The Matricellular Protein CCN1 Is Essential for Cardiac Development

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Abstract—The matricellular protein CCN1 (formerly named CYR61) regulates cell adhesion, migration, proliferation, survival, and differentiation through binding to integrin receptors and heparan sulfate proteoglycans. Here we show that Ccn1-null mice are impaired in cardiac valvuloseptal morphogenesis, resulting in severe atrioventricular septal defects (AVSD). Remarkably, haploinsufficiency for Ccn1 also results in delayed formation of the ventricular septum in the embryo and persistent ostium primum atrial septal defects (ASD) in ~20% of adults. Mechanistically, Ccn1 is not required for epithelial-to-mesenchymal transformation or cell proliferation and differentiation in the endocardial cushion tissue. However, Ccn1 deficiency leads to precocious apoptosis in the atrial junction of the cushion tissue and impaired gelatinase activities in the muscular component of the interventricular septum at embryonic day 12.5, when fusion between the endocardial cushion tissue and the atrial and ventricular septa occurs, indicating that these defects may underlie the observed AVSD. Moreover, human CCN1 maps to 1p21-p31, the chromosomal location of an AVSD susceptibility gene. Together, these results provide evidence that deficiency in matrix signaling can lead to autosomal dominant AVSD, identify Ccn1<sup>+/−</sup> mice as a genetic model for ostium primum ASD, and implicate CCN1 as a candidate gene for AVSD in humans. (Circ Res. 2006;99:961-969.)

Key Words: apoptosis ■ cardiac development ■ cardiovascular disease ■ integrin ■ matricellular genes ■ matrix metalloproteinases ■ transgenic mice

Atrioventricular septal defect (AVSD) is a common family of genetic disorders, accounting for ~7.5% of the recognized congenital heart disease (CHD) in humans.<sup>1</sup> During cardiac development, the endocardial cushion tissue expands through formation of the cardiac jelly, a specialized extracellular matrix (ECM) between the endocardium and myocardium, into which endocardial cells that have undergone epithelial-to-mesenchymal transformation (EMT) invade. Subsequent growth and remodeling of the cushion tissue, coupled with its fusion with the developing atrial septum and muscular interventricular septum, lead to formation of the definitive atrioventricular (AV) septa and valves.<sup>2,3</sup> Defects in this process are manifested in a spectrum of abnormalities with varying severities, ranging from partial forms of AVSD with ostium primum atrial septal defects (ASD) to complete forms with absence of the AV septa and lack of partitioning of the AV valve into separate mitral (left) and tricuspid (right) valves. Although AVSD is frequently associated with Down’s syndrome (trisomy 21), nonsyndromic AVSD also occur. Although nonsyndromic AVSD may be a sporadic trait or the result of multifactorial inheritance, strong evidence also support the presence of susceptibility genes for AVSD that are autosomal dominant and incompletely penetrant.<sup>4–6</sup>

CCN1 (formerly named CYR61 [CYsteine-Rich angiogenic inducer 61; NM_001554]) is a secreted, cysteine-rich protein associated with the ECM. A dynamically expressed protein, CCN1 serves regulatory rather than structural roles and can be considered a matricellular protein.<sup>7</sup> CCN1 promotes cell adhesion, migration, proliferation, differentiation, and survival or death in a cell type–dependent manner.<sup>8</sup> CCN1 also induces angiogenesis in vivo, and Ccn1-null mice undergo embryonic lethality, in part, because of placental vascular insufficiency and compromised embryonic vessel integrity.<sup>9,10</sup> Mechanistically, CCN1 binds and functions through specific integrin receptors, requiring heparan sulfate proteoglycans (HSPGs) as coreceptors in some contexts. Whereas CCN1 promotes cell adhesion, migration, and proliferation in fibroblasts through integrins α<sub>1</sub>β<sub>1</sub>, α<sub>2</sub>β<sub>1</sub>, and α<sub>β</sub>5, respectively, the proangiogenic activities of CCN1 in endothelial cells are mediated through α<sub>β</sub>. Recently, the specific binding sites of CCN1 for these integrins and HSPGs have been identified.<sup>11,12</sup>

