Teratogenic and Molecular Methods

for the Study of Mouse

Gastrulation and Neurulation

By

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A thesis submitted in conformity with the requirements for the degree

Doctor of Philosophy

Graduate Department of Zoology

University of Toronto

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Teratogenic and Molecular Methods for the Study of Mouse Gastrulation and Neurulation

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Although gene homology is expected between Xenopus, Drosophila and mouse, it is likely that mouse specific pathways exist. The aim of my thesis is to characterize genes and pathways that are specific to gastrulation and neurulation in the mouse. Two approaches were carried out. Using lithium treated 2-cell mouse embryos I was able to generate axial defects similar to that observed with lithium treated frog embryos. This lead me to investigate the interaction of lithium with GSK and β-catenin and their role in mouse dorso-anterior development. Five days after treatment with lithium, a spectrum of defects were observed consistent with alterations in the developmental capacity of the mesoderm. The lag period makes it unlikely that lithium is affecting maternal mRNAs and proteins the same way that is observed in Xenopus. Therefore the most parsimonious explanation for my results is that alterations of the body plan, even in an embryo lacking localized maternal axial determinants, occurs because of changes mediated through alterations to the chromatin. Therefore a role for chromatin structure as a primary regulatory factor in development is implicated. A second approach using a differential screen with normal day 8.5 and mesoderm deficient embryo derived probes, led to the isolation of the D2 cyclin gene. Expression studies suggest a role in neural induction and a dependency on mesoderm. Expression in the neuroectoderm at day 7 expands as the underlying mesoderm forms, followed by expression in the metacephalon at day 11. Using PCR methods with mouse mutants deficient for mesoderm, we were able to verify that D2 cyclin expression is dependent on the formation of mesoderm during gastrulation. Gene expression requires that chromatin adopts an open conformation. These changes are dependent on an increase in cell cycle length, which is regulated by cyclins and their partner kinases. This study demonstrates that cyclin regulation is important for cell differentiation.
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Chapter 1. Introduction.

The establishment of the body axes is central to the overall organization of the embryo, and is a product of both inducing signals and the competence of cells to respond to such signals. Furthermore, proper inductive interactions during development require that cell-signaling events occur under stringent temporal and spatial parameters. Experiments have aptly illustrated that a defined window of time exists for both inducing and responding events. For example the chick node can no longer induce neural tissue beyond stage 7 and the ectoderm has lost its competence to form neural tissue by stage 4 (Streit et al, 1997). Therefore loss of competence to respond to signals precedes loss of induction capacities at least in this case. The establishment of proper signaling leading to the formation of the major axes (Dorsal-Ventral and Anterior-Posterior) in *Xenopus* and *Drosophila*, relies heavily on maternal factors for the initiation of proper inductive events. In contrast, evidence from mouse studies suggests that the mouse axes are established as a result of zygotic factors.

I hypothesize that the mouse does not require maternal factors for axes development and therefore must use pathways other than those characterized in *Xenopus*. I will discuss current models of axes development and the differences observed between mouse and *Xenopus*. Furthermore, based on the results of the thesis, I will discuss the possible gene regulatory role of chromatin and the cell cycle, and their impact on embryo development.
1.1 Axial development.

1.1.1 Prepatterning events.

In *Xenopus*, induction events leading to the establishment of the body axes are programmed early. The cortical rotation initiates the asymmetric distribution of maternal factors followed by 12 rapid cell divisions, which leads to their compartmentalization. *Drosophila* undergo 14 rapid nuclear divisions without cellularization, the absence of cell membranes allows for the establishment of gradients of maternal factors capable of imparting positional information. What about axis determination in the mouse? To date there is no evidence of maternal factors (RNA or protein) that provide positional information during cleavage stages in the mouse. The gap between fertilization and the first identifiable dorsal-ventral or anterior-posterior landmark is 5.5 days, too long for the survival of maternal factors. Another difference is that the mouse undergoes a very early differentiation event that results in the separation of cells into the trophectoderm (TE) and inner cell mass (ICM) lineages. Therefore early embryogenesis in mammals focuses on the formation of the extraembryonic tissues such as the placenta and allantois, which are important to the survival of the embryo. The lack of maternal positional information is also suggested by chimera studies, which indicate that mouse cleavage stage blastomeres are totipotent (Rossant and Vinh 1980 and Gardner 1992). Therefore a *Xenopus*-like model which is dependent on maternal factors for the establishment of body axis is unlikely to occur in the mouse. Whether the conveyor of positional information is maternally derived may not be critical, as zygotic forms of maternal factors exist. It is therefore possible that differences may occur between mouse and *Xenopus* regarding the source and timing of axis
induction, but the active molecules and pathways involved in axis formation may be conserved.

Although there has been great success in using Drosophila or Xenopus as models on which to base experiments for the study of mouse embryo development, differences must occur between the different species, and all genes and pathways may not be used in the same manner. Thus one might expect the mouse to have a unique set of development specific genes. These genes would fall into one of three possible categories I) unique genes with unique functions II) Similar genes and pathways to other vertebrate genes but with a different expression pattern (for example, engrailed-wnt regulation is conserved between mouse and Drosophila but the expression pattern is very different with the mouse engrailed-wnt pathway being involved in midbrain–hindbrain development) III) Similar genes but with different functions; (β-catenin is good example; in Xenopus, β-catenin is involved in cell signaling while in the mouse the only property attributed so far to β -catenin during embryogenesis is cell adhesion).

In this thesis two methods are used to explore some of the similarities and differences of gene utilization in the establishment of the body plan. The first approach is a study of the effects of lithium treatment of cleavage stage mouse embryos on axial development. Lithium is a well-characterized teratogen which disrupts axial development in Xenopus and Zebrafish (Schneider, 1997). Cleavage stage embryos were treated with lithium and post-gastrulation stages were observed for axial defects. We hypothesized that since lithium has proven to be a useful tool for the study of axis development in Xenopus, similar treatments with lithium would allow us to disrupt axial development in the mouse. It has been established that lithium has its effect by specifically disrupting the activity of the kinase GSK3β (glycogen synthase kinase). Thus
lithium treatment of cleavage stage embryos would allow us to determine if GSK-3β has an early role in mouse axial development. The second procedure involves analysis of the expression pattern of a gastrulation specific gene, the D2 cyclin gene. D2 cyclin is transiently expressed in the central nervous system during mouse embryogenesis. Recent studies have shown an increasingly important role for cyclins in the regulation of gene expression during development (Oshugi et al., 1997; Zavitz and Zipursky, 1997). Cells must temporarily exit the cell cycle in order to reprogram their genome and take on a new differentiated state. At the same time a return to a rapid cell cycle is required to make sure the new cell type is expanded to provide enough cells to form tissues and organs.

1.1.2 Embryo prepattern and cellular competence.

Spemann and Mangolds (1924) classic experiment in which they induced a second axis in amphibians by transplanting a group of blastomeres from the presumptive dorsal side to the ventral side of a host embryo illustrates the presence of axis determining molecules contained within the cells. These experiments were further refined by showing that injection of specific molecules into ventral blastomeres of UV treated embryos is able to rescue the ventralized embryos (Smith and Harland, 1991). Further work defined the role of inducing signals, competence of responding cells and competence modifiers.

Competence arises from the specification of individual cell fate which is due, in part, to internal gene programming as well as local cell-cell interactions. These two separate processes are integrated giving rise to cell competence that determines the cells’ ability to respond to signals. Interactions of like cells with each other and local interactions of dissimilar cells cause a
change in the intracellular composition of the cell. It has been shown with Notch and β-catenin that small differences in protein levels that arise between daughter cells are enough to result in cell differences and alter cell fate. Both β-catenin and Notch are active in different cell types and are able to work with other signals to regulate the cell's ability to respond to specific developmental cues. (Labell, 1997, Heitzler et al., 1993).

Besides cytoplasm composition differences, external signals contribute significantly to the establishment of the body plan. Some signals can directly induce cell fate while others act synergistically to modify the inducing signal. Therefore the final phenotype is dependent on the amount of signal, the timing of the signaling events, the presence of coactivating signals and the competence of the responding cells. The relationship of these elements is illustrated in various experiments carried out in *Xenopus*. For example, Xwnt-8 is unable to induce mesoderm but will work synergistically with Activin to form dorsal mesoderm i.e. it modifies the competence of the cells (Sokol and Melton, 1992). Xwnt-8 also modifies the activity of bFGF. Using identical concentrations of bFGF, animal caps from embryos injected at the two-cell stage with Xwnt-8 prior to FGF treatment produced dorsal mesoderm while animal caps treated with FGF alone produced ventral mesoderm. The combined actions of Xwnt-8 and bFGF indicate first, that the wnt signaling pathway is able to prepattern the cells and limit their response to FGF or activin, and secondly that the establishment of the dorso-anterior axis by the wnt pathway requires the assistance of growth factors (Doniach, et al., 1995). A study by Moon (1993) showing that a constant dose of activin will induce different tissue types in older animal caps illustrates the maturation process the cells go through as they are made competent to respond to a broad range of signals.
While the *Xenopus* experiments have revealed important molecular and cellular relationships, one must remember that these embryos differ from mammalian embryos in two respects. Early cleavage of a relatively large egg is rapid and synchronous with no net increase in size and there is a heavy reliance on maternally stored products during embryogenesis. The mode of early embryogenesis in mammals, on the other hand is characterized by very slow cleavage divisions of a small egg whose maternal products are eliminated early. Development of extraembryonic tissues precedes gastrulation, which occurs following an increase in mass after implantation in the uterus. This raises several important questions. Is there any evidence of a prepattern in mouse embryos that occurs prior to implantation besides the separation of the ICM from the TE, and when does the mouse embryo undergo axial determination? Work by Gardner (1997) revealed a morphological asymmetry in early embryos of the PO strain of mice. The polar body remains attached to the blastocyst at the inner cell mass:trophectoderm boundary. Gardner suggested that this mark depicts the future anterior-posterior axis. Does it in fact mark the future anterior-posterior axis and could this hint at an underlying molecular asymmetry?

There are insufficient studies in the mouse to conclude whether or not a prepattern exists. An analogous study in the mouse to that of Spemann and Mangold is currently unfeasible. Preimplantation mouse embryos are symmetrical and therefore it is impossible to accurately identify dorso-anterior vs ventral-posterior, in order to carry out similar blastomere transplantation studies.

Although nuclear transplant studies by various groups have indicated that the nuclei of
cleavage stage and blastocyst embryos are totipotent, these studies do not address the question of prepatternning potential. Tsuoda et al. (1997) transferred the nuclei of 4-cell stage or compacted morula into enucleated oocytes. In both cases development in vivo went to term. The fact that the oocyte cytoplasm is able to reprogram the morula nucleus is significant, although these experiments are really just addressing the developmental potential of the cytoplasm, and its ability to possibly reprogram a differentiated mouse nucleus. Single cells from the morula and ICM were aggregated into 8 cell embryos and were able to contribute to inner and outer cell lineages. Thus even blastocyst cells have some ability to populate all lineages (Rossant and Viji 1980, Gardner 1968). The routine use of embryonic stem cells (ES), derived from the ICM cells, for the construction of chimeras in which all but the extraembryonic lineages are produced, demonstrate that the pluripotent state can be maintained even after multiple cell divisions in vitro (Joyner, 1994).

Taken together, the transplant studies and the chimera studies suggest that the cells of even late stage mouse embryos are capable of contributing to a wide variety of cell lineages, but do not contain any molecules specifically capable of conferring a body plan. Furthermore, there is no evidence in the mouse embryo of a mRNA or protein that shows asymmetrical expression at the 2 or 8-cell stage as would be expected if the axes were determined at this stage. Therefore the fate of mouse blastomeres does not seem to be predetermined, as are cells of the Xenopus blastula. It is possible that the nuclei of mouse blastomeres are pre-programmed and have a limited competence under normal conditions, but the experiments described above cause a change in the gene expression patterns, possibly by altering the chromatin structure, and this accounts for the totipotency observed.
1.1.3 Genetic regulation of cell competence.

