Expression Analysis of Chick Wnt and Frizzled Genes and Selected Inhibitors in Early Chick Patterning

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Wnt signaling is an important component in patterning the early embryo and specifically the neural plate. Studies in *Xenopus*, mouse, and zebrafish have shown that signaling by members of the Wnt family of secreted signaling factors, their Frizzled receptors and several inhibitors (sFRP1, sFRP2, sFRP3/Frzb1, Crescent/Frzb2, Dkk1, and Cerberus) are involved. However, very little is known about the expression of genes in the Wnt signaling pathway during early anterior neural patterning in chick. We have performed an expression analysis at neural plate stages of several Wnts, Frizzled genes, and Wnt signaling pathway inhibitors using in situ hybridization. The gene expression patterns of these markers are extremely dynamic. We have identified two candidate molecules for anterior patterning of the neural plate, Wnt1 and Wnt8b, which are expressed in the rostral ectoderm at these stages. Further functional studies on the roles of these markers are underway. Developmental Dynamics 229:668–676, 2004.

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INTRODUCTION

A gradient of Wnt/beta-catenin signaling is required for anterior-posterior (AP) patterning of the embryo during gastrulation and neurulation (Kiecker and Niehrs, 2001; Davidson et al., 2002; Houart et al., 2002; Nordstrom et al., 2002; Lagutin et al., 2003). A posterior to anterior gradient of Wnt signaling, with Wnt5 being more highly expressed posteriorly and Wnt inhibitors being more highly expressed anteriorly, results in posterior to anterior allocation of cell fate in the neural plate. Known Wnt inhibitors of the extracellular pathway include molecules such as the sFRPs (secreted Frizzled Related Proteins; Leyns et al., 1997; Pfeffer et al., 1997), Cerberus (Bouwmeester et al., 1996; Rattner et al., 1997; Wang et al., 1997; Zhu et al., 1999; Esteve et al., 2000; Ladher et al., 2000) and Dkk1 (Dickkopf1; Glinka et al., 1998; Piccolo et al., 1999; Mukhopadhyay et al., 2001). These molecules act by means of at least two mechanisms to reduce Wnt levels in rostral neuroectoderm: sFRPs and Cerberus directly bind Wnts, preventing interaction with the Frizzled receptors (Fzs), whereas Dkk1 interacts with LRP5/6 (LDL-receptor-related proteins 5/6), which in turn acts as a high affinity antagonist to the Fzs (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001). More players in this intricate pathway are being uncovered: for example, Kremen, which interacts with Dkk1 to regulate AP patterning in *Xenopus* (Davidson et al., 2002; Mao et al., 2002). A large number of molecules interacting within the intracellular pathway are also able to influence Wnt signaling including, Axin, Tcf3 and the recently reported Chibby (Takemaru et al., 2003).
Studies in Xenopus have produced the clearest model of the extracellular Wnt pathway with regard to AP patterning (Kiecker and Niehrs, 2001; Davidson et al., 2002). Overexpression of Wnt inhibitors results in enlarged forebrain and head structures, and an ectopic head can be induced with the further addition of bone morphogenetic protein inhibitors (Glinka et al., 1997; Glinka et al., 1998). Inactivation of the inhibitory mechanism, however, results in loss of forebrain and head structures, with posteriorization of the remaining tissue. The secreted Frizzled-related Wnt antagonist Tlic is required in zebrafish to establish the telencephalon (Houart et al., 2002). Evidence suggests that Wnt18b is the target of the Wnt-inhibiting molecules (Kelly et al., 1995; Kim et al., 2001; Houart et al., 2002). In mouse, disruption of Dkk1 results in head truncations rostral to the midbrain (Mukhopadhyay et al., 2001). Similarly, disruption of Six3, which binds the Wnt1 promoter region and would normally repress Wnt1 expression, results in loss of the forebrain and posteriorizes the remaining mutant head (Lagutin et al., 2003). These data suggest a further method for modification of the pathway by direct inhibition of Wnt transcription. Moreover, whereas the Dkk1 mutant acts at gastrulation/early neurulation stages, the Six3 effect occurs at late neurulation/early somite stages, indicating that repression of Wnt signaling in the rostral embryo must be maintained over a long period of development to ensure that anterior patterning is not disrupted.

