A cell-autonomous role of Cited2 in controlling myocardial and coronary vascular development

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Introduction

New cardiac myocytes are efficiently created during foetal life, initially from cardiomyocyte progenitors and subsequently by cardiomyocyte division (reviewed in Bhattacharya et al.). In gross morphological terms, the thin-walled mouse embryonic ventricle begins to develop finger-like projections of trabecular myocardium at the endocardial surface by embryonic day (E) 10.5 (reviewed in Sedmera et al.). Thickening of the epicardial myocardium begins by E11.5 and results in the formation of the compact myocardium. This process occurs concomitantly with the formation of the coronary vessels (reviewed in Luttun and Carmeliet and Reese et al.). Many zygotic mutations associated with abnormal extra-embryonic tissue development also have abnormal myocardial development as a phenotypic feature, and complementation experiments indicate that myocardial development is dependent

Myocardial development is dependent on concomitant growth of cardiomyocytes and a supporting vascular network. The coupling of myocardial and coronary vascular development is partly mediated by vascular endothelial growth factor (VEGFA) signalling and additional unknown mechanisms. We examined the cardiomyocyte specific role of the transcriptional co-activator Cited2 on myocardial microstructure and vessel growth, in relation to Vegfa expression.

Methods

A cardiomyocyte-specific knockout of mouse Cited2 (Cited2lox/lox) was analysed using magnetic resonance imaging and histology. Ventricular septal defects and significant compact layer thinning (P < 0.02 at right ventricular apex, P < 0.009 at the left ventricular apex in Cited2lox/lox vs. controls, n = 11 vs. n = 7, respectively) were found. This was associated with a significant decrease in the number of capillaries to larger vessels (ratio 1.56 ± 0.56 vs. 3.25 ± 1.63, P = 2.7 × 10^-6 Cited2lox/lox vs. controls, n = 11 vs. n = 7, respectively) concomitant with a 1.5-fold reduction in Vegfa expression (P < 0.02, Cited2lox/lox vs. controls, n = 12 vs. n = 12, respectively). CITED2 was subsequently found at the Vegfa promoter in mouse embryonic hearts using chromatin immunoprecipitation, and moreover found to stimulate human VEGFA promoter activity in cooperation with TFAP2 transcription factors in transient transfection assays. There was no change in the myocardial expression of the left-right patterning gene Pitx2c, a previously known target of CITED2.

Conclusions

This study delineates a novel cell-autonomous role of Cited2 in regulating VEGFA transcription and the development of myocardium and coronary vasculature in the mouse. We suggest that coupling of myocardial and coronary growth in the developing heart may occur in part through a Cited2→Vegfa pathway.

Keywords

CITED2 • VEGFA • Myocardial development • Capillary growth

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on normal development of the extra-embryonic tissues. Myocardial compact layer growth abnormalities can also be secondary to abnormal vascular development. Endothelial-specific conditional knockout of several genes [e.g. NFI, Mapk7, Efhb2, and Casp8 (reviewed in Bhattacharya et al.1)], and pro-epidermal organ-specific knockout of Roxa, result in myocardial growth defects.6 Genetic evidence suggests that the coupling of coronary vessel development to myocardial growth occurs, at least in part, through the vascular endothelial growth factor (VEGFA),7 a known target of the hypoxia-activated transcription factor (HIF1A).8 Myocardial deletion of Vegfa results in fewer coronary microvessels and a thinned ventricular wall.7

