Mouse embryos in culture: models for understanding diabetes-induced embryopathies and gene function

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ABSTRACT Both the metabolic studies on diabetes and the genetic studies using antisense oligodeoxynucleotides clearly demonstrate the importance and usefulness of rodent whole embryo culture. Without this technique, these studies would be impossible and, consequently, our knowledge of both normal and abnormal development would not be as advanced as it is today. The culture system fills a unique niche in studies in the fields of developmental biology and teratology and these sciences would have been less well served without Dr. New's contribution.

KEY WORDS: *diabetes, glucose, somatomedin inhibitors, antisense, Wnt, engrailed, sonic hedgehog*

Introduction

Twenty three years ago, rodent whole embryo culture, as devised by Dr. New (New, 1978), became the mainstay of our research program. We modified the technique slightly from a roller bottle system to a rotator wheel (Fig. 1) and adapted it for mouse embryos instead of rats (Sadler, 1979; Hunter *et al.*, 1987a), but in all other aspects it has remained as originally developed by its inventor. Initially, we employed the technique to study the effects of altered maternal metabolism, produced by diabetes, on embryogenesis. More recently, we have combined the technique with antisense technology to study the roles of genes important for gastrulation, neurulation, and craniofacial development. Both sets of studies show how important embryo culture can be to investigating mechanisms of teratogenesis and to providing data important for prevention of birth defects.

Altered maternal metabolism

The culture system is ideal for studying the teratogenesis of altered maternal serum factors resulting from metabolic diseases such as insulin dependent (Type 1) diabetes. This disease is known to cause an increased incidence of birth defects (Molsted-Pedersen *et al.*, 1964; Soler *et al.*, 1986), and the majority of the abnormalities are induced during the 4th and 5th weeks of gestation (post fertilization) (Mills *et al.*, 1979) when comparably staged rodent embryos can be maintained in culture. However, the precise factors and mechanisms responsible for these abnormalities were not understood. *In vivo* models of the disease were available, but in these systems embryos were exposed to a multitude of metabolic factors, such that it was impossible to determine which were harmful to the conceptus. For example, diabetes could be induced chemically with alloxan or streptozotocin in pregnant rats and mice and offspring could then be examined for malformations (Angervall, 1959; Watanabe and Ingalls, 1963; Deuchar, 1977). However, these drugs produced the classical characteristics of diabetes, including hypoinsulinemia, hyperglycemia, ketoacidosis, increased levels of somatomedin inhibitors, etc. Consequently, it was impossible to determine which factor(s) produced the abnormalities. Modeling of the *in vivo* characteristics of the disease was possible in the culture system by using serum from diabetic rats as culture medium (Fig. 2). This serum was shown to be teratogenic with the frequency and the severity of malformations dependent upon the severity of diabetes induced in the rats just as it is in laboratory animals and humans (Sadler, 1980a). Furthermore, insulin administration to the diabetic animals prior to collection of their serum for culture reduced the incidence and severity of malformations, as occurs with similar therapy *in vivo* (Horii *et al.*, 1966; Eriksson *et al.*, 1982; Sadler and Horton, 1983). Thus, the culture system mimicked the characteristics of the disease observed in animals and humans.

The culture system allowed further advances in studies of the diabetic embryopathy by providing a venue for evaluating the teratogenicity of each altered serum factor independently or in combination with others (Fig. 2). Initial studies focused on glucose (Cockroft and Coppola, 1977; Sadler, 1980b), which caused malformations, but only at high concentrations (900 mg/dl in rats; 600 mg/dl in mice) representing 4-6 times normal blood glucose levels. Embryos could be protected from the effects of hyperglycemia by the addition of arachidonic acid or prostaglandin E2 to the culture medium, suggesting that excess glucose interfered with prostaglandin synthesis (Goldman *et al.*, 1985; Goto *et al.*, 1992). Additional work showed that glucose also inhibited myoinositol uptake and decreased inositol concentrations in the embryo (Baker *et al.*,