Human CCN1 has been mapped to chromosome 1p22-p31,<sup>13</sup> the same region (1p21-p31) as an AVSD susceptibility gene identified in a large kindred.<sup>8</sup> In this study, we show that Ccn1-null mice exhibit AVSD with complete penetrance. Remarkably, haploinsufficiency for Ccn1 also results in ostium primum ASD in 20% of adults, resembling a common form of partial AVSD recognized clinically in humans. These results implicate CCN1 as a candidate gene for AVSD. Ccn1
deficiency does not affect endocardial cushion tissue formation or EMT but results in accelerated apoptosis in the cushion tissue and reduced gelatinase activity in the developing ventricular septum and AV valves. These results show that the matricellular protein CCN1 is essential for heart septation and underscore the importance of matrix signaling in valvuloseptal morphogenesis.

Materials and Methods

Animals, Histology, and Immunohistochemistry
Generation of Ccn1-deficient mice, knock in of a lacZ reporter gene, and detection of β-galactosidase activity have been described previously.10 For histology, formalin-fixed embryos or postnatal hearts were embedded in paraffin, and 7-μm sections were stained with hematoxylin/eosin (H&E). For immunohistochemistry, embryos fixed in 4% paraformaldehyde were sectioned and stained with antibodies against α-smooth muscle actin antibody (clone 1A4; Sigma) or activated caspase-3 (5A1, Cell Signaling) and detected with alkaline phosphatase (AP) chromogen (Zymed). For proliferation assay, sections were stained with polyclonal anti-Ki67 antibodies (Novocastra Laboratory) and Alexa Fluor 594–conjugated secondary antibody (Molecular Probes) and counterstained with 4',6-diamidino-2-phenylindole diacetate (DAPI) (Sigma). Images were acquired through a fluorescence microscope using a digital camera, and superimposition of Alexa Fluor 594 (red) and DAPI (blue) staining was performed using the AxioVision (Zeiss) software.

In Situ Hybridization
Embryos were fixed in 4% paraformaldehyde and paraffin embedded; 20-μm sections were hybridized to digoxigenin-labeled Mox-1 antisense riboprobe (generous gift of Christopher Wright, Vanderbilt University, Nashville, Tenn) and visualized with AP-conjugated anti-digoxigenin antibody (Roche) and AP-chromogen. Sense riboprobe of Mox-1 was used as control (data not shown).

AV Cushion Explant Culture
AV cushion explants were cultured on collagen gels as described.14 Embryonic day 9.5 (E9.5) (somite no. 21–26) AV cushion tissues were microdissected and cultured on type I collagen (BD Biosciences) gels, then fixed and stained with fluorescein phalloidin (Invitrogen) and counterstained with DAPI after 48 hours.

TUNEL Assay and Zymography
Paraffin sections (10 μm) of 4% paraformaldehyde-fixed embryos were subjected to TUNEL assay using ApopTag Red In Situ Apoptosis Detection Kit (Chemicon) per the instructions of the vendor. In situ zymography was performed as described.15 H9c2 cardiomyocytes (American Type Culture Collection) were treated

Figure 1. Expression of β-galactosidase driven by the Ccn1 promoter. A, Intense X-gal staining in the myocardium (black arrow) of the bulbus cordis of the primitive heart of E8.5 Ccn1+/− embryos; less staining was seen at endocardium (black arrowhead). White arrow points to the foregut. B, Whole-mount embryo of E9.5 Ccn1−/− showing hypoplastic ventricles. Scale bar=200 μm. C, X-Gal staining at E10.5 embryo showing prominent cardiac staining (arrow). D, Whole-mount E10.5 Ccn1−/− embryo showing severe hypoplasia of the heart (chevron). Scale bar=200 μm. E, Expression of Ccn1 mRNA in E8.5 hearts was quantified by quantitative real-time RT-PCR, normalized to cyclophilin mRNA as internal control. No detectable signal was observed in Ccn1−/− (n=3 per genotype; P<0.03 between +/+ and −/−). Bars=200 μm.
with 4 μg/mL recombinant CCN1 protein in DMEM containing 0.1% BSA. Conditioned medium was collected after 1 to 3 days and subjected to gelatin gel zymography following standard protocol with precast 10% zymogram gel containing gelatin (Bio-Rad).

