ER71 Transcriptionally Activates Brachyury

A Study of Molecular Mechanisms Involved in Gene Regulation

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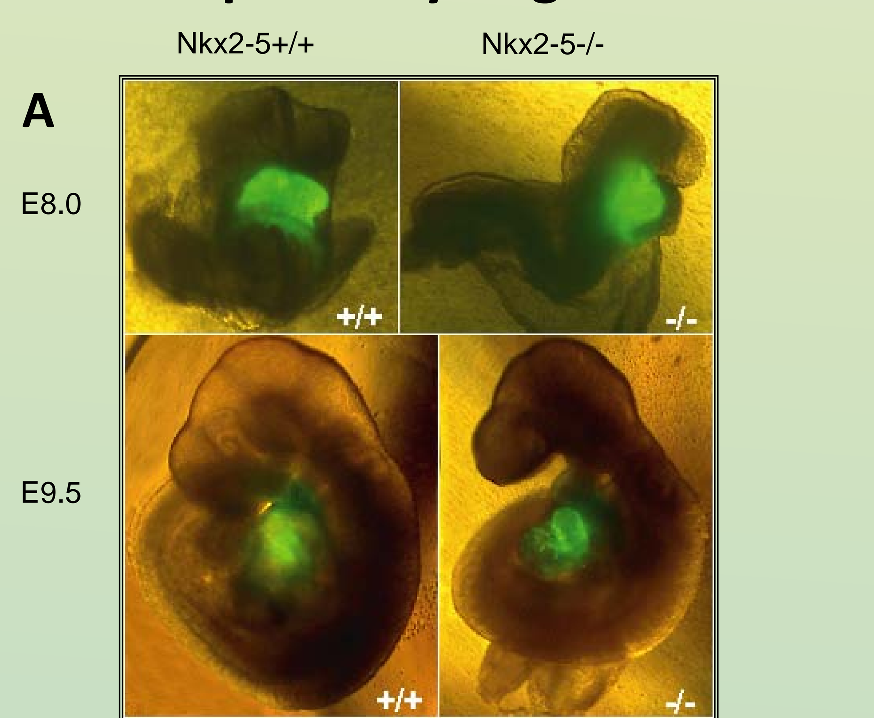
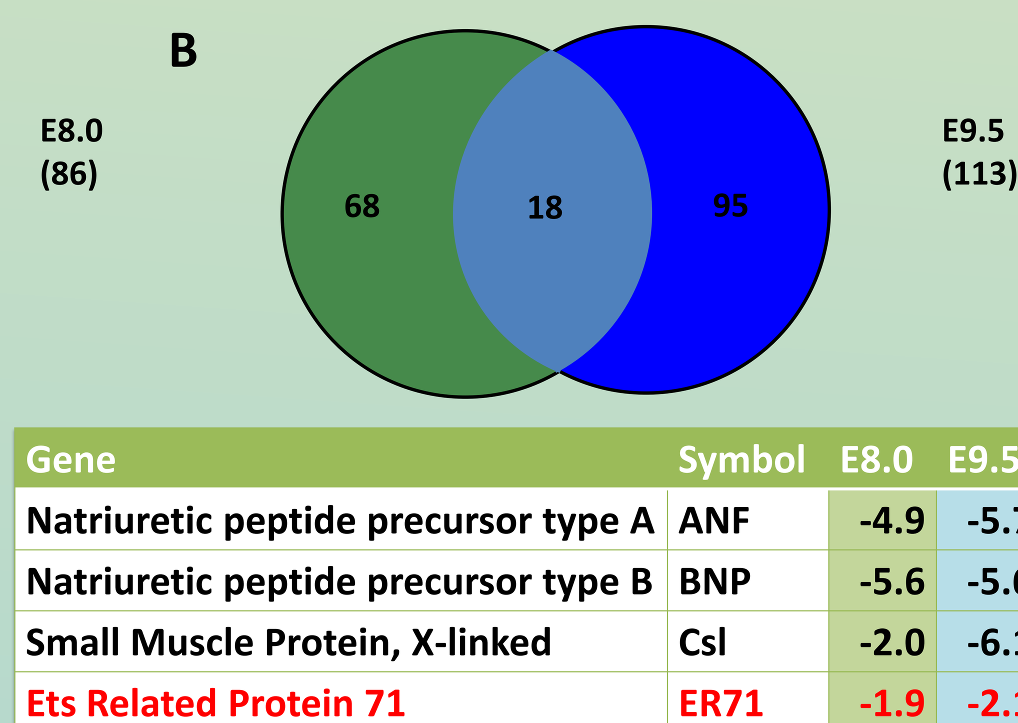
Abstract