In chick, investigation of the very early Wnt signaling effects leading to initiation of the primitive streak and organizer have been reported (Joubin and Stern, 2001; Skromne and Stern, 2001; Bertocchini and Stern, 2002). Also, a role for Wnt signaling in progressive induction of caudal neural identity has been investigated (Nordstrom et al., 2002). However, the expression patterns of the Wnt genes, their Frizzled (Fz) receptors and inhibitors has not been systematically determined at early stages of development.

Here we use in situ hybridization to map out the expression patterns of genes involved in Wnt signaling at early stages of development and are undertaking further functional studies to determine the role of the Wnt signaling pathway in AP patterning. Separation of the gastrulating embryo transversely into rostral and caudal blastoderm isolates does not affect rostral identity (Darrell et al., 1999). Our recent studies confirm that separating the rostral embryo from more caudal tissue containing the organizer does not result in the loss of anterior patterning, as determined by the induction and continued expression of the anterior neuroectoderm marker Ganf (Hess1 in mouse; Chapman et al., 2003). However, removal of the lower layer in these rostral blastoderm isolates does result in the loss of Ganf induction. Neural specification, marked by the expression of Sox2, which occurs just after stage 3c, is not affected by the removal of the lower layer (Chapman et al., 2003). Our in situ hybridization data presented here suggest that a mechanism for the loss of anterior identity in the neuroectoderm is the loss of Wnt inhibitors expressed in the lower layer, resulting in increased Wnt1 and/or 8b signaling.

RESULTS AND DISCUSSION
Wnt Genes Are Expressed in Rostral Tissue During Early Regional Patterning of the Neural Plate
To identify candidate Wnt signaling pathway molecules that may be involved in patterning anterior regional identity, we have conducted an analysis of the expression patterns of genes in the pathway. The stages of interest are those that directly follow neural specification: stages 3d, 4, 5, and 6. Genes studied by in situ hybridization include the Wnt genes, their Frizzled receptors and selected inhibitors (Table 1). Grouping these markers into any meaningful order is difficult. However, the Wnt genes can be usefully separated into those markers expressed in the primitive streak and completely excluded from rostral tissue, as compared with those that have some expression rostral to Hensen’s node. Many of these markers have been reported previously only at somite stages and later (Hollyday et al., 1995). However, improvements in the in situ hybridization technique have led to a reassessment of these early data, and in many cases, expression at younger stages is detected.

In agreement with Skromne and Stern (2001), we find expression of Wnt15a, Wnt18c, and Wnt11 from pre-streak stages (see Chapman et al., 2001, for a detailed explanation of staging in early chick embryos). However, Wnt11 and Wnt3a expression are also detected in pre-streak embryos, in contrast to earlier reports. Wnt5b and Wnt114 (also called Wnt19a) are expressed from stage 2; shortly thereafter, Wnt3 and Wnt14 transcripts are detected also in early linear stage-stamp embryos (stages 3a/b onward). Wnt6, 7a, 7b, and 13 (also called Wnt2b) are not expressed until stages 5–7. Wnt 8b is expressed from the earliest stage tested, stage 3.

Wnt3, 3a, 5a, 5b, and 8c are expressed in the primitive streak and ingressing mesoderm (Fig. 1A–O). However, within this domain, individual expression patterns are quite distinct. Wnt3 expression is confined to the streak at stages 4/5 but is excluded from Hensen’s node and is not expressed in mesoderm migrating away from the streak (Fig. 1A–C). Wnt13a, initially weakly expressed, becomes strongly expressed throughout the streak and node, and in the first mesoderm to migrate from the primitive streak (Fig. 1D–F). Wnt5a is excluded from the rostral third of the streak, with some expression in the mid-streak region and strongest expression in the caudal-most part of the streak and surrounding mesoderm population (Fig. 1G–I). Wnt5b staining is strong throughout the streak, node, and exiting lateral mesoderm (Fig. 1J–L). Wnt18c is expressed along most of the length of the streak, except for the posterior-most streak and Hensen’s node at stage 3d/4 (Fig. 1M). By stage 4/5, Wnt18c is a good marker for mesoderm migrating through the streak, with expression extending more rostrally toward the anterior of the
streak, but it is still excluded from the node in most embryos. In some embryos, there seems to be some asymmetrical expression on the left side of Hensen’s node (Fig. 1N, O).