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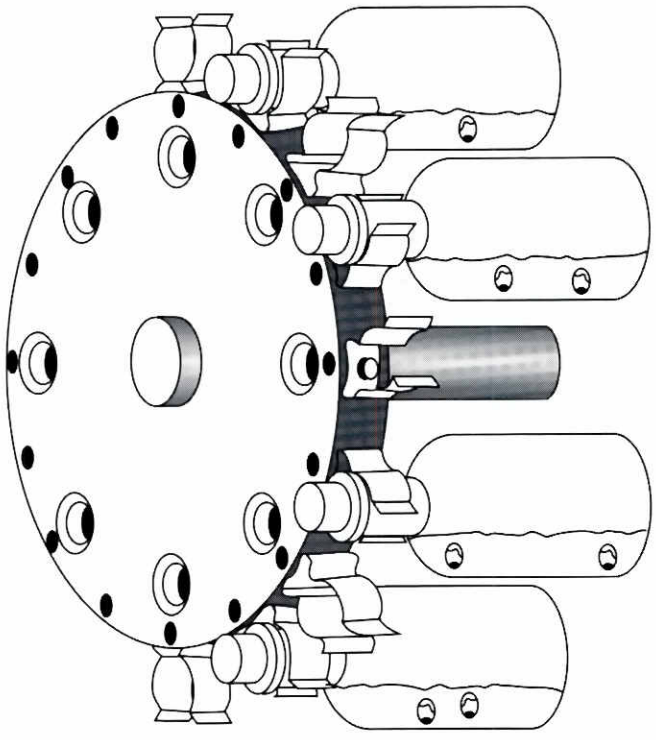

Fig. 1. Rotator culture system. *Drawing of the rotator wheel system used to culture rodent embryos. In this system, flasks containing embryos and medium are clipped onto a wheel that is mounted on the inside of a standard convection type incubator at 37°C. Power is provided by a small constant speed motor mounted on the outside of the incubator.*

1990; Weigensberg *et al.*, 1990). More recent studies have shown that hyperglycemia leads to free radical formation, which presumably damages cell membranes and macromolecules, and that these effects can be prevented by adding free radical scavengers such as superoxide dismutase, catalase, and glutathione peroxidase to the culture medium (Eriksson and Borg, 1991, 1993). Thus, the culture system was not only successful in identifying potential teratogens, but also mechanisms for their action.

Questions concerning the relevance of high levels of glucose necessary to produce terata in culture led to a continued analysis of the teratogenicity of diabetes-induced factors. The ketone, β-hydroxyubutyrate (BOHB), caused closure defects in the anterior and posterior neuropores and growth retardation in early somite stage mouse embryos exposed to high concentrations ranging from 4-32 mM (Horton and Sadler, 1983). Metabolic studies showed that in 5-6 somite embryos, metabolism of BOHB resulted in a change in redox potential that inhibited the pentose phosphate pathway and its production of ribose for nucleic acid synthesis (Hunter and Sadler, 1987; Hunter *et al.*, 1987b). Confirmation of the mechanism of teratogenesis was provided by demonstrating the protective effects of ribose supplementation to BOHB containing medium (Hunter *et al.*, 1987b). However, at earlier embryonic stages of development (2-3 somites), no effect of BOHB on the pentose phosphate pathway was demonstrable and yet these embryos were more sensitive to the ketone's teratogenic effects (Horton and Sadler, 1983). Subsequently, it was shown that BOHB, possibly through steric hindrance of the enzyme carbomoyl phosphate synthetase (Bhasin and Shambaugh, 1982), directly inhibited *de novo* synthesis of pyrimidines when embryos were exposed at the 2-3 somite stage (Shum and Sadler, 1990). The differences in mechanisms of action at the 2 stages, which represent approximately a 4 hour difference in development, is due to the fact that inhibition of the pentose phosphate pathway is dependent upon metabolism of BOHB to acetoacetate (Hunter *et al.*, 1987b). Older, 5-6 somite embryos have a 40% greater ability to complete this reaction than younger 2-3 somite embryos, thus accounting for the differences in effects of the compound at the 2 stages (Shum and Sadler, 1990). These studies illustrate the capacity of the culture system to determine mechanisms of action of compounds and to elucidate slight differences in response due to even minor variations in development, something that would be impossible to demonstrate *in vivo*. They also show how the system can be used for metabolic studies of substrates at early embryonic stages of development.