The inducing signal and cellular competence are dependent on gene expression. The specification of individual cell fate is due to internal gene programming resulting in local cell-cell interactions. Interactions of like cells with each other and local interactions of dissimilar cells lead to differentiation. As mentioned above, asymmetrical compartmentalization of maternal products, as occurs in *Drosophila* and *Xenopus*, leads to organized differences in neighboring cells.

Although there is a tendency to study isolated events, the study of induction and competence is becoming more interesting as the genetic interactions required for proper development are being deciphered (Ang and Rossant 1997). It is becoming apparent that the regulation of many genes is required for the proper development of the embryo. Although the simultaneous regulation of many genes seems like a daunting task to explain, current studies on chromosome configuration and the newly discovered role of chromatin proteins, such as histone H1 (Zlatanova and Holde, 1998), or the role of acetyltransferases and deacetylases as transcription regulators are providing a good model for global gene regulation (Kuo and Allis, 1998).

Histone H1 levels are found to be linked to mesodermal competence thus suggesting competence is not regulated only at the level of specific gene transcription but is also regulated more globally, via chromatin structure. *Xenopus* nuclei at the mid blastula transition (MBT)
undergo a change from maternal histone B4 to histone H1A. By artificially depleting histone H1A using specific ribozymes, both histone B4 association with the chromatin and competence to respond to mesoderm agents, was prolonged. Conversely, an increase in H1 histone resulted in shortening the time that animal cap cells are competent to respond to activin (Steinbach, Wolfe, Rupp, 1997). In mammals a similar remodeling of the chromatin occurs. As maternal mRNA is degraded there is a delay of one to two cell cycles before the new synthesis of histones occurs. The newly synthesized histones, which appear at the 4-cell stage, are different isoforms from those that occur in the oocyte and fertilized egg. It is possible, though unproven, that the new isoforms have specialized functions and represent a process similar to the histone B4 to histone H1 transition described in Xenopus (Clarke et al., 1998). The above data show that chromatin structure proteins have an important role in elaborating cellular competence.

1.1.4 Wnt, Gsk3β-Catenin and Lef-1. Their role in axes development.

Embryonic development is under the control of a large array of interacting biochemical pathways, which work simultaneously or sequentially with each other resulting in the continuous differentiation of the cell. The outcome of these interactions is not always immediately observable. For example, Larabell et al., (1997) show that β-catenin is found asymmetrically distributed in the two cell Xenopus embryo and that at the 16-cell stage, β-catenin localizes to the nuclei in the presumptive dorsal cells only. The maternal message and protein is positioned within a few key cells allowing those cells to become distinct from their neighbors with respect to their ability to induce surrounding cells. Figure 1 diagrams the wnt pathway in Xenopus. The accumulation of β-catenin is triggered by the activation of the wnt
pathway. Wnt activation leads to the inactivation of GSK3β, which allows for the stabilization and accumulation of β-catenin. In the absence of wnt activation, GSK3β is active and marks β-catenin, by phosphorylation, for destruction by adenomatous polyposis coli (APC). Thus, the asymmetrical accumulation of β-catenin could be due to either the activation of wnt or any downstream component of the pathway.

Since β-catenin does not contain a nuclear localization signal it must combine with the transcription factor Lef-1/Xtcf, a HMG box-containing protein. Lef-1 is an architectural protein, which has the ability to bind to DNA, and decreases the DNA angle from 120 degrees to 80 degrees (Behrens et al., 1996). The importance of Lef-1 to proper β-catenin action is illustrated in the experiments described below. Xtcf-3 is a Xenopus member of the Xtcf/Lef family. When the β-catenin binding site of Xtcf-3 was deleted and the modified mRNA was microinjected into the dorsal blastomere of a 4-cell embryo, normal cleavage stage development ensued but they failed to form a proper body axis. Furthermore, when the same construct was injected into ventral blastomeres along with β-catenin mRNA, the deleted Xtcf-3 could suppress β-catenin induced secondary axis formation.

Mouse studies are less clear. ES cells that have artificially increased levels of β-catenin have the ability to up regulate the expression of Lef-1 which is normally silent (Huber et al., 1996). Although β-catenin/Lef-1 interactions in adult mouse tissue occur (Novak et al., 1998), a role in embryo development has not been determined. The adhesion property of β-catenin has been studied extensively in cell culture and the spatio-temporal distribution found in mouse embryos is indicative of a role for cell adhesion in the morphogenetic movements of gastrulation and neural induction (Haegel et al., 1995). β-catenin null mice do not have defects
prior to gastrulation, presumably due to the activity of maternal β-catenin or to the buffering capacity of other adhesion molecules which can compensate for the lack of mouse β-catenin. By d4.5-5.5 maternal β-catenin is gone and all embryos hatched in vitro. Therefore all preimplantation development is normal and the first observable defect is at d7.0 in the ectoderm layer. The mesoderm is not formed and the epithelial organization of the embryonic ectoderm is lost. It is interesting to note that extra embryonic structures all form including the ectoplacental cone, parietal endoderm and the Reicherts membrane (a thick basement membrane that is non cellular). In β-catenin null mice Otx-2 expression is very weak and brachyury is negative, suggesting that gastrulation does not commence. By d7.5 the embryos are half the size of normal littermates with ectoderm cells shedding into the proamniotic cavity. At d8.5 increased cell death occurs and the embryos are very disorganized. This suggests that cell proliferation and cell adhesion are defective. The disorganization observed could be explained by the loss of the dorsal axis determining function associated with β-catenin or solely by cell adhesion defects caused by the loss of β-catenin. (Haegel et al., 1995). Further studies using stage specific, β-catenin null mice will clarify the matter.

1.1.5 Lithium and the disruption of the wnt pathway.

In amphibians lithium can respecify the body plan in one of two opposing ways, depending on the stage of treatment. Pre-MBT treatment leads to an enhancement of anterior structures, whereas post Mid Blastula Transition (MBT) treatment leads to anterior truncations (Kao et al, 1986). As outlined in a recent review by Kao and Elinson, (1998), early studies by Masui (1961) illustrated that the treatment of gastrula stage Triturus pyrohogastor with lithium
resulted in a reduction of anterior tissue. Explant studies where either the responding tissue (ectoderm) or inducing tissue (organizer) were treated with lithium and then co-cultured, demonstrated that lithium alters the competence of the ectoderm cells to respond to signals from the organizer. Furthermore, the treatment of the organizer tissue with lithium caused the mesoderm to develop ventral-posterior fates, not dorso-anterior fates. Thus the combined effects of lithium on both the organizer and the competence of the responding tissue led to a truncation of neural and mesoderm anterior structures. These effects are analogous to the late (post MBT) treatment of Xenopus embryos. In contrast to the above results, the treatment of 32-cell stage Xenopus embryo with lithium results in the over specification of mesoderm, causing dorsal axial development to occur from cells that would normally give rise to ventral structures. Injection of ventral vegetal cells with lithium caused axial duplication, while injection of dorsal vegetal cells or injection of animal cap cells had no effect (Kao et al, 1986). The dorsalizing effect of lithium is also evident in studies where UV treatment of Xenopus oocytes prior to fertilization prevents formation of dorsal structures including CNS, notochord, and somites. Lithium is able to restore dorsal structures to UV ventralized embryos (Kao et al., 1988).

Since myo-inositol can rescue lithium treated embryos the inositol pathway was implicated as the target of lithium (Busa and Gimilich, 1989). Under normal conditions the inositol pathway is triggered in response to the stimulation of platelet derived growth factor receptor or epidermal growth factor receptor, both are tyrosine kinase linked receptors. Their stimulation leads to the hydrolysis of the membrane lipid precursor, phosphatidylinositol 4,5-bisphosphate by phospholipase C, which gives rise to diacylglycerol (DAG)and inositol triphosphate (InsP₃). DAG stimulates Protein Kinase-C (PK-C) and InsP₃ causes release of
calcium from internal stores. The inositol pathway recycles by converting the various by-products into myo-inositol and then regenerates the membrane inositol lipid. There are a number of pathways that are involved in this regeneration process and two of the enzymes involved are lithium sensitive. Of these two enzymes, mono-phosphatase (IMP'ase) is more sensitive and occurs at the junction of two of the recycling pathways (Klein & Melton, 1996; Berridge et al., 1989). See Figure 2.

Using a specific inhibitor of IMP'ase, Klein and Melton (1996) demonstrated that Xenopus morphogenesis was not affected, therefore ruling out a role for InsP3 pathway as being a target of lithium induced axial disruption. They went on to show that lithium inhibits GSK-3β, and that a dominant negative GSK mimics the lithium phenotype. Although their results are very convincing they do not explain the ability of myo-inositol to rescue LiCl treated embryos. Further work by Hedgepeth et al., (1997) showed that, although the inhibition of IMP’ase does not alter normal development, myo-inositol can still rescue the effect of a dominant negative GSK3β. The inositol pathway is further implicated because the inactivation of GSK3β by wnt is regulated by PK-C, which in turn is regulated via the inositol pathway (Cook et al., 1996). Thus both papers suggest that inositol is connected to the wnt-GSK3β-β-catenin pathway via PK-C, at the point of GSK3β but the exact mechanism is unknown. In the case of the rescue of dominant-negative GSK3β by myo-inositol it is possible that the added myo-inositol feeds back to increase the overall activity of the PI cycle. This leads to increased levels of PK-C which in turn acts on endogenous GSK3β, possibly causing activation of stored, inactive GSK3β which overpowers the dominant-negative GSK3β. A second hypothesis is that PK-C substitutes for GSK3β and phosphorylates β-catenin itself.
1.1.6 Signaling pathways of the vertebrate organizer.

The nuclear translocation of β-catenin, in a complex with Xtcf/lef, occurs in a very wide area on the dorsal side of the *Xenopus* embryo. Possible downstream targets of the Xtcf/β-catenin complex, which delineate the Spemann organizer, that have been identified, include *Siamois, Xnr3, chordin* and *noggin*, with *siamois* and *Xnr3* having the earliest expression. A list of other molecules found in the organizer of vertebrates is found in Table 1. The broad area delineated by β-catenin translocation can be subdivided based on zygotic gene expression. The first cells to migrate during gastrulation, the anterior endomesoderm, express *cerberus*, and are unable to induce a second axis upon transplantation. These cells are followed by the head mesoderm cells, which express *chordin*, and *cerberus*, and are able to induce a second axis. The most posterior cells are the trunk organizer and they only express chordin. Thus the head and trunk organizer are a result of different downstream targets of the initial β-catenin nuclear translocation (Darras et al., 1997). This subdivision of the *Xenopus* organizer is somewhat similar to the separate mouse organizers. The mouse trunk organizer is attributed to the cells of the proximal posterior ectoderm while the head organizer is believed to occupy the proximal anterior endoderm, 180° from the trunk organizer. It has been suggested that the *Xenopus* trunk (ectoderm) and head (endoderm) organizers, although juxtaposed, are analogous to the two separate ectoderm and endoderm organizers of the mouse (Beddington and Robertson, 1998).

The recent discovery of a separate head organizer in the mouse has led to the re-examination of molecules thought to be only localized in the ectoderm of the node and primitive
streak (Beddington and Robertson, 1998). The discovery that many genes initially shown to be ectoderm and mesoderm specific are also expressed in the primitive endoderm very early in development has led to the conclusion that the head organizer in the mouse has equal organizing properties to that of the node.

Some of the head organizer molecules include nodal, a TGF-beta family member, and a secreted growth factor essential for gastrulation. Nodal is expressed in the posterior primitive streak and the visceral endoderm, just prior to primitive streak formation. Chimeric embryo analysis show that embryos missing endodermal nodal fail to form anterior neural structures. Thus endodermal nodal is responsible for anterior neural structures while ectodermal nodal expression is responsible for posterior structures, analogous to the proposed two-organizer model (Varlet et al., 1997).

*Hex-1* has a symmetrical expression pattern restricted to the primitive endoderm cells that lie between the inner cell mass (ICM) and the blastocoel cavity (d4.5). At d5.5 *Hex-1* is expressed in a few visceral endoderm cells at the distal end of the embryo. These cells migrate anteriorly and thus establish an asymmetry in the egg cylinder 24 hours prior to the formation of the primitive streak and brachyury expression (Thomas et. al., 1998).

