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Original Article

Genetic patterning of the posterior neuropore region of *curly tail* mouse embryos: deficiency of *Wnt5a* expression

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ABSTRACT The mouse mutant *curly tail (ct)* develops tail flexion defects and spina bifida as the result of delayed or failed closure of the posterior neuropore (PNP). With the aim of identifying genes involved in the chain of events resulting in defective neurulation, which can be detected at day 10.5 of development, we examined the expression patterns of a number of genes implicated in patterning of axial structures, mesoderm and neuroepithelium. The genes analyzed were *Shh, HNF3α, HNF3β, Brachyury, Hoxb1, Evx1, Fgf8, Wnt5a* and *Wnt5b*. No differences could be detected between non-mutant embryos and *ct/ct* embryos with normal PNP size for any of these genes. Comparisons between *ct/ct* embryos with enlarged PNP and phenotypically normal *ct/ct* or non-mutant embryos showed differences only for *Wnt5a*. Expression of this gene was greatly reduced in the ventral caudal mesoderm and hindgut endoderm. Analysis of younger embryos revealed that prior to the stage at which embryos at risk of developing neural tube defects can be detected, the same proportion of *ct/ct* embryos shows reduced *Wnt5a* expression. The proportion of embryos showing reduced expression and almost undetectable expression of *Wnt5a* reflects the proportions of tail defects and spina bifida seen at later stages. We suggest that deficiency of *Wnt5a* signaling in the ventral caudal region tissues is an important component of the mechanism of development of the defects in affected *curly tail* mutant mice, and that it is causally related to decreased cell proliferation within the ventral caudal region. A possible relationship between decreased *Wnt5a* expression and reduced levels of heparan sulphate proteoglycan is discussed.

KEY WORDS: *neural tube defects, posterior neuropore, mouse embryo, Wnt5a, in situ hybridization*

Introduction

The transition from primary to secondary axis formation in mammalian embryos involves fundamental changes in tissue organization and cell behavior. Gastrulation and neurulation, which during the primary axis formation are functions of the epiblast and its derived neuroepithelium, are taken over by the tail bud mesenchyme in the secondary phase. The remarkable outcome of this change is an apparently seamless continuity of axial development through the lumbosacral region and into the tail. Only the hindgut, which terminates just caudal to the point of transition, presents as a discontinuous structure.

The end of primary neurulation involves closure of the posterior (caudal) neuropore (PNP); failure or abnormality of this process results in spina bifida or vertebral defects. Spina bifida comprises a variety of pathological conditions, the most common form being myelomeningocele, which in the human embryo arises as a result of failed closure of the caudal neural tube at 26-30 days post-fertilization (Knepper and McLone, 1992). Epidemiological and experimental studies indicate that spina bifida is in most instances of multifactorial origin, involving multiple genetic and environmental factors (Hall *et al.*, 1988; Copp *et al.*, 1990; MRC Vitamin Study Research Group, 1991; Georges and McLone, 1995).

The *curly tail (ct)* mouse mutant is considered a good animal model of human neural tube defects (NTD), in particular spina bifida, showing similarities with the human condition with respect to location of the defects (Embury *et al.*, 1979). Like the human condition, *curly tail* NTD are multigenic in origin. The major gene *ct* is mapped to distal chromosome 4 (Neumann *et al.*, 1994), and three modifier loci are probably involved in determining its penetrance and expressivity, one of which has been mapped to chromosome 17 (Letts *et al.*, 1995). Environmental factors, such as vitamin A, mitomycin C, hydroxyurea and inositol, influence the incidence of NTD in the *curly tail* mutant (Seller and Adinolfi, 1981;

Abbreviations used in this paper: ct, curly tail; HSPG, heparan sulphate proteoglycan; NTD, neural tube defects; PNP, posterior neuropore; VER, ventral ectodermal ridge.

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Seller and Perkins, 1982,1983; Seller and Perkins-Cole, 1986; Chen *et al.*, 1994, Greene and Copp, 1997). Homozygous *ct/ct* mice develop spinal NTD in about 60% of cases, either as tail flexion defects (40 to 50%) or spina bifida with/without tail flexion defects (10 to 20%). The remaining 40% are morphologically normal.

In *curly tail* embryos the development of NTD, including tail flexion defects, results from delayed closure of the posterior neuropore (Copp *et al.*, 1982; Copp, 1985). During normal primary neurulation, the rate of apposition of the folds accelerates at the 23-25 somite stage, leading to closure of the PNP at around 30-32 somites (van Straaten *et al.*, 1992). In pre-NTD *ct/ct* embryos, neural tube closure proceeds normally until the 22-23 somite stage, after which it decelerates. The consequential delay in PNP closure is correlated with exaggerated ventral curvature of the developing caudal region, thought to result from imbalance in cell proliferation between a rapidly dividing neuroepithelium and more slowly proliferating ventral structures (notochord and gut) (Copp *et al.*, 1988; Brook *et al.*, 1991; Peeters *et al.*, 1996). Excess ventral curvature imposes mechanical strain upon the dorsal neural folds inhibiting elevation.

Understanding of the genetic mechanisms underlying the *curly tail* defect is still very limited. Identification of genes that may be part of the altered chain of events leading to the development of spinal NTD requires an understanding of genetic patterning of the tissues involved in the primary/secondary axis formation transition. The genes expressed in this region include *HNF3α*, *HNF3β*, *Shh* and *Brachyury* in the caudal notochord, developing floorplate and hindgut, which play key roles in induction, formation and differentiation of these structures at the time of PNP closure; *Wnt5a*, *Hoxb1*, *Brachyury*, *RARγ* and *Evx1* are expressed in the caudal mesoderm, identifying four subdomains within the morphologically homogeneous mesenchyme; patterns of expression of *Wnt5a*, *Evx1* and *Fgf8* reveal subpopulations of cells within the open neural plate and the recently closed neural tube (Gofflot *et al.*, 1997).