One of the most teratogenic factors produced by maternal diabetes are the somatomedin inhibitors (Sadler *et al.*, 1986). The inhibitors are small molecular weight (~1000 Da) serum factors that are present in serum from diabetics (Phillips *et al.*, 1983) and are also elevated in patients with renal disease (Phillips *et al.*, 1984). They are classified as somatomedin inhibitors because they antagonize somatomedin stimulated growth of cartilage in a bioassay (Phillips *et al.*, 1983). In culture, they produce severe growth retardation and neural tube defects in early somite stage embryos and craniofacial abnormalities at later stages (Sadler *et al.*, 1986). Whether or not they affect embryos directly is not clear, although

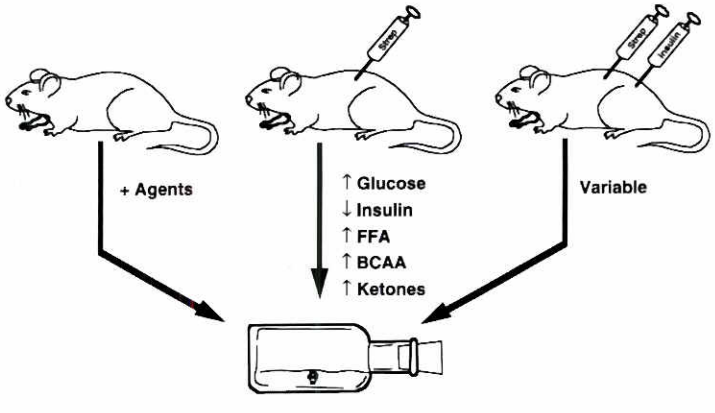

Fig. 2. Experimental design for diabetes research. *Cartoon of the experimental approach used to study the effects of maternal diabetes on embryogenesis using New's whole embryo culture system. The toxicity of the disease was studied by inducing diabetes in non-pregnant animals with streptozotocin injections to kill pancreatic β cells and then culturing embryos in the animals' serum. Insulin's ability to reduce the toxicity was tested by providing the hormone to animals with streptozotocin-induced diabetes and testing their serum for toxicity. Finally, the teratogenicity and mechanism of action of each of the many serum factors altered by the disease was analyzed by collecting serum from non-diabetic animals and adding the factors singly or together to that serum. Streptozotocin (Strep); free fatty acids (FFA); branched chain amino acids (BCAA).*

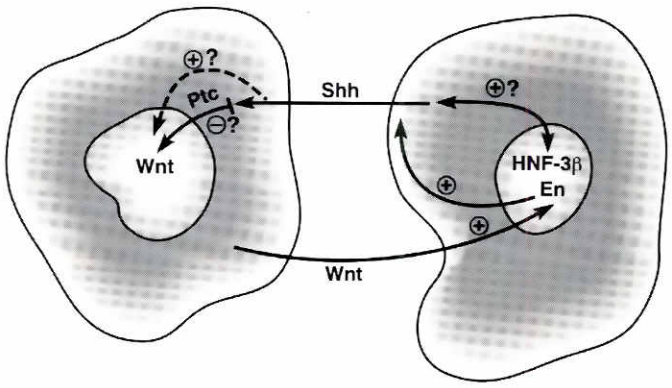

Fig. 3. Potential cell signaling pathways during gastrulation. *Cartoon of two cells communicating through a hypothetical Wnt-Engrailed (En) pathway, based on work from Drosophila and vertebrate embryos. Wnt expression regulates and maintains En in a neighboring cell, and, in turn, En maintains Wnt expression through another gene, sonic hedgehog (shh). Shh may regulate Wnt expression directly or by removing negative inhibition of Wnt by genes such as Patched (Ptc). Hepatocyte nuclear factor β (HNF-3β) may also play a role in regulating shh expression. Targeting these genes in whole embryo culture with antisense cDNA has begun to elucidate their roles in this pathway as it regulates gastrulation.*

early stage embryos have IGF-1/somatomedin receptors and contain this growth factor (Smith *et al.*, 1987). However, it is clear that the inhibitors reduce pinocytic activity by the visceral yolk sac that surrounds the embryo, thereby inhibiting processing of serum proteins by this tissue (Balkan *et al.*, 1989; Hunter *et al.*, 1991). As a result, proteins accumulate in the endoderm cells of the yolk sac and are not processed to their constituent amino acids. In turn, embryos are nutritionally deprived and fail to grow. Partial recovery is possible if a mixture of free amino acids are provided in the medium (Balkan *et al.*, 1989).

