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Examining the Cardiac NK-2 Genes in Early Heart Development

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Abstract

The cardiac NK-2 transcription factors are the vertebrate relatives of the *Drosophila tinman* gene. Without the *Drosophila tinman* gene, fruit flies fail to form their heart ("dorsal vessel"), and mutations or altered expression of cardiac NK-2 genes may lead to abnormal heart formation in vertebrates. Although the cardiac NK-2 gene *NKX2–5* is recognized as an important factor in cases of human congenital heart disease and heart development in vertebrates, the roles of the other cardiac NK-2 genes are less clear. This report reviews what is known about the cardiac NK-2 genes in cardiac development, comparing studies in several different model systems.

Keywords

Abnormal heart formation; Cardiac NK-2 genes; Cardiac NK-2 transcription factors; Congenital heart disease; Early heart development

One of the molecules critical to normal cardiac development across vertebrate species is the transcription factor Nkx2–5. Nkx2–5 is a member of a family of transcription factors that uses the *Drosophila tinman* protein, essential for dorsal vessel (the heart of the fly) formation in the fly, as a prototype. Mutations in NKX2–5 are among the most common genetic causes of nonsyndromic congenital heart disease in humans, and this homeodomain-containing transcription factor has been central to models of cardiac development.

This review aims to introduce Nkx2–5 and closely related proteins expressed in cardiac tissue. Although studies investigating a variety of organisms are discussed, we highlight studies examining this family of proteins that have used embryos of the South African clawed frog, *Xenopus laevis*.

What Characterizes the Nkx2–5 Family of Proteins?

Several features of Nkx2–5 make it recognizable across species (Fig. 1). The official name of Nkx2–5 is NK-2 transcription factor related, locus 5. This name refers to the grouping of homeodomain-containing proteins into 20 different classes, one of which is the NK-2 class.

The abbreviation NK refers to Nirenberg and Kim [26], who carried out a search for *Drosophila* genes containing homeodomain-encoding genes. The "x" in Nkx was added to designate vertebrate members of the family. Within the NK-2 genes expressed in vertebrates, a subset, referred to as the "cardiac group" [20], includes NKX2–3, NKX2–5, NKX2–6, nkx2–7, nkx-8, and nkx2–10.

At the outset, it is worth noting that NKX2–3, although found in zebra fish, frog, chicken, mouse, rat, and human, is only expressed in the cardiac region in frog and chicken [30]. The results from analysis of multiple species encourage a model in which NKX2–5 and at least one other family member is involved directly in early cardiac formation.

The protein domains that define the NK-2 family are shown in Fig. 1. The DNA-binding homeodomain is 60 amino acids long with a tyrosine at position 54. In addition to the homeodomain, these proteins have an amino terminal 10-amino-acid-long TN domain. In vertebrates, but not in *Drosophila tinman*, a 16-amino-acid NK-2 domain lies just beyond the C-terminal residue of the homeodomain. Typically, cardiac NK-2 proteins also have a C-terminal amino acid sequence of GIRAW. The sequence conservation in each of these domains is very high. Using the human NKX2–5 protein sequence as a comparative standard, the vertebrate members of the cardiac NK-2 protein family have an 80% or greater identity in the TN domain and the homeodomain, and more than a 64% identity in the NK2 domain.

How Do These Domains Relate to Protein Function?

Full understanding concerning the function of each of the domains remains a work in progress. However, ample evidence shows that the homeodomain itself is critical. The cardiac NK-2 genes encode DNA-binding transcription factors, with the consensus binding site of 5'T(C/T) AAGTG [9,37] bound by the homeodomain. Sparrow et al. [37] reported subtle differences using *in vitro* DNA-binding assays for direct comparison of the three cardiac NK-2 proteins found in *Xenopus*. Although these subtle differences may be selective for one NK-2 protein over another, the authors favor a model in which NK-2 regulation of specific genes depends on other modulators.