The purpose of the present study was to investigate whether alterations in the expression patterns in any of these genes were associated with the phenotypic differences seen within litters of *curly tail* mutant mice, i.e., whether they were associated with penetrance of the NTD phenotypes in this complex mutant. Differences in *RARγ* expression have been reported previously (Chen *et al.*, 1995), and are not described here. In this study, we have used only embryos with normal neuropore size (unaffected) and embryos with greatly enlarged neuropore size equivalent to category 4/5 of Copp (1985) (affected).

The most significant observation was a reduced level of *Wnt5a* transcripts specifically within the caudal mesoderm and hindgut of embryos with an enlarged PNP, with a slight reduction in the neuroepithelium. This effect was observed in the same proportion of *ct/ct* embryos analyzed at a stage earlier than that at which a morphological abnormality can be detected, suggesting that a defect of *Wnt5a* signaling may be causally associated with the onset of abnormal development.

Results

Comparison of non-mutant and phenotypically normal ct/ct embryos

Gene expression patterns in 27-32 somite-stage non-mutant embryos were compared with those of *ct/ct* embryos of the same somite number and similar neuropore size, using whole-mount *in situ* hybridization. No differences were observed, establishing that any differences identified in the affected *ct/ct* embryos were likely to be causally related to the abnormal phenotype, and not to genetic factors common to all embryos of the mutant stock. Non-mutant and phenotypically unaffected *ct/ct* embryos were both used for comparison of all hybridization results, but in general unaffected *ct/ct* embryos are used as a controls for illustration of the results. For day 10 embryos, in which morphological differences between unaffected and pre-NTD embryos are not yet apparent, both non-mutant and unaffected *ct/ct* embryos are illustrated.

Technique considerations

In comparing the hybridization results presented in Figures 1 and 2, certain limitations of the method need to be borne in mind: (a) there is a slight variation in background, even between non-mutant embryos of the same litter; (b) the hindgut is a blind-ended tube whose lumen contains protein, and it is very difficult to wash out all unhybridized probe; (c) there is some stage variation between the embryos, within the defined somite range. The following apparent differences between unaffected and affected paired specimens are not significant for these reasons: slight differences in background, which were also variable using sense probes (Fig. 1 A/B,C/D,I/J,K/L,O/P); age differences are reflected in expression of *Fgf8* in the hindlimb buds (Fig. 1M/N) and the size of the *Wnt5a* expression domain in the tail bud (Fig. 2A/B). Probe trapping in the hindgut should be noted and subtracted when interpreting Figures 2D,G,H and 3G-I. Differences referred to in the text were consistent whether or not any of these other variables were present. Figure 3 shows vibratome sections cut at 50 μm thickness. This thickness is necessary to detect the color intensity in sectioned whole-mount preparations; it gives poor morphological definition at the low magnification required for illustrating the whole section in one photomicrograph, and the textual description also includes information from observation at higher power.

Patterns of gene expression in axial structures (hindgut and notochord)

Four regulatory genes, *Shh*, *HNF3α*, *HNF3β* and *Brachyury*, which are expressed in the hindgut and notochord and are involved in either late stage of gastrulation and/or notochordal-neuroepithelial interactions, were compared in *ct/ct* affected and unaffected embryos (Fig. 1A-H). No differences were observed in either transcript levels or domains of expression of these genes. *Wnt5a*, which is expressed in the most caudal hindgut but not the notochord, is described in detail below.

Patterns of gene expression in the tail bud and caudal mesoderm

The tail bud mesenchyme, like the mesoderm cranial to it, is formed from cells that have undergone gastrulation through the primitive streak. *Brachyury*, *Hoxb1*, *Evx1*, *Fgf8*, *Wnt5b*, and *Wnt5a* are all expressed within the primitive streak and in the recently formed mesoderm, including the tail bud, each with specific patterns. At day 10.5, their expression is observed in all or part of the tail bud, and in different subdomains of the caudal region mesenchyme (Figs. 1,2). *Wnt5a* transcript levels are high throughout the most distal tail bud mesoderm (Fig. 2A) but sections reveal a ventral bias close to the caudal tip (not illustrated; see Gofflot *et al.*,