Two other genes that are involved in head development have been shown to have anterior endodermal expression at gastrulation. Initially *Lim-1* is expressed in the primitive streak and mesoderm wings while *Otx-2* is present in the endoderm. Just prior to gastrulation, expression patterns of both molecules change. *Lim-1* expression extends into the anterior
visceral endoderm and *Otx-2* expression becomes restricted to the anterior endoderm as expected for a head organizer (Acampora et al., 1995). Other genes that are expressed in the primitive endoderm just prior to gastrulation are *cerberus-like, VE-1*, and *HNF3β*. Null mutations show that *Otx-2, Lim-1, nodal* and *HNF3β* are required for anterior development.

The physical separation of the mouse head organizer and the trunk organizer contributes to the difficulty in applying a *Xenopus* model of dorso-anterior determination. Where would β-catenin nuclear localization occur? As discussed above, studies in *Xenopus* indicate that β-catenin translocation is the initial trigger for both the head and trunk organizer. We would then expect β-catenin to have two domains of nuclear translocation in the mouse coinciding with the two organizers.

### 1.2 Differentiation and Cell Proliferation

The obvious fact that genes reside on chromosomes and chromosomes have an organized structure is usually lost during discussions of the regulation of gene transcription. We tend to think of naked DNA waiting passively for whatever combination of transcription factors might alight. Recent work by the lab of Alan Wolffe has merged the science of chromosomal structural proteins and transcription factors by finding that histone H1 is able to act in either capacity (Steinbach et al., 1997, Vermaak et al., 1998). These results are novel and exciting and provide a possible mechanism to explain well known examples of chromosome based gene
regulation including position effect variegation in *Drosophila* (Henikoff, 1990) and X-chromosome inactivation in humans (Brown et al., 1991). Chromatin conformation is an important hurdle that differentiating cells must contend with. Since differentiation requires new gene expression, it is mandatory that the cell cycle slows and allows chromatin to take on a configuration that encourages proper transcription.

It has been well documented that differentiating cells must establish a longer growth phase of the cell cycle by lengthening either G1 or G2 in order to differentiate. Mitotic indices have been measured in different parts of the developing mouse embryo and show a strong correlation between differentiation and the lengthening of the cell cycle (Kauffman, 1968). Ohsugi et al., (1997), explored the relationship between the cell cycle and body pattern using chick limb buds. They examined the role of growth factors BMP, FGF and Shh on cell differentiation, by asking the question “Does pattern precede growth or does growth control lead to the elaboration of pattern?” In limb buds, anterior cells have a shorter cell cycle in relation to posterior cells. When the cell cycle of anterior cells was lengthened to reflect a more posterior cell cycle length they found that anterior cells began to transcribe posterior specific genes, specifically *Bmp-2*. Downstream genes *Hoxd-11* and *Fgf-4* also were expressed ectopically. This induction of *Bmp-2* by lengthening the cell cycle mimics the effect of *Shh* that normally induces *Bmp-2*, suggesting that *Shh* may function to control the cell cycle, which in turn-directs transcription of *Bmp-2*. Work by Tabin et al., (1997) reinforces this idea. They showed in the limb bud that Shh patterns the mesoderm and induces *Fgf-4*, while *Fgf-4* induces proliferation of the mesoderm and maintains Shh expression. Evidence from work in *Drosophila* suggests a second role for hedgehog. Through its interaction with *string*, a *Drosophila* homolog of cdc25,
it is capable of regulating the cell cycle (Follette and O’Farrell, 1997). Taken together there is a strong link between the need to control proliferation and the process of differentiation and patterning.

1.3 Aims of the thesis

My goal was to study body axis formation in the mouse and to determine whether the mouse uses similar molecules and pathways as *Xenopus* or whether a different mechanism is utilized. A comparison of cleavage stage development in the mouse and *Xenopus* reveal many differences. These include 1) a much slower cell cycle during mouse cleavage stage development relative to the frog 2) the activation of the mouse zygotic genome at the two cell stage versus the 4000 cell stage for *Xenopus* 3) The formation in the mouse of two distinct cell types early in development which give rise to the inner cell mass and the trophectoderm 4) The separation of the head and trunk organizer. These major differences, as discussed above, strongly suggest that any mechanism involved in primary axis formation must contend with these differences. Therefore it is possible that the establishment of the primary body axis during gastrulation in mouse will yield some novel mechanisms.

I undertook a teratogenic and a molecular approach for the study of mouse gastrulation and neurulation. Lithium, a well characterized teratogen, is capable of altering the competence of cells to respond to axis inducing signals. Lithium was used to disrupt normal gastrulation, which allowed me to speculate about a possible role for chromatin structure as a regulator of gene regulation and to investigate a possible role for β-catenin/Lef-1 in establishing cell
competence during cleavage stage development. The second approach involved studying the expression pattern of a gene, D2 cyclin, isolated from a differential hybridization gene screen using cDNA probes derived from normal and gastrulation deficient embryos.
Table #1  
Vertebrate early development genes

<table>
<thead>
<tr>
<th>GENE</th>
<th>MOLECULAR NATURE</th>
<th>ORGANISM</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goosecoid</td>
<td>Homeodomain protein</td>
<td>Chick, Mouse, <em>Xenopus</em>, Zebrafish</td>
<td>Craniofacial &amp; Skeletal; Axial development</td>
</tr>
<tr>
<td>Lim-1</td>
<td>Homeodomain protein</td>
<td>Mouse, <em>Xenopus</em></td>
<td>Head development</td>
</tr>
<tr>
<td>Xnot/ floating head</td>
<td>Homeodomain protein</td>
<td>Chick, <em>Xenopus</em>, Zebrafish</td>
<td>Notochord differentiation</td>
</tr>
<tr>
<td>Hnf3B</td>
<td>Forkhead-domain protein</td>
<td>Chick, mouse</td>
<td>Node &amp; Notochord development</td>
</tr>
<tr>
<td>Pintallavis</td>
<td>Forkhead-domain protein</td>
<td><em>Xenopus</em></td>
<td>Induces &amp; patterns neural tube</td>
</tr>
<tr>
<td>XANF1</td>
<td>Homeodomain protein</td>
<td>Mouse, <em>Xenopus</em></td>
<td>Dorsal development</td>
</tr>
<tr>
<td>Siamois</td>
<td>Homeodomain protein</td>
<td><em>Xenopus</em></td>
<td>Dorsal development</td>
</tr>
<tr>
<td>otx2</td>
<td>Homeodomain protein</td>
<td>Chick, mouse, <em>Xenopus</em>, Zebrafish</td>
<td>gastrulation, Axialmesoderm, rostral brain formation</td>
</tr>
<tr>
<td>XIPOu2</td>
<td>Pou-domain and Homeodomain protein</td>
<td><em>Xenopus</em></td>
<td>Neuralizes</td>
</tr>
<tr>
<td>noggin</td>
<td>Secreted protein</td>
<td>Mouse, <em>Xenopus</em></td>
<td>Dorsal and Axial development.</td>
</tr>
<tr>
<td>follistatin</td>
<td>Secreted protein</td>
<td>Mouse, <em>Xenopus</em></td>
<td>Induces neural tissue</td>
</tr>
<tr>
<td>chordin</td>
<td>Secreted factor</td>
<td><em>Xenopus</em></td>
<td>Dorsal and neuralizing activity</td>
</tr>
<tr>
<td>nodal</td>
<td>TGF-B family</td>
<td>Mouse</td>
<td>Primitive streak</td>
</tr>
<tr>
<td>Xnr3</td>
<td>TGF-B family</td>
<td><em>Xenopus</em></td>
<td>Dorsal development</td>
</tr>
<tr>
<td>ADMP</td>
<td>TGF-B family and BMP3 related</td>
<td><em>Xenopus</em></td>
<td>anti-dorsal, inhibits neural</td>
</tr>
</tbody>
</table>

Adapted from Lemaire and Kodjabachian, 1996, TIG 12, 525-532
Figure 1

Wnt

Dsh

LiCl → GSK

β-Catenin

Lef-1

Siamois activation

IMP'ase

Phospholipase C

Protein Kinase-C

APC

Degraded

β-Catenin
Chapter 2

Epigenetic alterations brought about by lithium treatment disrupt mouse embryo development
2.1 Abstract

The commonly accepted mechanism by which LiCl dorsalizes amphibian embryos is a respecification of ventral blastomeres, presumably through realignment of dorsal positional information in the embryo. An alternative mechanism, however, is an epigenetic change in the competence of cells to respond to cues they may be normally exposed to without effect. In order to test this hypothesis, we treated mouse preimplantation embryos, which do not possess any axial positional information, with LiCl, and observed axial abnormalities which must have been elaborated several days after treatment. We interpret this as support for the hypothesis that cellular competence rather than positional information is altered by LiCl, and suggest that this competence may be altered through the action of lithium sensitive enzymes that interact with chromatin.
2.2 Introduction

Epigenetic phenomena are widespread in nature, although not well understood. The most intensively studied examples in recent years are mammalian X chromosome inactivation (Brown et al., 1991), genomic imprinting, mainly in mammals (McGarth and Solter, 1984), and position effect variegation (PEV) in fruit flies (Henikoff, 1990). These are characterized by somatically heritable changes in genome activity that are independent of the state of differentiation of the cells. For example, imprinted genes are monoallelicly expressed, and often differentially methylated, depending on the parental origin of the allele. Similarly, PEV is characterized by random inactivation of sensitive alleles. Evidence in favour of a role played by chromatin structure in these phenomena is accumulating.

Epigenetic changes can also be induced by exogenously applied toxins, or by changes in the environment. For example, a temperature shift during larval development can act as a modifier of PEV in adult fruit flies (Henikoff, 1990), and temperature changes can alter paramutable alleles in plants (Mikula, 1995). Substances such as ether can produce phenocopies of bithorax mutations (Capdevila and Garcia-Bellido, 1974).

In amphibians, application of LiCl at very early stages of development can cause dorsalization of the embryo during gastrulation (Kao et al., 1986). One interpretation of these latter experiments is that LiCl alters the localization or activity of cytoplasmic determinants that control formation of the body axes. However, another explanation is that LiCl alters the competence of the blastomeres through a heritable change in chromatin structure. Because
extensive analysis of the totipotency of murine blastomeres and inner cell mass cells indicates that preimplantation mouse embryos do not possess positional information for the embryo body plan (Rossant, 1987; Lawson et al., 1991), these alternative mechanisms can be distinguished by examining the effects of LiCl on mouse embryos. If a cytoplasmically localized component is responsible for the effects of LiCl on axial development, LiCl should not affect the body plan of the mouse embryo. Alternatively, if LiCl affects chromatin structure in early embryos, then some changes in the body plan of the mouse embryo may be observed following exposure of cleavage stage embryos to LiCl. Our experiments indicate that treatment of two cell or eight cell mouse embryos with LiCl is not toxic, and has no effect on trophoblast development. However, LiCl treated embryos accumulate a number of defects consistent with alterations in the developmental capacity of the mesoderm commencing at gastrulation. Our results indicate that LiCl treatment of early cleavage stage embryos can alter subsequent development of the vertebrate body plan, even though such preimplantation embryos lack localized axial determinants. Thus, epigenetic changes of the genome may mediate the effects of LiCl in both amphibian and mammalian embryos.
2.3 Materials and Methods

Mouse Strains

CD-1 mice were purchased from Charles River, and maintained in a 12 hour light/dark cycle, fed on commercial rodent chow *ad libitum*. The recombinant inbred line LTXBO was originally obtained from Dr. J. Nadeau at the Jackson Laboratory, and has been bred in our mouse facilities for several years.