Another important result from embryo culture studies on diabetes was the observation that embryos require glucose at early somite stages, such that even minor episodes of hypoglycemia are teratogenic (Ellington, 1987; Sadler and Hunter, 1987). Thus, 1-2 h of exposure to a 30% reduction in normal glucose concentrations results in abnormal growth and development (Buchanan *et al.*, 1986; Akazawa *et al.*, 1989; Smoak and Sadler, 1990). Clinically these observations are important because the recommended therapy for diabetic women is to strictly regulate their metabolism with multiple insulin injections prior to conception and throughout gestation (Fuhrmann *et al.*, 1983; Burkart *et al.*, 1988). While this approach has reduced the incidence of birth defects in this cohort, it also increases the risk for multiple episodes of severe hypoglycemia (Rayburn *et al.*, 1986; Amiel *et al.*, 1987). Thus, overaggressive attempts to prevent hyperglycemia may create a new problem if glucose levels become too low during the period of neurulation (approximately the 3rd-4th weeks of gestation, post fertilization in humans). In fact, culture results showing that mild hyperglycemia is not teratogenic, whereas even short periods of hypoglycemia produce severe abnormalities, suggests that it is better to have slightly elevated glucose levels at this stage of development than depressed concentrations of this substrate.

Together, studies using whole embryo culture to evaluate factors and mechanisms responsible for the diabetic embryopathy have defined the critical periods of development when conceptuses are susceptible to the teratogenic effects of the disease. They have also demonstrated that the origin of the defects is probably multifactorial and that hyperglycemia is not the ultimate teratogen. In fact, embryos tolerate excess glucose well, especially at early stages of development. In contrast, hypoglycemia is more detrimental, even after short exposures. From a clinical standpoint, these studies indicate that women with diabetes need to plan their pregnancies and achieve the best metabolic balance possible prior to conception. Waiting until pregnancy is detected is too late to avoid most of the adverse effects of the disease on embryogenesis. Also, caution is warranted in being overaggressive with insulin therapy, since such an approach may lead to frequent, severe episodes of hypoglycemia.

Gene targeting with antisense oligodeoxynucleotides

In more recent studies, we have combined embryo culture and molecular techniques to study gene function during early stages of embryogenesis. Together with my colleague Edison Liu from the University of North Carolina Cancer Center and a graduate student, Karen Augustine, we have devised a mechanism of disrupting gene expression using antisense oligodeoxynucleotides (ODNs) injected into the amniotic cavity of day 9 (plug day= day 1), 4-6 somite mouse embryos (Augustine *et al.*, 1993). The ODNs are 20-25 bases long and are directed to specific sequences of targeted mRNA (Fakler *et al.*, 1994). The probes are phosphorothioated, to resist degradation by nucleases, begin to enter cells 30-45 min after injection by endocytosis (Loke *et al.*, 1989; Yakubov *et al.*, 1989), and appear to be distributed throughout the embryo (Augustine *et al.*, 1993). By 3 h they reach a peak concentration that remains