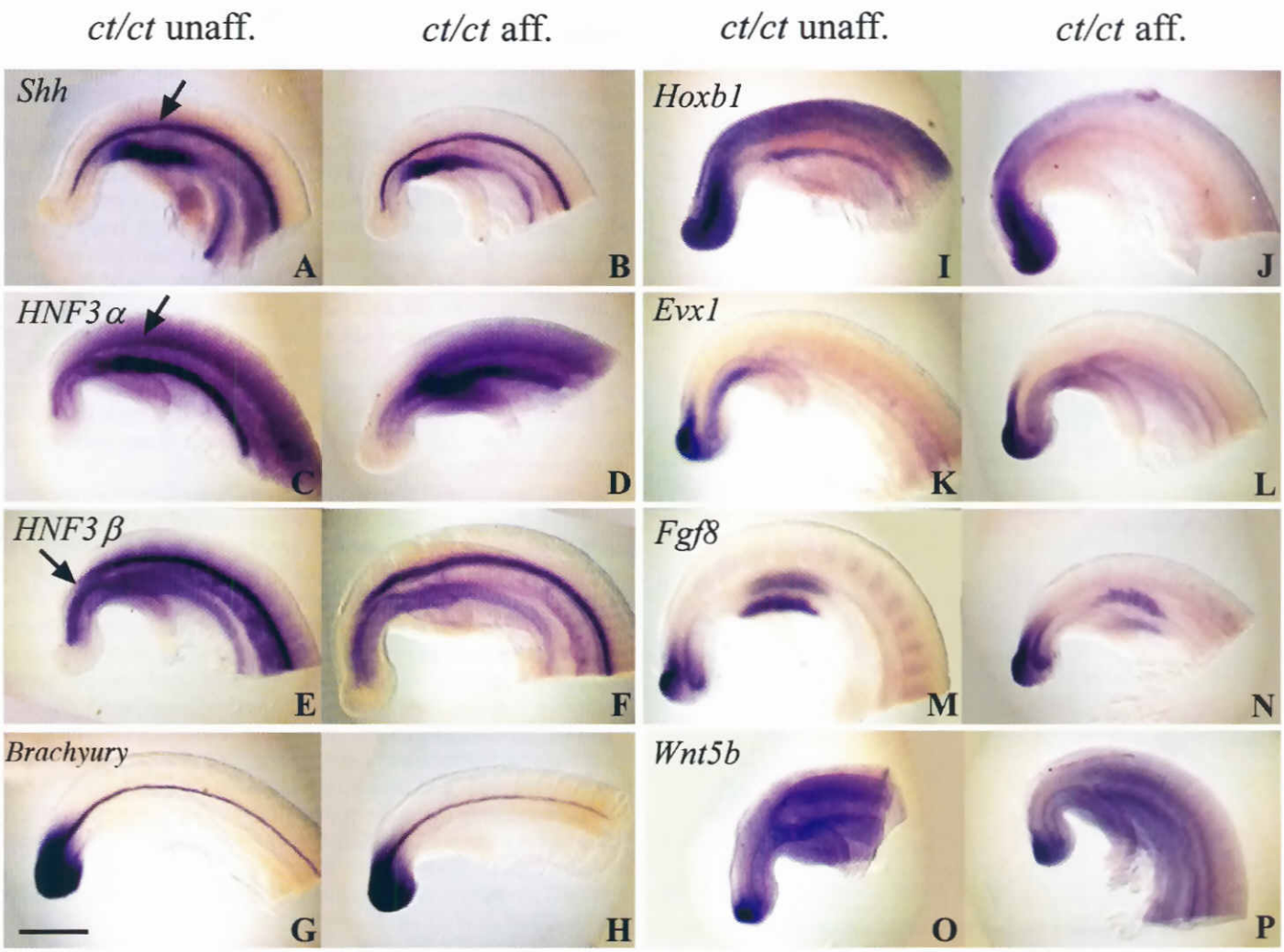

Fig. 1. Whole-mount *in situ* hybridization of day 10.5 *ct/ct* embryos (28 to 30 S) revealing expression of the genes indicated. *Tails have been isolated from the whole embryos by a cut cranial to the hindlimb buds. In all figures, caudal is on the left, and dorsal uppermost. ct/ct unaffected (unaff.) embryos are those displaying a neuropore similar in size to that of non-mutant embryos; ct/ct affected (aff.) embryos are those displaying an enlarged neuropore.* **(A-F)** *Shh, HNF3α and HNF3β transcripts are present in the hindgut and cloaca ventrally, the notochord, and the ventral part of the neural tube. Arrows in A,C,E indicate the respective caudal border of expression of these three genes in the floorplate, which is always cranial to the caudal extent of the notochordal domain. Patterns of expression of Shh, HNF3α and HNF3β are similar in the two groups of ct/ct embryos.* **(G-H)** *In both ct/ct unaffected and affected embryos, Brachyury expression is very high in the tail bud and the adjacent tail region. Cranially, it is expressed only in the notochord.* **(I-J)** *Hoxb1 shows a high level of expression in the tail bud and the tail region adjacent to it, with a border of expression located at the level of the ventral curvature; cranial to this border it is expressed mainly in the dorsal neural tube. Expression in ct/ct affected embryos is very similar, though at a lower level in some embryos, as shown here.* **(K-P)** *Evx1, Fgf8 and Wnt5b show a very restricted domain of expression in the distal part of the tail, including part of the tail bud, the open neural folds and the caudal extremity of the hindgut. Cranially Fgf8 is expressed in the developing apical ectodermal ridge of the hindlimb buds; domains and levels of expression are similar in the two groups of ct/ct embryos. Bar, 0.4 mm.*

1997). Mesodermal patterns of expression of all these genes except *Wnt5a* are very similar in *ct/ct* unaffected and *ct/ct* affected embryos (Fig. 1G-P; see below for *Wnt5a*).

Patterns of gene expression in the neuroepithelium: altered Wnt5a expression

Most of the patterns of gene expression observed within the neuroepithelium are similar in *ct/ct* affected and unaffected embryos. *Hoxb1* showed a slightly lower level in some embryos, but this observation was not consistent and will not be discussed further. At the 28-30 somite stage, levels of *Wnt5a* in the neuroepithelium of the caudal neuropore region were slightly reduced compared to non-mutant embryos (Fig. 2D). This difference was much more marked at the 20-22 somite stage, before differences in neuropore size between affected and unaffected embryos are initiated (Fig. 2H).

Alteration of Wnt5a expression in the mesoderm and hindgut

Analysis of whole isolated tails revealed a differential intensity of *Wnt5a* expression in the caudal region mesoderm of *ct/ct* affected embryos at the 27-32 somite stage (Fig. 2A-D). The cranial level of the expression domain in both affected and unaf-