Lithium Treatment

Fertilized embryos were obtained from superovulated females mated with males of the same strain. Females were superovulated by intraperitoneal injection of 5 units of Pregnant Mares Serum (PMS) and a second injection 46 hours later of 5 units of human Chorionic Gonadotropin (hCG). Fertilized two-cell embryos were flushed from the oviducts of pregnant females and used within 2 hours or cultured until the 8 cell stage before treatment. All embryos were cultured in PLG medium (90 mM NaCl, 4.8 mM KCl, 1.7 mM CaCl₂ 2H₂O, 1.18 mM KH₂PO₄, 0.57 mM MgSO₄, 0.55 mM Glucose, 0.25 mM Pyruvic Acid, 0.25% D,L Lactic Acid, 0.25% BSA fraction V, 25 mM NaHCO₃) prior to and following treatment. Embryos were treated in PLG medium in which the equivalent molarity of NaCl was replaced by 0.9mM, 9.0 mM or 90 mM LiCl, for 3-5 hours. Alternatively embryos were cultured in PLG medium supplemented with 300 mM lithium chloride for 5-6 minutes. As a control, an equivalent procedure was carried out using 300 mM KCl. Following treatment, embryos were washed four times in a large
volume of PLG, and then either cultured *in vitro* to monitor development to the blastocyst stage, or transferred into the oviducts of d0.5 pseudopregnant CD-1 females. Some 8 cell embryos which developed to the blastocyst stage were transferred into the uteri of d3.5 pseudopregnant females. Lithium treated embryos were autopsied on d9.5 of gestation and scored for developmental defects. Day 9.5 was chosen for easy comparison to previous studies on rats and mice (Jurand, 1988, Hansen et al., 1990). Some of the 2 cell treated embryos (300 mM) were autopsied on d7.5 and used in whole mount *in situ* hybridization with the *brachyury* gene (Conlon and Rossant, 1992).
2.4 Results

To check for any possible toxic effects of lithium we treated embryos, transferred them to pseudo pregnant females and calculated the implantation rate by observing the number of implantation sites at d7.5 for both lithium treated and control embryos. Treated embryos transferred immediately after treatment into pseudopregnant mothers implanted at the same rate as control embryos (Table 1). We conclude from these studies that LiCl exerts only negligible toxic effects on preimplantation development. Thus, we were able to examine the effect of both treatment regimens on later development by returning embryos to foster mothers immediately after treatment.

Postimplantation Development of Lithium Treated Embryos

Embryos transferred into d0.5 pseudopregnant mothers immediately after treatment were examined on d7 and d9 of gestation with respect to the mothers’ pregnancies. Both extraembryonic and embryonic tissues were carefully examined, and the d7 conceptuses were further processed for whole mount in situ hybridization. In all cases, no defect in size or morphology of extraembryonic tissues (ectoplacental cone, extraembryonic ectoderm, yolk sac) could be observed. Indeed, in some cases, the only part of the conceptus that remained recognizable was the extraembryonic tissue. In both the control and experimental groups some empty deciduae were found. The frequencies of these did not differ among the various groups (Table 1).
Table 1  *In vivo* development of control and LiCl treated embryos

<table>
<thead>
<tr>
<th>Two-cell treated CD1</th>
<th>2 cell in (%)</th>
<th>Decidua (%)</th>
<th>Normal (%)</th>
<th>Abnormal (%)</th>
<th>Resorptions (%)</th>
<th>Empty (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d9 Control</td>
<td>76 (84%)</td>
<td>64 (92%)</td>
<td>59 (92%)</td>
<td>0</td>
<td>0</td>
<td>5 (8%)</td>
</tr>
<tr>
<td>0.9 mM LiCl 5 hr.</td>
<td>48 (83%)</td>
<td>40 (88%)</td>
<td>35 (88%)</td>
<td>0</td>
<td>0</td>
<td>5 (12%)</td>
</tr>
<tr>
<td>9.0 mM LiCl 5 hr.</td>
<td>48 (73%)</td>
<td>35 (89%)</td>
<td>31 (89%)</td>
<td>0</td>
<td>0</td>
<td>4 (11%)</td>
</tr>
<tr>
<td>90 mM LiCl 5 hr.</td>
<td>361 (76%)</td>
<td>273 (72%)</td>
<td>196 (72%)</td>
<td>19 (7%)</td>
<td>36 (12%)</td>
<td>22 (8%)</td>
</tr>
<tr>
<td>300 mM LiCl 5 min.</td>
<td>44 (91%)</td>
<td>40 (91%)</td>
<td>25 (63%)</td>
<td>4 (10%)</td>
<td>8 (20%)</td>
<td>3 (8%)</td>
</tr>
<tr>
<td>300 mM KCl 5 min.</td>
<td>48 (65%)*</td>
<td>31 (87%)</td>
<td>27 (87%)</td>
<td>0</td>
<td>0</td>
<td>4 (13%)</td>
</tr>
<tr>
<td>d7 300 mM LiCl 5 min.</td>
<td>66 (68%)</td>
<td>45 (68%)</td>
<td>8 (12%)</td>
<td>5 (8%)</td>
<td>8 (12%)</td>
<td></td>
</tr>
<tr>
<td>2 cell treated LTXBO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 mM LiCl 5 hr</td>
<td>42 (86%)</td>
<td>36 (39%)</td>
<td>14 (33%)</td>
<td>12 (33%)</td>
<td>5 (14%)</td>
<td>5 (14%)</td>
</tr>
<tr>
<td>8 cell treated CD1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90/300 mM LiCl</td>
<td>107 (50%)</td>
<td>54 (50%)</td>
<td>20 (19%)</td>
<td>33 (31%)</td>
<td>incl. Resorp.</td>
<td>incl. Resorp.</td>
</tr>
<tr>
<td>Control</td>
<td>29 (66%)</td>
<td>19 (66%)</td>
<td>0</td>
<td>10 (34%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*transfers into pseudo pregnant mothers for all d9 LiCl groups were done by one person.

For the KCl group transfers were done by two persons, which may account for the lower success rate.

The frequency of intact LiCl treated embryos recovered at d7 was similar to that of LiCl treated embryos recovered at d9 (Table 1). The resorptions at d7 were solid masses of tissue (8%), and not just trophoblast remnants, thus indicating that a certain proportion of embryos died prior to d7 but after implantation. Morphologically, d7 recovered, LiCl treated conceptuses were either indistinguishable from control embryos, or displayed mild abnormalities, such as an enlarged allantois or a mildly malformed extraembryonic ectoderm and ectoplacental cone.
(Figure 2). The frequency of abnormal embryos scored by phenotype was 8/66 (12%).

At d9 only two treatment regimens, 90 mM LiCl for 5 hours or 300 mM LiCl for 5 minutes resulted in abnormal embryos and resorptions (Table 1). Lower concentrations of LiCl and 300 mM KCl had no apparent effect. In a number of cases, we treated 8 cell CD1 embryos with LiCl, cultured them \textit{in vitro} to the blastocyst stage, and transferred them into the uteri of pseudopregnant mothers. This was done to determine whether the epigenetic effects were specific to the two cell stage. The frequency of abnormal d9 embryos which resulted from treatment of 8 cell embryos was significantly higher than that obtained with treated 2 cell embryos, although the spectrum of defects observed was the same (Table 2).

<table>
<thead>
<tr>
<th>Figure 1</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G(\text{H})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Cell</td>
<td>1 (4.5%)</td>
<td>3 (13%)</td>
<td>3 (13%)</td>
<td>3 (13%)</td>
<td>6 (26%)</td>
<td>2 (8.5%)</td>
<td>5 (22%)</td>
</tr>
<tr>
<td>8-Cell</td>
<td>2 (10%)</td>
<td>1 (5%)</td>
<td>5 (25%)</td>
<td>2 (10%)</td>
<td>2 (10%)</td>
<td>1 (5%)</td>
<td>7 (35%)</td>
</tr>
</tbody>
</table>

In a limited series of experiments, we examined the influence of genetic background on lithium teratogenesis. Table 1 shows that the lithium effect is significantly enhanced in LTXBO mice, a recombinant inbred strain of mice derived initially from LT/Sv and C57Bl\6. The phenotypes observed all fell into the more severely affected range with 8\(\text{I}\)2 abnormal embryos being of the type seen in Figure 1 D. Table 1 shows that the rate of implantation is the same in LTXBO and CD1 mice.
The resorptions at d9 consisted of embryonic remains typically seen as a large disorganized mass of embryonic tissue equivalent in size to a d7-8 embryo. The cause of death was not evident due to degeneration. No resorptions were observed in the controls. Although there appeared to be fewer resorptions at d7 than at d9, there was no significant difference by contingency Chi square analysis.

Abnormal embryos displayed a wide range of defects and could be divided into several broad groups based on the severity of the defects as illustrated by examples in Figure 1. Embryos in group A represented the most severely affected while those in group H were the least affected. Table 2 shows the distribution of the defects observed. All of these defects can be characterized as a preferential loss of dorsal anterior structures. Groups A and B represented embryos that showed no evidence of primitive streak formation although they possessed well developed extraembryonic membranes (Figure 1A;B). These groups consisted of a few embryos that had either no epiblast (A) or that resembled d7.5 embryos with thickened endoderm and ectoderm (B). The latter also had an abnormal constriction that formed between the embryonic ectoderm and the extra embryonic ectoderm, a feature observed in embryos harbouring a homozygous mutation in HNF3β (Ang and Rossant, 1993). Embryos in group C showed some anterior/posterior axis formation. Embryos in this category had developed head folds, but lacked an allantois and somites and were phenotypically similar to early d8 embryos (Figure 1C). Embryos in group D had a more distinct head fold and neural tube and an enlarged allantois (Figure 1D). The underlying tissue in these embryos had no structure, and no somite formation was seen. Embryos in group E had distinct somites, but abnormal heads. The embryo shown in Figure 1E has fused somites across the midline, a feature shared by only one other
embryo in this study. Fused somites have been observed in $HNF3\beta^{-/-}$ mouse embryos (Ang and Rossant, 1994; Weinstein et al, 1994) and in $floating\ head$ mutant zebrafish embryos (Halpern et al., 1995). The $floating\ head$ mutation results in the loss of notochord and fusion of the somites along the midline. The remaining embryos reached the size equivalent of a d9.5 embryo, and possessed either an open anterior neuropore (Figure 1F), an open cranium (Figure 1G), or a pronounced lack of facial structures (Figure 1H). The embryo in Figure 1G lacked eye cups and showed facial malformations. Some embryos in this group retained eye cups and had normal faces. Finally as shown in Figure 1H, some embryos had lost their most anterior structures resulting in a cleft face. The cranium was closed as was the neural tube. The most severely affected embryos showed no or very little embryonic structures and no axial formation. Some embryos exhibited a truncated anterior-posterior axis and incomplete dorsal-ventral axis. In less severely affected embryos, dorso-anterior structures were more affected than posterior or ventral structures. Interestingly, of 13 pups born from the only litter that went to term from 2-cell treated embryos, all appeared outwardly normal, but 5 died within 48 hours.
Figure 1  Morphology at d9.5 of Lithium Treated Two Cell Embryos

A: Represent embryos which showed no evidence of an epiblast although they possess well developed extra embryonic membranes

B: Resemble d7.5 embryos with thickened endoderm and ectoderm and an abnormal constriction between the embryonic ectoderm and the extra embryonic ectoderm. (arrow)

C: Embryos show some anterior posterior axis formation with abnormal head folds, and no allantois. Phenotypically they are similar to early d8.5 embryos.

D: Distinct head fold and neural tube are present over a disorganized cell mass. An enlarged allantois is present.

E: Distinct somites, but abnormal heads. Two embryo, including the one shown have fused somites across the midline. The remaining embryos reached the size equivalent of a d9.5 embryo.

F: Normal except for an open anterior neuropore. (arrow)

G: All embryos have open craniums (top arrow) and some are lacking eye cups and showed facial malformations. (bottom arrow)

H: The embryos have a cleft face and severe cases are lacking eye cups.

I: Control d9.5 embryo

a= allantois, hf= head fold, nf= neural fold, nt= neural tube, s= somites, ys= yolk sac
Scale Bar= 100 mm
Brachyury expression in d7.5 embryos treated with LiCl

The phenotypes of LiCl treated embryos suggested that gastrulation was affected and the abnormalities observed were due to changes in axial patterning. To study this further we looked at the expression of an axial midline gene, *brachyury*, in LiCl treated d7 embryos. The *brachyury* gene is expressed in the primitive streak, commencing at approximately d6 of gestation, and later, its expression is restricted to the notochord (Herrmann et al., 1994). We subjected both lithium treated embryos and embryos from natural matings to RNA whole mount in situ hybridization at the same time, with the same probe, under identical conditions. Figure 2 shows the results of these experiments. LiCl treatment at the 2 cell stage caused a general reduction in *brachyury* expression at d7, although there was no evidence of ectopic expression. Expression was always midline, but in about 40% of embryos, expression was weak and sometimes diffuse, suggesting that fewer cells were expressing *brachyury* in treated embryos. Both morphologically normal and abnormal embryos exhibited reduced expression.
Figure 2 Brachury expression in lithium treated 2-cell embryos at d7.5.