Wnt1 is not expressed in either the streak or rostral embryo, but at stage 4, it is expressed very weakly in Hensen’s node and in the caudolateral border between the area pellucida and area opaca (Fig. 1P, R). Wnt1 is thought to play a role in the paraxial mesoderm caudalizing-signaling pathway (Nordstrom et al., 2002). Expression that initially marks the rostral tip of Hensen’s node extends into the population of paraxial mesoderm cells migrating laterally away from the rostral and mid-streak area just after stage 4 (Fig. 1Q).

After neural specification at stage 3d/4, Wnt genes expressed in rostral tissue include Wnt 1, 8b, 4, and 14 (Fig. 1S–Z, a–d). The earliest expression of Wnt1 previously reported is at stage 7, and for Wnt8b, not before stage 9 (Hollyday et al., 1995). In contrast to these earlier findings, Wnt1 and 8b are initially expressed in the streak by stage 3d/4, with Wnt8b transcripts seeming to be more abundant (cf. Fig. 1S, V). Expression of Wnt8b is also detected in the rostral embryo, forming a band of expression across the presumptive rostral neural plate (Fig. 1V). By stage 5/6, expression of both genes marks the shaping (i.e., converging and extending) neural plate, with Wnt1 now being the stronger marker (Fig. 1T). Wnt4 (also previously reported only at stage 7) is expressed...
Fig. 1. Wnt gene expression in early chick embryos. For all figures: whole-mount embryos have rostral toward the top of the page and are dorsal views unless otherwise stated; midline sagittal sections at cut at 50–70 µm, with rostral toward the top and with ectoderm toward the right of the page. First image in each row has the stage indicated in top right hand corner. Asterisks mark Hensen’s node in sections. In this figure, stage 4 embryos are positioned above the later (4+ or 5) stage embryos. A–O: Wnt3, 3a, 5a, 5b and 8c are all expressed in the primitive streak. Sections C, F, I, L, O from whole-mounts A, D, G, J, N, respectively. Sections F and I show the caudal streak, with Hensen’s node at the top of the figure. M is centered at the level of Hensen’s node (asterisks) in both cases. P–R: Wnt11 is expressed in Hensen’s node (asterisks) and later (Q), in mesoderm migrating away from the streak. Section R is from the whole-mount embryo shown in P. S–X: Wnt 1 and Wnt8b expression. Section U is from the whole-mount embryo T. Section X from the whole-mount embryo V; note weaker expression in both layers between the node and rostral expression band. Y–Z,a–d: Wnt4 and 14 are expressed in the rostral crescent bordering the area opaca. Between stage 4 and 5, the streak begins to regress and the gap between streak expression and area opaca expression enlarges. Wnt14 expression does not directly abut the area opaca border rostrally (border indicated by arrowheads). Section a is from whole-mount embryo Y, and section d is from whole-mount embryo b.
Frizzled Receptor Genes Are Expressed in Rostral Tissue

Frizzled (Fz) genes code for Wnt receptors and the expression of several Fz genes in the cranial placodes has been reported previously (Stark et al., 2000a). However, the expression of these genes at neural specification stages has not been examined previously. Our in situ hybridization results suggest that the majority of the Fz genes are expressed from prestreak stages onward and in rostral tissues during neural specification, with Fz10 excluded rostrally (Table 1; Fig. 2).

Fz1 is expressed at low levels over a broad domain of rostral ectoderm and within the rostral streak at stage 4 (Fig. 2A). Although excluded from the rostral endoderm as seen in sections, expression is detected in the ingressing axial mesoderm and rostral-most ingressing lateral mesoderm of the stage 4/4+ embryo (Fig. 2C). By stage 5, expression becomes restricted to a rostral domain of ectoderm and the ingressing axial mesoderm (Fig. 2B).