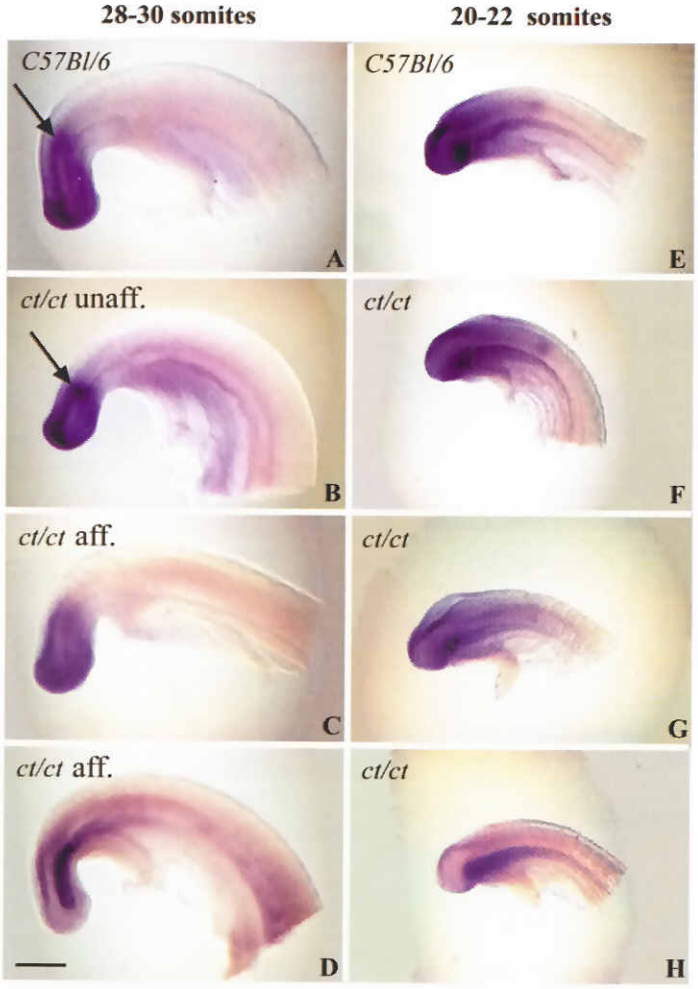

Fig. 2. *Wnt5a* expression in day 10.5 (A-D) and day 10 (E-H) non-mutant (C57Bl/6) and *ct/ct* embryos revealed by whole-mount *in situ* hybridization. *Day 10.5 ct/ct embryos are classified as unaffected (unaff.) or affected (aff.) according to the size of the open neuropore, whereas no morphological landmarks are available for day 10 embryos.* **(A)** *Wnt5a is expressed at a uniform level in the distal part of the tail, with a cranial border located at the level of the ventral curvature and of the last segmented somite (arrow; 28 somite-stage embryo).* **(B)** *The pattern and intensity of expression of Wnt5a in ct/ct unaff. embryos are similar to that in non-mutant embryos, with the border of expression at the level of the last segmented somite (arrow; 30 somite-stage embryo, in which the domain is shorter than at 28 somites).* **(C)** *In 46% of ct/ct aff embryos, a lower level of expression is observed in the caudal mesoderm, although the pattern of expression is similar to that of non-mutant embryos.* **(D)** *In 23% of ct/ct affected embryos, a very low level of expression is observed in the caudal mesoderm, whereas expression is still present at a high level in the open neural folds.* **(E)** *In day 10 embryos, the intensity of Wnt5a expression is very similar to that of day 10.5, but with a less clearly defined cranial border of expression.* **(F-H)** *ct/ct embryos show variable levels of expression of Wnt5a: 55.1% show a level of expression similar to non-mutant embryos (F); 40.8% show a reduced level of expression in the distal extremity of the tail (G); 4.1% show almost no expression in caudal tissues (H). Staining in the hindgut is mainly due to probe trapping. Bar, 0.3 mm*

fected *ct/ct* embryos and in non-mutants was at the level of the most recently segmented somite (arrowed in Fig. 2 A,B; the smaller domain size in B is due to the fact that this embryo has 30 somite pairs, and the last somite pair is closer to the tail bud than in A, which has 28). Affected embryos showed either a decreased level of transcripts with no change in the size of the domain (Fig. 2C) or such a low level of transcripts that a clear signal above background could only be detected in the neural folds (Fig. 2D; ignore probe trapping in the hindgut).

Thirteen affected *ct/ct* embryos were serially sectioned at 50 μm to analyze the differences in *Wnt5a* expression at the tissue level. Detailed analysis of the sections indicates that in unaffected and non-mutant embryos, the pattern of expression of *Wnt5a* in the mesoderm and hindgut endoderm is very dynamic in relation to the axial level (Fig. 3A-C). Taking the sections in caudal to cranial order, the mesodermal structures showing the highest levels of transcripts (arrowed) are (A) the ventromedial mesenchyme lying between the ventral ectodermal ridge (VER) and hindgut, (B) the ventrolateral subectodermal domain, and (C) the future dermamyotome. Expression in the hindgut endoderm is strongest in the most caudal sections (Fig. 3A), decreasing progressively at more cranial levels (Fig. 3B and C).

Sections from *ct/ct* affected embryos show differences in some or all of these areas. Four of the thirteen embryos (31%) show a pattern of expression similar to that observed in control embryos (not illustrated). Six embryos (46%) show a reduced level of expression in the ventromedial mesoderm overlying the VER (arrowed in Fig. 3D) and in the lateral subectodermal mesoderm, and the somites do not show the normal higher level of labeling in the dermamyotome; expression in the hindgut shows the same pattern as that of normal embryos, but the level is reduced (Fig. 3D-F). In the remaining three embryos (23%), *Wnt5a* expression in the ventromedial mesoderm is greatly reduced compared with non-mutant embryos, and barely detectable in the epithelium of the VER and in the ventral subectodermal mesoderm (Fig. 3G-I, which is the same embryo as Fig. 2D); the signal is also barely detectable in the hindgut endoderm (Fig. 3G-H).

Delay in neuropore closure is first apparent around the 22-23 somite-stage in *curly tail* embryos (van Straaten *et al.*, 1993). To examine the possibility that reduced *Wnt5a* expression may precede the morphological abnormality in *ct/ct* embryos we performed whole-mount *in situ* hybridization on *ct/ct* embryos with 20-25 somites. As no morphological marker can be used at these stages, all *ct/ct* embryos were pooled according to somite stage (20-22 somite- and 23-25 somite-stages). A total of 49 *ct/ct* embryos were hybridized; similar results were obtained for the two somite categories. The results were consistent with those from 27-32 somite stage embryos, showing variations in the level of expression of *Wnt5a* in *ct/ct* embryos as compared to non-mutant embryos. Embryos were separated into three groups according to the intensity of expression: 27 (55.1%) show a level of *Wnt5a* expression similar to non-mutant embryos (Fig. 2F); 20 (40.8%) show a lower level of expression (Fig. 2G); in the remaining 2 (4.1%) embryos, *Wnt5a* expression in the tail bud and caudal mesenchyme is undetectable (Fig. 2H). Allowing for the relatively small numbers of embryos and the subjectivity of classification on the basis of color intensity, this result is comparable with a recent assessment of NTD at day 14 of development carried out in the same *ct/ct* stock: unaffected 46%, tail flexion defects 44.5%, spina bifida 9.4% (Chen *et al.*, 1994).