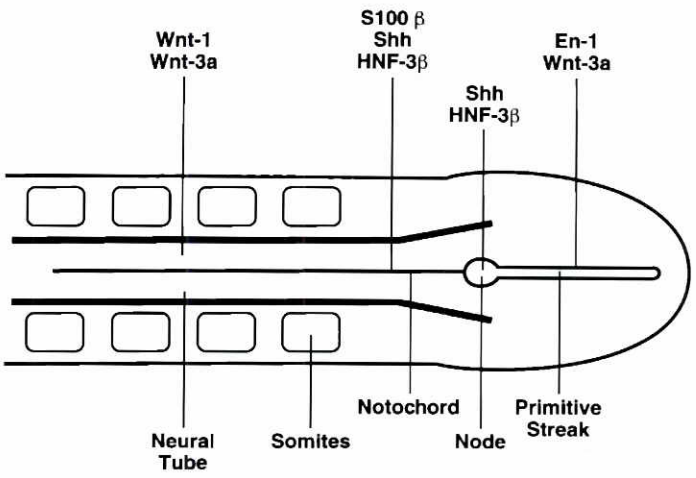

Fig. 4. Gene expression patterns in the neural tube and primitive streak. *Cartoon of the caudal half of an early somite stage mouse embryo undergoing neurulation and gastrulation. At this stage, the neural tube has initiated closure in the cervical region, 4-6 somite pairs are present, and formation of germ layers, notochord, and axis extension are occurring by ingression of cells through the primitive node and streak. Genes involved in a potential Wnt-En signaling pathway (see Fig. 3) are expressed in the streak, node, notochord, and neural tube as indicated, suggesting that they play a role in regulating the processes of axis formation and neural tube patterning. Targeting these genes by creating knockout mice or using antisense oligodeoxynucleotides in whole embryo culture supports this hypothesis (see Fig. 5).*

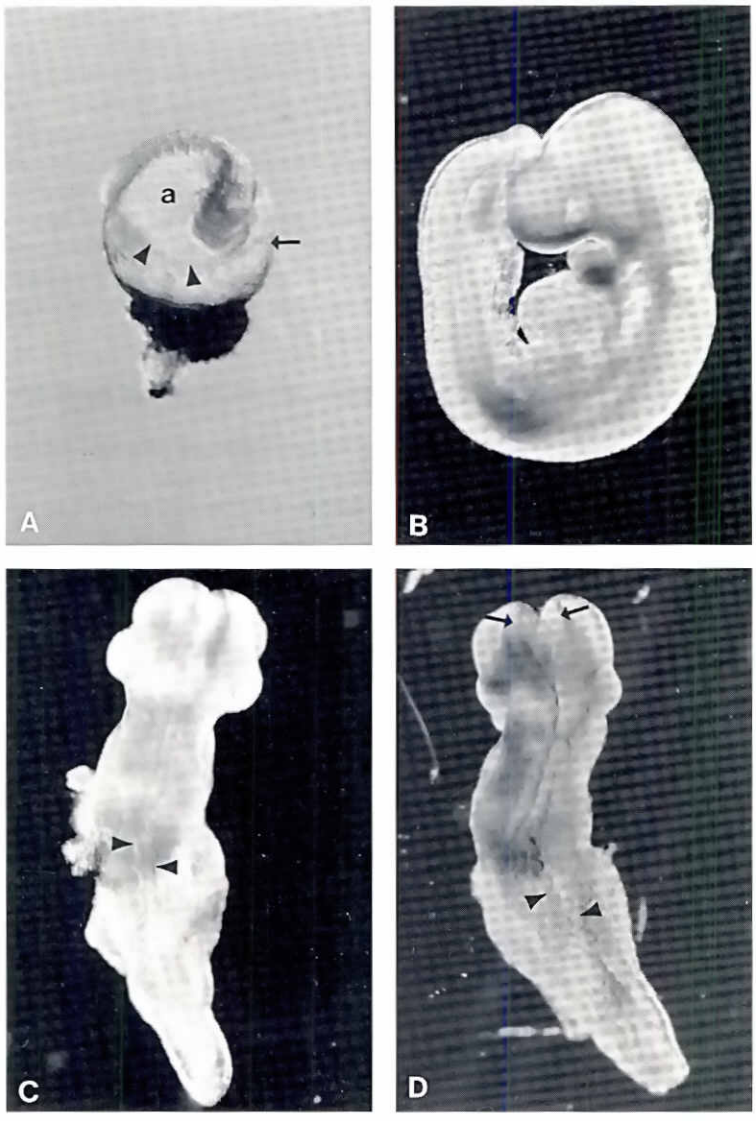

Fig. 5. Effects of antisense targeting of HNF-3ß and shh. (A) *Four-6 somite mouse embryo as prepared for whole embryo culture. Antisense oligodeoxynucleotides are injected through the yolk sac (arrow) and amnion (arrowhead) into the amniotic cavity (a) with a glass needle.* **(B)** *Mouse embryo cultured for 24h following injection of 100 nl of phosphate buffered saline at the 4-6 somite stage. Growth and development are normal with closure of the neural tube, somitogenesis, establishment of brain vesicles, and other morphogenetic events occur as they would in vivo over the same time period.* **(C)** *Embryo injected at the 4-6 somite stage with 60 μM antisense oligodeoxynucleotides to HNF-3ß and cultured 24 h. Axis extension is limited, somites are indistinct, and the neural tube (arrowheads) is wavy and disorganized.* **(D)** *Embryo injected at the 4-6 somite stage with 60 μM antisense oligodeoxynucleotides to shh and cultured 24 h. Axis extension is limited resulting in caudal dysgenesis, cranial neural folds (arrows) have failed to close, somites are indistinct, and the neural tube (arrowheads) is kinked and disorganized.*