Congenital cardiac malformation is the most frequent birth defect which contributes to advanced heart failure in the pediatric and adult population. An enhanced understanding of the transcriptional networks that direct cardiac progenitors during heart development will have important therapeutic applications for the treatment of congenital heart disease. Furthermore, a number of parallel transcriptional pathways or networks have been proposed for the generation and regeneration of tissues such as the heart. For these reasons we predict that the definition of the transcriptional regulatory mechanisms of cardiac progenitor cells in the developing heart will enhance our understanding of cardiogenesis, congenital heart disease and myocardial regeneration. Previously, using transcriptome and RT-PCR analysis we found that ER71 was dysregulated in Nkx2-5 null embryos at both E8.0 and E9.5 in comparison to their WT littermates. This data established that ER71 is a direct downstream target of the homoeodomain protein Nkx2-5. Here, we will focus on transcriptional regulation of cardiogenesis by Nkx 2.5, Etsrp71, and Brachyury (T protein).

Nxk2-5 Transcriptionally Regulates ER71





Gene	Symbol	E8.0	E9.5
Natriuretic peptide precursor type A	ANF	-4.9	-5.7
Natriuretic peptide precursor type B	BNP	-5.6	-5.6
Small Muscle Protein, X-linked	Csl	-2.0	-6.1
Ets Related Protein 71	ER71	-1.9	-2.1

Figure 1: Transient expression of Etsrp71 in the endocardial/endothelial lineage is downregulated in Nkx2–5 null cardiac progenitors. (A) Six-kilobase Nkx2–5-EYFP Tg:Nkx2–5± and Nkx2–5± mice were mated to generate EYFP⁺ Nkx2–5+/+ and EYFP⁺ Nkx2–5–/– littermates at E8.0 and E9.5. Note that the WT and Nkx2–5 mutant embryos are indistinguishable at E8.0 while at E9.5 the Nkx2–5–/– embryo has severe growth retardation compared to the WT littermate. (B) Transcriptome analysis showing differential gene expression value between Nkx2-5 null and WT littermates at E8.0 and E9.5. ER71 expression was significantly down regulated including other known downstream target genes of Nkx2-5. Images obtained from Ferdous et. al. PNAS 2009. (106) 814-819.

Hypothesis

We have characterized a highly conserved Ets binding element (EBE) within the first -2kb of the mouse Brachyury promoter. Using the rVISTA database we have found that this EBE is conserved between several organisms. Previous microarray data show activation of Brachyury in EBs overexpressing the transcription factor ER71.

Based on these data we have cloned 1.7kb of the Brachyury promoter into a pGLT vector driving luciferase. With the high conservation of the EBE in the Brachyury promoter and the EB microarray data we predict that overexpression of ER71 will cause an upregulation of luciferase expression in a gene reporter assay. With this information we hypothesize that ER71 transcriptionally regulates Brachyury during mesodermal differentiation. Furthermore, using chromatin immunoprecipitation we will demonstrate the ability of ER71 to bind the EBE within the regulatory region of the Brachyury promoter.

Results

Overexpression of ER71 in an inducible EB system results in an increase of Brachyury transcription. Further analysis of the Brachyury regulatory sequence identified a highly conserved Ets binding element (EBE). A 1.7 kb sequence including this EBE was cloned into a pGLT luciferase reporter vector to determine the effect of ER71 on Brachyury reporter expression in C2C12 mouse myoblast cells. Furthermore, chromatin immunoprecipitation was used to determine the ability of ER71 to bind the previously described Ets elements.

