To Activate or Not to Activate
The Existential Dilemma of an Enhancer

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Fibroblast growth factor 10 gene regulation in the second heart field by Tbx1, Nkx2-5, and Islet1 reveals a genetic switch for down-regulation in the myocardium

Watanabe et al

In a recent study, Watanabe et al identify a novel enhancer in the fgf10 locus that is necessary and sufficient for expression in anterior second heart field. Their results suggest a competitive mechanism in which Islet1 and Nkx2-5 bind the enhancer to activate or repress this enhancer, respectively.

In the last decade, new insights into heart development have demonstrated the existence of a second heart field, which gives rise to the second heart lineage.1,2 The first heart lineage is defined as the first cells to differentiate within the early cardiac crescent and forming heart tube, gives rise to a majority of cells within the left ventricle, and contributes to both atria. The second heart field progenitors actively proliferate and are progressively added to the heart to give rise to the right ventricle, outflow tracts, and to a majority of cells within the atria. As second heart field progenitors enter the heart, proliferation is downregulated concomitant with differentiation. Gene networks which regulate the transition from second heart field progenitor to differentiated myocyte have been the subject of intense scrutiny, particularly for anterior second heart field progenitors, which will contribute to the anterior pole of the heart.3,5

Pioneering work with an nLacZ transgene fortuitously integrated into the Fgf10 locus revealed the mammalian anterior second heart field.6 Expression of endogenous Fgf10 occurs first in the second heart field medial to the differentiating cells of the first lineage in the cardiac crescent. Its expression was later observed in the anterior second heart field comprising pharyngeal mesoderm dorsal and anterior to the heart, including core mesoderm of the first 2 pharyngeal arches.7,8 Fgf10 cooperates with Fgf8 to drive proliferation of the second heart field.9 To shed new light on gene networks regulating the transition from second heart field progenitor to differentiated myocyte, Watanabe et al.9 have performed a series of elegant experiments to analyze transcriptional regulation of Fgf10.

Using reporter transgenes, a 1.7-kb enhancer in the first intron of Fgf10 was found to be both necessary and sufficient for LacZ transgene expression in the anterior second heart field. Expression of the LacZ transgene is also observed in outflow tract and throughout right ventricle, in contrast to endogenous Fgf10 which is not expressed in outflow tract and is only in a restricted domain within the right ventricle. An assumption is made that this expression reflects perdurance of the LacZ transgene as previously observed for the original Fgf10 insertion transgene.

Because Nkx2-5 is an important regulator of the second heart field, Watanabe et al.9 examine expression of the 1.7-kb enhancer in Nkx2-5 mutants, finding that enhancer expression is decreased in pharyngeal mesoderm and ectopically localized throughout the Nkx2-5 mutant single ventricle, which has left ventricular identity. Intriguingly, these results demonstrate that Nkx2-5 contributes both to activation of the Fgf10 enhancer within pharyngeal mesoderm and to its repression in left ventricle. Electrophoretic mobility shift assays were performed to examine direct binding of Nkx2-5 to 8 predicted homeodomain binding sites within the enhancer. Results demonstrated that Nkx2-5 binds most avidly to sites 2 and 4 and more weakly to sites 3, 5, and 7. Sites 2 and 7 correspond to previously described Nkx2-5 consensus sites. Mutagenesis of all these sites results in reduced expression of enhancer transgenes in pharyngeal mesoderm and outflow tract in embryos at E9.5. Somewhat surprisingly, however, loss of homeodomain consensus sites does not result in ectopic reporter gene expression in left ventricle, as observed in Nkx2-5 mutants. These data suggested a possibility that, in Nkx2-5 mutants, another homeodomain protein might use these sites to activate expression of the transgene in the ventricle.