Discussion

We have analyzed patterns of gene expression in the caudal region of *curly tail* mouse embryos with normal and enlarged PNP

to gain further insight into the mechanism of development of the NTD mutant phenotype. The only consistent difference noted was in *Wnt5a* expression, for which greatly reduced transcript levels were observed in the ventral and ventrolateral caudal mesoderm, the VER and the hindgut, but normal or near-normal levels in the neuroepithelium of the enlarged PNP of affected embryos. This observation is functionally significant in several respects: (1) it was observed in embryos prior to the stage at which the morphological abnormality can be identified; (2) Wnt signaling is known to be associated with cell proliferation (Parr and McMahon, 1994); (3) cell proliferation is specifically altered in the ventral half of the tail bud of affected *ct* embryos (Peeters *et al.*, 1998); (4) Wnt/wingless signaling is associated with binding of the secreted Wnt/wg proteins to extracellular matrix proteoglycans, especially heparan sulphate (HSPG) and heparin (Cumberledge and Reichsman, 1997); (5) specific HSPG degradation causes enlargement of the PNP (Tuckett and Morriss-Kay, 1989).

Timing and localization of Wnt5a deficiency

At 10.0 days of development (20-25 somite-stage), before morphological differences can be detected among *ct/ct* embryos, they can be classified into three groups on the basis of the level of expression of *Wnt5a* in the caudal region. The incidence of normal, moderately reduced and almost undetectable levels of *Wnt5a* in these embryos reflects the incidence of straight tail, curly tail with closed neural tube, and spina bifida in *curly tail* mice in a previous study (Chen *et al.*, 1994), suggesting that differences in *Wnt5a* expression may be causally linked to the onset of the dysmorphogenesis leading to NTD.

The primary sites of alteration of *Wnt5a* expression are the mesenchyme and the hindgut; changes of expression in the neural plate are minimal. Within the mesoderm, the effects are principally in the ventromedial domain overlying the VER (which is itself affected) and the ventral subectodermal domain. These ventral and ventrolateral sites show the highest *Wnt5a* transcript levels in normal embryos, and the highest cell proliferation rates (Gofflot *et al.*, 1997). Reduced *Wnt5a* transcript levels were also observed in the dermamyotome of the most caudal somites. This effect is too far rostral to be the primary cause of the enlarged neuropore, but where the neuropore is already greatly enlarged, reduced cell proliferation in the dermamyotome (which has not been investigated) would affect the ability of the mesenchyme underlying the abnormally open neural folds to support their further elevation.

Wnt5a is a member of the Wnt gene family, encoding secreted cystein-rich glycoproteins; Wnt proteins have been implicated in regulation of pattern formation during embryogenesis, differentiation, and mammary carcinogenesis (Parr and McMahon, 1994). Two subgroups are recognized: the *Wnt-1* class, which induces a secondary axis when injected into *Xenopus* embryos, and the *Wnt-5a* class, which produces anterior truncations in *Xenopus* (Du *et al.*, 1995; Torres *et al.*, 1996; Moon *et al.*, 1997). The *Wnt5a* class includes also *Wnt4* and *Wnt11*.

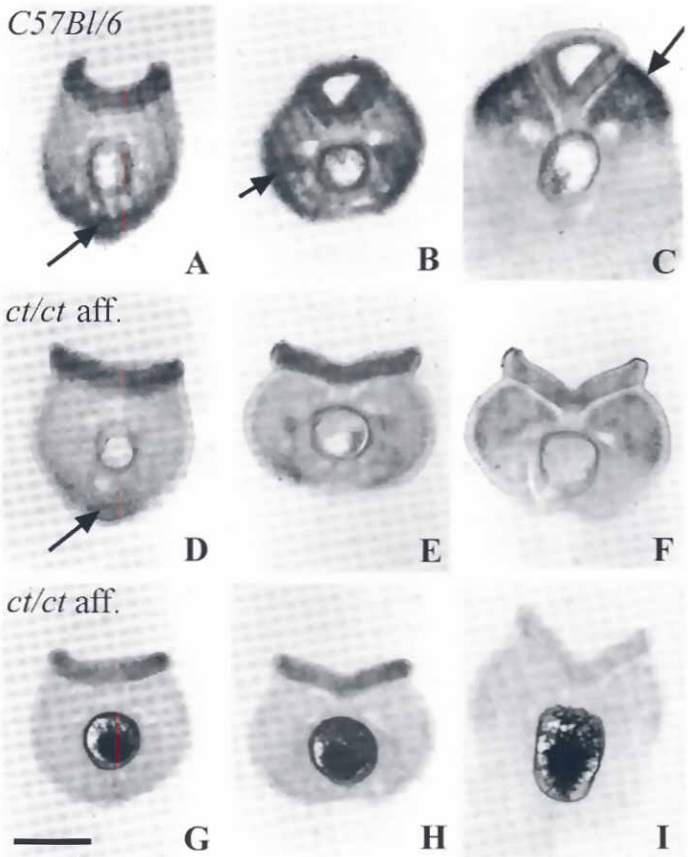

Fig. 3. *Wnt5a* expression observed on transverse vibratome sections of isolated tails from day 10.5 non-mutant and mutant embryos after whole-mount *in situ* hybridization. *Sections are presented in a caudal to cranial sequence (from left to right), each section being approximately 150 μm cranial to the previous one.* **(A-C)** *Wnt5a is expressed in the open neural plate and recently closed neural tube, and in the hingdut endoderm. In the mesoderm the main domains of expression are the ventral mesoderm (arrow in A), the subectodermal domain (arrow in B) and the dermamyotome (arrow in C).* **(D-F)** *In 46% of the ct/ct affected embryos, expression in the lateral subectodermal mesoderm, the mesoderm overlying the VER (arrowed in D), the dermamyotome, and the hindgut endoderm is strongly reduced, while the neuroepithelium is unaffected.* **(G-I)** *In 23% of the ct/ct affected embryos no expression is detected in the mesoderm or the hindgut endoderm, whereas Wnt5a transcripts are still present at a slightly reduced level in the open neural plate. (Probe trapping is the cause of the staining within the hindgut lumen). Bar, 130 μm.*