Expression of Fz2 transcripts is observed throughout the area pellucida and seems to be graded, with weakening expression anterior to posterior. High levels of expression are detected in Hensen’s node (Fig. 2D). At stage 5, staining increases in intensity, becoming restricted to the presumptive rostral boundary of the neural plate, underlying endoderm, head process, laterally to the heart mesoderm, and caudally to the primitive streak (Fig. 2E,F). Expression is down-regulated in the caudal neural plate.

Fz4 has been reported previously only from six somites (stage 9−) of development, starting in the kidney rudiment (Stark et al., 2000a,b), whereas we find expression from prestreak stages. At stage 4, weak expression is detected in the ectoderm and the primitive streak (AP gradient; Fig. 2G,I). By stage 4+/5, expression is more defined, with transcripts in the ingressing axial mesoderm and throughout the streak, in both neuroectoderm and underlying endoderm at the limit of the neural plate (Fig. 2H). Of interest, there seems to be a gap in the ectoderm expression domain just rostral to the streak.

Fz7 is expressed in a gradient of strong anterior to weaker posterior expression along the streak, with widespread expression in all tissue layers surrounding Hensen’s node (Fig. 2J). By stage 5, however, expression is more defined and is excluded from the caudal streak (Fig. 2K,L).

Fz8 expression is quite distinct, with strong expression in Hensen’s node and an expanding fan-shaped region of endoderm (prechordal plate) rostral to the node (Fig. 2M−P). As this rostral region increases in size, Hensen’s node expression diminishes (stage 5, Fig. 2O).

Fz9 at stage 4 is expressed in the rostral streak, spreading more caudally with high levels of transcripts in Hensen’s node (Fig. 2Q−T). Some weak mosaic expression is detected in the surrounding ectoderm, but early ingressing axial mesoderm has high levels of transcripts (Fig. 2Q,T). Fz10 was first reported to be expressed only from stage 6 in the caudal mesoderm and later extending rostrally in dorsal ectoderm (Kawakami et al., 2000), but we detected expression in embryos from prestreak stages. By stage 4, expression is in the primitive streak and mesoderm ingressing through the caudal streak (Fig. 2U,W). Expression is down-regulated in the rostral streak by stage 5 (Fig. 2V). Although staining in whole-mount preparations suggests that transcripts are initially in the node, section analysis shows that lower levels of rostral-streak transcripts are found only in the mesodermal component (Fig. 2W).

Analysis of Fz gene expression reveals that all of these markers are expressed in rostral tissue during patterning of the regional identity of the neural plate, except for Fz10, which is specifically excluded from the rostral embryo. These data indicate that Wnt signaling at these early stages in neural specification and regional patterning may be critically important. However, there are several Wnt inhibitors known, and this component requires careful analysis in relation to Wnt expression to provide insight into potential roles of Wnts in patterning.

Inhibitors of Wnt Signaling Are Expressed Dynamically in the Rostral Embryo

To address the potential role of Wnt signaling in regional patterning of the neural plate, the dynamic expression patterns of several Wnt inhibitor genes, sFRP1, sFRP2, sFRP3/Frzb1, Crescent/Frzb2, Dkk1, and Cerberus, were analyzed (Table 1; Fig. 3). sFRP1, Crescent/Frzb2, Dkk1, and Cerberus expression is detected from prestreak stages, with sFRP2 and sFRP2/Frzb1 weakly detected from HH stage 2. Expression shifts...
Fig. 2. Frizzled gene expression at stages 4 and 5. In Fig. 2, stage 4 embryos are positioned to the left of later (4+ and/or 5) stage embryos. A–C: Fz1 expression. Section C is from whole-mount embryo A. Widespread expression occurs in all layers. D–F: Fz2 expression is similar to that of Fz1, with broad expression becoming more defined at stage 5. Section F is from whole-mount embryo E. G–I: Fz4 expression at stage 4 is weak in the streak, Hensen's node (asterisks) and ingressing mesoderm. Section I is from whole-mount embryo G. J–L: Fz7 expression. At stage 5 Fz7 is strongly expressed in Hensen's node. Section L is from whole-mount embryo K. M–P: Fz8 is not expressed in the streak except for in Hensen's node. Fan-shaped expression in prechordal plate endoderm (and less so in overlying ectoderm, just rostral to the node). Section P is from whole-mount embryo O. Q–T: Fz9 is strongly expressed throughout the node and along rostral third of the primitive streak. Later expression extends along the length of the primitive streak, with rostral expression occurring in the endoderm. Section T is from whole-mount embryo Q. U–W: Fz10 is not expressed in the rostral embryo. Section W is from whole-mount embryo U.

