The effect of antisense oligonucleotides to *Cdx2* on the development of mouse embryos *in vitro*

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ABSTRACT The *Drosophila* homeobox gene *caudal* is thought to play a role in segmentation and the formation of posterior structures in the fly. One of the mouse homologs, *Cdx2*, is first expressed in embryonic tissue at 8.5 days, being detected in the tail bud, neural tube and hindgut. To gain insight into the role of *Cdx2* in mouse development, we have applied antisense technology to the mouse embryo culture system. Two antisense and two mismatch (control) oligonucleotides were injected into the amniotic cavity of 5-7 somite embryos, thus coming into direct contact with the dorsal surface of the embryo. The results showed that all constructs were biologically active, the nature of the abnormalities produced probably reflecting the developmental stage at which the embryo was exposed. However, the antisense constructs were clearly more potent than their mismatch controls and we conclude that some specificity of action is responsible for this effect. In our view, this does not necessarily imply that *Cdx2* has a direct role in brain morphogenesis.

KEY WORDS: *Cdx2, phosphorothioate oligonucleotide, whole embryo culture*

Introduction

The *Drosophila* homeobox gene *caudal (Cad)* was isolated and characterized by Mlodzik *et al.* (1985) and by Mlodzik and Gehring (1987). Both maternal and embryonic gene transcripts are active during development and null mutants indicate that the gene is involved with segmentation and the development of posterior structures in the fly (Macdonald and Struhl, 1986).

Three mouse homologs of *Cad* have been described. *Cdx1* (Duprey *et al.*, 1988) is expressed in embryonic and adult intestine and is first detectable at 7.5 days of gestation in the ectoderm and endoderm of the primitive streak. Thereafter, it is demonstrable in the caudal neural tube, somites and forelimb buds (Meyer and Gruss, 1993). *Cdx2* (James and Kazenwadel, 1991) is expressed in the trophoblast lineage, as well as in the posterior gut and neural tube, but not in the primitive streak embryo (Beck *et al.*, 1995). Patterns of *Cdx4* expression overlap those of the other two, being expressed in the primitive streak, embryonic hindgut and neural tube (Gamer and Wright, 1993); its possible expression in the extra-embryonic membranes has not been investigated.

We have examined the effects of two oligonucleotides antisense to *Cdx2* on the growth and development of early head fold mouse embryos in whole embryo culture (New, 1978). Previous studies (Augustine *et al.*, 1993, 1995a,b; Sadler *et al.*, 1995) using antisense applied to embryos in culture have confirmed information obtained from pre-existing gene knockout studies by targeted recombination of Wnt1, Wnt3a, Engrailed-1 and Engrailed-2 (McMahon and Bradley, 1990; Thomas and Cappechi, 1990; Joyner *et al.*, 1991; Takada *et al.*, 1994; Wurst *et al.*, 1994). The present experiments were done without prior knowledge of the phenotype exhibited by *Cdx2* inactivation. The results illustrate the difficulties associated with *de novo* studies using this methodology and highlight the difficulties of distinguishing specific effects from the non-specific actions of biologically active material injected into the amniotic cavity.

Results

The effects of *Cdx2* antisense and *Cdx2* mismatch oligonucleotides on whole embryo development in culture are set out in Tables 1 and 2. The principal abnormalities seen after 48 h of post-injection culture were non-expansion of the telencephalic vesicles, together with gross underdevelopment of the midbrain and a smaller but significant number of cases in which the hindbrain was not normally expanded (Fig. 1). Further defects included non-closure of the neural tube. Minor defects often associated with non-optimal preparation of the culture serum, such as small irregularities of the neural tube and slight caudal truncation were occasionally seen. Other parameters of development (heart, somites, circulatory system, etc.) were generally normal. They were carefully recorded and are reflected in the morphological scores (using the criteria described by Brown and Fabro, 1981 for the rat). Interestingly, cardiac malformations were not a feature of this

Abbreviations used in this paper: PBS, phosphate buffered saline; AS1, antisense 1; AS2, antisense 2; MM1, mismatch 1; MM2, mismatch 2.