Although NK-2 proteins are capable of binding DNA as monomers, the association is much stronger with DNA as a dimer [25]. The NK-2 proteins can form homodimers and heterodimers. Heterodimer-binding partners may be other NK-2 proteins or a variety of other transcription factors including GATA4 and Tbx5 [1,15,22]. Once a protein complex containing an NK-2 protein is bound to a DNA target site, transcription may be stimulated, as seen for the ANF gene and alpha cardiac actin, or inhibited, as seen for β-catenin [35].

At least part of the regulatory posture assumed by the NK-2 proteins is due to the choice of the heterodimer binding partner. For example, ANF transcription is stimulated by a Nkx2–5 GATA4 dimer [14] and inhibited by an Nkx2–5-Tbx-2 dimer [19]. Dimer formation is coordinated via the C-terminus of the homeodomain and enhanced by amino acids that extend beyond the C terminus [23,25]. The NK-2 domain (also called the NK-2-SD domain) is thought to mediate interaction with other proteins, and some assays indicate a role when NK-2 family members repress rather than activate gene expression [36].

Synteny Among Cardiac NK-2 Family Members

The growing catalog of genomic sequences from a variety of organisms opens the possibility of performing syntenic analysis. In the context of this review, synteny is used to describe the conservation of placement among other genes in the genome. Conservation of protein domains does not guarantee conserved function, and neither does synteny. However, synteny does imply a common genetic past for a group of genes. Using the program Metazome hosted by the Joint

Genomic Institute, it is possible to perform such an analysis on the cardiac group of NK-2 genes (Fig. 2). This type of analysis inspects the genes that surround each of the NK-2 genes and color codes gene identity. Among vertebrates, the genes identified as *NKX2–5* and *NKX2–3* assemble into separate syntenic groups. The analysis suggests that, using the human *NKX2–6* gene as the positional reference, the chicken *Nkx2–8*, *Xenopus nkx2–10*, and the zebra fish *nkx2–7* gene are possible syntenic matches. We note that the search using NK-2 as a keyword did not identify a chicken *Nkx2–3* or opossum *Nkx2–6* for syntenic analysis. However, with these data in mind, it may be reasonable to think about the cardiac NK-2 genes as defined by three syntenic groupings: *NKX2–3, NKX2–5*, or *NKX2–6*.

Assaying Cardiac NK-2 Genes in Developing Embryos

Functional analysis of cardiac NK-2 proteins supports both unique and redundant roles in cardiac development. Although temporal and spatial expressions are overlapping for different genes, expression patterns are not identical, according to *in situ* analysis of varied organisms. The answer to the question "How important is each individual gene in heart development?" may depend on the organism and how the question is addressed. For example, if the question is asked in relation to genetic causes of congenital heart defects in humans, *NKX2–5* stands out as a key factor for normal morphologic and physiologic development [6], and *NKX2–6* is implicated in normal great vessel development [21].

The mouse homolog of *NKX2–5* also is critical for heart development [7,9,15,24,25,28,29]. Mice null for *Nkx2–5* start to form a heart and express a variety of cardiac specific genes but fail to progress much beyond the linear heart tube stage [29]. Mice carrying *Nkx2–5* mutations have phenotypes that include compromised physiology, morphology, and function of the heart. Alternatively, mice null for *Nkx2–6* are not reported to have a cardiac phenotype [38].

Tanaka et al. [38] postulated that the loss of *Nkx2–6* was compensated by an increase in *Nkx2–5* expression. In zebra fish, *nkx2–5* and *nkx2–7* are expressed in the heart-forming region [10]. Tu et al. [39] recently published studies concluding that loss of *nkx2–5* expression alone has little effect on heart development but that loss of *nxk2–7* or both *nkx2–7* and *2–5* had significant effects. In their studies, the morpholino-induced loss of nkx2.5 and 2.7 protein led to defects that could be at least partially rescued by adding back either nkx2.5 or nkx2.7 mRNA, although nkx2.7 was effective at much lower doses. Reminiscent of *Nkx2–6* loss in mouse, *nkx2–7* inhibition in zebra fish increases expression of *nkx2–5*. Studies comparing mouse and zebra fish showed a difference in which cardiac NK-2 protein is most important. However, the loss of both did not lead to a complete loss of cardiac structure in either organism, so neither is both necessary and sufficient for at the least initial steps in cardiac formation.