high for at least 9 more hours. After 24 h, concentrations are decreased, but significant levels of intact probe can still be detected (Augustine *et al.*, 1993).

A 20-25 base probe is sufficient to confer specificity (Dash *et al.*, 1987) and is usually targeted to the ATG initiation codon, although targeting other regions of the mRNA has also been effective (Fakler *et al.*, 1994). Once inside cells, probes bind to the targeted mRNA and the resulting double stranded region of the nucleic acid is degraded by RNAse-H, leaving a cleaved, non-functional mRNA molecule and freeing the ODN to bind again (Helene and Toulme, 1990; Wagner, 1994). Ultimately, the result is a decrease in the protein that would have been synthesized from translation of the targeted mRNA.

While a number of genes have been successfully disrupted using whole embryo culture and antisense technology, most of our efforts have focused on those involved in gastrulation. It now appears that a signaling pathway involving *Wnt*, the mouse homolog of the *Drosophila* gene *wingless*, and the homeobox gene *engrailed* is important for the process of axis development, including formation of paraxial mesoderm. In *Drosophila*, this pathway involves neighboring cells, whereby *wingless* expression in one cell regulates and maintains *engrailed* in a neighboring cell (Fig. 3). In turn, *engrailed* regulates a reciprocal pathway that maintains *wingless* expression (Ingham, 1993; Ingham and Hidalgo, 1993). Other genes involved in the pathway include *sonic hedgehog (shh)* (Ingham, 1993; Ingham and Hidalgo, 1993) and, possibly, *hepatocyte nuclear factor β (HNF-3β)* (Fig. 3). Interestingly, these genes are expressed in the node, notochord, and primitive streak during gastrulation suggesting that they play a role in this process (Fig. 4).

Targeting these genes has been conducted by injecting 25-70 μM concentrations of antisense ODNs in 100 nl volumes into the amniotic cavity of 4-6 somite neurulating mouse embryos. Embryos were then cultured for 24-30 h and examined for abnormalities. Phosphorothioated, 20-25 base ODNs were employed and each included the ATG initiation codon. PBS injected embryos and those exposed to ODNs in which 4 bases were changed from the original antisense sequences (4 base-mismatch) were employed as controls. Effects on targeted mRNA were documented using PCR strategies.

Targeting *En-1*, *shh*, and *HNF-3 β* resulted in similar alterations of axis formation and neural tube development in approximately 60-70% of treated embryos (Augustine *et al.*, 1995a; Denno and Sadler, 1995; Sadler *et al.*, 1995). Embryos were shorter than controls with the caudal end terminating in a stump similar to the severe form of caudal dysgenesis in humans (Fig. 5). In addition somites were irregularly formed and not well segmented (Fig. 5C,D). The neural tube showed numerous kinks, particularly in the region of the forelimb buds. Approximately 10% of controls injected with PBS were malformed compared to 17-20% of those injected with mismatch probes and the defects were not as severe or similar to antisense injected specimens.

Antisense ODNs targeted to *Wnt-1* caused abnormalities of the midbrain and hindbrain regions in approximately 70% of the treated embryos (Augustine *et al.*, 1993) that were similar to those observed in knockout mice, in which the gene is eliminated by homologous recombination strategies (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). No defects of the neural tube or axis were observed despite the fact that *Wnt-1* is expressed in the roof of the closed neural tube (Wilkinson *et al.*, 1987; McMahon *et al.*, 1992). Targeting *Wnt-3a*, which is expressed in the brain, closed neural tube, and primitive streak (Parr *et al.*, 1993), produced forebrain defects and occasional kinks in the neural tube, but no shortening of the axis or