Fig. 3. Expression of Wnt-signaling pathway inhibitors at stages 4 and 5. In Fig. 3, stage 4 embryos are positioned to the left of later (4+ and/or 5) stage embryos. A–D: sFRP1, with section D from embryo in A. At stage 4, the endoderm has stronger expression than ectoderm, with the definitive endoderm just rostral to Hensen's node with the highest levels of transcripts. E–H: sFRP2, section H from embryo E. Weaker staining around the node, with rostral ectoderm expression much stronger and no expression in the endodermal layer. I–L: Frzb1, section L from whole-mount embryo L. A band of rostral expression quickly up-regulates and becomes refined to the neural plate domain. M–P: Whole-mount embryo M used to produce section P. The staining in the midline is prechordal plate endoderm and not axial mesoderm. Q–T: Dkk1, Q and R are stage 4 embryos. Embryo R is double stained, with Dkk1 in red and Ganf in purple (asterisk marks the node). Section T from embryo S at stage 4/H11001/5. Note the level of ingressing axial mesoderm (arrow). U–X: Cerberus, with stage 4 embryos in U and V. Embryos V and W double stained for Cerberus (blue) and Chordin (Hensen's node and ingressing axial mesoderm marker) in red. The section in X is from similar embryo to U. Cerberus only marks the prechordal plate endoderm, with early ingressing axial mesoderm just beginning to emerge from Hensen's node negative for transcript at this and later stages.
temporally, spatially, and in intensity during development and embryos need to be carefully matched to ensure that equivalent stages are compared.

sFRP1 expression is detected in embryos from prestreak stages onward (Esteve et al., 2000). In the stage 4 midline sagittal section shown here, low levels of transcripts are detected in the rostral ectoderm, whereas the endoderm has a larger number of transcripts, with the definitive endoderm just rostral to the streak having the highest levels (Fig. 3A–D). Expression is excluded from Hensen’s node and the primitive streak. This dynamic expression rapidly becomes more refined with a band of rostral ectodermal tissue and ingressing axial mesoderm and later derivatives, the prechordal plate mesoderm and notochord expression (Terry et al., 2000) becoming dominant by stage 5 (Fig. 3C).

sFRP2 expression is detected weakly from HH2, but up-regulates dramatically in both neural and epidermal ectoderm at stage 4 (Fig. 3E,H). By stage 5, expression is becoming restricted to the neural plate and is specifically excluded from the streak (Fig. 3F,G; Terry et al., 2000).

sFRP3/Frzb1 expression begins at HH2 but is very weak across the epiblast until stage 4 when a broad band of expression in the rostral neuroectoderm is detected (Fig. 3L,L). The initial broad expression becomes more defined as the neural plate forms, and it is almost identical to sFRP2 expression in the neural plate until stage 7, after which the patterns diverge (Fig. 3J,K; Ladcher et al., 2000).

Crescent/Frzb2 transcripts are detected from prestreak stages in the hypoblast and, by stage 3, in ingressing definitive endoderm (Pfeffer et al., 1997; Chapman et al., 2002). Expression is excluded from the most caudal endoderm, indicating that this tissue may either have different origins from other endodermal regions or is already exhibiting differences in patterning. As ingestion of the endoderm continues, expression becomes restricted to the rostral crescent of hypoblast and anterior definitive endoderm (Fig. 3M–P). Staining with HNK-1 antibody followed by section analysis indicates that expression is only in the endodermal layers and is excluded from the ingressing axial mesoderm (not shown). By stage 5, staining is progressively restricted to the prechordal plate endoderm, the only tissue to maintain expression during subsequent stages (Fig. 3N,O). Crescent is a known inhibitor of Wnt8c (Marvin et al., 2001).