All embryos were dissected out at d7.5 and then stained with antisense or sense brachyury. The sense probe did not result in any signal. (Data not shown). The antisense results are illustrated below. All hybridization times, temperatures and washes were identical for the controls and the LiCl treated embryos.

Figure 2A; All 4 embryos are from treatment of 2 cell CD1 embryos with 300 mM LiCl for 5 min. The 2 embryos on the left show normal phenotype with weak brachyury expression. The smallest embryo is abnormal for both phenotype and brachyury expression and the last embryo has very weak brachyury expression in the distal area, and lacks significant expression in the proximal posterior of the embryo.

Figure 2B; Control d7.5 embryos stained with brachyury antisense probe as in A.
2.5 Discussion

The mechanism by which LiCl respecifies axial patterning is unclear, and may be due either to a change in a maternally inherited dorsal determinant, or to a change in the competence of cells to respond to inductive signals. We tested these alternatives by treating cleavage stage mouse embryos with LiCl, reasoning that, since they lack maternally inherited axial determinants, any effects on axial development must have been mediated by changes in cellular competence. Our results clearly support this latter view. A short treatment with LiCl at the 2 cell or 8 cell stage causes mouse embryos to develop axial defects similar to those observed in some mutations that adversely affect gastrulation.

Lithium Treatment Affects Gastrulation in Mouse Embryos

LiCl appears to cause phenocopies of gastrulation mutations, notably *HNF3β* -/-.. Mutant embryos homozygous for an *HNF3β* null allele lack a node and notochord and one of the results is the induction of somites, but without proper patterning (Ang & Rossant, 1994, Weinstein et al., 1994). Similar observations were made in *floating head* mutant zebrafish embryos. Using mesoderm markers, the phenotype observed in *floating head* mutants was shown to be due to a respecification of midline mesoderm to paraxial fate. Therefore the precursor notochord cells are converted to a somite fate and the somites fuse along the midline underneath the neural tube (Halpern et al., 1995). It is clear from these studies that secondary structures like somites and neural tube can be induced in the absence of a notochord. The similarity of phenotypes observed in lithium treated embryos and axial midline mutants suggests that lithium disrupts axial
patterning. Thus, lithium can affect axial development even in embryos that lack a localized cytoplasmic axial determinant in the egg.

**Mouse and Amphibian Eggs are Different**

In many amphibians, the time between fertilization and gastrulation is short, only 10 hours in *Xenopus*, whereas in mouse embryos, gastrulation commences at approximately 6.5 days post fertilization. Early cleavage divisions in amphibians are rapid and synchronous, whereas in mouse embryos, the first cleavage alone takes 20 hours, and cell division is asynchronous from the beginning. Amphibians activate their embryonic genome at the mid-blastula transition (MBT) when there are approximately 4000 cells (Newport & Kirschner, 1982). In mouse embryos, the embryonic genome becomes activated as early as the second cell stage, and is accompanied by rapid degradation of maternal RNAs during the second cell cycle (Paynton et al., 1988, Latham et al., 1991a). For amphibian embryos it is possible to project a fate map on the newly fertilized egg, indicating the existence of some kind of positional information cues at this early stage. However, it is not possible to project a fate map onto mouse preimplantation embryos. Positional information with respect to embryonic pattern is not elaborated until just before gastrulation on d6 - d6.5 (Lawson et al., 1991). Finally, there is an approximately 500 fold increase in mass in mouse embryos between preimplantation stages and gastrulation stages (Rugh, 1968), leading to a comparable dilution of any surviving maternal components, while amphibian embryos maintain a constant mass throughout embryogenesis. Moreover, only a subset of preimplantation blastomeres contribute progeny cells to the embryo proper, with the others forming extraembryonic tissues. Because of this, the mammalian egg is not well suited to
the specification of body axes by a system of localized determinants in the egg, an observation amply supported by numerous experiments demonstrating the pluripotency of cleavage stage blastomeres and inner cell mass cells (Rossant, 1984, 1987). In addition, murine embryos degrade maternal mRNAs at the 2 cell stage, and there is a five day lag between the 2 cell stage (time of lithium treatment) and the onset of gastrulation (time when defects begin to appear). Thus, it is highly unlikely that maternally inherited RNAs and proteins directly affect mouse gastrulation. If such molecules are involved in mediating the effects of Li treatment, they must exert their effects very early, and those effects must be somatically inherited through approximately 12 - 14 cell divisions before any phenotypic consequences are realised.

**Lithium Acts Transiently**

Retention of lithium is also unlikely to be a factor. The effective concentration of LiCl is believed to be 0.5-2.5 mM (Breckenridge et al., 1987). Studies on human erythrocytes which were loaded with 40 mM lithium for 3 hours or 17.5 hours resulted in an internal lithium concentrations of 8% or 32.5% respectively, of the external concentration (Hughes, 1991). Therefore in our study the internal lithium concentration would be well below the 90 mM or 300 mM external concentration. Even assuming no net export of LiCl over the intervening 5 days, a 500 fold dilution would result in a concentration of LiCl below the measured effective concentration. It is formally possible that a small group of committed preimplantation cells either retains a damaged maternal component or maintains an effective concentration of lithium up to the point of gastrulation. However, as noted above, no positional information can be observed in the preimplantation embryos.
Cellular Competence and Chromatin Structure

The lack of axial determinants at the two cell stage, and the time lag between lithium treatment and developmental effects argues in favour of a somatically heritable change in the competence of two cell blastomeres. One mechanism to account for the effects of lithium on mouse embryos is a stable change in chromatin structure, possibly mediated by biochemical modifications (Thompson et al., 1995; Wolffe, 1994).

Nucleo-cytoplasmic interactions in cleavage stage mouse embryos which alter the competence of the genome have been demonstrated. Latham et al. (1991a, 1991b, 1994) have shown that one cell cytoplasm has a deleterious effect on the competence of eight-cell-blastomere nuclei to support development, and to express certain genes. During the 2nd cell cycle, 45% of approximately 500 proteins analyzed display increased expression, with the remainder showing either transient increases or decreases. It has been noted that a small fraction of proteins undergo changes in post translational modification, specifically phosphorylation during the second cell cycle (Latham et al., 1991a, Poueymirou and Schultz, 1987, Howlett, 1986). Any number of these proteins could be targets for second messenger induced enzymes such as kinases, and cause alterations in chromatin structure.

LiCl and the Inositol Second Messenger Pathway

Extensive biochemical investigation has revealed that lithium disrupts the inositol pathway, leading to an increase in the activity of PK-C (Berridge et al., 1982, 1989). Since manipulations
of inositol concentrations can either rescue (increased inositol - Busa and Gimlich, 1989), or mimic (depleted inositol - Cockcroft et al., 1992) the teratogenic effects of LiCl, there is reason to suspect that the inositol pathway is involved in the developmental changes brought about by lithium. Further, Otte and Moon (1992) showed that the overexpression of PK-C in *Xenopus* laevis causes ventral ectoderm to develop an equivalent competence for neural induction as dorsal ectoderm. The chromatin associated proteins topoisomerase I and II are activated by PK-C. The action of topoisomerase I and II is to alter the conformation of DNA and has been implicated in the activation of genes. Phorbol ester differentiation of cell lines is thought to act through the activation of protein kinase C and this differentiation is blocked by topoisomerase inhibitors (Cockerill and Garrard, 1986, Sahyoun et al., 1986). These and other chromatin proteins may be the targets of the LiCl sensitive inositol-PKC pathway and maybe subject to phosphorylation/ dephosphorylation through the activity of endogenous protein kinase/phosphatase cascades. These altered chromatin proteins may remodel the genomes of early cleavage stage blastomeres, thereby altering the competence of their cellular decedents.

Our understanding of chromatin structure is limited although there have been some interesting advances made in recent years. Studies of phenomena like position effect variegation (PEV) in *Drosophila* have revealed that a large number of genes may play a role in the formation of heritable chromatin states (Reuter et al., 1992). Several of these genes, referred to collectively as suppressors and enhancers of PEV, are DNA-binding or chromatin-binding proteins, and share homology with the Polycomb group of genes whose function is maintenance of proper homeotic gene expression patterns (Becker, 1994, Wolffe, 1994b). Other modifiers of PEV such as suvar(3)6 and suvar(2)1 are enzymes which modify the activity of other proteins;
su(var)6 is a protein phosphatase1 (Dombradi et al., 1989) and suvar(2)1 is probably a histone deacetylase (Reuter et al., 1992). Chromatin structure is not static and must undergo dynamic changes during each cell division. Agents that affect either structural or enzymatic components of the system might cause heritable changes that ultimately alter the developmental competence of cells many cell divisions later. If lithium is interacting with some of the enzymatic components of the cell which affect chromatin maintenance, this may explain the long range developmental consequences of exposure of cleavage stage murine embryos to lithium. Similar interactions may then be proposed in amphibian embryos, raising the possibility that the apparent alterations in positional cues may in fact reflect alterations in cellular competence.
Mouse blastocyst development *in vitro* is inhibited by the lack of NaCl in the culture medium and rescued by the addition of LiCl in a cell cycle dependent manner.
3.1 Abstract

Lithium chloride (LiCl) has profound effects on many stages of embryo development in many different organisms. LiCl is capable of inducing alterations in cell cycle, oocyte maturation, gastrulation and cell fate. Previously we have shown that the treatment of two cell mouse embryos with LiCl results in normal in vivo cleavage stage development followed by gastrulation defects 5 days post treatment. This delay in the lithium effect prompted us to investigate more closely the effect of LiCl treatment on cleavage stage embryos. Using an in vitro culture system we investigated its effect on the development of embryos to the blastocyst stage. We conclude that lithium has no adverse effect on the rate of blastocyst formation but that the lack of sodium results in arrested development.
3.2 Introduction

The classically described two cell block in development in mouse is strain specific and occurs when zygotes of some random bred and inbred strains are removed from the oviduct prior to or during S phase of the first cell cycle (Goddard and Pratt, 1983: Lawitts and Biggers, 1991). The result varies with each affected strain, with some undergoing first cleavage only, while others will undergo multiple divisions but will not proceed to the blastocyst stage. Depending on the strain and the culture medium used, partial rescue through the 2-cell block can occur, but this does not mean that the embryos will all develop to the blastocyst stage. Studies suggest that the two-cell block and the inability to form blastocysts *in vitro* is a complex genetic trait requiring protein synthesis (Wang and Latham, 1997).

Different culture media have been tested for their ability to allow embryos to proceed past the two-cell block, and more importantly to proceed to the blastocyst stage. For example, glucose is inhibitory from the 2 cell to morula stage, but is advantageous from the morula to blastocyst stage (Chatot et al., 1990). By lowering the glucose and decreasing the sodium and potassium levels, the 2-cell block was alleviated (Lawitts and Biggers, 1991). A later study by Summers *et al.* (1995) revealed that high glucose is not inhibitory in a lower sodium environment. Many of the early media had sodium concentrations of 140 mM which has proven to be detrimental. These high concentrations cause a decrease in protein synthesis, a requirement for development past the two-cell block. By decreasing the sodium concentration to 85 mM, an increase in the rate of protein synthesis and embryo development was observed. The most profound effect occurred if embryos were cultured in low sodium prior to the morula stage.
(Anbari and Schultz 1993). These studies clearly indicate a key role for sodium in determining *in vitro* embryo survival. Experiments that measured the rate of protein synthesis of 4-cell embryos grown in 125 mM NaCl or 85 mM NaCl showed a 55% reduction in protein synthesis at the higher sodium levels.

Becchetti and Whitaker, (1997) explored the effect of lithium on the PI pathway and the cell cycle in Sea Urchins. As expected, by replacing sodium with lithium in the seawater, treatment of zygotes within 3 hours of fertilisation caused an arrest at the 2-cell stage. Timed lithium injection studies and rescue with myo-inositol led to the conclusion that lithium is preventing nuclear envelope breakdown by interfering with the PI pathway.

