embryonic stem cells (mESCs), and the results suggest that CCK-8 does not act as a differentiation modulator of cardiomyocyte subtypes. Collectively, these findings indicate that proCCK is regulated at the transcriptional level by TBX5-MEF2 and neurohormonal signaling, informing use of proCCK as a biomarker and future strategies for upstream manipulation of cholecystokinin signaling in the heart and other tissues.

1. Introduction

Natriuretic peptide hormones secreted by the heart in response to increases in volume or pressure overload serve as biomarkers of heart failure [11,22,45,50,65,79]. These secreted cardiac peptides include atrial natriuretic peptide (ANP), encoded by the Nppa gene, and brain natriuretic peptide (BNP), encoded by the Nppb gene [11]. Nppa and Nppb are expressed in the heart during embryogenesis, and transcription of these genes is regulated by developmental cardiac transcription factors GATA4, TBX5, and NKX2-5 [6,19,60,84]. Importantly, fetal gene re-activation governed by these transcription factors underlies maladaptive cardiac hypertrophy, necessitating further study into fetal transcriptional programs and novel biomarkers resulting from their re-activation [12,17,20].

In addition to canonical cardiac natriuretic peptides, recent studies have described the expression of non-canonical cardiac peptides in the heart, such as adrenomedullins [64,75], apelin [74], ghrelin [59], and procholecystokinin (proCCK) [23,24]. Recently, it was observed that proCCK was expressed in the cardiomyoblast H9c2 line and increased upon isoprenaline treatment [24]. Procholecystokinin (proCCK) peptide was present in rodent and porcine hearts, and chamber-specific peptide levels reflected hemodynamic changes occurring during adaptation to postnatal life [18,23]. Furthermore, in clinical studies of heart failure patients, an increase in plasma CCK was associated with mortality in elderly females [25]. To date, there have been no reports of upstream regulators of proCCK in the heart underlying these expression patterns. Additionally, it is unclear whether differential peptide levels are due to regulation at transcriptional, translational, or secretory levels.

Though initially identified as a digestive peptide promoting pancreatic enzyme secretion and satiety, the role of CCK has been extended to that of a neurotransmitter, growth factor, and ubiquitous messenger [62]. CCK peptides are processed into secreted CCK-58, -33,

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https://doi.org/10.1016/j.peptides.2020.170459
Received 24 September 2020; Received in revised form 16 November 2020; Accepted 17 November 2020
Available online 26 November 2020

-22, and -8 forms, and this processing is cell-type specific [62]. Intriguingly, the cardiac form of the peptide is cleaved into a unique, truncated form lacking the N-terminal domain in a process distinct from the intestine and brain [24]. However, all forms have a biologically conserved, C-terminal tetrapeptide sequence (Trp-Met-Asp-Phe-NH_2), and this is also the active domain of the homologous peptide gastrin [62]. In addition, both CCK and gastrin are reported to be natriuretic and diuretic peptides which bind to both the cholecystokinin A receptor (CCKAR) and cholecystokinin B receptor (CCKBR) [8,78]. Thus, CCK is a multi-functional peptide hormone with context-dependent processing.

Several studies have reported the effects of exogenously administered CCK peptide on the heart. Exogenous CCK octapeptide (CCK-8) administration induced changes in heart rhythm, and this was blocked by CCK receptor antagonists, suggesting that CCK-8 affects the heart by acting on CCK receptors [21,49]. Additionally, CCK-8 increased mean arterial blood pressure in both the normal state and in the context of shock [4,21]. In rainbow trout, administration of physiological levels of CCK increased cardiac output and gastrointestinal blood flow [68]. This was shown to be a result of an increase in contractile force of the bulbus arteriosus, ventricle, ventral aorta, and brachial arteries [67]. Thus, CCK signaling induces multiple effects on cardiovascular physiology and hemodynamics relevant to cardiovascular biology and disease.

Despite its significance in multi-organ physiology, little is known about the transcriptional regulation of proCCK in cardiac or non-cardiac tissues. In promoter-luciferase experiments conducted in intestinal cell lines under serum-free conditions, hydrolyzed protein induced the expression of proCCK-luciferase, suggesting nutrient intake affects transcription of proCCK [10,14]. In PC12 neuroblasts, a 100bp human cholecystokinin promoter sequence was shown to be induced by KCl and forskolin, indicating that proCCK transcription is regulated by increases in intracellular levels of calcium and cAMP via protein kinase A (PKA) and extracellular signal-regulated kinase (ERK) signaling pathways [26]. A CCK-LacZ reporter showed activity of the proCCK promoter in embryonic tissues, including mouse hearts [43]. However, full characterization of temporal cardiac expression of endogenous proCCK mRNA or protein during embryonic development was not performed, and there are no indications of upstream regulators of developmental proCCK expression.

We here report the presence and chamber-specific transcriptional regulation of cholecystokinin signaling in the developing and postnatal heart. The expression of proCCK mRNA and protein was assessed during mouse cardiogenesis via whole mount *in situ* hybridization (ISH), immunohistochemistry (IHC), and quantitative real time PCR (qRT-PCR), detailing a dynamic expression range paralleling and diverging from the classical cardiac natriuretic peptides Nppa and Nppb. Additionally, whole mount ISH and optical projection tomography (OPT) were performed in neonatal mouse hearts to obtain a three-dimensional representation of proCCK, CCKAR, and CCKBR chamber-specific expression domains in the postnatal heart. Transcription factor motif analysis was conducted in a 10 kb region surrounding the proCCK transcriptional start site, leading to the identification of developmental cardiac transcription factors TBX5 and MEF2C as candidate regulators of proCCK expression. Reporter gene assays confirmed the activation of proCCK by cardiac developmental transcription factors TBX5 and MEF2C, demonstrating that proCCK is a target gene and candidate effector of transcriptional mediators associated with cardiovascular disease. proCCK regulatory regions were also activated by endothelin-1 and adrenergic signaling in neonatal cardiomyocytes, and endogenous proCCK mRNA was further examined following endothelin-1 treatment and cyclic mechanical stretch *in vitro*. Additionally, proCCK mRNA downregulation was observed in the left ventricles of rats following myocardial infarction (MI), in contrast to natriuretic peptides Nppa and Nppb. Finally, the effects of exogenous treatment with CCK-8 were examined in the spontaneous differentiation of mouse embryonic stem cells (mESCs). Collectively, our data indicate that proCCK may serve as a biomarker and candidate effector of TBX5-MEF2 activity in congenital and adult disease.

2. Materials and methods

2.1. Whole mount in situ hybridization

Embryos from wild type mouse litters (C57BL/6JOlaHsd) were dissected for ISH from healthy mothers, and whole embryos were fixed in 4% PFA and dehydrated in a methanol series. Similarly, hearts from C57BL/6JOlaHsd neonatal mouse pups (P1) were dissected and dehydrated in a methanol series prior to further analysis. The following PCR primers were used to amplify templates for probe synthesis from cDNA generated from mouse embryonic hearts.

proCCK: F: 5'-CCTCAACTTAGCTGGACTGCAG-3'
R: 5'-CGGTCACTTATTCTATGGCTGG-3'
CCKAR:F: 5'-GCTGCCACCTGGTGCCTCTC-3'
R: 5'-CCGCCAGGCATTGGCACTGA-3'
CCKBR:F: 5'-GCCGGGTCCGAAACCAAGGG-3'
R: 5'-TCCCTCAGCCAGGTCCCAGC-3'

Probes were labelled with digoxigenin-11-UTP (Roche) and whole mount ISH was performed as described previously [15]. mRNA was detected using an anti-dioxgen antibody and stained with BMPurple. For ISH of whole neonatal hearts, the time of permeabilization with PBS-Tween (0.1 %) was extended to four days. Additionally, pre-hybridization was extended to eight hours with extra washes and hybridization was extended to twenty-four hours. Finally, post-hybridization washes were extended to eight hours and post-antibody staining washes were extended to four days.

2.2. Optical projection tomography

Optical projection tomography was performed according to the published protocol [71]. In brief, samples were embedded in agarose, cleared, and imaged on a Bioptonics 3001 device. Bright field scanning was used to capture the BMpurple signal, and scanning of specimens on the GFP1 channel was used to capture the overall anatomy of the heart. Images were reconstructed using NRECON to generate 3D image stacks that were analyzed using AMIRA 5.0 (ThermoFisher).

2.3. Immunohistochemistry

For preparation of samples for IHC, embryos were cryosectioned (15 μM) and stained with anti-cholecystokinin (Cloud-Clone Corporation, PAA802Mu01) according to the manufacturer's instructions. A goat anti-rabbit HRP-conjugated secondary antibody (Jackson Immunoresearch) was developed using DAB substrate, and counterstaining was performed using hematoxylin. Stained tissue sections were imaged on a Pannoramic Digital Slide Scanner (3DHISTECH).

2.4. Embryonic tissue harvesting

Embryonic day 10 (E10) embryonic hearts, primary pharyngeal arches, and secondary pharyngeal arches were removed from wild type embryos (C57BL/6JOlaHsd). Primary and secondary pharyngeal arches, atria and ventricles were collected into TRIzol reagent (ThermoFisher Scientific), and RNA was extracted according to the manufacturer's instructions. RNA purity was improved using the RNeasy MinElute Cleanup kit (Qiagen). Each biological replicate consisted of tissue from four embryos. For representative *ex vivo* culture experiments, arches were grown as previously described [1,72]. Explants were fixed in 4% PFA, permeabilized with 0.5 % Triton-X and stained with anti-Cardiac Troponin T (ThermoFisher Scientific) and an Alexa-Fluor conjugated secondary antibody (ThermoFisher Scientific). Explants were imaged on a Leica DMi8 inverted fluorescent microscope.

