PERSPECTIVE

Switching on the notochord

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The notochord is a defining characteristic of the chordate embryo. It is a dorsally located rod of tensile mesodermal tissue that lies immediately beneath the neural tube. In vertebrates the notochord functions as a skeletal element during early embryogenesis and as a source of signals that pattern the neural tube and paraxial mesoderm. Despite the recent identification of mutations affecting notochord development in zebrafish, the rather modest progress in isolating genes expressed in the notochord during its differentiation has limited our understanding of the molecular mechanisms underpinning its structure and function. In a recent study, Takahashi et al. (1999), using embryos of the primitive chordate, the ascidian, have redressed the balance, identifying a large number of notochord-specific genes by subtractive cloning. Their approach, although not novel in principle, is remarkable for its efficacy and promises to set the stage for significant advances not only in our appreciation of the formation and function of the notochord but also in understanding how this important structure has evolved.

Notochord specification and the role of *Brachyury* in mesodermal patterning

In vertebrates, the notochord develops from the axial mesoderm of the gastrula via inductive interactions involving the transforming growth factor β (TGF β)-superfamily and fibroblast growth factor (FGF)-family signaling molecules. After neurulation the notochord lies beneath the floor plate of the neural tube, above the endoderm, and between the paired somites that extend the length of the trunk and the tail. Critical to its function, the notochord expresses transcription factors encoded by the *Brachyury*, *HNF-3 β* and *floating head* genes (Smith et al. 1991; Ruiz i Altaba and Jessell 1992; Talbot et al. 1995), as well as the secreted factor Sonic hedgehog, which patterns the somites and the neural tube (for review, see Ingham 1995). Studies in the mouse, *Xenopus*, and zebrafish have demonstrated that *Brachyury* is required for differentiation of axial midline mesoderm into notochord as well as for the formation of posterior mesodermal tissues (Chesley 1935; Halpern et al. 1993; Schulte-Merker et al. 1992; Conlon et al. 1996).

Ascidian development is highly autonomous, yet in-

ductive interactions are nevertheless critical for the establishment of some cell lineages (Nishida 1997). In these organisms, notochordal fate is induced by vegetal blastomeres in just 10 cells of the 110-cell embryo (Nakatani and Nishida 1994). These 10 cells then divide twice to produce the entire set of 40 cells that comprises the larval notochord. Initial expression of the *Ciona intestinalis* homolog of the vertebrate *Brachyury* gene, *Ci-Bra*, coincides precisely with the restriction of early blastomeres to a notochordal fate (Yasuo and Satoh 1993). Expression of *Brachyury* is then maintained exclusively in the notochord, in contrast to the situation in vertebrate embryos, where *Brachyury* is expressed in both notochord and ventral-posterior mesoderm (Fig. 1). Moreover, misexpression of ascidian *Brachyury* in cells of non-notochordal lineages is sufficient to impart to them with the morphological characteristics of notochord cells without the need for inductive interactions with vegetal blastomeres (Yasuo and Satoh 1998). Takahashi et al. (1999) have now extended this observation and demonstrated that misexpression of *Brachyury* in the endoderm of ascidian embryos is sufficient to induce fully differentiated ectopic notochord. This contrasts with the induction of ventral-posterior mesoderm observed when *Brachyury* was misexpressed in *Xenopus* animal caps (Cunliffe and Smith 1992): Only by coexpressing *noggin* with *Brachyury* could notochord be induced in this tissue (Cunliffe and Smith 1994). Significantly, Noggin is known to function by inhibiting bone morphogenetic protein (BMP) signaling pathways (Zimmerman et al. 1996); BMP4 can both ventralize dorsal mesoderm and induce epidermal fate in dissociated animal cap cells, and at high concentrations BMP4 is sufficient to induce ventral mesoderm in animal cap tissue (Dale et al. 1992; Jones et al. 1992; Sasai et al. 1995; Schmidt et al. 1995; Wilson and Hemmati-Brivanlou 1995). Thus, coexpression of Noggin with *Brachyury* in animal caps removes the ventralizing constraint of BMP signaling on mesodermal fate induced by *Brachyury*, yielding notochord and somitic muscle.