Ets Binding Element is Conserved in the Brachyury Gene Promoter

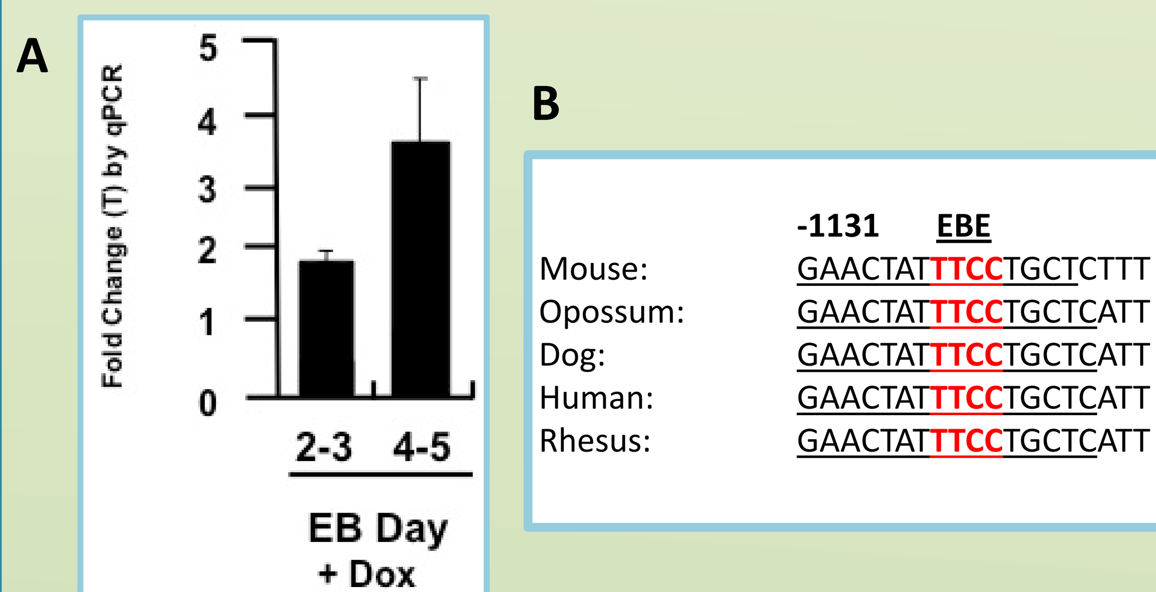


Figure 2: To further analyze whether ER71 plays a role in fate decisions, we genetically engineered an ES cell population to overexpress ER71 under control of doxycycline. ES cells were differentiated into embryoid bodies. Overexpression was induced for 24 hours at distinct times and analyzed immediately afterwards. Brachyury was upregulated during both time points. (A) EBs were induced with doxycycline at days 2-3 and days 4-5 and analyzed by qPCR. A significant fold change was observed in the expression of Brachyury gene. (B) Ets binding element (EBE) and conservation in T-gene promoter. Schematic alignment produced using rVISTA database shows conserved EBE in mouse, opossum, dog, human, and rhesus macaque at position -1131 base pairs from the ATG Brachyury start site (mouse). The EBE is highlighted in red and the conserved flanking regions are underlined.