2.5. Acute myocardial infarction

Myocardial infarction was produced by ligation of the left anterior descending (LAD) coronary artery in anaesthetized rats [34,66]. The sham-operated rats underwent the same surgical procedure without LAD ligation. Rats were anesthetized with ketamine (50 mg/kg, intraperitoneal) and xylazine//medetomidine (10 mg/kg, intraperitoneal). The animals were sacrificed at one week, hearts were excised and left ventricular samples were processed for expression characterization. Total RNA from the left ventricle lower transversal mid-section was isolated by the guanidine thiocyanate-CsCl method [66].

2.6. Gene expression analysis

qRT-PCR reactions were performed using the Fluidigm Biomark HD or Roche 480 LightCycler and taqman gene expression assays (Supplementary Fig. S1). Final values were calculated using a reference gene (Actb for embryonic samples, Tbp for infarction samples) [5].

2.7. Motif analyses

DNA motif analyses in the non-coding regions surrounding the proCCK transcriptional start site were conducted in R. Position frequency matrices were obtained for signaling pathway transcriptional effectors and transcription factors using MotifDb (Bioconductor). Motif enrichment analysis was conducted using Motif Counter [40]. Analysis was restricted to a 10,000 bp region encompassing the transcriptional start site (-8000/+2000 bp). The ubiquitously expressed beta-actin promoter was used for estimating the background presence of motifs (non-developmental/cell-type specific). For determination of relevant regions within the 10,000 bp sequence surrounding the transcriptional start site, the 10 kb region was separated into 500 bp increments and motif enrichment was again performed for MEF2 and TBX5 motifs. Tbx5_3p and Tbx_5p motifs represent motifs discovered in a previous study [47]. All other motifs were obtained via MotifDb.

2.8. Reporter gene assays

A 2500bp regulatory region encompassing the transcriptional start site of the mouse proCCK gene (-1500/+1000) was amplified from mouse genomic DNA and cloned upstream of Firefly luciferase in the pGL3-Basic backbone using Gibson Assembly. HEK293 cells were co-transfected with the Renilla control vector pRL-TK-d238 [28], FUdeltaGW-rtTA (Addgene #19780), and transcription factor constructs tetO-TBX5 (Addgene #46032), tetO-MEF2C (Addgene #46031), and tetO-GATA4 (Addgene #46030). HEK293 cells were grown in DMEM +/- 10 % FBS and transfections were carried out in 96-well plates at 20, 000 cells/well using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. After transfection with plasmids, cells were cultured in medium with or without serum containing 2 μg/mL doxycycline to induce transcription factor expression from tetO plasmids. After 24 h, luciferase activity was measured using a Promega Dual luciferase reporter kit and a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific). The firefly/renilla luciferase ratio was used as final measurement to avoid artifacts due to differential viability and transfection efficiency.

2.9. Primary cardiomyocyte cell culture and differentiation assays

Neonatal rat cardiomyocytes were isolated from Wistar rats as previously described [35]. Cardiomyocytes were transfected with Nppb-540-luciferase [60] and proCCK-luciferase (-1500/+1000bp) plasmids using Lipofectamine 3000 reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. Transfected cells were stimulated with endothelin-1 (ET-1) (Sigma-Aldrich) and isoprenaline HCl (Sigma-Aldrich). Cyclic mechanical stretch was conducted as described previously [80]. RNA was isolated from cardiomyocyte cultures using the NucleoSpin® RNA isolation kit (Macherey-Nagel). mESC differentiation assays were conducted as described previously [44] and fluorescence signal was measured using a BD LSRFortessa flow cytometer. Sulfated CCK-8 was obtained from Tocris and stocks were diluted in cell culture medium prior to administration.

2.10. Statistical analysis

Statistical analysis was performed in R using the indicated statistical tests. For qRT-PCR experiments, values were normalized to a reference gene (Actb or Tbp). Values from reporter gene and differentiation assays were normalized to indicated controls. All replicates are biological replicates and/or independent experiments.

3. Results

3.1. proCCK is differentially expressed in the four-chambered heart of E10 mouse embryos

We were prompted to examine proCCK in the heart based on re-analyses of mRNA sequencing of TKX5 knockout (KO) cardiomyocytes published in a previous study [47]. TBX5 is a well-known regulator of cardiac development and causative factor in congenital and adult heart disease [6,55,61]. Intriguingly, proCCK was downregulated in cardiomyocytes differentiated from TBX5 KO mESCs compared to wild type and NKX2-5 KO controls, suggesting proCCK is a downstream target of the transcription factor TBX5, but not NKX2-5 (Supplementary Fig. S2a). In the same experiment, cardiac natriuretic peptides Nppa and Nppb were downregulated in both TBX5 KO and NKX2-5 KO cardiomyocytes, indicating divergent regulation of proCCK compared to natriuretic peptides (Supplementary Fig. S2a). In our re-analysis of a separate study, proCCK was also downregulated in TBX5 KO embryonic hearts (Supplementary Fig. S2b) [81]. Intrigued by the expression of proCCK in embryonic cardiomyocytes, as well as a potential connection to the TBX5 transcription factor and its previously reported phenotypes, we sought to explore proCCK as a novel marker of newly differentiated cardiomyocytes, potentially under the direct transcriptional control of the cardiac developmental transcription factor TBX5.

As the expression of proCck in the embryonic heart has not been fully characterized, we first examined proCck mRNA levels by qRT-PCR in embryonic day 10 (E10) mouse pharyngeal arches and hearts. Primary pharyngeal arches (PA1) contain non-cardiac progenitor cells, whereas secondary pharyngeal arches (PA2) contain Isl1+ second heart field multipotent cardiac progenitor cells (CPCs) which migrate out of arches into the newly formed heart and differentiate into cardiomyocytes (Fig. 1a) [72]. As a confirmation of this model, we observed upregulation of mRNA of progenitor marker Isl1 in pharyngeal arches and upregulation of cardiomyocyte marker Tnnt2 in the atria and ventricles (Fig. 1b). Importantly, proCck mRNA was absent in PA1 and PA2 tissues, but was upregulated in the newly formed heart, suggesting proCck is a marker of differentiated, rather than progenitor cells (Fig. 1b). Additionally, the onset of proCck expression coincided with the upregulation of cardiomyocyte markers (Tnnt2), endothelial markers (Pecam1), the natriuretic peptide Nppa, and cardiac developmental transcription factors Tbx5, Nkx2-5, and Gata4 (Fig. 1b). Notably, proCck mRNA was upregulated in the ventricles compared to the atria (2-fold), implying a chamber-specific role of proCck in the embryonic ventricle (Fig. 1b). Furthermore, proCck mRNA was positively correlated with mRNA of Nppa, Tnnt2, and Tbx5 in embryonic structures (Fig. 1c). Thus, developmental proCck expression coincides with heart formation/cardiomyocyte differentiation, the upregulation of core cardiac developmental transcription factors, and the biomarker Nppa.