Assays on the Trio of Cardiac NK-2 Genes in *Xenopus*

Compromised expression of *NKX2–3* in human and mouse gives rise to defects in gut and spleen [32,40]. However, in frog and chicken, it joins *nkx2–5* and *nkx2–10* (or *Nkx2–8* in chicken) among NK-2 genes expressed in the heart. Although initial studies of *nkx2–10* suggested that it was transiently expressed during early cardiac development [31,37], a more recent analysis suggests that after a transient decrease, expression returns [3]. All the *Xenopus* cardiac NK-2 genes can be found in RNA isolated from adult heart, liver, and gut. *Nkx2–3* also was expressed in skin, whereas *nkx2–10* was present in the eye (Fig. 3 [3]).

Overexpression of nkx2–3 or nkx2–5 via injection of mRNA in early embryos gives a transient increase in both cell number and size of the heart with little known consequence to noncardiac tissue [12]. A similar nkx2–5 dose-dependent increase in heart size was reported using zebra fish [10], although reduction in heart size and morphologic defects with further increases in

nkx2–5 levels also has been reported [39]. Overexpression of *Xenopus* nkx2–10 has no obvious cardiac effect [3,31].

In frog, the loss of function of each cardiac NK-2 gene has been tested [3,17,18]. Grow and Krieg [18] generated dominant negative nkx2–3 and nkx2–5 through a single mutation in the homeodomain that disrupted DNA binding. Their dominant effects would be through binding to and potentially titrating away proteins that normally would be interacting with nkx2–5 (or nkx2–3) bound to DNA. They judged the consequences for heart development by monitoring the reduction of several cardiac-specific mRNAs with *in situ* hybridization (cardiac troponin 1, myosin light chain 2, and alpha cardiac actin). Dominant negative nkx2– 3 or nkx2–5 reduced expression of the tested cardiac genes and reduced heart size (to no heart at all in some cases) in 40% to 60% of the embryos scored. The effect of the dominant negative was partially mitigated by co-injection with wild type nkx2–5 mRNA. Importantly, these authors reported few consequences of the dominant negative mutations unrelated to cardiac development.

A slightly different approach was taken by Fu et al. [17]. Their dominant negative construct comprised the isolated homeodomain of nkx2–3 or nkx2–5 fused to the engrailed repressor. They allowed a repressor to occupy promoter sites that normally would have bound to any of the cardiac NK-2 proteins. The expression of either nkx2–5 or nkx2–3 repressor fusion reduced cardiac differentiation as assayed by decreased expression of cardiac marker genes. When both repressor fusion proteins were added together, cardiac development was even worse.

Studies examining nkx2–10 loss of function used oligonucleotide-mediated inhibition of protein expression [3]. In these studies, the loss of nkx2–10 led to both general anterior and cardiac defects. The defective hearts were smaller than control hearts and had decreased trabeculation as well as abnormal anterior repositioning of the atria. Leftward bending of the outflow tract occurred, although the outflow tract was clearly underdeveloped. The loss of nkx2–10 did not seem to change the quantitative embryonic expression of either *nkx2–3* or *nkx2–5* when these genes were assayed after lateral field fusion. This is a different result than reported for the loss of either Nkx2–6 in mouse or nkx2–7 in zebra fish, in which a loss led to an increase in expression of *Nkx2–5*.

These studies indicate that in *Xenopus*, all three cardiac NK-2 genes are playing an active role in cardiac development.