TABLE 1

NUMBER OF 27-32 SOMITES EMBRYOS USED IN THE HYBRIDIZATION EXPERIMENTS

Genes	Nº embryos hybridized			Nº embryos sectioned		
	non-mutant	ct/ct unaff.	ct/ct aff.	non-mutant	ct/ct unaff.	ct/ct aff.
Shh	21	15	21	8	/	4
HNF3α	15	7	9	3	/	3
HNF3β	14	8	11	3	/	3
Brachyury	10	10	10	5	/	6
Hoxb1	18	18	18	3	/	3
Evx1	18	15	12	3	/	3
Fgf8	9	9	9	3	/	3
Wnt5b	8	8	8	3	/	3
Wnt5a	40	40	40	13	9	13

Wnt5a was first identified in mouse, where it maps to centromeric chromosome 14; it is expressed in the distal mesenchyme of most outgrowing structures during embryogenesis, where it is associated with high levels of cell proliferation (Gavin *et al.*, 1990). Loss of function of *Wnt5a* confirms that it is required for outgrowth of these structures (Yamaguchi *et al.*, 1997). The null mutants die postnatally, with multiple malformations. Earlier in development, the embryos show a progressive shortening of the tail, limbs, facial processes and pinnae. These observations support the idea that *Wnt5a* is required for mesenchymal cell proliferation, either acting as a direct mitogen or being responsible for the competence of cells to respond to mitogens. The hindgut is also affected in Wnt5a$^{-/-}$ embryos, the caudal end being formed by a mass of cells that fail to elongate (T. Yamaguchi, personal communication).

Wnt5a, cell proliferation, and the ct/ct tail region

The pattern of development in *Wnt5a* null mutants is consistent with our data showing that domains of expression of *Wnt5a* in the caudal mesoderm correlate directly with domains of cell proliferation (Gofflot *et al.*, 1997). Cell proliferation is specifically reduced in the hindgut endoderm of *ct/ct* affected embryos as compared to non-mutant embryos, and not in the neuroepithelium of the PNP (Copp *et al.*, 1988); this dorsal-ventral difference in proliferation is thought to create a growth imbalance resulting in the excessive ventral curvature that is responsible for enlargement of the PNP (Brook *et al.*, 1991; Peeters *et al.*, 1996). A dorsal-ventral imbalance also exists in the caudal region mesenchyme: proliferation is specifically reduced in the ventral half of the caudal region (tail bud plus PNP region), where a prolonged cell cycle is associated with increased length of the G_1 phase (Peeters *et al.*, 1998). Our observation that altered *Wnt5a* expression precedes the morphological defect, being detectable in 20-25-somite embryos, is mirrored by the observation of Peeters *et al.* (1998) that prolongation of the cell cycle in the ventral caudal region mesoderm is already detectable in 21-25 somite-stage embryos. The clear possibility of a causal relationship between these two observations is further supported by our finding that differences in *Wnt5a* expression was greater at this early, pre-neuropore enlargement stage, than at the 28-30 somite stage. This stage-dependent decrease in the effect on *Wnt5a* expression correlates with the stage-dependent loss of cell cycle effect reported by Peeters *et al.* (1998).

Wnt5a, the extracellular matrix, and neurulation

Several recent studies have demonstrated a requirement for extracellular matrix proteoglycans, specifically heparin or heparan sulphate proteoglycan (HSPG), in signaling of vertebrate Wnt and *Drosophila* wingless (wg) proteins (reviewed by Cumberledge and Reichsman, 1997). Wnt signaling, including that of *Wnt5a*, is inhibited by the highly sulphated polyanions suramin and pentosan polyphosphate, which are thought to compete with the binding of Wnt protein to HSPG and effect its release into the medium (Burrus and McMahon, 1995). Proteoglycans, most likely HSPGs, are required for the maintenance of *Wnt11* expression in the ureter tips during branching morphogenesis in mouse kidney development (Kispert *et al.*, 1996). This observation is particularly significant in the context of our observations, since *Wnt11* is a *Wnt5a* class member (Du *et al.*, 1995), and may act in a similar fashion.

HSPG is known to be essential for mammalian neurulation. The HSPG of neuroepithelial basement membranes can be degraded by heparitinase (Tuckett and Morriss-Kay, 1989) or its synthesis inhibited by β-D-xyloside (Morriss-Kay and Crutch, 1982) in cultured embryos: both treatments result in exencephaly and enlarged PNP. The status of HSPG synthesis in the PNP region of *ct/ct* mouse embryos is not known. Copp and Bernfield (1988) found no difference between total sulphated glycosaminoglycans present in the PNP region of unaffected and affected *ct/ct* embryos; however, HSPG-specific detection was not carried out either biochemically or immunohistochemically. In our sections, it is clear that the extracellular matrix of the PNP region is poorly organized, and we have shown decreased levels of laminin immunoreactivity (M. Hall, F. Gofflot and G.M. Morriss-Kay, unpublished observations).

The possibility of an interaction between *Wnt5a* expression, HSPG localization, and cell proliferation in the caudal region of curly t*ail* mice clearly needs to be tested. The specific localization and timing of onset of NTD to the last phase of primary neurulation implicates the caudal region mesenchyme, rather than the neuroepithelium, as the key tissue. The fact that the majority of NTD in *ct/ct* embryos result in closed defects affecting only caudal axial levels supports this hypothesis. The normal pattern of expression of *Wnt5a* in the caudal tissues, its deficiency in the ventromedial and ventrolateral mesoderm and hindgut rather than the neuroepithelium of *ct/ct* embryos, and the correspondence of this observation with the cell proliferation data of Peeters *et al.* (1998), suggest that these tissues are the sites in which altered HSPG localization would be most functionally significant. The next stage of this study will be immunohistochemical detection of HSPG and experimental modulation of HSPG levels in *curly tail* mutant embryos, to further elucidate the relationship between *Wnt5a* expression, HSPG, cell proliferation and morphogenesis.