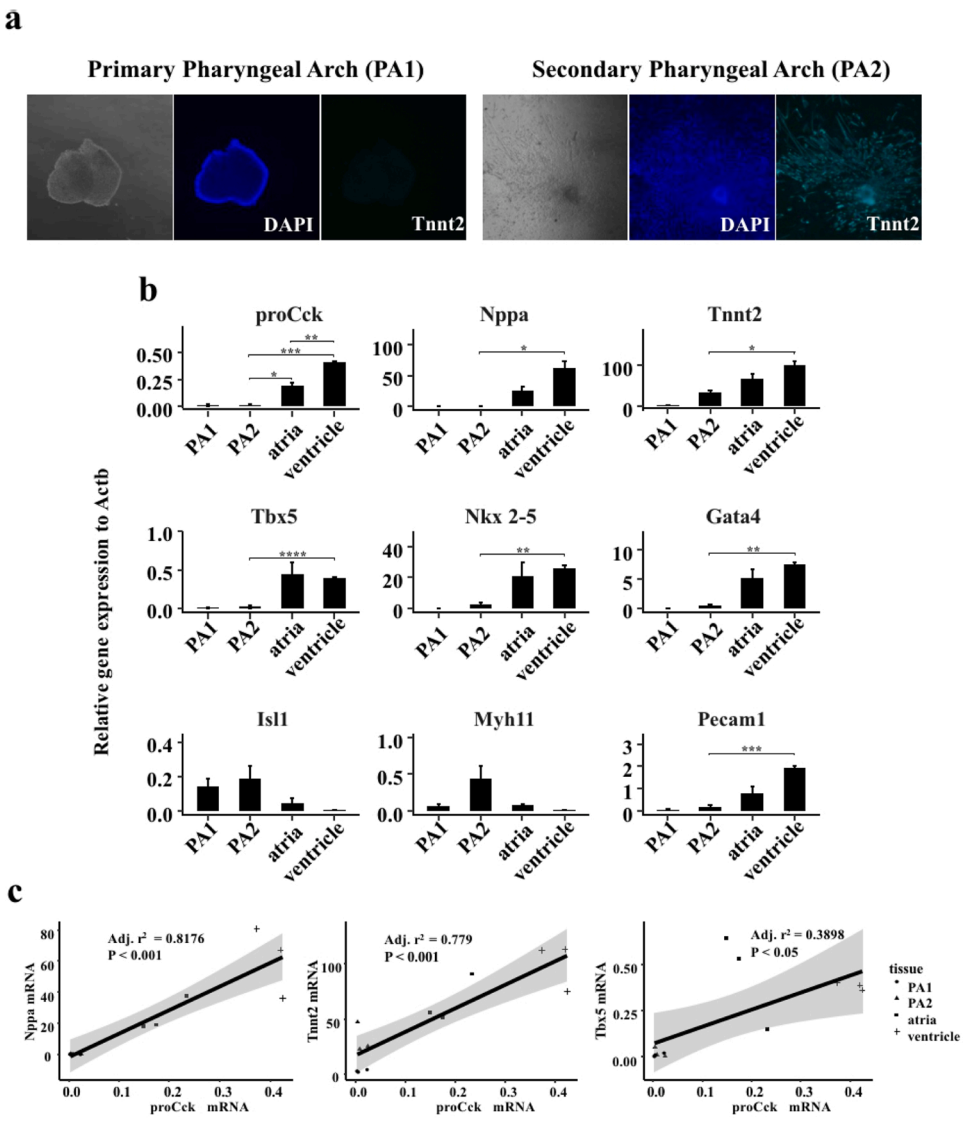

Fig. 1. Procholecystokinin expression in the developing heart. **a** *Ex vivo* culture of pharyngeal arches dissected from embryonic day 10 mice. Migration of cardiac progenitors from secondary pharyngeal arches (PA2) to become differentiated cardiomyocytes expressing cardiac troponin T (Tnnt2). Note the absence of differentiated cardiomyocytes in primary pharyngeal arches (PA1), but migration of cardiomyocytes from the secondary pharyngeal arches (PA2). This illustrates the model for qRT-PCR analysis of undifferentiated vs differentiated native cardiac progenitors. **b** qRT-PCR analysis of proCCK and known early cardiac markers in primary pharyngeal arches (PA1), secondary pharyngeal arches (PA2), atria, and ventricles of E10 mouse embryos. Data is presented as mean + SEM (n = 3). *p < 0.05 **p < 0.01 ***p < 0.001 ****p < 0.0001 (t-test, PA2/atria, PA2/ventricle, atria/ventricle). **c** Linear models of proCck mRNA expression in embryonic tissues versus Nppa, Tnnt2, and Tbx5 showing positive correlation. Gray areas represent a 95 % confidence interval. procholecystokinin (proCck), natriuretic peptide A (Nppa), cardiac troponin T (Tnnt2), T-box transcription factor 5 (Tbx5), NK2 homeobox 5 (Nkx2-5), GATA binding protein 4 (Gata4), ISL1 transcription factor, LIM/homeodomain (Isl1), myosin, heavy polypeptide 11, smooth muscle (Myh11), platelet/endothelial cell adhesion molecule 1 (Pecam1).

3.2. proCCK is expressed at the onset of cardiogenesis and in subpopulations of the developing mouse heart

In order to obtain a more detailed representation of proCCK expression in the developing heart, we further characterized the anatomical localization of proCck mRNA during embryogenesis via whole mount ISH. Strikingly, proCck mRNA was observed in the priomordial linear heart tube of E8.0 embryos, and at this stage no expression was observed outside the heart, suggesting that proCck is a cardiac-specific marker during early embryogenesis (Fig. 2a). Importantly, proCck expression began at the border of the primitive left ventricle with the forming right ventricle and outflow tract, the site of the future interventricular septum (IVS) and a structure whose formation is dependent on TBX5 [41]. In E8.5-9.0 embryos, expression continued in the future IVS, the forming left/right ventricles, and the base of the forming atria (Fig. 2b-g). At E9.5, proCck was still expressed in the atria, and expression of proCck mRNA expanded to the right side of the heart from the IVS (Fig. 2h). Though mostly cardiac-specific, expression of proCck in the somites was also observed at this stage. At E9.75, cardiac expression of proCck continued, though proCck mRNA was also observed in the cranial ridge and hindbrain regions (Fig. 2i-j).

In order to confirm that proCCK protein is also present during cardiogenesis, IHC was performed in E9.5 and E14.5 embryos. Similar to proCck mRNA, proCCK protein was detected in the ventricle and the base of the atria of E9.5 embryos, but was not observed outside of the heart at this stage (Fig. 2k). Additionally, proCCK protein was detected in all four chambers of the hearts of E14.5 embryos (Fig. 2l). Thus, proCCK protein is also present during cardiogenesis, suggesting a functional role of this protein and supporting its use as a marker of early cardiogenesis.

In order to obtain a three-dimensional representation of domains characterized by proCck mRNA expression, whole mount ISH and optical projection tomography (OPT) were performed on the E10.5 embryonic heart. proCck mRNA was observed in specific areas of both the right and left ventricles, the base of the atria, the base of the outflow tract region, and the forming IVS (Fig. 2m-p, Supplementary Video S1). Thus, consistent with qRT-PCR and IHC results, this provides confirmation of proCCK as a marker of early cardiogenesis in the mouse. Furthermore, its expression in first heart field-derived left ventricle/atria and IVS is consistent with regulation by TBX5, but expression in the right ventricle (devoid of Tbx5) suggests that additional regulators might control cardiac expression of proCCK.

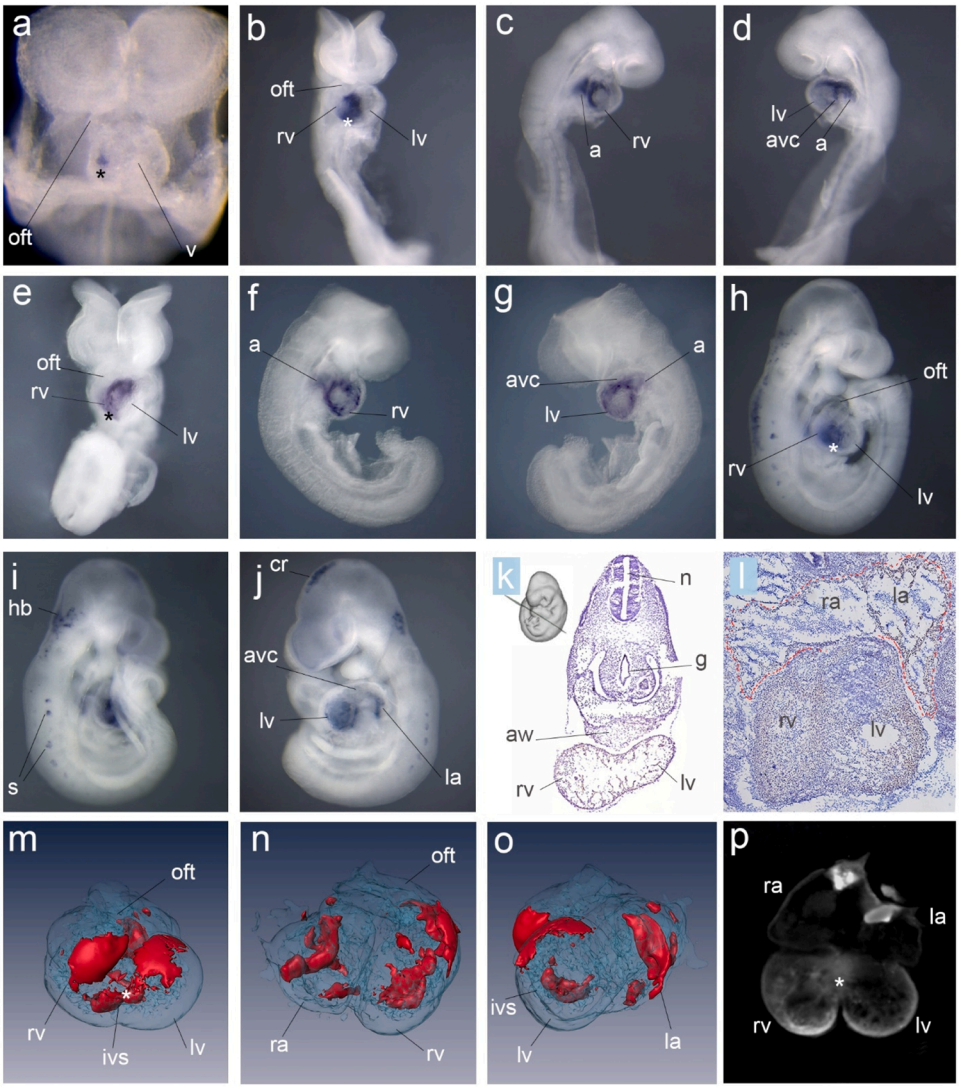

Fig. 2. proCck mRNA and protein localization during mouse cardiogenesis. In situ hybridization (ISH) for procholecystokinin (proCck) (*mRNA shown in purple*), *denotes proCck mRNA localization in the future interventricular septum **a** E8.0 (frontal), **b** E8.5-9.0 (frontal) **c** E8.5-9.0 (right) **d** E8.5-9.0 (left) **e** E8.5-9.0 (frontal) **f** E8.5-9.0 (right) **g** E8.5-9.0 (left) **h** E9.5 (right) **i** E9.75 (right) **j** E9.75 (left). Immunohistochemistry for anti-proCCK (DAB, brown) and counterstain (hematoxylin, purple) in **k** E9.5 (transverse) and **l** E14.5 (transverse) cryosections (Embryonic atria are outlined in red). Reconstruction of proCck mRNA expression (*shown in red*) using optical projection tomography (OPT) in **m** E10.5 heart (front), **n** E10.5 heart (right) **o** E10.5 heart (left) **p** E10.5 heart (four-chamber view, virtual section), * denotes interventricular septum. outflow tract (oft), ventricle (v), right ventricle (rv), left ventricle (lv), atria (a), atrioventricular canal (avc), hindbrain (hb), somites (s), cranial ridge (cr), atrial wall (aw), notochord (n), gut (g), right atria (ra), left atria (la), interventricular septum (ivs).