The ubiquitously expressed transcriptional co-factor CITED29 can inhibit hypoxia-activated transcription by blocking recruitment of the histone acetyltransferase CREBBP/EP300 to HIF1A.10–12 CITED2 also acts as a co-activator for transcription factors, such as TFA2P, LHX2, PPARA, and SMAD2/3,10,13–16 in part by recruiting CREBBP/EP300. Genetic evidence indicates that Cited2 is essential for cardiac left-right patterning via regulation of the left-right patterning Nodal-Pitx2c pathway.17–20 Zygotic and epiblastic deletion of Cited2 results in atrioventricular septation, outflow tract, and aortic arch defects, and also in left-right patterning defects such as right isomerism.18,19 Loss of Cited2 results in lack of expression of the Nodal target genes Pitx2c, Nodal, and Lefty2 in the left lateral plate mesoderm, explaining the left-right patterning defects. Cited2 is also essential for adrenal, neural crest, liver, lung, lens and placental development and is a regulator of adult haemato poetic stem cells.17,21–26 This early requirement of Cited2 in left-right patterning and in placental development makes it difficult to identify a later specific role in myocardial development. In this study, we therefore investigated the role of Cited2 in the myocardium by conditional deletion in cardiomyocyte precursors.

**Methods**

**Mice**

Cited2<sup>−/−</sup> mice (Cited2<sup>tm1Bha</sup>)<sup>17</sup> Nkx2.5<sup>Cre</sup> mice (Nkx2-2.5<sup>tm1[cry1flu]</sup>)<sup>27</sup> and Cited2<sup>flox/flox</sup> females (Cited2<sup>tm2Bha</sup>)<sup>19</sup> were crossed to create Cited2<sup>−/−</sup> and wild-type control embryos, and Cited2<sup>−/−</sup>;Nkx2.5<sup>Cre</sup> (control) and Cited2<sup>−/−</sup>;Nkx2.5<sup>Cre</sup> (tissue-specific deletion, referred to as Cited2<sup>−/−k</sup>) embryos. Embryos were collected at the indicated time after detection of a vaginal plug [embryonic day (E) 0.5] and genotyped using allele-specific polymerase chain reaction (PCR; details of primers available on request). All studies were performed in accord-
normal Mendelian frequency, and survived at least to weaning (\(Cited^{+/-} = 15\), \(Cited^{-/-} = 16\), \(Cited^{-/-};Nkx2.5Cre = 17\), \(Cited^{-/-};Nkx2.5Cre = 11\), total 59 mice) (Supplementary material online, Table S1). Examination of embryo genotypes revealed that there was no loss of \(Cited^{-/-}\) embryos in late gestation at E15.5 (\(Cited^{+/-} = 10\), \(Cited^{-/-} = 17\), \(Cited^{-/-};Nkx2.5Cre = 7\), \(Cited^{-/-};Nkx2.5Cre = 12\), total 46 E15.5 embryos) (Supplementary material online, Table S1). Examination of hearts from E9.5 embryos bearing a \(Nkx2.5Cre\) recombinated \(Cited^{flox}\) allele (where the \(Cited\) promoter drives expression of lacZ) showed that recombination was highly efficient in myocardial cells, appearing complete in the common atrium, atrioventricular canal, primitive ventricle, bulbus cordis, and outflow tract (Figure 1).

Analysis of E15.5 embryos by MRI and by histology, however, revealed a spectrum of cardiac defects affecting septation (Figure 2, Figure 3A–C). From 11 \(Cited^{Nkx}\) embryos examined, 6 showed ventricular septal defects (VSD), of which 2 also had atrioventricular septal defects. None of the mutant embryos had left-right patterning defects, seen when \(Cited\) is deleted earlier in development. Furthermore, no outflow tract or aortic arch patterning defects were observed. No cardiac defects were seen in control littermates (\(n = 7\)).
Cited2\textsuperscript{Nkx} embryos had smaller thoracic cavities ($P < 0.009$) ($n = 7$) than their littermate controls ($n = 11$), and smaller transventricular diameters ($P < 0.02$; Figure 3D and E). However, correcting the heart size for thoracic diameter, using the cardiothoracic ratio (defined as the maximum transverse diameter of the heart across the ventricles divided by the maximum diameter of the thorax in the section), the hearts were of the same size relative to the embryo size (Figure 3F). For all subsequent measurements, the value obtained was divided by the thoracic diameter to correct for this.