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experimental series, possibly because direct access of heart cells to the oligonucleotides is not available in the present procedure. In all the experiments, the nature of the abnormalities observed were similar for the antisense and mismatch series. This suggests that the effects seen represent a 'final common pathway' of abnormal development, rather than a primary effect of *Cdx2* antisense. However, it is clear that severe retardation of brain development is far more common in embryos exposed to either antisense construct than it is in those exposed to mismatch control constructs (e.g. p= 0.0036 for antisense 1 versus mismatch 1). Nevertheless, the injection of mismatch oligonucleotide resulted in a significantly higher severe abnormality rate than did injection of PBS alone (e.g. p= 0.035 for mismatch 1 versus PBS). The injection procedure itself appeared to be without effect as shown by the generally similar outcome of embryonic growth in PBS injected and untreated control animals.

Discussion

The present experiment highlights the difficulties inherent in the antisense approach to investigating development using whole embryo culture. A small but definite level of abnormal development is inevitable in the control untreated explants and this is somewhat increased in the control series injected with PBS. Injection of antisense oligonucleotide clearly has a major biological effect resulting in abnormal development of 79% of embryos injected with antisense 1 (AS1) and 86% of those injected with antisense 2 (AS2). However, 42% of embryos injected with mismatch 1 (MM1) and 50% of those injected with mismatch 2 (MM2) were also abnormal and it is apparent that the mismatch oligonucleotides also disturb development. Nevertheless, there is a statistically significant difference (p= 0.00106) in the proportion of affected conceptuses between the AS1 and MM1 constructs and the abnormalities themselves are more severe in the antisense treated group.

Augustine *et al.* (1993, 1995a,b) and Sadler *et al.* (1995) have performed carefully executed experiments using antisense to Wnt1 and 3a and to Engrailed 1 and 2 in mouse whole embryo cultures. In these studies, as in ours, raised levels of abnormal development were observed in control (mismatch and/or sense treated) embryos. This raised the question of the specificity of the abnormalities observed following treatment with antisense oligonucleotides. In the experiments performed by Augustine *et al.* and by Sadler *et al.*, knowledge of the expected phenotypes is available, since gene inactivation by targeted disruption has been performed (Thomas and Capecchi 1990; Joyner *et al.*, 1991; Takada *et al.*, 1994; Wurst *et al.*, 1994). It is clear that a correlation between the results obtained from antisense and those reported from targeting experiments does not always exist. Thus, Wnt3a antisense inhibition produced forebrain malformations and abnormalities of the spinal cord at the level of the forelimb buds, while Wnt3a null mutants were characterized by total caudal dysgenesis, but the neural tube anterior to the forelimbs was normal (Takada *et al.*, 1994). Furthermore, many of the cultured embryos treated with antisense to Wnt1 at optimum concentrations exhibited cardiac malformations which would probably not have been compatible with survival to term, whereas Thomas and Capecchi (1990) reported that only one in six homozygous Wnt1 negative embryos were dead just before the end of gestation. It was also observed (as in our experiments) that high doses of control (sense or mismatch) oligonucleotides produced similar malformations to those seen at lower doses in antisense treated embryos.