3.3. Three-dimensional analysis of the expression of proCCK and its receptors in the postnatal mouse heart

Intrigued by the possibility of continued expression of endogenous CCK signalling components postnatally, we next performed whole mount ISH and OPT for proCCK in P1 neonatal mouse hearts. proCck mRNA was broadly observed in the left ventricle, whereas there was only localized expression of proCck in the right ventricle in a specific region near the interventricular wall (Fig. 3a-h, Supplementary Video S2). Notably, proCck mRNA was observed in both the right and left atria, including the region in the right atria encompassing the sinoatrial node (SAN). Furthermore, proCck expression was observed at the base of the great arteries and was also prominently observed in the valves, especially the mitral valve separating the left atria and ventricle. Thus, proCck mRNA is present in specific cardiomyocyte subpopulations of the neonatal mouse heart.

In order to gain insight into not only domains of proCCK expression, but also areas of the postnatal heart which might be suspectible to CCK peptide secreted by other tissues, mRNA of CCK receptors Cckar and Cckbr were examined by whole mount ISH and OPT. Cckar expression was observed in only small domains of the right and left atria, in addition to the base of the aorta (Supplementary Fig. S3). However, Cckbr was observed in broad regions in the right atria encompassing the SAN, in addition to the base of the great arteries and the valves (Fig. 3i-p, Supplementary Video S3). Interestingly, Cckbr was mostly absent from the left atria and ventricles. Therefore, our results suggest that Cckbr is the predominant receptor for CCK signaling in the postnatal mouse heart, and that published effects of exogenous CCK-8 on cardiac rhythm might be due to the localized expression of Cckbr in the right atria [4,21,49].

3.4. Cardiac transcription factor motifs for TBX5 and MEF2C are enriched in the proCCK regulatory region

Due to the interesting expression patterns of proCCK in the embryonic and postnatal heart, and in order to generate further hypotheses regarding the transcriptional effectors governing proCCK expression, a 10 kb region surrounding the transcriptional start site of proCck (8 kb upstream, 2 kb downstream) was analyzed for upregulation of transcription factor binding sites. For this analysis, we applied a motif enrichment method utilizing a compound Poisson model that identifies motifs upregulated versus a background sequence [40]. A total of 446 motifs were included in this analysis, representing transcriptional effectors of retinoid, Wnt, Tgf-β, notch, Rtk, hedgehog, cytokine, hippo, and insulin signaling pathways. Additionally, transcription factors within the known cardiac developmental gene regulatory network were included (TBX5, NKX2-5, GATA4, MEF2C, SRF, IRX4, IRX5, HEY1, HEY2, TBX1, MEIS1, MEIS2, FOXC1, FOXC2, MESP1, MEF2A, MEF2C,

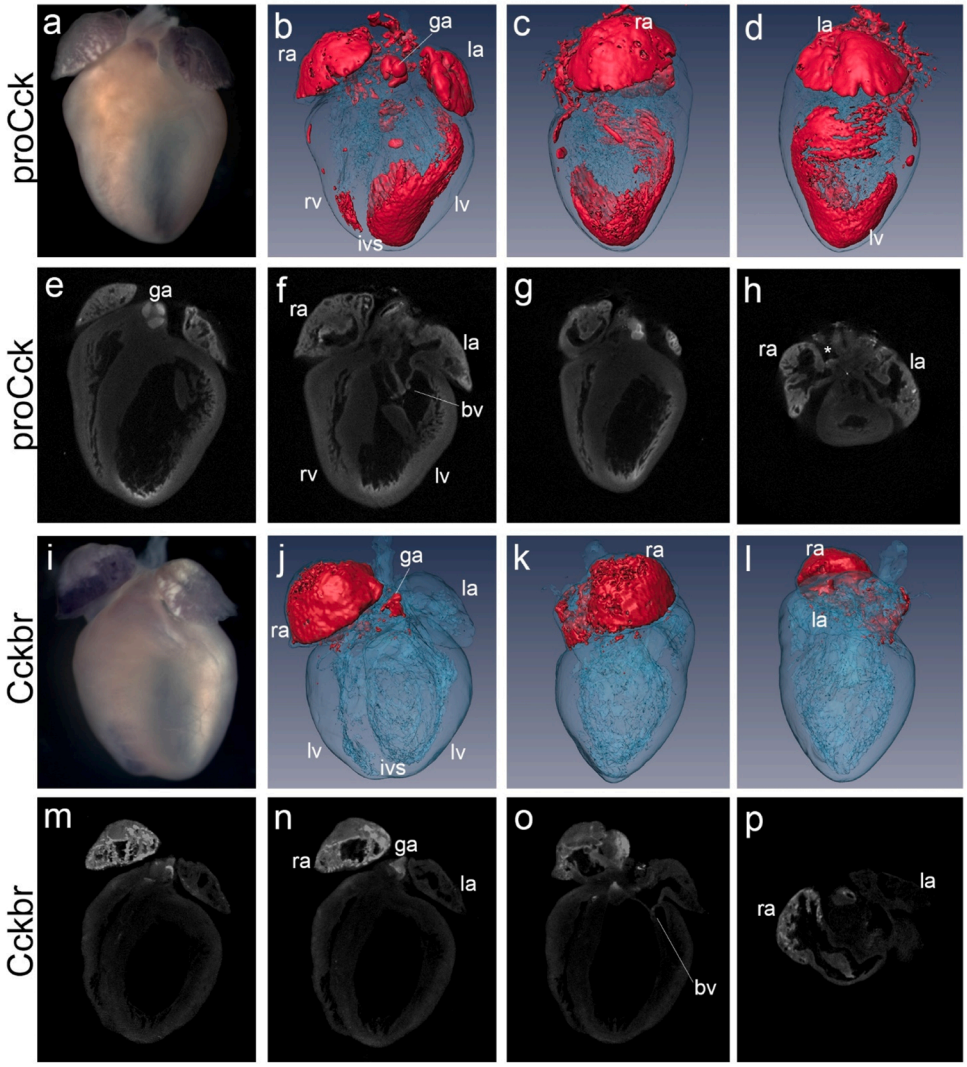

Fig. 3. proCck (a-h) and Cckbr (i-p) mRNA localization in the three-dimensional neonatal mouse heart. *in situ hybridization (ISH)* and optical projection tomography (OPT) for procholecystokinin (proCCK) **a** brightfield image of whole heart (*mRNA expression in purple*) **b** OPT reconstruction (frontal view, *mRNA in red*) **c** OPT reconstruction (right view, *mRNA in red*)) **d** OPT reconstruction (left view, *mRNA in red*) **e** OPT virtual section (frontal, *mRNA in white*) **f** OPT virtual section (frontal, *mRNA in white*) **g** OPT virtual section (frontal, *mRNA in white*) **h** OPT virtual section (transverse, *mRNA in white, *denotes the region containing the sinoatrial node)*. ISH and OPT for Cckar mRNA shown as **i** brightfield image of whole heart (*mRNA expression in purple*) **j** OPT reconstruction (frontal view, *mRNA in red*) **k** OPT reconstruction (right view, *mRNA in red*) **l** OPT reconstruction (left view, *mRNA in red*) **m** OPT virtual section (frontal, *mRNA in white*) **n** OPT virtual section (frontal, *mRNA in white*) **o** OPT virtual section (frontal, *mRNA in white*) **p** OPT virtual section (transverse, *mRNA in white*). right atria (ra), great arteries (ga), left atria (la), right ventricle (rv), interventricular septum (ivs), left ventricle (lv), bicuspid valve (bv).

ISL1, NR2F1 and NR2F2).

Motifs for a total of 35 transcriptional effectors were upregulated based on the cutoff value ($p < 0.05$) (Supplementary Fig. S4). Cardiac transcription factor motifs which were upregulated include TBX5, IRX4, IRX5, MEF2A, MEF2C, MESP1, FOXC1, FOXC2, SRF, and TBX1 (Fig. 4a). Importantly, motifs for the muscle master regulators MEF2A/MEF2C were the most upregulated cardiac motifs in the proCck regulatory sequence (Fig. 4a). Due to the similarity in MEF2A/MEF2C DNA binding motifs, it is not possible to differentiate between the binding sites of these factors. In addition to cardiac developmental transcription factors, signaling pathway effectors for cytokine (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6), hippo (TEAD1, TEAD3, TEAD4), insulin (FOXO1), retinoid (RARB, RXRA), Rtk (EHF, ELF3, ELF4, ELF5, ETV2, FEV, FLI1, SPI1), Tgf-β (SMAD2, SMAD3), and Wnt (LEF1, TCF4) signaling pathways were also upregulated (Supplementary Fig. S5). These results suggest the integration of multiple signaling pathways and cardiac developmental transcription factor motifs in non-coding DNA surrounding the proCck transcriptional start site.

We next analyzed 500 bp segments within the broader 10 kb regulatory region for enrichment of TBX5 and MEF2 binding sites (Fig. 4b-c). Importantly, TBX5 and MEF2 sites were upregulated in a 2500 bp region flanking the transcriptional start site of proCck, suggesting this DNA sequence contains regulatory elements under the control of a TBX5-MEF2 axis. Importantly, the presence of these sites is only suggestive, and itself does not indicate biological activity.

3.5. The proCCK cis-regulatory region is activated by cardiac transcription factors TBX5 and MEF2C

To test our hypothesis that proCCK is downstream of cardiac transcription factors TBX5 and MEF2A/MEF2C, the 2500 bp candidate regulatory region was cloned upstream of a luciferase reporter in order to conduct reporter gene assays. This construct showed similar transcriptional activity in transfected neonatal cardiomyocytes to a 540 bp Nppb reporter construct compared to backbone-only vectors (Fig. 5a). Co-transfection assays with proCCK-luciferase, TBX5, MEF2C, and GATA4 were also conducted. Though not upregulated in motif analyses, GATA4 was also included in reporter gene assays, as TBX5, MEF2C, and GATA4 have been previously shown to participate in transcriptional synergy and to be sufficient to induce the cardiomyocyte fate via transcriptional activation of cardiomyocyte genes [32]. As the proCCK promoter has been previously reported to be responsive to protein in cell culture medium [14], we also conducted reporter gene assays in serum- and serum-free conditions.