Dkk1 is expressed in prestreak embryos in hypoblast tissue and during streak formation and rostral extension. By stage 3d, expression is also detected in a mosaic pattern in the endoderm beneath the presumptive rostral neural plate. At stage 4, this expression is refined, with section analysis revealing a gap in expression in the lower layer directly beneath the Ganf-expressing zone in the rostral neuroectoderm (Fig. 3Q–T). Transcripts remain in Hensen’s node and the ingressing mesoderm at stage 4+, with a crescent of endoderm expression at the rostral extent of the neural plate (Fig. 3S,T; Chapman et al., 2002). The significance of the complimentary expression between Ganf and the Dkk1 domain remains to be functionally analyzed.

Another potent Wnt inhibitor is Cerberus. Cerberus is expressed in the hypoblast of prestreak stage embryos, and high levels of transcripts are produced in the streak at stages 2 and 3, after which expression is down-regulated, except for in the definitive endoderm. As the definitive primitive streak forms at stage 4 expression in the midline rostral tissue is dramatically down-regulated, leaving two lateral patches of expression with no midline transcripts by the time ingestion of axial mesoderm begins at stage 4+ (Fig. 3U–X; Chapman et al., 2002).

These data suggest that there is a balance between the expression of Wnt inhibitors and Wnts in the signaling pathway that plays an important role in patterning regional identities in the neural plate.

**Wnt Genes Are Candidates for Regional Patterning of the Neural Plate at Early Stages**

A role for Wnt pathway molecules in patterning anterior identity in the neuroectoderm in chick has not been investigated previously, although Wnt3a, Wnt8c, and Wnt11 signaling in patterning more caudal regions of the neural plate in stage 4 and stage 5 embryos has been examined (Nordstrom et al., 2002). The lack of candidate Wnt molecules expressed rostral to the primitive streak at appropriate time points is most likely why the role of the Wnt pathway in anterior patterning has not been investigated. Moreover, the role of the Wnt-signaling pathway in embryonic patterning at these stages is not restricted to regional identity of the neural plate. Inhibition of Wnt signaling is required for promoting heart formation in anterior heart mesoderm. For example, Crescent, Dkk1, and Frzb1 are able to inhibit Wnt3a, 5a, 8c, allowing cardiogenesis to progress, even in more posterior mesoderm that would normally give rise to blood derivatives (Baranski et al., 2000; Marvin et al., 2001). The opposite effect was elicited when Wnt3a or Wnt16c were overexpressed in heart mesoderm, causing posteriorizing effects such as blood development and suppression of heart muscle formation (Marvin et al., 2001). The effect of the Wnt signaling pathway in multiple patterning mechanisms within a similar temporospatial domain makes investigating anterior patterning a complex problem.

The expression patterns reported here also raise the question of the binding specificity between Wnt ligands, receptors, and inhibitors. Little data are available in regard to this issue in vertebrates in general and especially in chick. Future functional studies involving manipulation of the Wnt pathway though gain and loss of function experiments will provide further insight into interactions of Wnt pathway members in induction and patterning events. Importantly, Wnts are known to act in several pathways—canonical, noncanonical planar cell polarity pathway, and Wnt/calcium pathway (van Es et al., 2003). We have identified two new candidate Wnt signaling molecules (within the canonical Wnt signaling subgroup) that may have a role in patterning anterior identity in epiblast and neuroecto-
In Situ Hybridization

In situ hybridization was performed as described previously (Chapman et al., 2002). Embryos were then cleared in 80% glycerol/phosphate buffered saline, embedded in 20% gelatin, fixed with 4% paraformaldehyde, and sectioned by using a Leica Vibratome at 50–70 μm. Embryos were imaged with a SPOT, Coolsnap, or Zeiss Axilocam digital camera.

Important Web Sites for Wnt Gene Information

For additional information on the expression of components of the Wnt-signaling pathway, see the following: the Wnt gene Homepage—http://www.stanford.edu/~rmsusse/wntwindow.html, Geisha—chick cDNA gene expression patterns http://geisha Biosci.arizona.edu/data, and Chickexpressions—expression patterns of several chick genes, including Wnts, Frizzled, and inhibitors from prestreak embryos onward—http://www.neuro.utah.edu/chickexpressions.

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