We previously treated embryos from CD-1 mice with LiCl and transferred the embryos into pseudopregnant females (Rogers and Varmuza, 1996). The recovery rate of postimplantation embryos indicated that the treatment did not interfere with development up to the blastocyst stage *in vivo*. The apparent lack of effect on preimplantation stages was unexpected given the results of studies described above.

In the current study we show that the addition of 300 mM lithium chloride has no effect on the cell cycle as long as 90 mM NaCl is present. Further we are able to slow down and in some cases completely block the cell cycle of two-cell embryos by the removal of sodium, therefore extending the window of time that a two cell block can be induced. Partial rescue of this phenomenon with LiCl, but not KCl, mirrors the window established for the cytoplasmic injection rescue studies of Pratt and Muggleton-Harris (1988).
3.3 Materials and Methods

Mouse Strains

CD-1 mice (6 to 8 weeks old) were purchased from Charles River, and maintained in a 12 hour light/dark cycle, fed on commercial rodent chow and water *ad libitum*.

Treatment

Fertilised zygotes were obtained from superovulated females mated with males of the same strain. Females were superovulated by intraperitoneal injection of 5 IU of Pregnant Mares Serum Gonadotropin (PMSG) (Sigma) followed by a second injection of 5 IU of human Chorionic Gonadotropin (hCG) (Sigma) 46 hours later. Fertilised one or two-cell embryos were flushed from the oviducts of pregnant females at 18, 31, 36, 41 and 46 hours post hCG, and all embryos were cultured in PLG medium (90 mM NaCl, 4.8 mM KCl, 1.7 mM CaCl$_2$-H$_2$O, 1.18 mM KH$_2$PO$_4$, 0.57 mM MgSO$_4$, 0.55 mM Glucose, 0.25 mM Pyruvic Acid, 0.25% Lactic Acid, 0.25% BSA fraction V, 25 mM NaHCO$_3$) prior to and following treatment. Embryos were treated in PLG medium in which the 90 mM NaCl was replaced by 90 mM LiCl, KCl or CsCl for 5 hours. Alternatively embryos were cultured in PLG medium supplemented with 300 mM LiCl, or KCl for 5-6 minutes. Following treatment, embryos were washed four times in a large volume of PLG, and then cultured *in vitro* to monitor development to the blastocyst stage. All embryos were scored at 120 hours post hCG for development stage. Dead embryos were removed during the culture period.
Statistics

A contingency chi square test was performed using one degree of freedom. A p-value of 0.05 was considered significant.

Combined Nuclear and Fragmented DNA labelling:

Terminal transferase mediated DNA labelling (TUNEL) was carried out to determine if the embryos were undergoing death mediated by apoptosis or necrosis. Embryos were fixed in 4% paraformaldehyde diluted in phosphate buffered saline (PBS). Embryos were mounted on microscope slides (Fisherbrand Superfrost) and allowed to air dry. The slides were washed in PBS and preincubated in One-Phor-All Buffer (Pharmacia) supplemented with 0.1% triton-X. Embryos were overlaid with reaction cocktail of 0.1% triton X-100, One-Phor-All buffer, 0.25 U/ml terminal transferase (Pharmacia), 6 mM dATP, and 3 mM Bio-dUTP (Sigma). Reactions were carried out in a humidified chamber for 1 hour at 37°C. After washing slides in PBS, incorporated biotinylated nucleotides were detected with streptavidin-Texas Red conjugate (Calbiochem) for 30 min at 4°C. After three washes in cold PBS, slides were stained with 4,6-Diamidino-2-phenylindole (DAPI) (Sigma) at concentrations of 0.2 μM for 10 min. Slides were examined using a Leica fluorescent microscope. Assessment of cell death was recorded based on DNA condensation, fragmentation and nuclear morphology as previously described (Jurisicova et al., 1996).
3.4 Results

It has been well documented that some random bred and inbred strains of mice experience an inability to proceed to the blastocyst stage if they are removed from the uterine environment prior to or during S phase of the second cell cycle (Goddard and Pratt 1983). To characterize the developmental capacity of zygotes from the blocking strain of mice (CD1), we flushed out embryos at various times post hCG and scored embryos for the ability to form blastocysts on d4.5 (Table 1). As defined by Howlett (1986) we observed that the second cell cycle starts at approximately 28-30 hours post hCG (Figure 1). As expected, zygotes flushed out at 18 hours post hCG all failed to develop to the blastocyst stage (Table 1). At 31 hours post hCG, 28% of the embryos reached the blastocyst stage on d4.5, while 35% remained at the 2-8 cell stage. Embryos flushed at 36 hours post hCG showed a significant improvement in developmental potential with only 10% remaining at the two cell stage. Interestingly, embryos flushed at 41 and 46 hours post hCG did not exhibit any 2-cell block, with the majority of embryos reaching the blastocyst stage. These data indicate that two-cell CD1 embryos in our culture system do not progress to the blastocyst stage when flushed during or prior to S phase of the second cell cycle.

Lack of sodium, not the presence of lithium, induces a G2 block.

We carried out treatment of flushed embryos under two different regimens. Substitution of NaCl by other monovalent cations dramatically reduced the rate of development of two cell embryos to blastocyst stage (Table 1), although lithium seemed to afford some measure of
protection from the loss of sodium. In contrast, supplementation of PLG with any of these ions in the presence of sodium does not alter development to the blastocyst stage (Table 1). The different regimens were chosen to assess preimplantation development because we previously reported that treatment of embryos by substituting sodium for lithium in the culture medium or by the addition of lithium while keeping the sodium level constant, both resulted in gastrulation defects (Rogers and Varmuza, 1996). Results presented here suggest that the loss of sodium rather than the addition of other ions, is responsible for the induced two-cell block, and that lithium is able to substitute for NaCl, at least partially.

A prolonged block leads to cell necrosis.

Embryos exposed to medium that lacks sodium resulted in a block at the 2-8 cell stage. These embryos survive in culture up to 110 hours post hCG without dividing and appear indistinguishable from untreated two cell embryos. At 110 hours post hCG the embryos begin to degrade. We performed TUNEL on blocked embryos to assess whether apoptosis or necrosis is responsible for cell death. The extensive cytoplasmic and nuclear staining for both DAPI and TUNEL were observed in all samples. This suggests that necrosis, not apoptosis is the cause of cell death (Jurisicova et al., 1996).
Table 1. Effect of zygote age and culture medium composition on blastocyst formation.

<table>
<thead>
<tr>
<th>Hour post hCG</th>
<th>Ion</th>
<th>Blastocyst (%)</th>
<th>Morula (%)</th>
<th>2-8 cell (%)</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>18 (control)</td>
<td>90 mM NaCl, 4.8 mM KCl</td>
<td>0</td>
<td>0</td>
<td>14 (92)</td>
<td>15</td>
</tr>
<tr>
<td>31 (control)</td>
<td>90 mM NaCl, 4.8 mM KCl</td>
<td>22 (28)</td>
<td>29 (37)</td>
<td>28 (35)</td>
<td>79</td>
</tr>
<tr>
<td>31</td>
<td>0 mM NaCl/ 4.8 mM KCl/ 90 mM LiCl</td>
<td>1 (1)*</td>
<td>22 (29)</td>
<td>54 (70)</td>
<td>77</td>
</tr>
<tr>
<td>36 (control)</td>
<td>90 mM NaCl, 4.8 mM KCl</td>
<td>28 (48)</td>
<td>24 (42)</td>
<td>6 (10)</td>
<td>58</td>
</tr>
<tr>
<td>36</td>
<td>0 mM NaCl/ 94.8 mM KCl</td>
<td>0 (0)*</td>
<td>0</td>
<td>99 (100)</td>
<td>99</td>
</tr>
<tr>
<td>36</td>
<td>0 mM NaCl/ 4.8 mM KCl/ 90 mM LiCl</td>
<td>6 (4)*</td>
<td>112 (68)</td>
<td>44 (28)</td>
<td>162</td>
</tr>
<tr>
<td>41 (control)</td>
<td>90 mM NaCl/4.8 mM KCl</td>
<td>253 (68)</td>
<td>119 (32)</td>
<td>0 (0)</td>
<td>372</td>
</tr>
<tr>
<td>41</td>
<td>0 mM NaCl/ 94.8 mM KCl</td>
<td>0 (0)*</td>
<td>1 (1)</td>
<td>115 (99)</td>
<td>116</td>
</tr>
<tr>
<td>41</td>
<td>0 mM NaCl/ 4.8 mM KCl/ 90 mM LiCl</td>
<td>91 (44)</td>
<td>103 (50)</td>
<td>13 (6)</td>
<td>270</td>
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<tr>
<td>41</td>
<td>90 mM NaCl/ 4.8 mM KCl/ 300 mM Choline Cl</td>
<td>50 (90)</td>
<td>6 (10)</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
<td>41</td>
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<td>17 (89)</td>
<td>2 (11)</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>41</td>
<td>90 mM NaCl/ 4.8 mM KCl/ 300 mM LiCl</td>
<td>162 (77)</td>
<td>27 (13)</td>
<td>21 (10)</td>
<td>210</td>
</tr>
<tr>
<td>46 (control)</td>
<td>90 mM NaCl/4.8 mM KCl</td>
<td>17 (89)</td>
<td>2 (11)</td>
<td>0 (0)</td>
<td>19</td>
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<td>56</td>
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<tr>
<td>46</td>
<td>0 mM NaCl/4.8 mM KCl/ 90 mM LiCl</td>
<td>99 (86)</td>
<td>15 (13)</td>
<td>1 (1)</td>
<td>115</td>
</tr>
</tbody>
</table>

a) $\chi^2$ Test; P<0.05

*Significantly different from controls.

b) Dead embryos were removed during cell culture. Average 5% per group.
Figure 1.

The timing of the cell divisions is based on Howlett (1986) and confirmed by observing a population of CD-1 embryos in vitro. Fertilization takes place about 8 hours post hCG injection in CD-1 mice with the first cell division occurring at 28 hours post hCG.
Figure 2.

Murine embryos flushed at 42 hours and treated with 90 mM LiCl for 5 hours were cultured for 72 hours followed by TUNEL and DAPI staining. DAPI staining is diffuse which is characteristic of necrosis. The same embryo, after terminal transferase mediated DNA end labelling (TUNEL) using biotinylated nucleotides and streptavidin-Texas Red conjugate, indicates necrosis and not apoptosis.
3.5 Discussion

Mouse embryos will develop a cleavage stage block at the second cell cycle if exposed to PLG medium (+NaCl) prior to early G2 phase of the second cell cycle. A similar block can be induced by replacement of sodium in the medium with other ions at late G2 phase of the second cell cycle. This later block can be partially rescued by the addition of LiCl but not KCl or CsCl. These results contrast with those of Becchetti and Whitaker (1997) who observed that the replacement of sodium with lithium in the sea water of sea urchin embryos blocked the first cell cycle.

The timing within the cell cycle with respect to the sodium and lithium sensitivity is consistent with studies showing stage specific positive effects of either the oviduct environment or the state of the cytoplasm on the success of in vitro blastocyst development. Minami et al., (1992), showed that embryos co-cultured with oviducts during mid to late G2 phase of the second cell cycle developed to blastocyst four times more frequently than control embryos. Goddard and Pratt (1983) showed that CFLP outbred embryos require the oviduct environment up to 36 hours post hCG to successfully develop past the 2 cell stage in vitro. Flushing prior to this point resulted in 61% blocked embryos. Our results mirror these data.

The inability to develop to the blastocyst stage can be rescued by the injection of cytoplasm of non blocking embryos (Pratt and Muggleton-Harris 1988). These authors showed that the best rescue occurred when the recipients (blocking strain) were in early G2 phase of the second cell cycle. The donor cytoplasm was also most effective at G2 phase of the second cell
cycle. These studies emphasize the importance of G2 phase of the second cell cycle in determining success of blastocyst development, and suggest that a cell cycle regulated zygotic gene may be expressed at this time that promotes further cleavage. In contrast, we were able to flush embryos at 41 and 46 hr post hCG, and re-establish the two cell block by the transient removal of sodium from the culture medium. Of the monovalent cations tested, only lithium is able to replace sodium. In our previous study, a similar removal of sodium followed by transfer of the embryo into the oviduct of pseudopregnant females also rescued the development of the embryos as judged by the rate of implantation measured at d7 autopsy (Rogers and Varmuza, 1996).