**Quantitative Real-Time RT-PCR**
cDNA reverse transcribed from total RNA isolated from E12.5 embryonic hearts was quantified using a MyIQ single-color real-time PCR detection system (Bio-Rad). PCR in triplicate contains IQ SYBR Green Supermix (Bio-Rad), cDNA template and 200 nmol/L primer pairs for either Ccn1 or cyclophilin (internal control). Sense (S) and antisense (AS) primers are: Ccn1 S, 5'-GGCGAGACAGCCCAAGATCC-3'; Ccn1 AS, 5'-TTCTGTGCTGCAGGATTGTG-3'. Cyclophilin S, 5'-GGCAATACTGGGACAAAC-3'; cyclophilin AS, 5'-TTCTGGTCTGCAGAGGTGTG-3'.

**Statistical Analysis**
Values are expressed as mean±SD. Comparisons were made by Student’s t test; statistical significance was set at P<0.05.

**Results**

**Expression of Ccn1 During Heart Development**
We have previously placed the lacZ gene under the control of the endogenous Ccn1 promoter, thereby allowing Ccn1 expression to be monitored by following β-galactosidase activity. As judged by X-gal staining, Ccn1 is expressed in the E8.5 myocardium and endocardium of the bulbus cordis region in the primitive heart (Figure 1A). Prominent cardiac expression of Ccn1 was seen at E10.5 (Figure 1B), especially in the truncus arteriosus (arrowhead, Figure 1C), which later divides to form the aorta and pulmonary trunk. Ccn1 expression was also seen around the AV canal at later stages and in the AV cushion tissue and septum (Figure 1D and 1E). At E13.5, expression around the atrial junction had subsided but was prominent in the developing AV valvular structure (arrow in B). Ccn1 expression was high in the muscular component of the ventricular septum and the membranous mesenchyme of the cushion tissue and between the mesenchymal cap of the atrial septum and the AV cushion tissue (Figure 1F). By E13.5, expression around the atrial junction had subsided but was prominent in the developing AV valvular leaflets (Figure 1G). Expression was high in the vessel wall of the aorta, although little or no expression was seen in the pulmonary and aortic valve leaflets (Figure 1H). Thus, Ccn1 expression coincides with the temporal and spatial pattern of key events in atrioventricular valvuloseptal morphogenesis.

The levels of Ccn1 mRNA expressed in E12.5 embryonic hearts were examined by quantitative real-time RT-PCR, confirming a true null phenotype in Ccn1−/− embryos and haploinsufficiency in Ccn1+/− embryos (Figure 1I).

**Ccn1-Null Mice Exhibit AVSD**
Given the cardiac expression of Ccn1 described above, we examined the effects of Ccn1 deficiency in the heart. The atrial and ventricular septa were formed by E14.5 in wild-type (WT) embryos, as were the AV valves (Figure 2A and 2B). Ccn1−/− embryos, however, exhibited AVSD with 100% penetrance, with 55% (10/18) showing complete AVSD and 45% (8/18) showing VSD (Figure 2E through 2G). Severe malformation of the atrial and ventricular septa and defects in AV valve morphogenesis were evident. During AV septation and valve formation, the common AV canal is divided to form the aorta and pulmonary trunk.

**Figure 2.** Atrioventricular septal defects in Ccn1−deficient embryos. Transverse sections of E14.5 embryonic hearts were H&E stained. A and B, WT embryos showed well developed 4-chambered hearts, with complete closure of the ventricular septum (arrowhead in A) and extended AV valvular structure (arrow in B). C and D, Ccn1−/− embryos displayed VSD (arrowheads in C), ASD (arrow in D), and dysplastic atrioventricular valves (arrow in D). E and F, Ccn1+/− hearts exhibited VSD (arrowhead in E), ASD (arrow in E), and AV valve orifice (arrow in F). G, High-magnification views of dashed box in F and its adjacent sections reveal evidence of AV valve orifice, including gaps between superior (SC) and inferior (IC) endocardial cushions of AV canal (green arrows) with blood cells in between the 2 AV cushions (red arrows). H, left, Diagram illustrates the partitioning of the AV canal (AVC) into tricuspid (TV) and mitral (MV) valves, viewed from the atrial perspective. A hallmark of complete AVSD is the presence of a common AV valve orifice resulting from impaired fusion between the inferior (IC) and superior (SC) cushion tissue. Right, the plane of section in sections shown in G, showing how the inferior and superior cushion tissue are viewed. Bars=100 μm.
partitioned by the fusion of the inferior and superior endocardial cushion tissues into separate left and right components, which undergo further morphogenesis to form the mitral and tricuspid valves. Fusion of the inferior and superior cushion tissue was impaired in affected Ccn1<sup>−/−</sup> embryos, as evidenced by gaps between the 2 endocardial cushions, resulting in a common AV valve orifice that is a hallmark of complete AVSD (Figure 2F and 2G). Nearly all Ccn1-null embryos showed defects in maturation of the interventricular septum (IVS), which forms by the fusion of a muscular component that extends as an outgrowth of the ventricular wall and is a membranous component that forms from the AV cushion tissue. This fusion is defective in Ccn1-null embryos (Figure 2E), consistent with the expression of Ccn1 in the junction between the muscular component of the septum and the membranous mesenchyme (black arrow, Figure 1F).