TABLE 1

DEFECTS PRODUCED BY ANTISENSE 1 AND MISMATCH 1

		Antisense 1	Mismatch 1	PBS	Control
Dose 25 μM	Total abnormal embryos (%)	8/13 (62%)	5/11 (45%)[1]	1/10 (10%)[1]	2/11 (18%)
	Severe brain defects (%)	3/13 (23%)[2]	0/11 (0%)[2]	0/10 (0%)	0/11 (0%)
	Total brain defects (%)	8/13 (62%)	5/11 (45%)[3]	0/10 (0%)[3]	2/11 (18%)
	All other defects (%)	1/13 (8%)	2/11 (18%)	1/10 (10%)	0/11 (0%)
	Mean morphological score±S.E.	45.5 (S.E. 0.7)	45.8 (S.E. 0.7)	48.8 (S.E. 0.9)	47.8 (S.E. 1.1)
Dose 35 μM	Total abnormal embryos (%)	34/43 (79%)[4]	13/31 (42%)[4,5]	2/25 (8%)[5]	5/22 (23%)
	Severe brain defects (%)	21/43 (49%)[6]	5/31 (16%)[6,7]	0/25 (0%)[7]	0/22 (0%)
	Total brain defects (%)	34/43 (79%)[8]	13/31 (42%)[8,9]	2/25 (8%)[9]	3/22 (14%)
	All other defects (%)	8/43 (19%)	3/31 (10%)	0/25 (0%)	2/22 (9%)
	Mean morphological score±S.E.	40.6 (S.E. 1.5)[10]	46.2 (S.E. 0.8)[10,11]	49.0 (S.E. 0.4)[11]	48.9 (S.E. 0.4)

TABLE 2

DEFECTS PRODUCED BY ANTISENSE 2 AND MISMATCH 2

		Antisense 2	Mismatch 2	PBS	Control
Dose 35 μM	Total abnormal embryos (%)	19/22 (86%)[12]	10/20 (50%)[12]	7/20 (35%)	4/14 (29%)
	Severe brain defects (%)	11/22 (50%)	7/20 (35%)[13]	0/20 (0%)[13]	0/14 (0%)
	Total brain defects (%)	19/22 (80%)[14]	10/20 (50%)	7/20 (35%)[14]	4/14 (29%)
	All other defects (%)	3/22 (14%)	1/20 (5%)	2/20 (10%)	0/14 (0%)
	Mean morphological score±S.E.	41.6 (S.E. 1.0)[15]	43.9 (S.E. 1.1)[15]	46.6 (S.E. 0.5)	46.3 (S.E. 0.6)

1) p= 0.039; 2) p= 0.089; 3) p= 0.039; 4) p= 0.0011; 5) p= 0.014; 6) p= 0.0036; 7) p= 0.035; 8) p= 0.0011; 9) p= 0.014; 10) p= 0.005; 11) p= 0.0013; 12) p= 0.011; 13) p= 0.0036; 14) p= 0.011; 15) p= 0.084.