Materials and Methods

Mouse strains and embryo selection

The *curly tail* mutation arose spontaneously in the GFF strain, and was subsequently transferred onto a CBA/Gr background (Gruneberg, 1954). Since then, the strain has been maintained as a closed, entirely in-bred colony of homozygous *ct/ct* individuals. Experimental litters were obtained from matings between males with tail flexion defects and females that were either straight tail or curly tail. Mice were paired for mating overnight, after which females were checked for vaginal plugs. The day of finding the plug was designated as day 0 of gestation. For most of the experiments females were sacrificed by cervical dislocation on day 10.5. Embryos were dissected free of their extraembryonic membranes. The number of somites was counted and only embryos with 27-32 somites were processed further. Embryos were subdivided according to the size of the neuropore in three categories as defined by Copp (1985): 1/2 (unaffected), 3, 4/5 (affected). The neuropore size of category 4/5 embryos at the 27-32 somite stage ranged from 500 to more than 800 μm, in contrast to 0 to 400 μm in category 1/2 and 0 to 200 μm in non-mutant embryos. Category 3 embryos were excluded from this study. For some of the experiments, females were sacrificed early on day 10. At that stage, affected and unaffected embryos are indistinguishable based on the size of the neuropore; they were therefore pooled in groups with either 20 to 22 somites, or 23 to 25 somites. Embryos of other somite stages were discarded. After classification, the embryos were fixed in 4% paraformaldehyde overnight at 4°C. The following day, they were washed in phosphate buffered saline with 0.1% Tween 20 and dehydrated through a graded series of ethanols, then stored in 100% ethanol at -20°C. *C57Bl/6* embryos were used as non-mutant controls and processed similarly.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was carried out on whole embryos using digoxigenin-labeled riboprobes according to Wilkinson (1992), with

the following minor modifications. Embryos were permeabilized with proteinase K (5 μg/ml) before hybridization and no RNaseA treatment was used afterwards. When the color reaction was complete, the embryos were bleached in 100% ethanol to intensify the reaction product and decrease the background. They were rehydrated and stored in 50% glycerol. For analysis and photography, tails were isolated from the body by a cut just cranial to the hindlimb buds. Details on the number of non-mutant, unaffected and affected embryos hybridized for each probe are presented in Table 1 (see text for day 10 embryos). For each probe and each stage analyzed, the same procedure was carried out using sense probes on at least three equivalent embryos. Non-mutant, *ct/ct* unaffected and *ct/ct* affected embryos were hybridized in parallel in the same experiment, using the same solutions including probes and antibodies.

Plasmids and probes

Plasmids for *in situ* hybridization were kindly provided by the following: *Shh*, *Wnt5a*, *Wnt5b*, A. McMahon; *HNF3α*, *HNF3β*, B. Hogan; *Hoxb1*, P. Chambon; *Brachyury*, R. Beddington; *Fgf8*, I. Mason; *Evx1*, M. Dush and G. Martin. The specific digoxigenin-labeled riboprobes were synthesized from linearized plasmids in a standard T3, T7 or Sp6 polymerase reaction.

Vibratome sections

After observation and photography, the whole tails were embedded in gloop (egg albumin (57 g), gelatin (0.75 g) and sucrose (30 g) in phosphate buffer 0.1 M pH 7.3 (150 ml), mixed 10:1 with glutaraldehyde 25 %). Freshly embedded specimens were cut on a vibratome at 50 μm. Sections were air-dried and mounted in DPX medium for observation.

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References

BROOK, F.A., SHUM, A.S.W., VAN STRAATEN, H.W.M. and COPP, A.J. (1991). Curvature of the caudal region is responsible for failure of neural tube closure in the *curly tail (ct)* mouse embryo. *Development 113*: 671-678.

BURRUS, L.W. and MCMAHON, A.P. (1995). Biochemical analysis of murine Wnt proteins reveals both shared and distinct properties. *Exp. Cell Res. 220*: 363-373.

CHEN, W.H., MORRISS-KAY, G.M. and COPP, A.J. (1994). Prevention of spinal neural tube defects in the *curly tail* mouse mutant by a specific effect of retinoic acid. *Dev. Dynamics199*: 93-102.

CHEN, W.H., MORRISS-KAY, G.M. and COPP, A.J. (1995). Genesis and prevention of spinal neural tube defects in the *curly tail* mutant mouse: involvement of retinoic acid and its nuclear receptors RAR-β and RAR-γ. *Development 121*: 681-691.

COPP, A.J. (1985) Relationship between timing of posterior neuropore closure and development of spinal neural tube defects in mutants *(curly tail)* and normal mouse embryos in culture. *J. Embryol. Exp. Morphol. 88*: 39-54.

COPP, A.J. and BERNFIELD, M. (1988). Accumulation of the basement membrane-associated hyaluronate is reduced in the posterior neuropore region of mutant (curly tail) mouse embryos developing spinal neural tube defects. *Dev.Biol. 130:* 583-590.

COPP, A.J., BROOK, F.A. and ROBERTS, H.J. (1988). A cell-type-specific abnormality of cell proliferation in mutant *(curly tail)* mouse embryos developing spinal neural tube defects. *Development 104*: 285-295.

COPP, A.J., BROOK, F.A., ESTIBEIRO, J.P., SHUM, A.S. and COCKROFT, D.L. (1990). The embryonic development of mammalian neural tube defects. *Progr. Neurobiol. 35*: 363-403.

COPP, A.J., SELLER, M.J. and POLANI, P.E. (1982). Neural tube development in mutant *(curly tail)* and normal mouse embryos: the timing of posterior neuropore closure *in vivo* and *in vitro*. *J. Embryol. Exp. Morphol. 69*: 151-167.

CUMBERLEDGE, S. and REICHSMAN, F. (1997). Glycosaminoglycans and WNTs: just a spoonful of sugar helps the signal go down. *Trends Genet. 13*: 421-423.

DU, S.J., PURCELL, S.M., CHRISTIAN, J.L., MCGREW, L.L. and MOON, R.T. (1995). Identification of distinct classes and functional domains of wnts through expression of wild type and chimaeric proteins in *Xenopus* embryos. *Mol. Cell Biol. 15*: 2625-2634.

EMBURY, S., SELLER, M.J., ADINOLFI, M. and POLANI, P.E. (1979). Neural tube defects in curly-tail mice. I. Incidence, expression and similarity to the human condition. *Proc. R. Soc. Lond. [Biol.] 206*: 95-107.