Results from reporter gene assays are shown in Fig. 5b. A 1.5-fold increase in proCCK-luciferase expression was considered as evidence for biological significance. proCCK-luciferase activity increased in response to both TBX5 and MEF2C in serum-free medium (Fig. 5b). Additionally, in serum-free medium the combined effect of both factors was greater than that of either alone, indicating moderate transcriptional synergy (Fig. 5b). Surprisingly, TBX5/MEF2C co-activation was

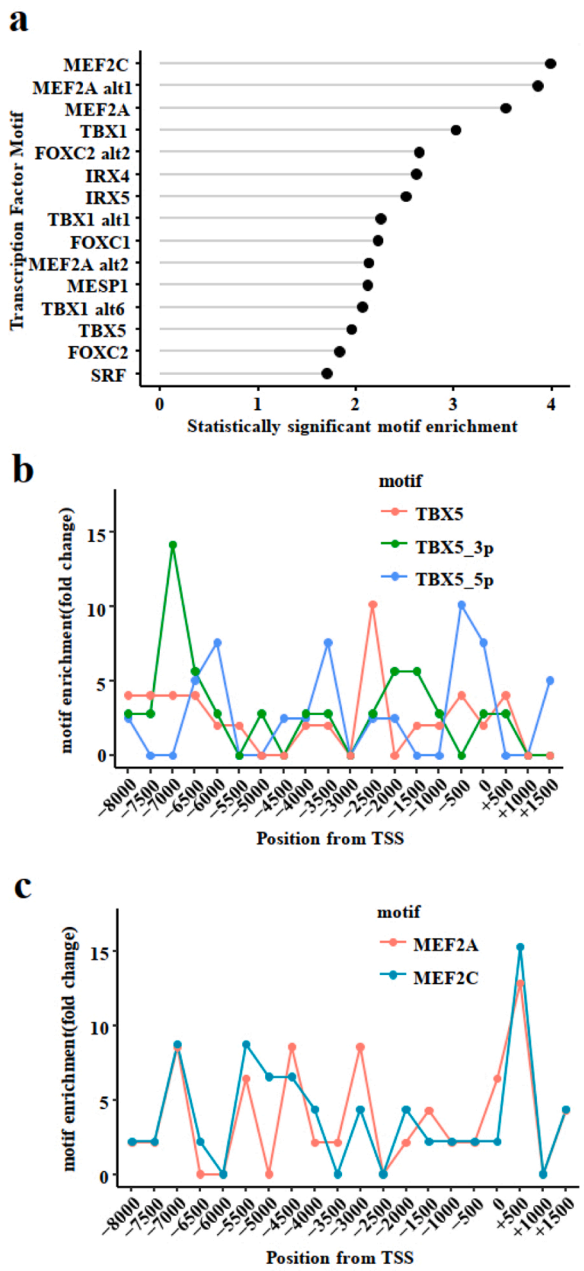

Fig. 4. Transcription factor motif analysis near the procholecystokinin (proCCK) transcriptional start site. **a** Cardiac developmental transcription factors with statistically significant ($p < 0.05$) transcription factor binding enrichment in 10 kb region (8000bp upstream/2000bp downstream) flanking the proCCK transcriptional start site. Myocyte enhancer factor 2C (MEF2C), myocyte enhancer factor 2A (MEF2A), T-box 1 (TBX1), forkhead box C2 (FOXC2), iroquois homeobox 4 (IRX4), iroquois homeobox 5 (IRX5), forkhead box C1 (FOXC1), mesoderm posterior 1 (MESP1), T-box 5 (TBX5), serum response factor (SRF). **b** Breakdown of 10 kb proCCK regulatory region into 500bp fragments to indicate location of regions with enrichment of TBX5 and **c** MEF2 binding sites.

attenuated in the presence of serum, potentially indicating repressive effects of serum on TBX5/MEF2C-mediated transcription (Fig. 5b). Interestingly, serum response factor (SRF) motifs were present in the proCCK regulatory region, though the role of this transcription factor in proCCK-luciferase expression was not further explored. GATA4 did not induce significant proCCK transcription, but attenuated both MEF2C and TBX5 activation of the proCCK construct in both serum-free and serum-containing medium. These results, in conjunction with *in silico* motif analyses and downregulation of proCCK in TBX5 knockout mESC-derived cardiomyocytes and linear heart tubes, indicate that proCCK is a transcriptional target gene of developmental cardiac transcription factors MEF2C and TBX5.

3.6. Regulation of proCCK occurs at the transcriptional level in pathological contexts

Endothelin and adrenergic signaling are involved in pathological compensatory processes during heart failure [33,37]. Due to the known roles of MEF2C and TBX5 as mediators of cardiovascular disease, in addition to reports of proCCK as a biomarker of heart failure [25], proCCK-luciferase activity was also measured after 24 h of stimulation with endothelin-1 and isoprenaline in transfected neonatal cardiomyocytes. Strikingly, ET-1 (100 nM) stimulated a nearly 3-fold increase in proCCK-luciferase activity (Fig. 5c). In turn, isoprenaline (100 nM) induced a nearly 2-fold non-significant increase in proCCK-luciferase activity (Fig. 5c). These results indicate that regulatory elements necessary for neurohormonal activation of proCCK exist in the 2500bp region under study. These results are also in line with previously reported increases in proCck mRNA following isoprenaline treatment in the H9c2 cardiomyoblast cell line [24].

To further explore the role of proCCK as a target of pathological signaling and potential biomarker of cardiovascular stress, changes in endogenous proCCK mRNA were explored in neonatal cardiomyocytes in response to early (4 h) and late (24 h) stimulation with ET-1 and cyclic mechanical stretch (Fig. 6a-d). Importantly, ET-1 is a key regulator of cardiovascular disease progression, hypertrophy, and natriuretic peptide expression [54]. Nppa and Nppb mRNA levels were also analyzed, as these are established biomarkers of the ET-1 and mechanical stretch responses. After 4 h of ET-1 treatment, there was a tendency for Nppb to increase, while proCck and Nppa mRNA levels were not changed (Fig. 6a). In response to 24 h of ET-1 treatment, there was a trend for proCck, Nppa, and Nppb mRNA levels to increase compared to control cells (Fig. 6b). After 4 h cyclic mechanical stretch, proCck and Nppb mRNA levels slightly increased, whereas after 24 h Nppa was the only gene to increase, though this change was not statistically significant (Fig. 6c-d). Correlations between changes in proCck and Nppa mRNA as well as proCck and Nppb mRNA were statistically significant after ET-1 treatment (Fig. 6e). However, only the correlation between changes in proCck and Nppb mRNA were statistically significant in stretched neonatal cardiomyocytes (Fig. 6f). Therefore, proCCK shows similar transcriptional changes as Nppa and Nppb in response to ET-1 stimulation, but the response to mechanical stimuli may be divergent. Importantly, this is the first examination of proCck mRNA in isolated primary neonatal cardiomyocytes.

We next sought to determine the role of proCck mRNA in adult rats post-myocardial infarction in samples taken from the left ventricular wall one week after operation. Interestingly, proCck mRNA was downregulated (>2-fold) post-myocardial infarction, in contrast to Nppa and Nppb, which were both upregulated (Fig. 7a). Negative correlations between proCck and Nppa as well as proCck and Nppb were statistically significant (Fig. 7b-c). Stable levels of cardiomyocyte marker Tnnt2 suggest that these changes do not reflect increased death of cardiomyocytes or an increase in the proportion of endothelial cells (Pecam1) (Fig. 7a). However, smooth muscle marker Myh11 was upregulated, suggesting more cells adopt a smooth muscle phenotype post-myocardial infarction (Fig. 7a). Importantly, no changes were observed in the mRNA levels of Tbx5 and Nkx2-5 post-infarction, whereas there was a trend for Gata4 levels to increase (Fig. 7a). Collectively, these results confirm that proCCK is expressed in the adult rat ventricle, and that it is downregulated post-myocardial infarction, in contrast to the cardiac natriuretic peptides Nppa and Nppb.