The compact layer of myocardium was significantly thinner in Cited2\textsuperscript{Nkx} embryo hearts than their littermate controls at both the RV and LV apex ($P < 0.02$ and $P < 0.009$, respectively; Figure 3G). The trabecular layer, however, was the same thickness (Figure 3H). When the myocardial thickness was examined as a ratio of compact to trabecular layers, it was significantly reduced in...
Cited2Nkx hearts at both the RV (P < 0.024) and LV (P < 0.007) apex (Figure 3I), indicating a selective developmental defect in the compact layer compared with the trabecular layer in both ventricles.

**Cardiomyocyte deletion of Cited2 causes abnormal microvessel development**

Abnormal myocardial compact layer development is often secondary to abnormal vascular development. To examine possible causes of defective compact layer development, the number of vessels was counted in each ventricle at the LV and RV apex and septum. There was a significant decrease in the number of total vessels seen (mean 56 ± 24 in control vs. 41.5 ± 20 in Cited2Nkx, P = 0.018; Figure 4C) (control n = 7, Cited2Nkx = 11 hearts examined). When actual vessel types were examined, it was found the number of large vessels was unchanged but there was almost half the number of capillaries present (large vessels, 15.7 ± 11.0 vs. 17.4 ± 9.9, P = 0.56 and capillaries, 40.7 ± 18.4 vs. 24.1 ± 12.3, P = 0.002; control vs. Cited2Nkx, respectively for each; Figure 4D). The ratio of capillaries to large vessels was also examined, and found to be significantly decreased in the Cited2Nkx vs. control hearts (1.56 ± 0.56 vs. 3.25 ± 1.63, P = 2.7 × 10⁻⁶; Figure 4E). This demonstrates that there is a reduced presence of capillaries relative to larger vessels.

**Cited2 positively regulates Vegfa expression in the developing heart**

As deletion of Cited2 in cardiomyocytes caused VSDs, abnormal myocardium and abnormal vessels, a candidate gene approach was taken to identify potential Cited2 target genes. We tested two candidates that have previously been linked to Cited2. These are Pitx2c and Vegfa. Embryo hearts were isolated at E13.5, just prior to completion of ventricular septation. To ensure Cited2 knockout in our conditionally knocked out samples, we first performed a qPCR analysis for Cited2. There was a 4.7-fold decrease in Cited2 expression between control (n = 12) and Cited2Nkx (n = 12) hearts, (Figure 5A) validating our samples for further analysis. We next performed qPCR for Pitx2c and for Vegfa. There was no significant difference seen in Pitx2c expression (control = 10 and Cited2Nkx n = 11), (Figure 5B). A significant 1.5-fold decrease in Vegfa expression levels was found in the Cited2Nkx hearts compared with controls (P < 0.02, n = 12 for each genotype) (Figure 6A).

Next, we tried to ascertain if this decrease in Vegfa could have arisen from a decrease in Hif1α expression, this being a major regulator of Vegfa expression. To ensure consistency, we performed these experiments on the same conditionally deleted Cited2Nkx hearts, but observed no difference in Hif1α expression (Figure 6B).

To determine whether Vegfa expression was affected in zygotic deletion of Cited2, we analysed by qPCR a separate series of C57Bl6/J congenic Cited2⁻/⁻ hearts and wild-type littermate controls (n = 12 for each genotype). Here, there was a non-significant increase in Vegfa expression, compared with control (Figure 6C). We also performed a qPCR for Hif1α in these samples; there was no significant change observed between congenic Cited2⁻/⁻ hearts and wild-type littermate controls (Figure 6D).