Although as in vertebrates, BMP signaling is implicated in epidermal induction and the inhibition of neural fate in ascidians, it appears to have no obvious role in the dorsoventral patterning of the mesoderm of these animals: Misexpression of BMP4 does not ventralize dorsal mesoderm, and in any case it is not expressed in the appropriate cell lineage (Miya et al. 1997). Similarly, studies of the expression pattern of *BMP2/4* in the

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Figure 1. Expression of *Brachyury* in ascidian and *Xenopus* embryos. (a) In situ localization of *Ci-Bra* mRNA in mid-tailbud embryo of *C. intestinalis*. Signal is strongest in posterior notochord cells (1997). Anterior is to the left; dorsal is up. (b) Notochord-specific expression of the *lacZ* transgene under the control of the 3.5-kb *Ci-Bra* promoter in the mid-tailbud embryo of *C. intestinalis* (a,b) Reprinted, with permission, from Corbo et al. 1997. Anterior is to the left; dorsal is up. (c) In situ localization of *Xbra* mRNA in late gastrula stage *Xenopus* embryo. Posterior view; dorsal is up. The signal is visible in two domains: notochord and ventral-posterior mesoderm.

cephalochordate *Amphioxus* indicate a function for this gene in specification of ectodermal fate but not in dorsoventral patterning of the mesoderm (Panopoulou et al. 1998). The absence of a ventralizing function of ascidian BMP4 may therefore explain how misexpression of *Brachyury* in ascidian endoderm leads directly to the formation of ectopic notochord.

Taken together, the observations of *Ci-Bra* and *Xenopus Brachyury* (*Xbra*) function have implications for the evolution of mesodermal patterning mechanisms. It is possible that the earliest evolutionary role for *Brachyury* in chordate development was to specify notochord, and that of BMP signaling may have been to induce epidermal fate. Subsequently, the function of BMP signaling in evolution may have been modified to influence the regulatory program under control of *Brachyury*. This would then have enabled the specification and patterning of several types of mesodermal tissues in the vertebrates in addition to notochord, including somite, mesenchyme, and mesothelium, in a BMP concentration-dependent manner.

The simple switch effect of *Brachyury* in ascidians has been exploited by Takahashi et al. (1999) to stunning effect. A high efficiency electroporation technique was used to introduce plasmid DNA encoding *Ci-Bra* under the control of the ascidian *fork head/HNF-3 β* (*Ci-fkh*) promoter. Because the combined efficiencies of the electroporation technique and promoter construct used resulted in extremely high levels of *Ci-Bra* transcription and conversion of a large part of the embryo into notochord, these embryos represent an excellent source of material for subtractive hybridization approaches.

Novel genes involved in notochord specification and function

A subtractive library comprising 599 cDNA clones was produced, 501 of which were up-regulated in embryos expressing the *Ci-fkh/Ci-Bra* construct. Of these, 38 exhibited a notochord-specific expression pattern by in situ hybridization, representing a frequency of 6% of the clones in the original library. Another 84 (14%) induced cDNA clones exhibited expression in notochord and other discrete locations in the embryo, giving a total frequency of 20% notochord-induced cDNAs. However, a further 81 (14%) of the induced clones exhibited specific expression patterns in locations other than the notochord, suggesting that signaling by ectopic notochord induces gene expression in other tissues.

Twenty of the 38 notochord-specific genes are suggested by Takahashi et al. (1999) to encode proteins with no sequence similarities in available databases. Given the number and size of the existing model genome and expressed sequence tag (EST) databases, this suggests that there are likely to be many specialized aspects of notochord function that remain to be understood. The remaining 18 genes encode products with sequence similarity to proteins of known function. These include extracellular matrix proteins, cell adhesion molecules, and cytoplasmic signaling pathway components. One gene that is expressed relatively late in notochord formation is a member of the ezrin-radixin-moesin (ERM) family of polypeptides, which tether components of the plasma membrane to the actin cytoskeleton (Tsukita and Yone-mura 1997). Perhaps this ERM protein plays a role in cell intercalation, maintaining the integrity of the notochord or producing the changes in notochord cell shape that contribute to tail extension.

Other *Brachyury*-inducible genes

Tada et al. (1998) previously used a subtractive hybridization approach to isolate genes that were activated when *Xbra* was misexpressed in *Xenopus* animal cap tissue. Because no notochord is induced in this scenario, the functions of these putative *Xbra* target genes are unlikely to be related to those characterized by Takahashi et al. (1999). Of 37 cDNA clones from the subtracted library, 4 were identified as *Brachyury* inducible. One cDNA clone that was isolated identified a family of homeodomain protein-encoding cDNAs related to the *Mix.1* family of genes. This cDNA, *Bix1*, is expressed in mesoderm and endoderm at late blastula and gastrula stages but it is excluded from the dorsal marginal zone of the early gastrula that is fated to form the notochord. Misexpression of *Bix1* in the dorsal marginal zone prevented notochord formation, whereas misexpression of *Bix1* in animal cap tissue was sufficient to induce formation of ventral mesoderm. Thus *Bix1* may be a direct target for *Brachyury* in the presence of BMP signaling and would not therefore be predicted to be activated by *Ci-Bra* in ascidians.