ER71 Activates the Brachyury Promoter

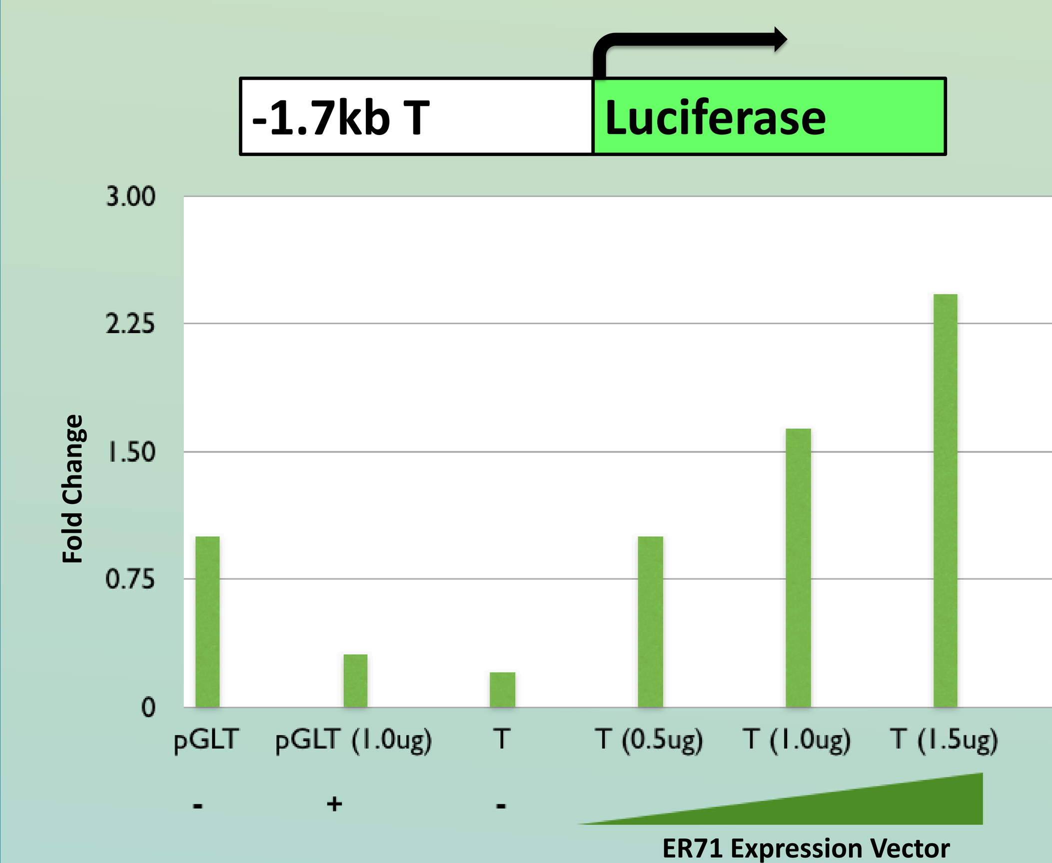


Figure 3: -1.7kb of the Brachyury promoter containing the EBE characterized in Figure 2B was fused to luciferase in the pGLT vector backbone. The backbone was linearized in the pGLT construct and in the empty control. The reporter genes along with ER71 expression vector and pRLTK, an internal control, were transfected into C2C12 mouse myoblast cells. A dose dependent response of luciferase expression was observed with increasing concentrations of ER71 vector. (-) corresponds to cells not transfected with ER71 expression vector, (+) corresponds to cells transfected with ER71 expression vector, and dose response with increasing concentration of ER71 expression vector is indicated with green wedge.

References

Ferdous, A, Caprioli A, Iacovino A, Martin CM, Morris J, Richardson JA, Latif S, Hammer RE, Harvey RP, Olson EN, Kyba M, and Garry DJ. Proc Natl Acad Sci U S A. 2009 January 20; 106(3): 814–819.

Huber TL, Kouskoff V, Fehling HJ, Palis J, Keller G. Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. Nature. 2004 Dec 2; 432(7017):625-30

Acknowledgements

Thanks to members of the Garry lab and Lillehei Heart Institute for assistance and support, and thank you to the UROP program for funding this project.