**CITED2 positively regulates Vegfa expression via TFAP2 transcription factors**

Our previous work established that CITED2 and TFAP2 family members physically interact, and that CITED2 acts as a co-activator of TFAP2 transcription factors. Vascular endothelial growth factor expression has been shown to be

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**Figure 4** Blood vessel analysis in Cited2Nkx hearts. (A and B), Haematoxylin and eosin-stained transverse sections of control and Cited2Nkx hearts at ×10 magnification. (A’ and B’) Enlargement of boxed areas in (A) and (B). Capillaries (vessels 1 red cell in width) are indicated by black arrowheads. Large vessels (>1 red cell in width) are indicated by white arrowheads. (C) Total number of vessels counted in control (n = 7) and in Cited2Nkx hearts (n = 11). (D) Large vessel and capillary counts in control and in Cited2Nkx hearts. (E) Ratio of large vessels to capillary counts in control and Cited2Nkx hearts. Bars indicate the mean and lines standard error of the mean.
**Figure 5** Cited2 and Pitx2c expression in E13.5 Cited2^{Nkx} hearts. Quantitative reverse transcriptase polymerase chain reaction was used to assess Cited2 and Pitx2c RNA expression in control and in Cited2^{Nkx} hearts. (A) A 4.7-fold decrease in Cited2 expression in Cited2^{Nkx} hearts compared with controls was found (n = 12 control, n = 12 Cited2^{Nkx}, P = 1.07 × 10^{-5}). (B) No significant difference was seen in Pitx2c expression levels (n = 10 control, n = 11 Cited2^{Nkx}). For each panel, each dot represents data from a single E13.5 heart of the relevant genotype. All expression levels are relative to 18s RNA expression levels. Lines indicate mean and standard error of the mean.

**Figure 6** Vegfa and Hif1a expression in E13.5 Cited2^{Nkx} and Cited2^{+/+} hearts. Quantitative reverse transcriptase polymerase chain reaction was used to assess RNA levels. (A) Vegfa expression in control and in Cited2^{Nkx} hearts showing a 1.5-fold reduction in Cited2^{Nkx} hearts (P < 0.02) (n = 12 control, n = 12 Cited2^{Nkx}). (B) Hif1a expression in control and in Cited2^{Nkx} hearts (n = 12 control, n = 12 Cited2^{Nkx}). (C) Vegfa in wild-type control and in congenic Cited2^{−/−} hearts (n = 12 control, n = 12 Cited2^{−/−}). (D) Hif1a in wild-type control and in Cited2^{−/−} hearts (n = 12 control, n = 12 Cited2^{−/−}). For each panel, each dot represents data from a single E13.5 heart of the relevant genotype. All expression levels are relative to 18s RNA expression levels. Lines indicate mean and standard error of the mean.
positively\textsuperscript{31–35} or negatively\textsuperscript{36} modulated by TFAP2 factors in other cellular systems. Analysis of the human VEGFA and mouse Vegfa promoters using the transcription factor database (http://www-bimas.cit.nih.gov/) showed that both human and mouse promoters contain multiple AP2 consensus-binding sites (Figure 7A), consistent with previous observations.\textsuperscript{37} Using chromatin immunoprecipitation, CITED2 could be detected at the Vegfa promoter in developing wild-type mouse embryonic hearts (Figure 7B). We next tested, by transient transfection assays, whether CITED2 and TFAP2 could modulate the transcriptional activity of the human VEGF promoter. These co-transfection experiments showed that CITED2 cooperates with TFAP2C to co-activate the human VEGF promoter by \(\approx 2.5\)-fold in comparison with the promoter transfected with the control vectors (Figure 7C). These results indicate that endogenous CITED2, a transcriptional co-activator, is present at the Vegfa promoter, and that CITED2 is a positive regulator of Vegfa transcription, working at least in part through TFAP2 sites in the Vegfa promoter.

### Discussion

In this study, we have identified, by conditional deletion of Cited2 from the developing myocardium, a cell autonomous role for Cited2 in mouse cardiac myocytes. We show that Cited2 is required for normal myocardial thickening, ventricular septation, and coronary vascular development. We also show that conditional deletion of Cited2 in the mouse myocardium results in deficiency of the mouse Vegfa transcript, that CITED2 is found at the mouse Vegfa promoter in vivo, and that CITED2 transactivates a VEGFA promoter in cells. The defect in myocardial compact layer formation...
observed at E15.5 is at a time in which myocyte proliferation in this layer is the predominant type of growth seen,\textsuperscript{38–40} and we hypothesize that defective myocardial proliferative growth is the most likely mechanism for this observation. This may be secondary to the lack of a supporting vascular network required to support the rapid increase in cell number.