Regulation of the Cardiac NK-2 Genes in the Heart

Most of the studies on the promoters of cardiac NK-2 genes have focused on regulation of homologs of *NKX2–5*. Transcription of this gene demands both temporal and spatial control during embryogenesis. Transacting regulation is mediated through a variety of transcription factors including BMP/Smad, GATA family members [8,34,37] FGF [4], and NFAT [11]. Other reviews (e.g., [1,13,16,27]) and their references can be consulted for a more complete picture of *NKX2–5* regulation. However, the coordinated regulation of cardiac NK-2 genes in a single background is relatively unstudied beyond those using *in situ* hybridization. In *Xenopus*, relatively detailed *in situ* hybridization of all three cardiac NK-2 genes has been presented [33,37], indicating both overlapping and unique spatial and temporal regulation of each gene.

A relatively new technique, chromatin immunoprecipitation (ChIP) followed by deep sequencing (ChIP-seq), can be used to generate genome-wide maps of chromatin modifications and transcriptional activity. The analysis of such an experiment with *Xenopus tropicalis* embryos [2] provides an interesting insight into the regulatory state of the cardiac NK-2 genes of *Xenopus* just before gene activation can be detected by *in situ* hybridization. Antibodies directed against trimethylated histone H3 lysine 27 (H3K27me3) were used to identify regions

repressed by Polycomb repressor proteins. Antibodies against trimethylated lysine 4 of the same protein (H3K4me3) were used to identify active promoters, whereas antibodies directed against RNA polymerase II (RNAPII) identified transcribed regions.

In addition, cDNA obtained from embryonic polyadenylated mRNA was sequenced to identify mature transcripts (RNA). The profiles of the sequences captured are displayed over the genomic regions of the cardiac NK-2 genes in Fig. 4. All three cardiac NK-2 genes are decorated with H3K27me3, indicating Polycomb-mediated repression. Concordantly, *nkx2–5* and *nkx2–10* are not transcribed at this early stage of embryogenesis. However, the promoter of *nkx2–3* is relatively depleted for the H3K27me3 marker, is enriched for the active histone modification (H3K4me3), and recruits RNA polymerase 2. In addition, some nkx2–3 RNA was detected in these early embryos (Fig. 4). Moreover, the *nkx2–3* promoter and the promoter proximal region respectively recruit TATA binding protein, a key transcription initiation factor, and monomethylated histone H3 lysine 4, a marker for putative enhancers (data not shown).

Together, these data indicate that the first of the three cardiac NK-2 genes activated in the frog embryo is *nkx2– 3*. The other two NK-2 genes are repressed. The promoter regions of *nkx2– 5* and *nkx2–10* are covered by H3K27me3, consistent with the need for chromatin remodeling as a necessary step before their activation. Ample precedent has been found for the regulation of cardiac genes by dynamic chromatin modification [5]. The ChIP-seq profiles suggest that neither *nkx2–5* nor *nkx2–10* contain the stalled or paused RNA polymerase reported for many developmentally important genes in *Drosophila* [41], whereas *nkx2–3* shows strong enrichment for RNA polymerase II directly at the transcription start site but much less enrichment further into the gene body, suggestive of pausing. It will be interesting to determine whether the genes regulated by the cardiac NK-2 genes are associated with repressive chromatin or whether they have paused polymerase in anticipation of activation. Another important direction for further research is to determine whether the sequential activation of *nkx2–3* and *nkx2–5/nkx2–10* represents a cross-regulatory hierarchy in which the cardiac gene regulatory network is progressively established or whether the early activation of *nkx2–3* is reflective of a parallel regulatory pathway that, independently of the other NK-2 genes, contributes to the cardiac program.

Conclusion

Although a great deal has been learned about the role of *NKX2–5* in heart development, less is known about the other cardiac NK-2 proteins. Unification of their roles in cardiac development may not be possible, but we suggest that it is worth examining these genes as members of three syntenic groups. It is unclear which, if any, combination of cardiac NK-2 genes will be responsible for full activation of cells that can contribute to heart development. However, a great deal still remains to be uncovered before we understand all the roles cardiac NK-2 genes assume in normal heart development.