ER71 binds to the Brachyury promoter

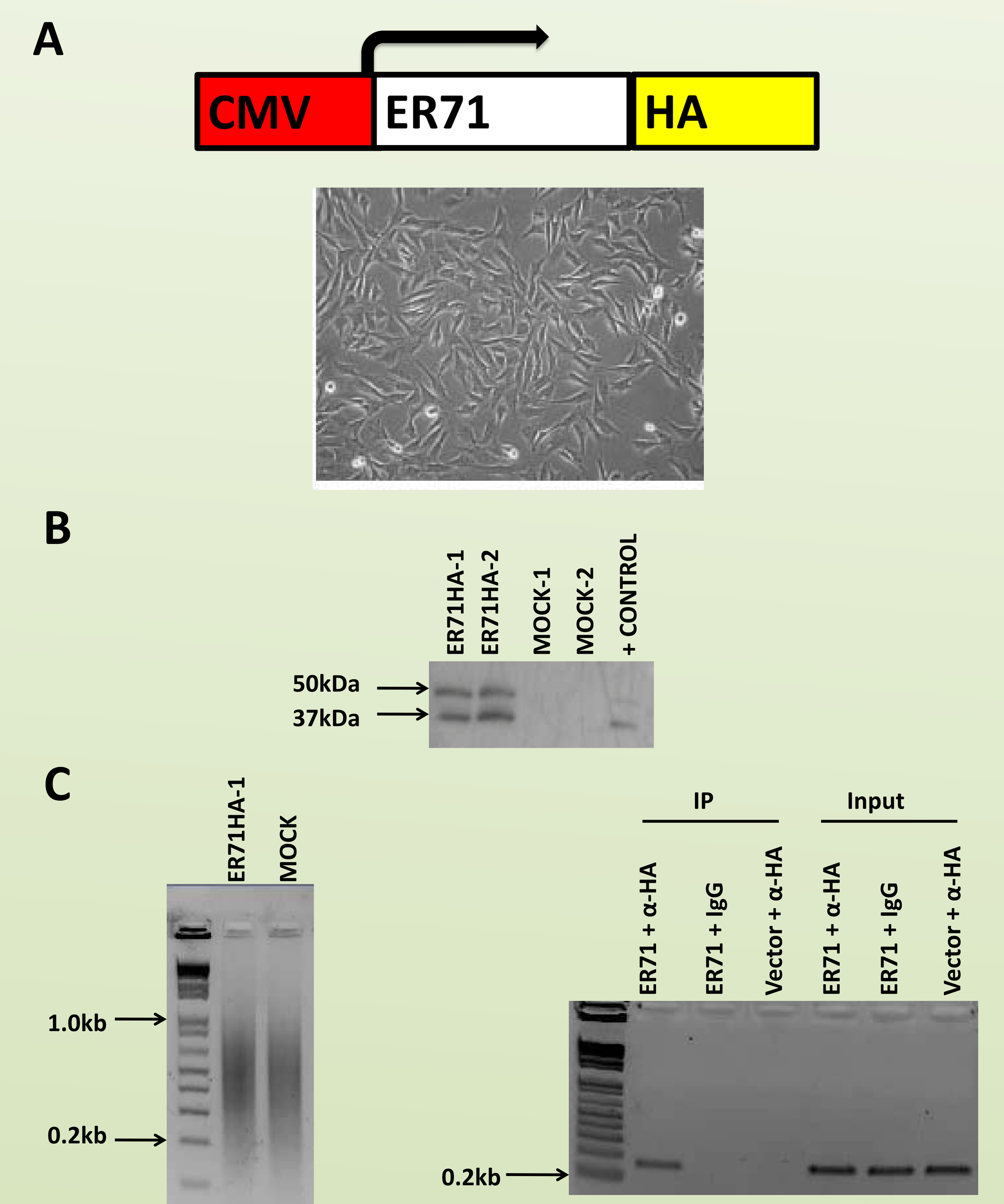


Figure 4: C2C12 mouse myoblast cells were transfected with ER71 overexpressing vector tagged with HA in order to demonstrate the ability of ER71 to bind the previously described EBE in the Brachyury promoter. (A) This represents a schematic diagram of the ER71-HA expression vector transfected using lipofectamine into C2C12 mouse myoblast cells. (B) A Western assay was performed using harvested C2C12 cells 24 hours post-transfection. The primary antibody used was goat polyclonal anti-HA (Y-11) and the secondary was polyclonal rabbit anti-goat IgG conjugated to HRP. (C) Cells were also harvested from the same transfection for use in the ChIP assay. Sonication was performed on total chromatin and fragment sizes ranged from 0.2 kb to 1.0 kb. Lysates were immunoprecipitated with the same anti-HA (Y-11) antibody used in the Western blot and an additional control IgG.

Future Directions

Several different mouse models have been studied that contain mutations in the Brachyury gene. In 2004, Gordon Keller's group engineered a mouse model with GFP cDNA targeted to the Brachyury gene locus. The GFP-Brachyury heterozygous mice have a short tail phenotype similar to previously studied Brachyury mouse models. The phenotype of the null GFP-Brachyury mice have not been analyzed, but it is most likely embryonic lethal. We plan to isolate the GFP+ cells to determine if Brachyury and ER71 are coexpressed *in vivo.* We also plan to cross these mice with ER71 knockout mice and compare the phenotypes of the embryos at primitive streak stages. In doing this, we hope to show an *in vivo* relationship between ER71 and Brachyury.

Figure 5: Images of Brachyury T/+ adult mice and embryos. (A) T/+ adult mouse showing abnormal truncated phenotype characteristic of a mutation in the Brachyury gene. Image obtained from Jackson Labs database. (B) GFP expression in the primitive streak of an GFP–Bry+/- embryo at the neural plate stage. Image obtained from Huber et al. Nature 2004; 432: 625-629.

Conclusions

1. Overexpression of ER71 leads to an increase of Brachyury expression as seen in ER71 inducible EBs by qPCR.
2. The Brachyury gene promoter contains a highly conserved Ets binding element within -2 kb of the ATG start site.
3. ER71 transcriptionally activates Brachyury in a dose dependent manner as seen in luciferase assays.
4. ER71 binds directly to the conserved EBE in the Brachyury regulatory region as seen by chromatin immunoprecipitation.