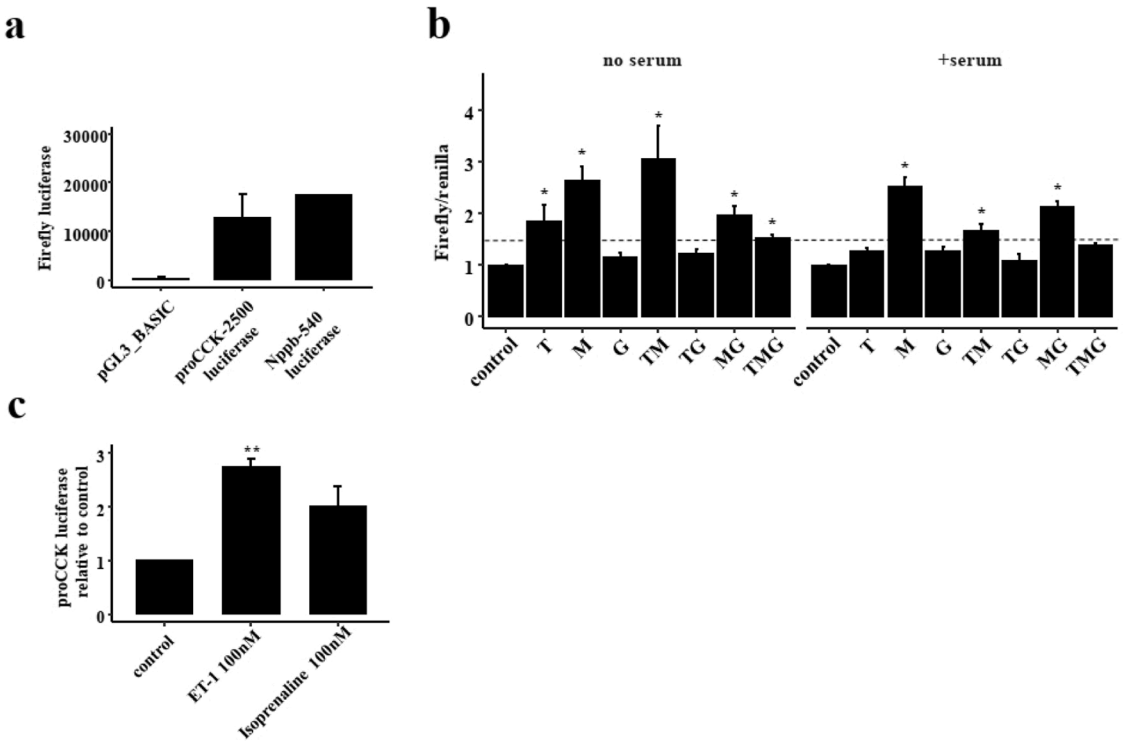

Fig. 5. Regulation of proCCK-luciferase (-1500/+1000) transcription by developmental cardiac transcription factors, endothelin-1, and isoprenaline. **a** Expression of pGL3-BASIC backbone, proCCK-luciferase, and Nppb-540-luciferase in neonatal ventricular cardiomyocytes show activation of regulatory elements. Raw firefly luciferase values are presented as mean + SEM (n = 2-3). **b** Reporter gene assays for proCCK-luciferase in HEK293 cells in response to MEF2C, TBX5, and GATA4 overexpression. TBX5 (T), MEF2C (M), GATA4 (G), TBX5/MEF2C (TM), TBX5/GATA4 (TG), MEF2C/GATA4 (MG), TBX5/MEF2C/GATA4 (TMG). Firefly/renilla luciferase was normalized to control and data is presented as mean + SEM (n = 4). *p < 0.05 (Wilcoxon test vs control). **c** Activation of proCCK-luciferase construct in response to ET-1 and isoprenaline in neonatal ventricular cardiomyocytes following 24 h treatment. Firefly luciferase was normalized to control. Data is presented as mean + SEM (n = 3). **p < 0.01 (T-test vs control).

3.7. Exogenous CCK-8 does not modulate the differentiation of stem cells to the cardiac fate

Endogenous progenitors in the adult heart are known to differentiate to smooth muscle cells, endothelial cells, and rarely cardiomyocytes [76], and this process may be significant to cardiac repair. Interestingly, exogenous CCK-8 was reported to affect cell fate decisions of pancreatic acinar cells and the differentiation of subpopulations of CD4 + T cells [16,85]. In light of the differential expression of proCCK during progression of undifferentiated progenitors (PA2) to differentiated cardiomyocytes, in addition to chamber-specific expression in both embryonic and neonatal hearts, we examined whether the addition of exogenous CCK-8 affects the differentiation of mouse embryonic stem cells (mESCs) to chamber-specific (atrial/ventricular) cardiomyocytes. To this end, a dual reporter mESC line for atrial (atr-RFP) and ventricular (ven-GFP) cardiomyocyte fates was used to conduct differentiation assays. Several parameters were measured, including the %ven-GFP cells, ven-GFP mean fluorescent intensity (ven-GFP MFI), %atr-RFP cells, atr-RFP mean fluorescent intensity (atr-RFP MFI), atrial/ventricular ratio, and atrRFP MFI/venGFP MFI. Results are shown in Fig. 8. No statistically significant changes were observed in the expression of these markers in D2-D12, D2-D5, D6-D9, and D9-D12 treatment windows at 200 ng/mL or 400 ng/mL of exogenous CCK-8 (Fig. 8a-f). These results suggest that exogenous CCK-8 has little to no effect on the differentiation of mESCs to the cardiomyocyte fate, but is instead a cell marker of differentiation to cardiomyocytes.

4. Discussion

Peptides secreted by the heart serve as serum biomarkers of diverse cardiovascular phenotypes [11,22,65,79]. Additionally, they modify cardiovascular function via autocrine/paracrine signaling. In the present study, CCK signaling components were characterized in the developing and postnatal heart. Interestingly, proCck mRNA and protein were detected in the heart during the earliest stages of cardiogenesis in the ventricle and interventricular septum prior to detection in other tissues. Based on these results, proCCK mRNA or secreted peptide could be used together with sarcomeric markers to characterize cardiomyocyte subpopulations differentiated from pluripotent stem cells or reprogrammed from non-cardiomyocyte stromal cells. Furthermore, there may be an early window in which this marker is specific to cardiomyocytes of the interventricular septum. Intriguingly, this early embryonic cardiac-specific expression of proCCK may itself be of therapeutic relevance, as recent reports suggest that levels of other developmentally regulated peptides such as ANP and BNP may be used as maternal biomarkers of congenital heart malformations, fetal arrhythmias, and fetal heart failure [52,53]. It would be interesting to examine CCK peptide levels in the context of fetal disease, particularly those commonly arising from mutations in transcription factors identified in the present study to regulate proCCK expression.

Anatomical localization of CCK signalling components in postnatal mouse hearts reflects known effects of exogenous delivery of CCK-8 peptide. Previous studies demonstrated that CCK signaling regulates heart rhythm in cardiac explants, and that this effect was blocked by CCK receptor antagonists [49]. In our analysis of neonatal mouse hearts, mRNA of proCck and Cckbr were broadly expressed in the right atria in regions including the sinoatrial node, suggesting CCK signalling might be an endogenous regulator of action potential generation. Additionally, exogenous CCK-8 delivery has been reported to induce increases in mean arterial blood pressure [4,21]. We observed distinct expression of proCck and its receptors at the base of the great arteries. As CCK-8 has been reported to induce contraction of smooth muscle cells in the

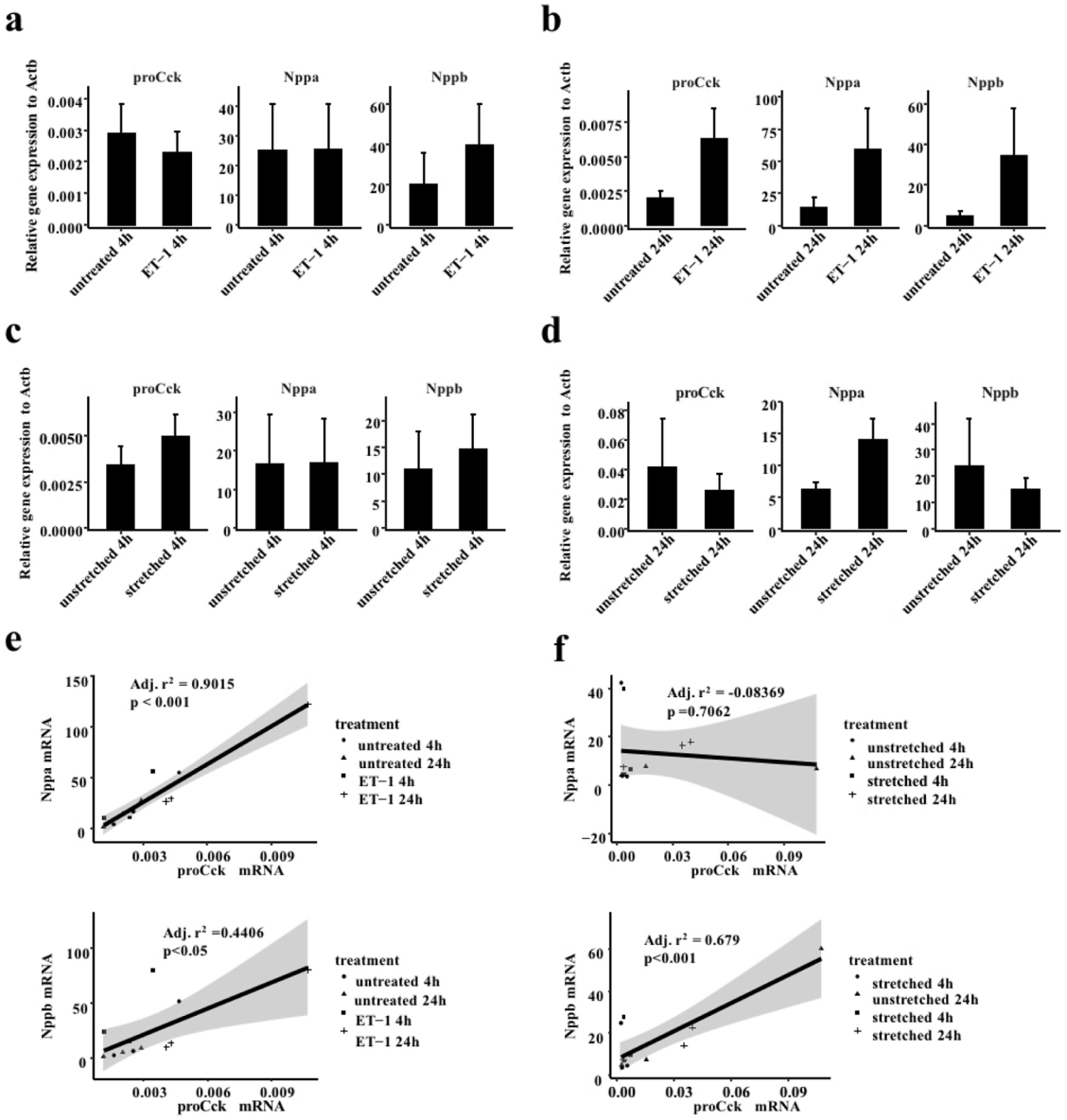

Fig. 6. Regulation of endogenous proCck, Nppa, and Nppb mRNA in rat cardiomyocytes in response to **a** 4 h ET-1 (100 nM) **b** 24 h ET-1(100 nM) **c** 4 h cyclic mechanical stretch **d** 24 h cyclic mechanical stretch. Data is presented as mean + SEM (n = 3). Linear model showing relationship between proCck and Nppa mRNA and proCck and Nppb mRNA upon **e** ET-1 (100 nM) treatment and **f** cyclic mechanical stretch. procholecystokinin (proCCK), natriuretic peptide A (Nppa), natriuretic peptide B (Nppb). The gray areas represent a 95 % confidence interval.

digestive system, it would be interesting to examine if CCK-8 induces contraction of arterial smooth muscle cells to exert effects on mean arterial blood pressure [13].