Acknowledgments

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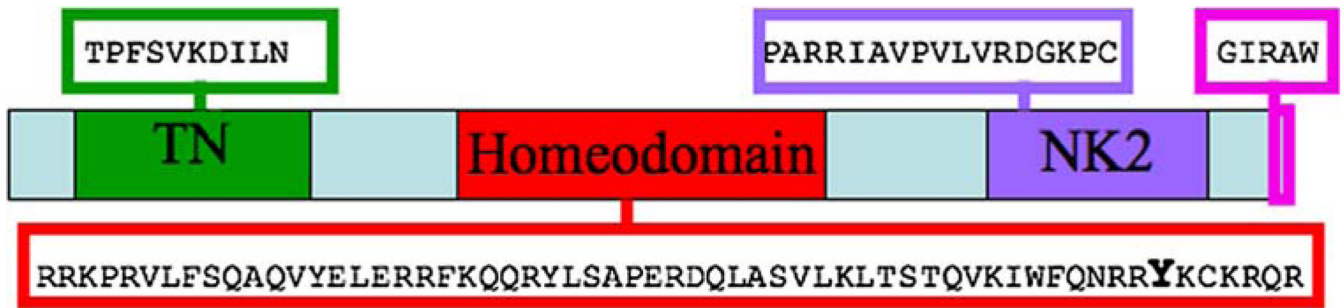
Fig. 1.
General domain structure of the cardiac NK-2 family of proteins. Four conserved motifs are found consistently in the NK-2 family of proteins. The domains and the amino acid sequence from human NKX2–5 corresponding to each domain starting from the amino terminus of the protein are the TN domain, the homeodomain, the NK-2 domain, and the sequence GIRAW. One of the distinguishing features of the homeodomain is the presence of a tyrosine (Y) at amino acid position 54
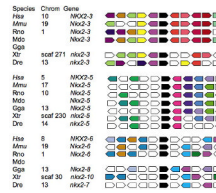
Fig. 2.