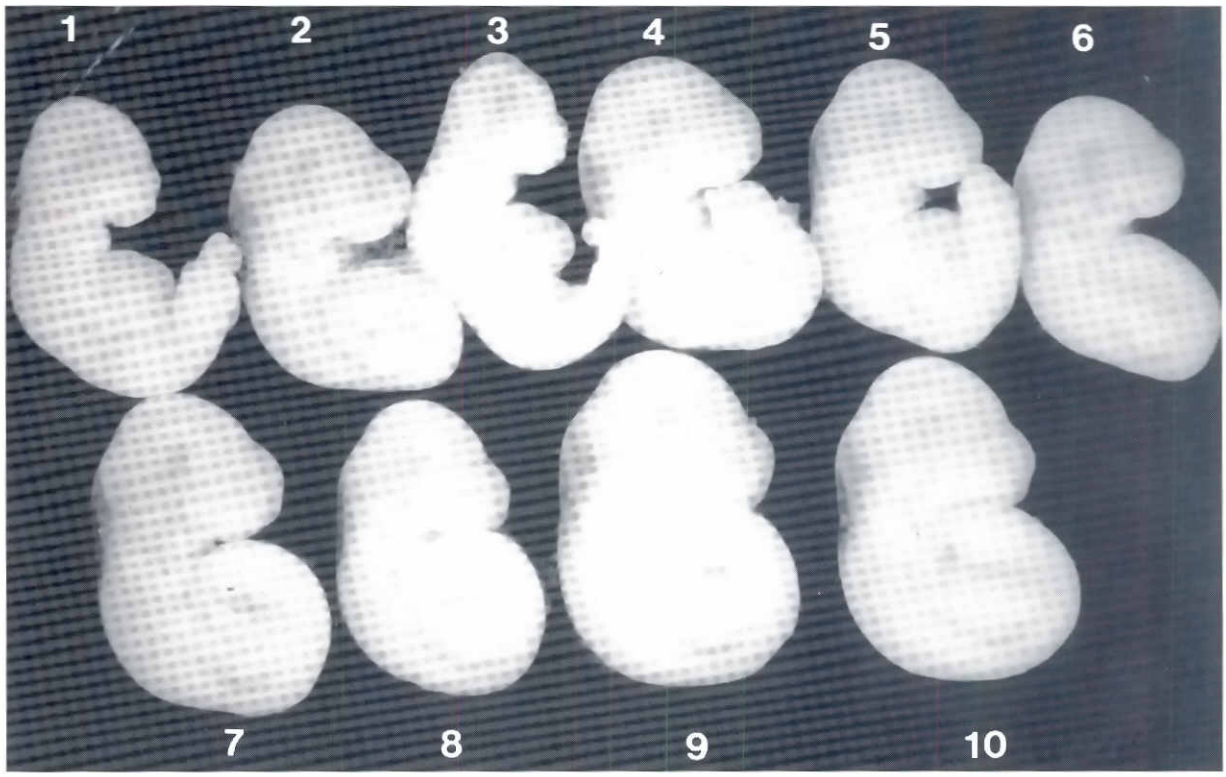

Fig. 1. Results of a typical experiment in which 5/7 somite embryos treated with AS1 or MM1 were cultured for 46 h. *Embryos 1-3 treated with AS1 show abnormal expansion of the cerebral vesicles and caudal truncation. Embryos 4-6 treated with MM1 are much better developed, but still have some caudal truncation and marginally deficient brain development. PBS treated embryos 7-8 are normally developed, but 8 is slightly smaller than usual. Untreated embryos 9 and 10 have developed normally.*

An increasing body of literature indicates that many of the biological effects of antisense oligonucleotides do not result from inhibition of mRNA activity mediated by RNAse H breakdown of the double stranded product of specific nucleotide sequences to target message. Both native and phosphorothioate modified sequences bind strongly to proteins within cells (Tonkinson *et al.*, 1994) and are, therefore, likely to interfere with a catholic range of cellular processes. Perhaps even more importantly, the negative charge on these compounds causes them to interact with a variety of extracellular material, including growth factors, fibronectin and laminin (Gura 1995; Guvakova *et al.*, 1995). Consequently, basic developmental processes, including cell migration and cell to cell adhesion, are likely to be compromised.

The time period covering whole embryo culture extends from Theiler (1989) stage 12 to stage 16. During this period, a cascade of developmental cues results in the appearance of a normal 33 somite embryo. Interference with the orderly progression of these events at a variety of different points is likely to result in similar phenocopies at the end stage because a 'final common pathway' (e.g. of neural tube closure or of forebrain enlargement) is involved. Hence, it is possible to explain the similarities in the effects produced by the antisense and by the control constructs.

Our experiments indicate that much of the effect of intra-amniotic injection of *Cdx2* antisense oligonucleotides is non-specific. However the significantly enhanced efficacy of both antisense constructs compared to their controls is evidence of some specificity, probably acting at the level of *Cdx2* mRNA.