GAVIN, J., MCMAHON, J.A. and MCMAHON, A.P. (1990). Expression of multiple novel Wnt-1/int-1-related genes during fetal and adult mouse development. *Genes Dev. 4*: 2319-2332.

GEORGES, T.M. and MCLONE, D.G. (1995). Mechanisms of mutant genes in spina bifida : a review of implications from animal models. *Pediatr. Neurosurg. 23*: 236-245.

GOFFLOT, F., HALL, M. and MORRISS-KAY, G.M. (1997). Genetic patterning of the developing mouse tail at the time of posterior neuropore closure. *Dev. Dynamics 210*: 431-445.

GREENE, N.D.E. and COPP, A. (1997). Inositol prevents folate-resistant neural tube defects in the mouse. *Nature Med. 3*: 60-66.

GRUNEBERG, H. (1954). Genetical studies of the skeleton of the mouse. VIII. curly tail. *J. Genet. 52*: 52-67.

HALL, J.G., FRIEDMAN, J.M., KENNA, B.A., POPKIN, J., JARWANDA, M. and ARNOLD, W. (1988). Clinical, genetic and epidemiological factors in neural tube defects. *Am. J. Hum. Genet. 43*: 827-837.

KISPERT, A., VAINO, S., SHEN, L., ROWITCH, D.H. and MCMAHON, A.P. (1996) Proteoglycans are required for maintenance of *Wnt-11* expression in the ureter tips. *Development 122*: 3627-3637.

KNEPPER, P.A. and MCLONE, D.G. (1992). Development of the spinal cord: normal and abnormal neurulation. In *Spinal Dysraphism*. (Ed. T.S. Park). Balckwell Scientific Publications, Boston, pp.1-18.

LETTS, V.A., SCHORK, N.J., COPP, A.J., BERNFIELD, M. and FRANKEL, W.N. (1995). Mapping modifier genes for tail defects in curly tail mutant mice. *Genomics 29*: 719-724.

MOON, R.T., BROWN, J.D. and TORRES, M. (1997). WNTS modulate cell fate and behavior during vertebrate development. *Trends Genet. 13*: 157-162.

MORRISS-KAY, G.M. and CRUTCH, B. (1982). Culture of rat embryos with β-D-xyloside: evidence of a role for proteoglycans in neurulation. *J. Anat. 134*: 491-506.

MRC VITAMIN STUDY RESEARCH GROUP. (1991). Prevention of neural tube defects: Results of the Medical Research Council Vitamin Study. *Lancet 338*: 131-137.

NEUMANN, P.E., FRANKEL, W.N., LETTS, V.A., COFFIN, J.M., COPP, A.J. and BERNFIELD, M. (1994). Multifactorial inheritance of neural tube defects: localization of the major gene and recognition of modifiers in *ct* mutant mice. *Nature Genet. 6*: 357-362.

PARR, B.A. and MCMAHON, A.P. (1994). Wnt genes and vertebrate development. *Curr. Opin. Genet. Dev. 4*: 523-528.

PEETERS, M.C.E., SCHUTTE, B., LENDERS, M.H.J.N., HEKKING, J.W.M., DRUKKER, J. and VAN STRAATEN, H.W.M. (1998). Role of differential cell proliferation in the tail bud in aberrant mouse neurulation. *Dev. Dynamics 211:* 382-389.

PEETERS, M.C.E., SHUM, A.S.W., HEKKING, J.W.M. and VAN STRAATEN, H.W.M. (1996). Relationship between altered axial curvature and neural tube closure in normal and mutant *(curly tail)* mouse embryos. *Anat. Embryol. 193*: 123-130.

SELLER, M.J. and ADINOLFI, M. (1981). The curly tail mouse: an experimental model for human neural tube defects. *Life Sci. 29*: 1607-1615.

SELLER, M.J. and PERKINS, K.J. (1982). Prevention of neural tube defects in curly-tail mice by maternal administration of vitamin A. *Prenatal Diagnosis 2*:297-300.

SELLER, M.J. and PERKINS, K.J. (1983). Effect of hydroxyurea on neural tube defects in the curly-tail mouse. *J. Craniofac. Genet. Dev. Biol. 3*: 11-17.

SELLER, M.J. and PERKINS-COLE, K.J. (1986). Effect of mitomycin C on the neural tube defects of the curly-tail mouse. *Teratology 33*: 305-309.

TORRES, M.A., YANG-SNYDER, J.A., PURCELL, S.M., DEMARAIS, A.A., MCGREW, L.L. and MOON, R.T. (1996). Activities of the *Wnt-1* class of secreted signaling factors are antagonized by the *wnt-5A* class and by a dominant negative cadherin in early *Xenopus* embryos. *J. Cell Biol. 133*: 1123-1137.

TUCKETT, F. and MORRISS-KAY, G.M. (1989). Heparitinase treatment of rat embryos during cranial neurulation. *Anat. Embryol. 180*: 393-400.

VAN STRAATEN, H.W.M., HEKKING, J.W.M., CONSTEN, C. and COPP, A.J. (1993). Intrinsic and extrinsic factors in the mechanism of neurulation : effect of curvature of the body axis on the closure of the posterior neuropore. *Development 117*: 1163-1172.

VAN STRAATEN, H.W.M., HEKKING, J.W.M., COPP, A.J. and BERNFIELD, M. (1992). Deceleration and acceleration in the rate of posterior neuropore closure during neurulation in the *curly tail (ct)* mouse embryo. *Anat. Embryol. 185*: 169-174.

WILKINSON, D.G. (1992). Whole-mount *in situ* hybridization of vertebrate embryos. In *In situ Hybridization* (Ed. D.G.Wilkinson). IRL Press, Oxford, pp. 75-83.

YAMAGUCHI, T.P., JONES, S.N., BRADLEY, A. and MCMAHON, A.P. (1997) *Wnt5a* is required for distal patterning and growth along the body axes during mouse embryogenesis. *Dev. Biol. 186*: 328 (Abstr.).

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