**Haploinsufficiency in Ccn1 Leads to Atrial Septal Defects**

Surprisingly, Ccn1<sup>+/−</sup> embryos also display AVSD. This finding is significant because autosomal dominant inheritance has been observed in nonsyndromic AVSD in humans. Histological analysis showed that ~67% of Ccn1<sup>+/−</sup> embryos (18/27) exhibited cardiac defects of varying severity at E14.5, including complete AVSD (4/27), VSD (11/27), or dysplastic mitral valves (3/27) (Figure 2C and 2D). Although VSD phenotypes in Ccn1<sup>+/−</sup> hearts were conspicuous at E13.5 and E14.5, they were not observed after E15.5 (n = 35), indicating that IVS formation is delayed in Ccn1<sup>+/−</sup> embryos but not persistently impaired. However, ~20% of adult Ccn1<sup>+/−</sup> mice (5/25) show persistent ASD (Figure 3). Although cardiac septal defects might form as secondary effects of placental abnormalities, these cardiac defects are not likely related to the placenta because the Ccn1<sup>+/−</sup> placenta develop normally.

Septation of the common atrial chamber begins at E10.5 with the formation of the septum primum, which approaches the AV cushion tissue from the atrial chamber roof. The opening between the 2 atrial chambers, the ostium primum, is obliterated on fusion of the septum primum with the AV cushion and is replaced by the ostium secundum, which forms by apoptosis in the septum primum. The septum secundum then forms, with the foramen ovale serving as the opening between the atria. Postnatal fusion of the septum primum and septum secundum closes the openings of both septa and ensures complete separation of the left and right atria after birth when pulmonary circulation is established (Figure 3A and 3B). In affected Ccn1<sup>−/−</sup> neonates, the septum primum failed to fuse completely with the cushion tissue and the ostium primum remained (Figure 3C and 3D). Consistent with a role in this fusion event, Ccn1 was expressed in the AV cushion, where fusion with the mesenchymal cap of the septum primum occurs (Figure 1F). Although formation of the septum secundum was successful, postnatal fusion of the 2 septa was precluded by the defective septum primum. Consequently, blood cells were trapped between the atria as the hemodynamic flow was in disarray (Figure 3C and 3D) and the foramen ovale remained patent in affected adult Ccn1<sup>−/−</sup> mice (Figure 3E and 3F).

**EMT and Differentiation of Cushion Mesenchyme**

Central to valvuloseptal morphogenesis is the AV cushion tissue, which forms when endocardial cells undergo EMT and invade into the cardiac jelly. We therefore examined whether EMT occurs in AV cushion tissue explants isolated from E9.5 Ccn1<sup>−/−</sup> embryos. Endocardial cells that successfully undergo EMT are capable of migrating into collagen gel on which the explant is cultured. The percentages of cells migrating into collagen gel in WT and Ccn1<sup>−/−</sup> cushion explants were indistinguishable (Figure 4E), indicating that Ccn1 is not essential for EMT. We next investigated the differentiation of the mesenchymal cells within the cushion tissue using molecular markers. Smooth muscle α-actin is expressed as mesenchymal cells gain characteristics of smooth muscle–like myofibroblasts, and Mox-1 is a marker of more differentiated mesenchymal cells in the cushion. Expression of both smooth muscle α-actin and Mox-1 was similar in WT and Ccn1<sup>−/−</sup> embryonic hearts (Figure 4F), indicating that EMT and further differentiation of cushion tissue mesenchymal cells were successful in Ccn1<sup>−/−</sup> embryos.
Cell Proliferation