Materials and Methods

Culture and micro-injection techniques

RB Swiss mice were mated overnight and a positive mating was indicated by the presence of a vaginal plug the following morning. The mice are designated 0.5 day pregnant on the day of plug detection and are taken for experimentation on day 8.5. The dissection procedure of early organogenesis stage embryos follows that described by Sturm and Tam (1993) based on the technique developed by New (1978). Basically, the conceptus was removed from the uterus and decidual tissue leaving the embryo, visceral yolk sac and ectoplacental cone intact, but removing Reichert's membrane. The embryos were injected and placed into 15 ml glass culture bottles containing 1 ml culture medium per embryo. Medium for the first 24 h contained 50% immediately centrifuged rat serum (Sturm and Tam, 1993) and 50% DMEM (high glucose). The bottles were placed on a rotating culture unit (B.T.C. Engineering, UK) and were continuously gassed with 5% O_2, 5% CO_2 and 90% N_2 for the first 18 h. The gas mixture was changed to 20% O_2, 5% CO_2 and 75% N_2 from 18 h to 42 h post-explantation and to 40% O_2, 5% CO_2 and 55% N_2 for the remaining period of 4 h. The culture medium was replenished after 24 h, 1.5 ml per embryo at a concentration of 75% rat serum and 25% DMEM. The embryos were scored for morphological development based on the technique described by Brown and Fabro (1981) for the rat, and crown-rump lengths and somite numbers recorded before being fixed in either Bouin's fixative or 4% paraformaldehyde.

Micro-injections were performed by hand using glass pipettes (20 μm diameter, bevelled tip) connected by Silastic tubing to a silicone oil-filled syringe. The needles were inserted into the amniotic cavity via the yolk sac avoiding the embryo proper. A volume of 100 nl was injected into the cavity. This volume was previously calibrated by using a standard curve to measure injections of a dye.

Oligodeoxynucleotides

Antisense and mismatch scrambled control phosphorothioate oligonucleotides complementary to portions of the *Cdx2* gene sequence were synthesized on an Applied Biosystems model 380A automated DNA synthesizer using standard ß-cyanoethyl nucleoside phosphoramidites on a 1 μmole scale with the 5' trityl group being retained on the last nucleotide. Sulfurization of the phosphite backbone was effected using tetraethylthiuram (TETD) in acetonitrile. The oligonucleotides were purified by reversed-phase liquid chromatography on a Synchrom C_{18} RP (250x10 mm i.d.) column using a linear gradient (0→50% B over 30 min) consisting of 0.1 M sodium acetate, pH 7.0 (A) and acetonitrile (B) with the eluant being monitored spectrophotometrically at 260 nm. Detritylation was affected by treating the eluate containing the oligonucleotides with 50% acetic acid for 10 min and then dialyzing immediately against deionized water (3x3 L). The amount of oligonucleotide was determined by UV spectrophotometry at 260 nm. Each solution was filter sterilized, lyophilized and then made up in sterile PBS to the appropriate concentration. Solutions were kept at -20°C throughout the course of the study and their purity confirmed by gel electrophoresis.

Two sequences were chosen for the antisense studies. The first sequence was 22 nucleotides long and targeted the *Cdx2* mRNA in the homeodomain region between helix I and helix II.

Antisense (AS1) 5'CTCACTTTTCCTCCTGATGGTG3' (734→755)
Mismatch control (MM1) 5' CTCTCTTTACCTCGTGATCGTG 3'

This sequence was chosen as it was found to contain insignificant homology with the related *Cdx1* gene.

The second sequence was 20 nucleotides long and was designed to target the *Cdx2* transcript in the AUG initiation region.

Antisense (AS2) 5' CTCACGTACATGGTGGCGAG 3' (95→104)
Mismatch control (MM2) 5' CTAACGTCCACGGTGGTGAG 3'

Numerous dose levels were used; optimal effects were obtained at doses of 25 μM and 35 μM in volumes of 100 nl and these results are presented.

Examination of cultured embryos

Embryos were examined under a dissecting microscope and only perfectly developed specimens with vigorously beating hearts and morphological scores appropriate to the developmental stages achieved at harvesting were classified as normal. Assessment of normal brain expansion is necessarily somewhat arbitrary when very small deviations from the norm are considered. In all such doubtful specimens, the embryos were scored as abnormal. This accounts for the relatively high levels of suboptimal development reported for PBS injected and uninjected control embryos.

Statistical analysis

The morphological scores were compared using Kruskal-Wallis H nonparametric analysis of variance. The proportions of defective embryos were compared using Pearson chi-square analysis. Significance was assumed at $p<0.05$.

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