It is possible that the change in osmolarity brought about by the treatment could account for the developmental block, as increased osmolarity causes inhibition of mouse embryo development (Dawson and Baltz, 1997). However this is an unlikely explanation because the replacement of sodium with potassium keeps the osmolarity unchanged from that of the controls, but results in an increase in the frequency of 2-cell block. In contrast, the addition of 300 mM ion has no effect on development, although the short duration of this treatment may account for the lack of affect.

Our data suggest a dependence of the two cell embryo on sodium during the first half of the second cell cycle for proper development. The fact that the requirement for sodium and the partial rescue by lithium is cell cycle dependent and follows the same pattern as that for oviduct factors and cytoplasm injections, indicates a common mechanism. The rescue effect of lithium could come simply from the fact that lithium is able to substitute for sodium. Richelson (1977)
demonstrated that lithium can substitute for sodium in mouse neuroblasts to induce an action potential. Richelson also suggested that lithium is using the sodium channel to enter the cell. The same has been shown in erythrocytes (Hughes, 1991).

The second cell cycle is characterised by extensive maternal RNA degradation (Paynton et al., 1988) and major zygotic genome activation (Latham et al., 1992). The importance of protein synthesis in cell cycle progression was demonstrated by Wang and Latham (1997) who showed that protein synthesis is required for proper embryonic gene activation at the two cell stage, possibly through the recruitment of maternal mRNAs encoding transcription factors. Maternally inherited stores of the transcription factors Sp-1 and TATA binding protein (TBP) are limiting in the two cell embryo and protein synthesis is required to maintain levels of these transcription factors (Ho et al., 1995). Therefore the progression of the cell cycle may require a minimum level of a specific protein. In vitro culture conditions may not be optimal for protein synthesis for some strains of mice. Non-blocking strains may have higher levels of critical mRNAs than blocking strains that compensate for the low levels of protein synthesis that occur in culture. These high levels of mRNA may be due to either more efficient zygotic gene activation or a higher level of stored maternal RNA. Thus the excess mRNA provides the embryo with the critical edge to push it into M phase. The oviduct factors reported in other studies (Minami et al., 1992), and the manipulations of the medium that results in blocking strains proceeding past the block (Du and Wales, 1993), may be having the indirect effect of improving the efficiency of transcription and/or protein synthesis. Indeed Ho et al., (1994) and Anbari et al., (1993) report that altering sodium concentration increases the stability of RNA and protein synthesis. Our studies, like those above, show that the G2 phase of the second cell cycle

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is a critical point in determining the survival of an embryo past the two cell stage and that sodium plays a critical role at this point in determining successful in vitro embryo development.
LiCl disrupts axial development in mouse but does not act through the β-catenin/Lef-1 pathway.
4.1 Abstract

*Xenopus* axes are determined early due to the asymmetric distribution of maternally derived factors in the one cell zygote. In contrast, chimera and cell marking studies suggest that axial determination in mouse embryos occur at post implantation stages. In our earlier study we used lithium chloride (LiCl) to perturb development of mouse axes. Here we investigate whether the lithium induced axial defects in mouse are being mediated by the β-catenin/Lef-1 pathway as in *Xenopus*. In lithium treated embryos we did not observe any changes in the amount or localization of β-catenin protein. Furthermore, the lack of Lef-1 mRNA in treated and untreated embryos indicates the LiCl induced axial defects in the mouse are not mediated by the β-catenin/Lef-1 pathway.
4.2 Introduction

The establishment of the dorsal-ventral and anterior-posterior axes is one of the primary events of embryonic development. This complex process is well characterized in *Xenopus*, which has become a paradigm for studies in other organisms. The *Xenopus* embryo undergoes a cortical rotation which is key in setting up an asymmetry of maternal determinants within the one cell embryo. Molecules in the vegetal cortex are moved towards the dorsal-vegetal area, where they activate zygotic genes at the Mid Blastula Transition (MBT), leading to the formation of the Spemann organizer (Ding et al., 1998, Heasman, 1997). Members of the Wnt/β-catenin signaling pathway are candidates for the maternal dorsal determinants, specifically GSK3β (Marikawa et al., 1997). The addition of a dominant-negative GSK (DN-GSK) to ventral vegetal blastomeres induces a second axis, thus establishing a negative regulation role for GSK-3β (He et al., 1995, Dominguez et al., 1995). Studies by Larabell et al., (1997), Pierce and Kimelman, (1995) and Pai et al., (1997) have all shown that GSK-3β acts by targeting β-catenin for degradation. Inactivation of GSK3β leads to the stabilization of β-catenin (Yost et al., 1996) followed by its association with Lef-1 and the translocation of the β-catenin/Lef-1 complex to the nucleus where it acts as a transcription factor (Behrens et al., 1996, Huber et al., 1996, Molenaar et al., 1996). One of the downstream targets of the β-catenin/Lef-1 complex in *Xenopus* is siamois (Fan and Sokol, 1997, Brannon et al., 1997). The action of the β-catenin cell signaling pathway, via siamois, is to activate genes of the Spemann organizer. A similar β-catenin cell signaling pathway seems to be present in the mouse. Huber et al., (1996), using mouse ES cells, showed that increased β-catenin protein levels induced Lef-1 protein and
a β-catenin/Lef-1 complex translocated to the nucleus.

LiCl has been very useful in the study of dorsal axial development and the wnt signaling pathway. In *Xenopus* and *Zebrafish*, lithium disrupts the formation of the midline axis with a characteristic gain of dorsal-anterior structures (Kao et al., 1986, Schneider et al., 1996). Injection of lithium into the ventral vegetal cells of a 32-cell *Xenopus* embryo results in axis duplication (Kao et al., 1986). The lithium phenotype mimics the phenotype seen by injection of various members of the wnt pathway, including wnt, dsh, DN-GSK3β, β-catenin, siamois and Xtcf-3 (Reviewed in Heasman, 1997). Although targets of LiCl are diverse (Berridge and Irvine, 1989, Berridge et al., 1989) it was established by Klein and Melton (1996) that the mechanism is likely mediated by the inhibition of the GSK3β. There is also evidence that the inositol pathway plays a role, probably by regulating other members of the wnt-pathway (Hedgepeth et al., 1997).

We previously used LiCl as a tool to study mouse axial development. Although treatment does result in axial defects, significant differences in the mouse occur which merit further study. First, treatment of cleavage stage mouse embryos resulted in a loss of dorso-anterior structures, not the over specification of dorsal mesoderm as was observed with the treatment of cleavage stage *Xenopus* embryos. Second, treatment of 2-cell embryos was followed by a 5 day lag before observation of defects. Preimplantation stages and extraembryonic tissues developed normally but primitive streak formation was truncated, as observed by reduced brachyury staining (Rogers and Varmuza, 1996).

The fact that treatment of 2-cell embryos resulted in axial defects was surprising. Many
studies have demonstrated the totipotency of early mouse blastomeres, which suggests that there is no axis determining asymmetry found in cleavage stage embryos (Tsuoda et al., 1997, Rossant and Vijh, 1980). In a recent paper, Gardner (1997) suggested that morphological landmarks exist which can be related to the anterior-posterior axes in the mouse, although no molecules have been associated with these early embryo asymmetries. In this paper we investigate the possible role of β-catenin in mediating the axial defects caused by the treatment of two-cell mouse embryos with LiCl. Our results strongly suggest that a wnt/β-catenin signaling pathway similar to the one involved in early Xenopus axial determination is not responsible for the lithium mediated axial defects in the mouse.
4.3 Experimental procedures

Immunohistochemistry

Whole mount immunostaining was carried out using Transduction Labs monoclonal antibody to β-catenin according to Huber et al., (1996), which allows detection of nuclear β-catenin. Mouse embryos were fixed in 0.5% paraformaldehyde in PBS for 20 min and post fixed in methanol (-20°C) for 10 min followed by permeabilization for 10 min in 0.5% Triton X-100 in PBS. Embryos were blocked for 30 minutes in 10% swine serum/PBS and primary antibody incubation was carried out at room temperature for 60 minutes. Primary antibodies against β-catenin were either a monoclonal antibody (Transduction Laboratories) or a polyclonal antibody (provided by Dr. B. Gumbiner) and were diluted in 1% swine serum/PBS. Anti PCNA (proliferating cell nuclear antigen) monoclonal antibody was used as a control (Cedarlane). Embryos were washed in PBS/1% serum/ 0.1% Triton X-100/ 0.5 mM MgCl2, 5 times for 10 minutes each. Samples were incubated in biotin conjugated secondary antibody (Multilink:DAKO). Incubation times were the same as for the primary antibody, and samples were washed as described above. After the washes, detection was carried out using Texas Red conjugated streptadivin. Following incubations with Texas red, samples were washed 3 times for 5 min followed by a 5 min incubation with 10 µm 4’6’-Diamidine-2 phenolinodole (DAPI). A second procedure with extended incubation times was also used to increase the sensitivity. Antibody incubations were done at 4°C overnight and washes were extended to 5 X 1 hr. Further alterations included an extended methanol post fix, up to 3 hours, which also helps to permeabilize the cells (Marikawa et al., 1997). In some cases the Triton X-100 step was extended for 2 hours.
Western blot

ES cells were treated with 300 mM LiCl for 5 minutes, washed and cultured for various times before harvesting. Protein extraction, gel electrophoresis and western blot procedures were performed according to Sambrook et al., (1989). All protein samples were quantitated using the Bradford reaction (Bradford, 1976). Following preincubation in 3% milk powder/PBS/0.1% Tween-20, membranes were incubated in the polyclonal primary antibody diluted in 3% milk powder/PBS/0.1% Tween-20 for 2 hours at room temperature. Filters were washed 5 X 10 minutes in PBS/tween-20 followed by incubation with biotinylated Multilink secondary antibody for 2 hours at room temperature and washed again. Detection was carried out according to the Amersham-ECL protocol.

RT-PCR

Embryos were harvested at 0 hr, 3 hr, 6 hr, and 12 hr. post treatment. Five treated or control embryos with the zona intact were lysed in 4 M GITC solution (Sambrook et al., 1989) and precipitated with ethanol. The RNA pellet was resuspended in 20-50 μl reverse transcriptase buffer (Gibco/BRL). Each reaction consisted of 25 μl total volume with 2μm of each gene specific primer (Lef-1 or PP1cy), 2 μm dNTPs, 1X PCR Buffer (Gibco/BRL), 20 units Taq polymerase (Gibco/BRL) and 2μl of RNA. All reactions were carried out at the melting temperature of the primers for 30 cycles. To control for genomic amplification, 2-cell embryos or blastocysts were lysed in GITC (Sambrook et al., 1989) and used in a reverse transcription reaction without the addition of reverse transcriptase enzyme. The sample was subjected to PCR as described above. D7.5 mouse embryo RNA was used as a positive control and untreated ES
cell RNA was used as a negative control for Lef-1. PP1cy primers were used to control for the quality of cDNA. Primer sequences are as follows:

PP1cy upstream: 5' gctgtggaaaaacgtcacag 3',
PP1cy downstream: 5' aaccatttcagcacatggc 3',
Lef-1 upstream: 5' agcgcctggatccgataggc 3,'
Lef-1 downstream: 5' tacatgtcaaatgggtcc 3'.
4.4 Results and Discussion

Under conditions which favor dorsalization in *Xenopus*, the wnt pathway triggers the inactivation of GSK3β and this leads to an increase in and subsequent translocation of the β-catenin and Lef-1 proteins to the nucleus (Figure 1). Together they cause the activation of siamois which leads to the activation of Spemann organizer genes like *gooseoid* (Gsc). Lithium has the ability to cause a respecification of ventral mesoderm into dorsal mesoderm in *Xenopus* by inactivating GSK3β ectopically. This inactivation of GSK3β by lithium allows the β-catenin protein to become stable, resulting in an ectopic β-catenin signaling (Schneider et al., 1996).