A likely candidate for this was another important regulator of the second heart field, the homeodomain protein Islet1, which is ectopically upregulated in Nkx2-5 mutant ventricle.10 To address this, Watanabe et al.9 performed additional electrophoretic mobility shift assay analysis to demonstrate that Islet1 can also specifically bind to homeodomain consensus sites within the Fgf10 enhancer, strongly binding to sites 1, 3, 4, 5, 7, and 8. Ectopic expression of Islet1 throughout the heart resulted in weak ectopic expression of the Fgf10 transgene in left ventricle, and atria, although not to robust levels observed for ectopic expression of the transgene in ventricle of Nkx2-5 mutants, suggesting perhaps competition between Nkx2-5 and Islet1 for binding the enhancer in wild-type left ventricle.
Watanabe et al. performed competitive electrophoretic mobility shift assay to demonstrate that Nkx2-5 and Islet1 compete for Fgf10 enhancer homeodomain sites in vitro. Chromatin immunoprecipitation assays of embryonic heart extracts at E9.5 demonstrated significant binding of Islet1, but not Nkx2-5, to Fgf10 enhancer sequences in pharyngeal mesoderm. Binding of both Nkx2-5 and Islet1 to the Fgf10 enhancer occurs in outflow tract/right ventricle, and left ventricle, contexts where the endogenous Fgf10 gene is normally downregulated. Binding of Islet1 to Fgf10 enhancer in left ventricle is puzzling, as expression of Islet1 protein in left ventricle is not observed by Western blot analysis or by immunohistochemistry, other than low level of expression at the junction of the right and left ventricles.

Together, these data suggest a model whereby multiple homeodomain consensus sites within the Fgf10 enhancer are bound by Islet1, or both Nkx2-5 and Islet1, in a context-dependent manner (Figure). Observations that Fgf10 is downregulated in Islet1 mutants, and that Islet1 binds to the 1.7-kb enhancer in pharyngeal mesoderm in vivo, are consistent with Islet1 binding to activate the Fgf10 enhancer in pharyngeal mesoderm, although it would be of interest to examine enhancer transgene expression in Islet1 mutants. In outflow tract, right and left ventricle, where the endogenous Fgf10 gene is not expressed, with the exception of a restricted domain within right ventricle, the enhancer is bound by both Islet1 and Nkx2-5, suggesting that binding of these proteins can confer repression. Why Nkx2-5 does not bind the enhancer in pharyngeal mesoderm where it is expressed at reasonable levels, and why Islet1 binds the enhancer in left ventricle where it is expressed at vanishingly low levels are not yet fully understood. Does this reflect much greater avidity of binding of Islet1 than Nkx2-5 to the enhancer in vivo, and if so, what are the molecular determinants of binding avidity?

The dual role of Nkx2-5 in the regulation of Fgf10, both being required for activation in anterior second heart field and for repression in ventricles, begs for further investigation into the exact nature of this regulatory mechanism. Although from chromatin immunoprecipitation studies Nkx2-5 does not directly bind the Fgf10 enhancer in pharyngeal mesoderm, expression of the enhancer in this domain is reduced in Nkx2-5 mutants, despite normal levels of Islet1 expression in this region. Therefore, downregulation of reporter expression in pharyngeal mesoderm in the absence of Nkx2-5 suggests that enhancer activity in pharyngeal mesoderm does not depend solely on Islet1, but on other factors downstream of Nkx2-5. It is also interesting to note that an Islet1-dependent Mef2c enhancer-LacZ transgene is not ectopically expressed in left ventricle of Nkx2-5 mutants. This may reflect greater sensitivity of the Fgf10 enhancer to Islet1, as might be expected from the greater number of Islet1 binding sites in the Fgf10 enhancer, or could reflect an additional mechanism underlying ectopic expression of the Fgf10 enhancer in the Nkx2-5 background. For example, an alternative hypothesis would be that in Nkx2-5 mutants ectopic expression of another transcription factor in the mutant ventricle may recognize sites within the Fgf10 enhancer other than the homeodomain sites to activate ectopic expression.

In this regard, in addition to 8 homeodomain consensus sites, the Fgf10 enhancer contains 6 Tbx consensus binding sites. Previous studies in Tbx1 mutants have demonstrated decreased expression of Fgf10, and, consistent with this, Watanabe et al. observe decreased expression of the Fgf10 enhancer in pharyngeal mesoderm and outflow tract of Tbx1 mutants. However, it must be noted that Islet1 expression is downregulated in Tbx1 mutants.