Our qPCR results indicate that the onset of proCCK expression coincides with the establishment of the prototypical cardiac transcription factor network including TBX5, NKX2-5, and GATA4. Furthermore, *in silico* motif analysis indicated the presence of TBX5 and MEF2 binding sites within the proximal regulatory region of proCCK. This was confirmed by reporter gene assays showing that proCCK is a direct target of TBX5 and MEF2C. Importantly, Nppa and Nppb promoters have long been valuable experimental models of pathological hypertrophy driven by reactivation of GATA4/NKX2-5/TBX5/MEF2C fetal gene regulatory networks. The identification of proCCK as a target of TBX5/MEF2C in the present study can serve as an alternative model of cardiac gene transcription driven by a subset of the cardiac gene regulatory network.

Loss of TBX5 from the onset of development is characterized by gross cardiac morphological defects, whereas cardiomyocyte-specific deletion of TBX5 in adults leads to an onset of arrhythmias, indicating that TBX5 is a master transcriptional regulator of both cardiac formation and maintenance of atrial rhythm [6,55]. Based on the known chronotropic effects of CCK-8, it is tempting to speculate that altered expression of proCCK in TBX5 mutants could contribute to TBX5-mediated effects on cardiac rhythm. This would place proCCK in a class of TBX5 target genes, including a variety of sodium channel genes, which regulate cardiac rhythm [2]. Direct lines of evidence would be needed to address this hypothesis via cardiac-specific knockout and overexpression of proCCK, beyond the scope of the present study. However, our data in cultured rat neonatal cardiomyocytes indicate that proCCK is upregulated by endothelin signaling, which itself was previously shown to control embryonic heart rate [36]. Importantly, exogenous ET-1 also induces expression of Tbx5, suggesting that upregulation of proCck mRNA upon ET-1 treatment observed during the current study might occur via upregulation of the TBX5 transcription factor [3].

In line with our observations of direct regulation of the proCCK

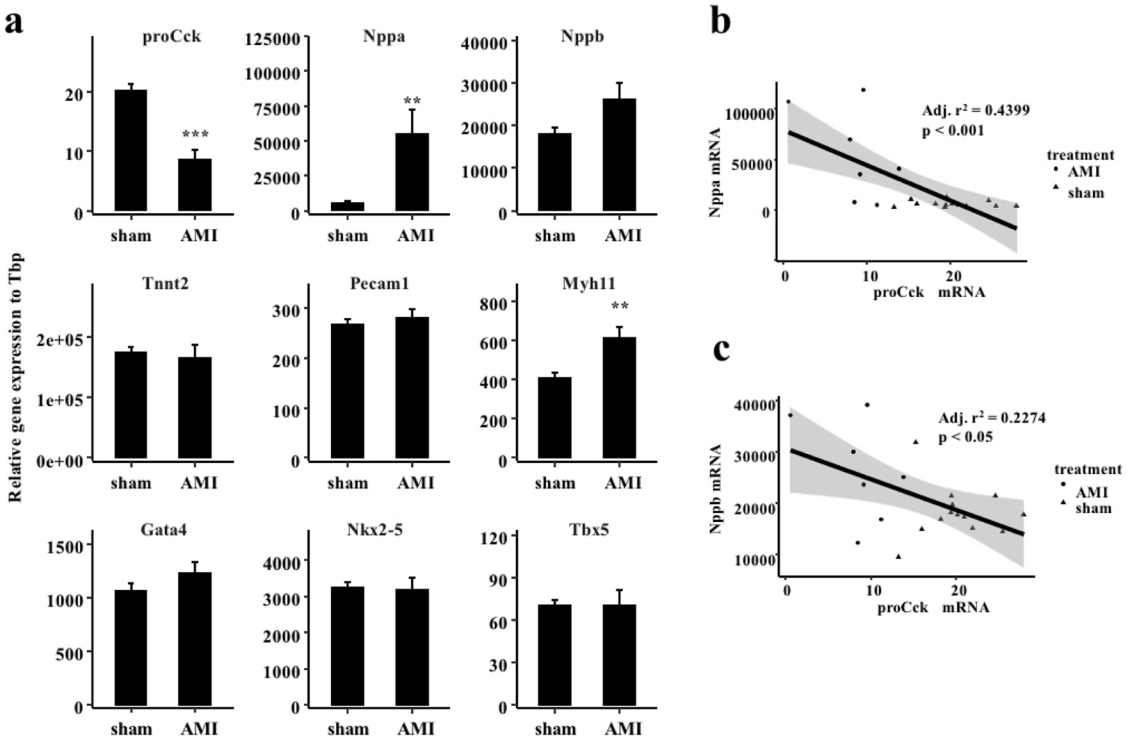

Fig. 7. **a** qRT-PCR analysis of rat hearts one week post-myocardial infarction. Data is presented as mean + SEM (sham, n = 13; AMI, n = 7). *p < 0.05 **p < 0.01 ***p < 0.001 (T-test). **b** linear model showing correlation between proCck and Nppa mRNA and **c** proCck and Nppb mRNA in the infarcted rat hearts. Gray areas represent a 95 % confidence interval. procholecystokinin (proCck), natriuretic peptide A (Nppa), natriuretic peptide B (Nppb), troponin T2, cardiac type (Tnnt2), platelet/endothelial cell adhesion molecule 1 (Pecam1), myosin, heavy polypeptide 11, smooth muscle (Myh11), GATA binding protein 4 (Gata4), NK2 homeobox 5 (Nkx2-5), T-box transcription factor 5 (Tbx5).

regulatory region by MEF2C, embryonic proCCK expression patterns resemble MEF2C expression patterns. MEF2C loss-of-function mutants exhibit gross cardiac morphological defects by E9.5 in which the right ventricle does not form, impeding cardiac looping and developmental progression [46]. Our results indicate that proCCK is strongly expressed in the embryonic right ventricle. Similar to TBX5, MEF2C is also upregulated in response to ET-1, indicating that it may also be involved in ET-1-mediated upregulation of proCCK transcription [31].

In addition to the heart, MEF2C also has significant roles in excitatory neurons of the nervous system [48]. proCCK is also enriched in developing neuronal populations, suggesting that MEF2C is a positive regulator of proCCK in both the cardiac and nervous systems. Importantly, altered CCK signaling is known to exhibit numerous effects on the central nervous system [7,29,39,57]. As MEF2C mutations are also associated with neuropsychiatric disorders, this MEF2C-CCK connection has potential implications for behavioral disorders such as autism, intellectual disability, and schizophrenia [27,82]. This could warrant the investigation of CCK signaling as a biomarker or pharmacological target in patient populations with MEF2C mutations.

Secreted CCK peptide has recently been proposed as a marker of heart failure in elderly female patients and in response to hemodynamic changes in the postnatal porcine heart [23,25]. In this line, our results suggest that proCCK is regulated at the transcriptional level in response to fetal transcription factors and neurohormonal signaling, both components of maladaptive cardiac remodeling [37,58]. These *in vitro* results, combined with previously published studies of endogenous proCCK levels *in vivo* [23], represent a growing body of evidence supporting the use of proCCK as a biomarker of cardiac diseases. However, due to the likely contribution of other tissues to serum CCK levels, the use of CCK as a biomarker in cardiovascular diseases would likely require assays specific to the cardiac form [24].

Interestingly, our results indicate that in contrast to Nppa and Nppb, proCck mRNA is downregulated in the left ventricle following acute myocardial infarction in rats. A decrease in proCCK mRNA levels in the left ventricles was also observed in an isoprenaline-induced myocardial ischemia injury model in rats within 72 h (Leigh et al., unpublished observation). This is in line with previously published observations that proCCK peptide levels decrease in ventricles of pigs following myocardial infarction [23]. Interestingly, a recent study involving gene expression profiling of Nppb + cells from the border infarct region revealed that there is a shift from MEF2-driven homeostatic expression programs to AP-1-driven injury expression programs [77]. Thus, decreased transcription of proCCK in the ventricles of infarcted hearts could be a result of decreased activity of MEF2 factors, though analysis of MEF2 binding at the proCCK promoter in samples isolated from the border infarct region would be necessary to determine this relationship. Additionally, we have not examined the effects of loss-of-function of MEF2 factors on proCCK levels in cardiomyocytes, and whether specific MEF family members (MEF2A, MEF2B, MEF2C, MEF2D) are responsible for its regulation. However, we have clearly shown that overexpression of MEF2C increases the expression of the proCCK promoter. Importantly, a limitation of this study is that atrial expression of proCCK following myocardial infarction was not examined. Additionally, only a single time point was analyzed, and transcriptional changes of proCCK following myocardial infarction likely have temporal changes. Indeed, we observed differences in proCCK transcription at four and twenty-four hours in response to ET-1 in cultured neonatal cardiomyocytes, and *in vivo* transcriptional changes during maladaptive remodeling might be even more dynamic.