This is distinct from the mechanisms of cardiac malformation and left-right patterning defects reported previously in Cited2 deficiency,\textsuperscript{18} as there is no deficiency in Pitx2c expression. Vegfa is essential for normal coronary development, and normal coronary development is required for normal myocardial development. The levels of VEGFA are tightly regulated during normal development, as evidenced by the fact that loss or gain of even a single allele results in cardiac malformation.\textsuperscript{41–43} The phenotype described here is similar to that found in a myocyte-specific deletion of VEGFA, where the myocardium was found to be the main source of VEGFA. The conditional knockout mice were viable but subsequently showed impaired function and reduced weight, with thin ventricular myocardium and reduced capillary number.\textsuperscript{7} We, therefore, suggest that one mechanism by which Cited2 deficiency affects myocardial and coronary development is, at least in part, via deficiency of VEGFA.

Our results, using a cardiac-specific conditional knockout, are the opposite of the previously reported increased cardiac Vegfa expression observed in zygotic deletion of Cited2.\textsuperscript{12} Based on this we interpret the results of zygotic Cited2 deletion on Vegfa as the effect of severe global embryonic hypoxia resulting from complex cardiac malformations or the abnormal placental development seen with zygotic deletion,\textsuperscript{25} and activation of Vegfa as the effect of severe global embryonic hypoxia resulting from complex cardiac malformations or the abnormal placental development seen with zygotic deletion,\textsuperscript{25} and activation of Vegfa (although this was not statistically significant). Tissue-specific deletion of Cited2 here permitted more focused study at a tissue-specific level, eliminating the gross effects of abnormal placental development and severe cardiac malformation and the further non-tissue-specific gene responses this would entail.

CITED2 is a key regulator of HIF1\textalpha, blocking its interaction with CREBBP/EP300, and hence HIF1\textalpha-mediated transcription. Our results would suggest that mechanisms regulating downstream target genes of HIF1\textalpha may be more complex. CITED2 directly blocks HIF1\textalpha-mediated transcription,\textsuperscript{10–12} but, by allowing transcription of a downstream target such as Vegfa, it may be protective in severe hypoxia, allowing some Vegfa transcription and vessel development but not at too high a level, preventing disorganized vessel growth and ensuring that not all Vegfa transcription is lost. Another model of cardiomyocyte-specific Cited2 deletion was reported recently, in which no septation, myocardial, or coronary defects were noted.\textsuperscript{16} The reasons for the differences from our study are not clear but could result from reduced efficacy of Cited2 deletion in cardiomyocytes or from the use of a mixed genetic background, either of which could result in reduced penetrance of phenotype.

There may be alternative mechanisms for the abnormalities in myocardial and coronary development observed in the myocardial-specific knockout of Cited2. Trp53 is an additional key regulator, coordinating myocardial and coronary growth via the HIF1\textalpha and VEGF pathway, and could be an important intermediary particularly as it has been shown to be a target of Cited2.\textsuperscript{23,45} However, previous complex mutants of Cited2 and p53 have been created without much effect visible on the cardiac phenotype,\textsuperscript{18} suggesting its role in the developing myocardium is unclear. The role of other regulators of embryonic myocardial and coronary development—such as GATA4 (reviewed in Bhattacharya et al.),\textsuperscript{1} and VEGFB—needs to be addressed in future studies.

Taken together, our results suggest that Cited2 controls the coupling of embryonic myocardial and coronary vascular development, at least in part via regulation of Vegfa transcription. We speculate that activation of the Cited2\rightarrow Vegfa pathway may be beneficial in heart failure or ischaemia, where such coupled myocardial and coronary growth may be desirable. Moreover, we speculate that Cited2 may also play a necessary role in tumour angiogenesis by ensuring tumour growth proceeds with a concomitant vascular network.

Supplementary material
Supplementary material is available at European Heart Journal online.

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References
Cited2 couples myocardial and coronary vascular development