**Figure.** A modular 1.7-kb enhancer within the Fgf10 gene is sufficient and required for reporter gene expression, resembling endogenous Fgf10 expression in anterior second heart field (SHF) and derived lineages. The enhancer comprises multiple homeodomain sites which have distinct affinities for Islet1 or Nkx2-5, as indicated by either red (high affinity to Nkx2-5), green (high affinity for Islet1), or orange/yellow (binds to both). The enhancer also contains multiple Tbx sites interspersed with the homeodomain sites, with as yet unaddressed binding affinities for distinct Tbx proteins (shown as white fill). In anterior SHF, high expression of Islet1 (green squares) activates, whereas in ventricle, high Nkx2-5 (red squares) represses the enhancer. Tbx1 in subsets of cells within the anterior SHF may directly bind and cooperate with Islet1, whereas other Tbx factors might function to corepress the enhancer with Nkx2-5 once SHF-derived cells enter the outflow tract. Other Tbx factors may also play a role in activation of the enhancer in the anterior SHF by directly binding to the enhancer to activate expression, or by interactions with Nkx2-5 to prevent its binding which would otherwise result in repression.
mutants, which could also account for decreased expression of the Fgf10 enhancer in this context. In addition, in terms of direct binding of Tbx1 to regulate the enhancer, it must be kept in mind that Tbx1 expression itself within the second heart field is relatively restricted, but that Tbx1 regulates expression of secreted growth factors, including Fgf10 and Fgf8, suggesting that mutation of Tbx1 will have noncell autonomous effects within adjacent second heart field populations.

Mutagenesis of Tbx consensus sites within the Fgf10 1.7-kb enhancer results in loss of enhancer transgene expression throughout pharyngeal mesoderm and outflow tract. This may reflect a requirement for direct binding by Tbx1 in those domains where it is expressed, and other Tbx proteins which may also be able to bind to activate the enhancer. In this regard, Tbx1, 2, and 3 have been shown to act redundantly during second heart field development, and Tbx1 mutants affect pharyngeal mesoderm expression of Tbx2 and Tbx3. Do Tbx consensus sites within the enhancer bind with distinct affinities to individual Tbx proteins, as observed for Nkx2-5 and Islet1 binding to homeodomain consensus sites? Do Tbx sites within the enhancer mediate its downregulation in pharyngeal mesoderm of Nkx2-5 mutants, where Islet1 binding to homeodomain consensus sequences?14,15 Islet1, and this Tbx protein might be downregulated in Nkx2.5 mutants. Alternatively, loss of Nkx2-5 may result in ectopic expression. Binding of a Tbx protein to the enhancer may be required for its activation in concert with Islet1, and this Tbx protein might be downregulated in Nkx2.5 mutants. Alternatively, loss of Nkx2-5 may result in ectopic activation of a Tbx protein in pharyngeal mesoderm, which can bind and repress the Fgf10 enhancer.

Transcription factors of the same family, including those of the homeodomain or T-box family, are characterized by a high degree of sequence conservation within DNA-binding domains, and accordingly often bind to the same sites in vivo, as recently shown for Tbx3 and Tbx5 on Scn5a enhancer sequences. In addition to DNA binding, homeodomain and T-box protein domains are involved in protein–protein interactions with each other, as well as other cofactors, also displaying promiscuity for cofactor binding between family members. In addition to Nkx2-5, Isll, and Tbx1, many other transcription factors are likely to be involved in the correct spatiotemporal expression of Fgf10, either by directly binding to DNA or via interaction with Nkx2-5, Isll, or Tbx1. As noted, Tbx2 and Tbx3 in second heart field may occupy T-box sites within the enhancer and have also been shown to physically interact with Nkx2-5. In outflow tract and ventricles, Nkx2-5 is coexpressed with Tbx5 and Tbx20, both of which can bind Nkx2-5. Transcription factor interactions may prove to be instrumental in recruitment of cofactors to enhancer sequences, or in modulation of DNA-binding affinities. Complexities of protein–protein interactions are therefore additional factors that must be taken into consideration when identifying mechanisms underlying activation or repression of enhancer elements.

Possibilities highlighted above illustrate the potential complexity of interactions between homeodomain and Tbx proteins, which must be further addressed to fully understand the workings of this modular enhancer. The comprehensive in vitro and in vivo studies by Watanabe et al provide a working model by which a single enhancer, comprising multiple consensus binding sites for distinct transcription factor families, can confer highly complex temporal and site-specific gene regulation, dependent on qualitative and quantitative variation in expression of transcription factor family members, and the affinity with which they bind each consensus sequence. The latter is also likely to be dependent on the presence of distinct cofactors (Figure). Altogether, these studies reveal complexities underlying gene regulation during cardiogenesis and provide an intriguing paradigm for further investigation.

Disclosures
None.

References
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