In the present study, we conducted in silico analysis of a 10 kb region flanking the proCCK transcriptional start site, in addition to in vitro assays within a smaller 2.5 kb fragment to show upregulation by neurohormonal signalling and cardiac transcription factors TBX5 and MEF2C. Importantly, we cannot exclude that additional regulatory elements might affect proCCK expression. Indeed, context-dependent (developmental vs postnatal, healthy vs diseased) transcriptional

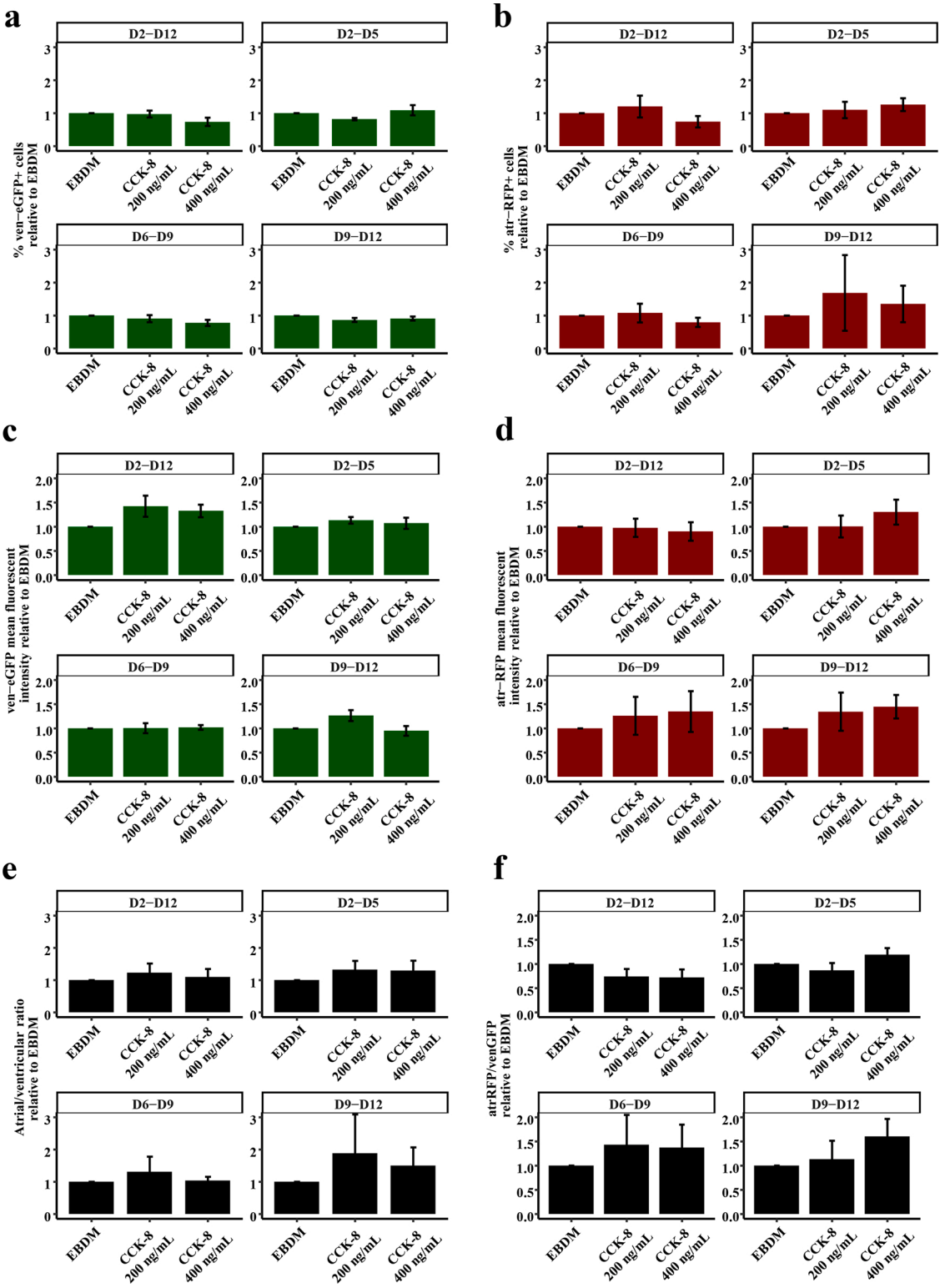

Fig. 8. Effects of exogenous CCK-8 on atrial and ventricular reporter gene expression during the spontaneous differentiation of pluripotent stem cells to cardiomyocytes. Effects of exogenous CCK-8 on **a** %ven-GFP + cells **b** %atr-RFP + cells **c** ven-GFP MFI **d** atr-RFP MFI **e** atrial/ventricular ratio **f** atr-RFP/ven-GFP MFI ratio. Raw values were normalized to control and are presented as mean +/- SEM (n = 5). ventricular reporter gene (ven-GFP, Myl2-eGFP), atrial reporter gene (atr-RFP, SMyHC3-TdTomato), mean fluorescence intensity (MFI).

regulation by distinct regulatory elements has been observed in the case of cardiac natriuretic peptides Nppa and Nppb [30,38,63,69,70]. Though out of the scope of the current study, it would be interesting to perform iterative investigation of proximal and distal proCCK regulatory elements in transgenic mouse assays to attempt to dissect regulatory features and determine overlap with embryonic and postnatal endogenous gene expression patterns observed in the present study.

Despite reported effects of CCK-8 on adult cardiovascular physiology, there are no reports of CCK-8 effects on differentiating cardiac progenitors. In light of robust developmental expression, and the known

roles of other peptides on heart formation and the differentiation of cardiac progenitors [42], we sought to characterize effects of CCK-8 on differentiating stem cells *in vitro*. We observed no salient effects of CCK-8 on cardiomyocyte differentiation, suggesting that the role of secreted CCK peptide *in utero* might be to regulate cardiac function, rather than formation. Importantly, it has been speculated that the CCK-8 form used in the current study is not the active form in the cardiovascular system as it is processed differently in the heart [24]. Indeed, the 1–24 or 25–94 CCK fragments might exert different functions in the heart, and it would be interesting to test these forms in differentiation assays and primary cardiomyocytes should they become available.

Interestingly, this study supports the broader role of gut hormones as potential regulators of cardiovascular function. We here detailed expression of the Cckb receptor in the right atria of postnatal hearts. Interestingly, gastrin, which binds the Cckb receptor, was reported to be cardioprotective in a rat model for myocardial ischemia [83]. Additionally, leptin, ghrelin, and glucagon-like peptide 1 signalling have been shown to exert effects on cardiovascular function [51,56,59]. Furthermore, the gastrointestinal hormone ghrelin was reported to reduce myocardial fibrosis following myocardial infarction, and ghrelin is also expressed both in the developing heart and the adult heart [9,73,59]. Investigation of the developmental and tissue-specific regulation of other traditional gut peptides and their receptors could uncover additional biomarkers and candidate regulators of cardiovascular function, in addition to indicating overlapping functional pathways of peptides known to exert effects on the heart. Moreover, gastrointestinal hormones may exert unexpected effects on cardiovascular function that are relevant to CVD progression. Importantly, analysis of effects of exogenous CCK-8 on embryonic cardiomyocyte contractility, proliferation, or survival were not undertaken in the present study.

In sum, we provide evidence for the use of proCCK as a novel marker of early differentiated cardiomyocytes, and place CCK signaling into a TBX5-MEF2-dependent regulatory framework. Additionally, our data support the further exploration of proCck mRNA and secreted CCK peptide as biomarkers of diverse cardiovascular disease states. Future studies will clarify potential roles of CCK signaling in cardiovascular homeostasis and disease, both in the embryo and in the adult.

Funding

This research was funded by R.S.L grants from Business Finland (Tekes, 3iRegeneration, project 40395/13), the Finnish Foundation for Cardiovascular Research, the Finnish Cultural Foundation; H.J.R. grants from the Academy of Finland (Project 266661), Business Finland (Tekes, 3iRegeneration, project 40395/13), Sigrid Juselius Foundation, the Jane and Aatos Erkko Foundation, and the Finnish Foundation for Cardiovascular Research; B.L.K, grants from Biocentrum Helsinki, Biocenter Finland, and Business Finland (Tekes, 3iRegeneration, project 40395/13).

Ethics approval

Animals used for obtaining samples were obtained via license number ESAVI-2028-041007-2014. Animal experiments were carried out in accordance with the 3R principles of the EU directive 2010/63/EU governing the care and use of experimental animals, and following local laws and regulations [Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013, Government Decree on the Protection of Animals Used for Scientific or Educational Purposes (564/2013)]. The protocols were approved by the national Animal Experiment Board of Finland.

CRediT authorship contribution statement

Robert S. Leigh: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Heikki J. Ruskoaho:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition. **Bogac L. Kaynak:** Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

We are grateful to Alicia Jurado Acosta, Liisa Konttinen, Annika Korvenpää, Lotta Pohjolainen, Kati Rautio, Sirpa Rutanen, and Jaana Rysä for valuable technical assistance. We also thank the Biomedicum Functional Genomics Unit, University of Helsinki for use of qRT-PCR facilities. We acknowledge the Histotechnology and Laboratory Animal Pathology facility, University of Helsinki for assistance with imaging of tissue sections. We thank Veli-Pekka Ronkainen (Biocenter Finland, University of Oulu Bioimaging core facility, Finnish Euro-BioImaging node) for assistance with Optical Projection Tomography.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.peptides.2020.170459.

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