Another target gene of *Xenopus Brachyury* is *eFGF*

(Casey et al. 1998), activity of which is required for *Brachyury* function in the specification of dorsal mesoderm including notochord (Isaacs et al. 1994; Schulte-Merker and Smith 1995; Conlon et al. 1996). In this case, it seems surprising that no FGF homologs were identified in the collection of subtracted cDNA clones by Takahashi and colleagues (1999); this may indicate a further difference between the function of *Brachyury* in ascidians and vertebrates that merits further investigation.

Endodermal competence and the role of T-box genes in endoderm formation

The discovery that *Ci-Bra* can convert ascidian prospective endoderm into notochord demonstrates that in this species, prospective endoderm is competent to realize a mesodermal fate. However, as in vertebrates, the ascidian endoderm is the source of the notochord-inducing signal (Nakatani and Nishida 1994). A mechanism should therefore exist that blocks self-induction of *Ci-Bra* in endoderm and thus prevents autodifferentiation into notochord.

No effects of misexpressing vertebrate *Brachyury* in *Xenopus* vegetal pole prospective endoderm have been reported, but the observation by Takahashi et al. (1999) of an experimentally produced endoderm-to-mesoderm fate switch is not without precedent in vertebrate embryos. Transcripts of the maternally expressed *Xenopus* T-box family member *VegT* are initially restricted in the early embryo to the vegetal hemisphere and later are distributed quite broadly throughout the mesoderm (Horb and Thomsen 1997; Lustig et al. 1996; Stennard et al. 1996; Zhang and King 1996). Misexpression of *VegT* in animal cap tissue causes mesoderm formation, but intriguingly, depletion of maternal *VegT* mRNA stores by oligonucleotide-mediated mRNA destruction results in the differentiation of prospective endoderm into mesoderm and of marginal zone prospective mesoderm into ectoderm (Zhang et al. 1998). Thus, whereas *VegT* fulfills one role in the mesoderm, it also is required in the prospective *Xenopus* endoderm for the specification of this germ layer.

Given that *VegT* and *Xbra* are highly related and can bind specifically to the same target sites (Tada et al. 1998), it is tempting to speculate that misexpression of *Ci-Bra* in ascidian endoderm diverts endoderm to a mesodermal fate both by competitive inhibition in the endoderm of a putative maternal *VegT* homologue, such as the gene described recently by Takada et al. (1998), as well as by promoting notochord differentiation.

This mutual competence of mesoderm and endoderm to form one or another tissue and the finding that they are controlled by related transcription factors are underscored further by the discovery that *Bix1*, which is inducible by both *Xbra* and *VegT* and is expressed in both mesoderm and endoderm, can specify differentiation of each tissue in animal cap explants (Tada et al. 1998). These observations raise the question of how these germ layers acquire such different fates while expressing very similar regulatory molecules.

Implications for future studies—a general approach to analyzing chordate tissue specification

In the zebrafish, screens for mutants have so far identified at least 29 genes that are required for notochord formation (Odenthal et al. 1996; Stemple et al. 1996). Genes such as *bozozok* and *floating head* control the formation of the axial midline mesodermal precursors, whereas *no tail* (zebrafish *Brachyury*) and *gno* are required for commitment to the notochord pathway of differentiation. Many of the other genes recovered in these screens appear to be involved in the differentiation, maintenance, and normal shape of the notochord and thus most likely act downstream of *Brachyury*. Because multiple alleles have been recovered at many of these loci, these aspects of notochord development may be close to saturation. Alternative approaches that augment the mutant analyses in the zebrafish will therefore be necessary if a complete description of notochord differentiation and function is to be realized. One route will undoubtedly be the characterization of vertebrate homologs of the genes isolated by Takahashi et al. (1999). It may turn out that some of these genes are homologs of those identified in the zebrafish screens for notochord-determining genes. Further insights into the function of the genes that lack corresponding mutant zebrafish homologs should emerge from studying their expression patterns in the notochord mutants produced in the Tübingen and Cambridge screens.

A frequency of 20% notochord-induced cDNA clones in the *Ci-Bra*-specific subtractive library indicates that the procedure of Takahashi and coworkers (1999) is reasonably efficient and may be more widely applicable to the identification of target genes that are under the control of other transcription factors and inducing factors.

Comparative evolutionary studies of developmental regulatory genes are becoming increasingly important for understanding how the mechanisms of developmental patterning operate. Moreover, studies of ascidian embryos are gaining popularity because their uncomplicated body plan is fundamentally similar to those of vertebrate embryos and because of their highly conserved homologs of key vertebrate patterning genes. Such features, along with the small genome size and the recently improved methods with which to study gene function in ascidians, suggest that rapid progress will be made with these organisms in understanding the conserved molecular mechanisms that specify the axes and components of the chordate body plan.

Acknowledgments

We are grateful to The Company of Biologists Limited for permission to use Figure 1a and 1b, and to Dr. J.C. Smith for permission to use Figure 1c. V.T.C. is a Lister Institute Research Fellow.

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Genes Dev. 1999, **13**:

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