abnormalities in soqmite formation (Augustine *et al.*, 1993, 1995b). Because of their overlapping expression patterns in the neural tube, it was hypothesized that the two genes served redundant functions in this region to maintain normal development when either of the genes was disrupted. This hypothesis was tested by targeting both genes simultaneously with antisense ODNs. These experiments resulted in 65% of treated embryos with numerous kinks in the neural tube and shortening of the embryonic axis, although the severity of caudal dysgenesis was less than that produced by disruption of *engrailed, shh* or *HNF-3 β* (Augustine *et al.*, 1995b). Thus, it appears that the 2 *Wnt* genes serve redundant functions and that both need to be altered to produce neural tube abnormalities.

Specificity of the antisense approach was assessed by isolating mRNA from PBS, mismatch, and antisense injected embryos 4-6 h after treatment. PCR techniques were then employed to determine ratios between targeted genes and β-actin, a housekeeping gene unaffected by the injections. Results from analysis of *Wnt-1* and *shh* showed that mRNA levels of these targeted genes was decreased by 40-70% following antisense injections, compared to exposures to PBS or mismatch probes (Augustine *et al.*, 1993). Additional proof of the specific effects of the antisense probes was provided by showing that 12-48 h following targeting of *En-1*, protein levels were significantly decreased in all regions where the protein is normally located (Augustine *et al.*, 1995a). Levels of *En-1* proteins were also decreased at similar times following antisense disruption of *Wnt-1*, substantiating the hypothesis that these 2 genes interact in a signaling pathway (Augustine *et al.*, 1995b).

The abnormal morphology of the neural tube produced by antisense targeting of these genes is similar to that observed in knockout mice in which these same genes have been disrupted. For example *Wnt-3a* (Takada *et al.*, 1994) and *HNF-3β* (Ang and Rossant, 1994; Weinstein *et al.*, 1994) knockout mice exhibit kinks in the neural tube. Furthermore, mice that overexpress *shh* (Echeland *et al.*, 1993) also show similar kinks suggesting that any disturbance in this gene results in abnormal signals and altered phenotypes. In most cases, however, the antisense approach has resulted in abnormalities in more organs and tissues and more severe defects than those observed in knockout mice. However, antisense-induced defects are always consistent with the expression patterns of the targeted gene. For example, in addition to having brain abnormalities, embryos in which *Wnt-1* is disrupted by antisense often have cardiac and facial defects that may be related to abnormalities in neural crest cells where the gene is expressed (Wilkinson *et al.*, 1987). However, no similar malformations were observed in knockout mice. In another example, caudal dysgenesis was a prominent feature of embryos exposed to antisense ODNs to *En-1*. This defect had not been observed in knockout mice for this gene (Wurst *et al.*, 1994) and, in fact, no expression pattern of this gene that would be consistent with this abnormality had been described. However, further analysis using immunohistochemistry and PCR approaches showed that, like *Wnt-3a, En-1* is expressed in the primitive streak region (Sadler *et al.*, 1995) and that the protein was down regulated after exposure to antisense probes (Augustine *et al.*, 1995b). Differences in phenotypes between knockout and antisense approaches may be due to compensation that can occur *in vivo* over a period of time. In this scenario, knockout mice that are missing the gene from the time of fertilization would have an extended period to compensate for the loss of the gene, whereas embryos in culture would not. Support for this hypothesis is derived from numerous studies in which genes have been targeted, but no phenotypes occur. Additional support is provided by recent studies in which mice with an *int-2* knockout often exhibit defects on only one side (Mansour *et al.*, 1993). This result suggests that compensation can occur and that even within the same animal it may be successful unilaterally or by degrees.

Results from all of the antisense studies show that rodent whole embryo culture can be used to study the roles of genes during organogenesis. In fact, combining antisense technology with embryo culture affords several advantages over knockout mice such as:

1) effects on targeted genes can be determined within 24-48 h instead of 1-2 years;
2) costs for the experiments are much lower;
3) multiple genes can be targeted simultaneously in an efficient manner. A major disadvantage is the limited time that embryos can be maintained in culture that precludes describing the ultimate expression of a phenotype. However, if targeted genes are selected appropriately, then early stages of a number of developmental abnormalities can be characterized.

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