The cardiac NK-2 factors can be sorted into three syntenic groups. Using the program Metazome (http://www.metazome.net/) and the genomic database at the Joint Genome Institute, a search for all chordate NK-2 genes was conducted, and a syntenic cluster analysis was performed. In each case, the cardiac NK-2 gene is indicated in black. Genes with a common identity are color coded, and the non-matching sequence is in white. Clustering based on *NKX2–3*, *NKX2–5*, and *NKX 2–6* for human (*Hsa*), mouse (*Mmu*), rat (*Rno*), opossum (*Mdo*), chicken (*Gga*), frog (*Xtr*), and zebra fish (*Dre*) are included in the analysis
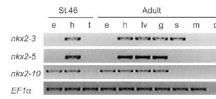
Fig. 3.
Examination of cardiac Nk-2 gene expression in *Xenopus laevis*. Findings show mRNA isolated from: *Xenopus* stage 46 embryo eyes (e), heart (h), and embryo tail (t) or *Xenopus* adult eye (e), heart (h), liver (lv), gut (g), skin (s), skeletal muscle (m); and negative control (c). This figure is modified from Allen, Allen-Brady, and Weeks [3]
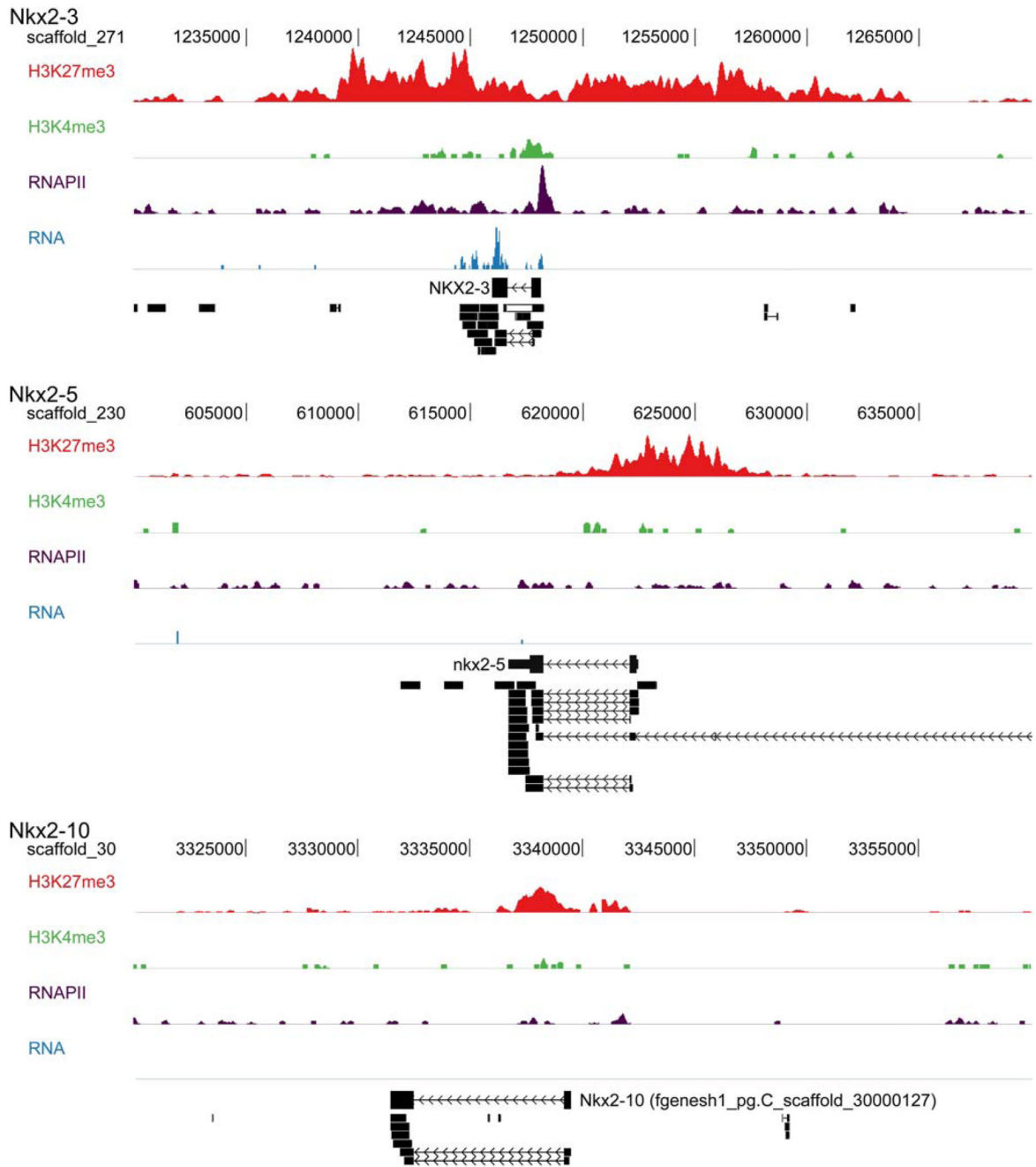
Fig. 4.
Analysis of proteins bound to genomic regions of the cardiac NK-2 genes of *Xenopus tropicalis*. A ChIP-seq analysis of gastrulastage embryonic chromatin was performed using antibodies to H3K27me3, H3K4me3, and RNApolII. Analysis of each antibody is presented for *nkx2–3*, *nkx2–5*, and *nkx2–10*. Deep sequencing results of cDNA (RNA-seq) are indicated as well as known gene annotation and sequenced ESTs (bottom of each panel). Scaffold positions are across the top of the analysis for each gene. Direction or transcription is always from right to left in this figure