Valvuloseptal morphogenesis begins with the expansion of the cushion tissue and fusion with the AV septa and valves. Coordinated with these morphogenetic events are highly regulated cell proliferation and programmed cell death in the cushion tissue, and bone morphogenetic protein (BMP)-4 deficiency causes septal defects attributable to reduced cushion mesenchymal cell proliferation. As judged by Ki67 staining, the percentages of proliferating cells in WT and Ccn1-null AV cushion were similar as the cushion tissue actively expands at E11.5 and E12.5 (Figure 5). Thus, cell proliferation in the AV cushion is not affected by Ccn1 deficiency.

Precocious Apoptosis in the Ccn1-Deficient Cushion Tissue

CCN1 can induce apoptosis in fibroblasts but promote endothelial cell survival in vitro. Consistent with its prosurvival function in endothelial cells, vascular cells of large vessels in Ccn1-null mice undergo apoptosis. As previously reported, very few apoptotic cells were detected in the WT AV cushion tissue as it began to fuse with the AV septa at E12.5 (Figure 6A and 6B). By contrast, a large number of TUNEL- or activated caspase-3–positive apoptotic cells were detected in the Ccn1-null cushion proximal to the atrial septum (Figure 6A through 6E and 6K through 6N), where Ccn1 expression was detected in E14.5 cushions and developing valves in both WT and Ccn1-null embryos by in situ hybridization. These results suggest that CCN1 is required for cushion tissue cell survival at this embryonic stage and that its absence results in apoptosis. This early onset of apoptosis in the E12.5 cushion tissue may be detrimental to atrial septum fusion, leading to the ASD phenotype. By E13.5, the atrial septum primum fusion has occurred, and expression of Ccn1 was diminished in the atrial fusion site of the AV cushion (Figure 1G), correlating with a dramatic increase in apoptosis in the WT cushion tissue (Figure 6F and 6G), consistent with previous reports. Thus, although programmed cell death occurs in the AV cushion on fusion with the atrial septum, precocious onset of apoptosis in Ccn1-null mice may prevent the fusion event from occurring successfully. Although Ccn1 deficiency also leads to VSD, no abnormal cell death was detected at the fusion site between the ventricular septum and the cushion tissue (Figure 6).
Gelatinase Deficiency in the Ccn1-Null Heart

Matrix metalloproteinases (MMPs) and their regulators are thought to be involved in aspects of cardiac development as regulators of growth factors activity and ECM composition. Several MMPs have been identified as key targets of CCN1-regulated signaling pathways in fibroblasts, prompting us to examine MMP activity in embryos. Gelatinases (MMP-2 and MMP-9) degrade type IV collagen in basement membrane and are of particular importance in tissue remodeling. Gelatinase activity was detected by in situ gelatin zymography in the E12.5 WT heart (Figure 7A and 7D). This activity was blocked by the inhibitor 1,10-phenanthroline (PNT), confirming that it is attributable to a metalloprotease (Figure 7B and 7E). By contrast, gelatinase activity was greatly diminished in the Ccn1−/− heart, particularly in the muscular component of the IVS and the valvular leaflets where defects are observed (Figure 7C and 7F). To test whether CCN1 is able to directly regulate the expression of gelatinases, which are synthesized as zymogens and are proteolytically cleaved to the active forms, we treated H9c2 cardiomyocytes with purified CCN1 protein. Indeed, CCN1 treatment dramatically increased both secreted pro-MMP-2 and activated MMP-2 (Figure 7G). Thus, CCN1 is an inducer of MMP-2 in cardiomyocytes, and a deficiency in gelatinase activities in Ccn1-null mice may compromise the matrix remodeling process that is crucial for fusion of the IVS and AV cushion tissue.
provide the first evidence that haploinsufficiency in an ECM protein can lead to autosomal dominant ASD and implicate CCN1 as a candidate gene for AVSD in humans.

CHDs are the most common group of birth defects in humans, encountered in ~1% of live births and 10% of stillborns. Consistent with the complex nature of cardiac development, nullizygosity of a multitude of genes in mice result in heart defects. Whereas human CHDs exhibit predominantly autosomal dominance rather than homozygous recessive modes of transmission, only a very few genes, encoding cardiac-specific transcription factors, have been identified to cause autosomal dominant CHD attributable to haploinsufficiency in mice. Heterozygous mutations in these transcription factor genes, including Tbx1 (DiGeorge syndrome), Thx5 (Holt–Oram syndrome), and Nkx2–5 (conduction defects, tetralogy of Fallot), also cause CHDs in humans. Because CCN1 is a matricellular protein that acts through binding to integrins and HSPGs, Ccn1 mutant mice provide a unique animal model for addressing the role of matrix signaling in heart development. Aside from Ccn1 and AVSD1, another genetic locus known to be associated with nonsyndromic AVSD is CRELD1, which encodes a predicted matricellular cell adhesion molecule based on its sequence. Although the activities of CRELD1 have not yet been examined, these findings are consistent with the notion that matrix signaling is crucial for AV septation and valvular formation.

The AV cushion tissue is central to valvuloseptal morphogenesis, and malformations in the cushion tissue, such as those caused by deficiency in versican, hyaluronan synthase 2, or the transforming growth factor (TGF)-β type III receptor, result in AVSD. Although Ccn1 and AVSD1, another genetic locus known to be associated with nonsyndromic AVSD is CRELD1, which encodes a predicted matricellular cell adhesion molecule based on its sequence. Although the activities of CRELD1 have not yet been examined, these findings are consistent with the notion that matrix signaling is crucial for AV septation and valvular formation.

Discussion

ECM signaling plays a critical role in developmental processes. As demonstrated in this study, the matricellular protein CCN1 is indispensable for cardiovascular development, and nullizygosity in Ccn1 results in severe AVSD and embryonic death. Although heterozygous Ccn1 mice are largely viable, 20% of them display ostium primum ASD. Furthermore, CCN1 maps to the same human chromosomal location (1p21-p31) as that of a susceptibility gene for nonsyndromic AVSD, AVSD1, mutations in which also lead to predominantly ostium primum ASD. These results provide the first evidence that haploinsufficiency in an ECM protein can lead to autosomal dominant ASD and implicate CCN1 as a candidate gene for AVSD in humans.

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involved in matrix remodeling in fibroblasts and upregulates MMP-2 levels and enhances its activation in cardiomyocytes (Figure 7G). Although deficiency in gelatinase activities may contribute to cardiac defects, it is by itself insufficient to cause cardiac abnormalities. No heart defects have been reported in mouse models with null mutations in MMP-2 (gelatinase A), MMP-9 (gelatinase B), or both. However, loss of MMP-2 and/or -9 protects against the tissue fibrosis seen following myocardial infarction in adults, demonstrating that these gelatinases do have significant effects on myocardioctyes in vivo.

Ccn2 (formerly named CTGF [Connective Tissue Growth Factor]) is a closely related homolog of Ccn1, and both genes are highly expressed in the myocardium and AV cushion during heart development and play a role in adaptation of the heart to cardiovascular stress. Although Ccn2-null mice show apparently normal heart development through birth, there may be significant functional redundancies between these genes that allow Ccn1 to compensate for the loss of Ccn2 in the cardiovascular system, because Ccn1 and Ccn2 have similar activities in vitro. Hence, although specific functions of CCN1 are clearly indispensable, the potential roles of Ccn2 and possible overlapping functions between Ccn1 and Ccn2 in cardiac development remain to be established.

How Ccn1 is integrated into the regulatory circuits that control heart development is currently unknown, although consensus binding sites for the cardiac transcription factors Nkx2.5 and GATA-4 are found on its promoter. Mechanically, CCN1 can function to regulate cell survival/death and the expression of genes involved in matrix remodeling. In addition, CCN1 may potentially modulate the activities of other regulatory pathways through interactions with growth or morphogenic factors or their receptors. Because haptoglobin sufficiency in Ccn1 leads to ostium primum ASD resembling that observed in humans, the possibility that mutations in Ccn1 may lead to clinical AVSD clearly warrants further investigation.

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Disclosures
None.

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