In order to determine whether lithium has a similar effect on mouse development, thus implicating the GSK3β/β-catenin pathway in mouse axial development, we previously treated two cell or eight cell mouse embryos with LiCl and observed axial defects. However the defects were opposite to those found in *Xenopus* embryos. Instead of an enhancement of dorso-anterior structures we observed a loss of dorso-anterior structures, and decreased *brachury* expression (Rogers and Varmuza 1996).

It has been well documented that lithium treatment of amphibian embryos at gastrulation results in a loss of dorso-anterior structures. (Nieuwkoop, 1970 and references within; Yamaguchi and Shinagawa 1989). Christian and Moon (1993) and Fredieu et al., (1997) have shown that *Xwnt-8* is capable of ventralization of the *Xenopus* embryo, and that lithium can phenocopy the effects of overexpression of *Xwnt-8*. Thus both pre-MBT lithium treatment, and post MBT lithium treatment phenocopies over expression of Xwnts. Whether or not the downstream elements of the post MBT Xwnt activity consist of GSK, β-catenin and siamois has
yet to be determined. In our study we looked at the expression patterns of β-catenin and Lef-1 to determine whether this part of the pathway is involved in lithium mediated axial defects in mouse embryos.

**Localization of β-catenin in the mouse preimplantation embryo**

In *Xenopus*, treatment with LiCl at the 16-cell stage leads to the ectopic translocation of β-catenin, shortly after MBT (Schneider et al., 1997). We investigated whether a similar mechanism might be involved in mouse gastrulation by treating 2-cell embryos with LiCl and looking at β-catenin translocation at various times post treatment. We expected that if lithium is acting through GSK3β and β-catenin to disrupt axis development, as it does in *Xenopus*, that we should see 1) LiCl induced translocation of β-catenin to the nucleus; 2) lithium induced stabilization of β-catenin and an increase in protein levels; 3) presence of Lef-1 in the embryo at the time of lithium treatment and β-catenin translocation; and 4) induction of Lef-1 due to the stabilization of β-catenin by LiCl (Huber et al., 1996).

A monoclonal antibody (Transduction Labs), and two different antibody incubation protocols (Huber et al., 1996 and extended incubation times) were employed to investigate the localization of β-catenin throughout preimplantation development. Treatment regimens are listed in Table 1. We used an antibody to proliferating cell nuclear antigen (PCNA) as a positive control for nuclear staining. We also performed similar experiments with mouse ES cells. Results for each antibody and each method are indistinguishable.
Table 1  Embryo age at time of lithium treatment and β-catenin detection times.

<table>
<thead>
<tr>
<th>Embryos/ES cells</th>
<th>Treatmenta</th>
<th>Ab Detectionb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (hours post hCG)</td>
<td></td>
</tr>
<tr>
<td>2 cell</td>
<td>42 hours</td>
<td>0,10,30,60,120 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4,6,8,12,14,20,24,48,120hr.</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>130 hours</td>
<td>0,10,30,60,120 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12,24 hr.</td>
</tr>
<tr>
<td>ES cells</td>
<td></td>
<td>0,10,30,60,120 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12,24 hr.</td>
</tr>
</tbody>
</table>

a) Treatment consisted of exposure of embryos/cells to 300 mM LiCl in PLG for 5 min followed by extensive washing to remove LiCl and culture for indicated times (column 3) in PLG.

b) All time points contained 10-15 embryos or 100+ cells and were tested three times. Two Ab detection protocols, a short incubation with the primary Ab (Huber et al., 1996) or a long incubation (4°C 16 hours) were employed. Thus each time point was tested 6 times. For each time point untreated control samples were also tested.

Treatment of 2-cell embryos with LiCl did not result in the expected nuclear translocation of β-catenin at any time following treatment. Figure 2A shows a mouse 2-cell embryo stained with DAPI to reveal the position of the nuclei and Figure 2B is the same embryo incubated with anti β-catenin antibody. In all cases studied the results are similar. The mouse two cell embryo has strong plasma membrane staining but no nuclear staining. Embryos
cultured in vitro for 24 and 48 hours developed to the 4 cell and 16 cell stage. All of these embryos had strong membrane staining without nuclear staining (data not shown). We never observed nuclear translocation, slowing of development or any gross morphological changes in the treated samples versus the untreated embryos. Blastocysts treated at 130 hours post hCG with LiCl and immunostained at various times all gave the same results. Strong membrane staining occurred but no nuclear staining. Figure 2C & 2D show a treated blastocyst immunostained six hours after lithium treatment. DAPI staining shows the position of the nuclei. All LiCl treatments, including untreated controls, and antibody staining protocols, produced indistinguishable results.
Huber et al., (1996) showed that embryonic stem (ES) cells are negative for Lef-1 and positive for β-catenin. In ES cells that are null for the β-catenin adhesion partner, E-cadherin, the cytoplasmic pool of β-catenin is increased and Lef-1 expression is positive, suggesting that Lef-1 is inducible by high levels of β-catenin. We treated wild type ES cells with lithium chloride. If lithium causes a stabilization of free β-catenin then we expected to see an induction of Lef-1 and nuclear localization of β-catenin. Figure 2E and F show treated ES cells immunostained at 6 hours post treatment. These results are the same for all time points and are identical to untreated samples. There is no indication of nuclear staining. Figure 2G and H show the results of immunostaining of blastocysts with an antibody to PCNA, a protein localized to the nuclei of dividing cells. Positive nuclear staining was obtained using the same conditions and secondary antibody as in the β-catenin immunostaining experiments. We were unable to detect any nuclear translocation of β-catenin suggesting that lithium may not be using the GSK3β/β-catenin pathway to cause axial disruptions in mice.

Levels of β-catenin protein remain constant in lithium treated ES Cells

The results from the immunostaining experiments indicate that lithium may not be acting through the β-catenin pathway. The lack of nuclear staining in our lithium treated mouse embryos could be the result of increased β-catenin protein levels in the absence of Lef-1 induction, resulting in an inability of β-catenin to move into the nucleus. It is also possible that β-catenin protein levels are not increasing, and this may account for the lack of nuclear localization. Larabell et al., (1997) found that the treatment of Xenopus embryos with lithium resulted in an increase of β-catenin protein levels detectable by western blot analysis. To
investigate whether lithium treatment of mouse embryos is inhibiting the ability of GSK-3β to degrade β-catenin, we treated ES cells with lithium, isolated protein at 3, 6, 12 and 24 hours post treatment and carried out western blots. All experiments consist of equally loaded lanes. All protein samples were quantitated using the Bradford reaction (Bradford, 1976) protein quantitation method. The western blot was repeated twice using independently treated samples. The results are shown in Figure 3. There is no detectable change in the levels of β-catenin at any time point in relation to the controls. The results of the western blot correlates with those of the immunostaining; there appears to be no increase in β-catenin protein levels and no consequent nuclear translocation.

**Lef-1 is not present in preimplantation embryos or ES cells and is not induced by Lithium treatment**

Lef-1 has been shown to interact with β-catenin and probably causes the nuclear translocation observed in *Xenopus* (Behrens et al., 1996, Huber et al., 1996). It is possible that the Lef-1 protein is present in the 2-cell mouse embryo, but, in concert with the absence of free cytoplasmic β-catenin, would result in the absence of nuclear β-catenin staining. We tested for Lef-1 mRNA by RT-PCR. Only d7.5 embryo RNA contained Lef-1 mRNA. This is expected as Lef-1 had been shown previously to be expressed in the primitive streak (Oosterwegel et al., 1993). ES cells were negative, confirming the studies by Huber et al., (1996). All 2-cell embryos and blastocysts, both lithium treated and untreated, were negative. We carried out studies on embryos harvested at 3, 6 and 12 hours post treatment. Figure 4 shows the results of the 6 hour time point. These samples were subjected to PCR with a set of positive control primers (PP1c γ) to verify the integrity of the cDNA. Lef-1 is part of the tcf/lef family. Other
members of this family have been isolated in mouse and although there are no data indicating their presence in cleavage stage embryos, it is possible that these other forms are present in the two-cell embryo and went undetected in our assay. Null mice generated from Lef-1 or tcf-1 mutations result in late stage developmental defects in T cells, teeth and skin, without apparent defects in the body axis (Korinek et al., 1998).

Taken together, the results from the immunostaining, western blots, and Lef-1 RT-PCR detection all reinforce the idea that, although lithium is able to disrupt axial development in the mouse, it is not acting through the inactivation of GSK3β and the stabilization of β-catenin in preimplantation embryos. The lack of Lef-1 in treated embryos coupled with the lack of a detectable increase in β-catenin protein is consistent with the failure to observe nuclear staining in lithium treated embryos.

In vivo, lithium has its first detrimental effect in mouse embryos at the point of gastrulation, some 5 days after the initial treatment, with no apparent effect on preimplantation stages. As there is no evidence of maternal axis determining factors in mouse (Rossant and Vijn, 1980, Duncan et al., 1997), this raises an interesting question regarding the mechanism by which lithium mediates its teratogenic effects. Exposure to other substances at early stages has also been observed to result in delayed defects beginning at gastrulation (Holiday, 1991, Generoso et al., 1991). These studies and ours clearly suggest that the cleavage stage embryos are sensitive to epigenetic alterations that do not affect cell viability or development at early stages, but only become apparent at the onset of gastrulation several days later. Whether the epigenetic alterations are affecting an as yet unidentified maternal positional cue, or the
competence of embryonic cells through alterations in genome activity remains to be determined.

We examined the role of β-catenin in mouse axial development using LiCl, a well characterized teratogen. Lithium’s ability to disrupt GSK-3β and β-catenin activity is a powerful tool for determining the mechanism of axial development in frog and fish. The results reported here establish that a lithium treatment which alters the axial program in mouse does not act by way of β-catenin and Lef-1. These results also indicate that while these molecules are involved in the dorsalization of *Xenopus*, their role in mouse axial development still needs to be determined.
Figure 1. Regulation of β-catenin.

The activation of the wnt pathway causes the down regulation of GSK3β. Since the phosphorylation of β-catenin by GSK3β targets it for destruction by adenomatous polyposis coli (APC), the inactivation of GSK3β results in the stabilization of β-catenin (Zeng et al., 1997). β-catenin is then free to complex with Lef-1 and together they move to the nucleus and in *Xenopus*, activate genes of the dorsal organizer such as siamois. Further regulation of this pathway comes from the inositol pathway where Protein Kinase C (PK-C) has been implicated in the regulation of GSK-3β (Hedgepeth et al., 1997, Cook et al., 1996). Active PK-C stimulates GSK3β and leads to the degradation of β-catenin which results in the establishment of a ventral fate. LiCl is able to inactivate the inositol pathway by blocking the function of IMP’ase, an enzyme required for the recycling of the inositol pathway. This leads to a down regulation of PK-C and dorsalization of the mesoderm.
Figure 1
Figure 2. Immunohistochemistry of mouse cleavage stage embryos with anti-β-catenin antibody.

Cleavage stage embryos were treated with 300 mM LiCl and stained with anti-β-catenin monoclonal antibodies. All cells shown were stained for β-catenin protein 6 hours post LiCl treatment (B,D,F). Cells were counterstained with DAPI to highlight the position of the nuclei (A,C,E,G). A&B, 2 cell mouse embryo, C&D, blastocyst stage, E&F, ES cells. An antibody to Proliferating Cell Nuclear Antigen (PCNA) was used as a positive control for nuclear staining (H). In all cases the same secondary antibody and Texas Red detection was used. PCNA gave a positive result under the same incubation and wash conditions used for β-catenin protein detection. Arrows in paired images highlight same cells.

Figure 3. Western Blot detection of β-catenin protein.

ES cells were treated with LiCl and harvested at t= 0, 3, 6, 12, 24 hours post lithium treatment. Equal amounts of protein were loaded in each lane of a SDS-PAGE gel and subjected to western blot procedures using the anti-β-catenin monoclonal Ab.

Figure 4. Lef-1 is not expressed in preimplantation embryos. RNA was extracted from 2-cell embryos (lanes 3,4,5,6) blastocysts (lanes 7,8,9,10), ES cells (lane 11) and d7.5 embryo (lane 12) and subjected to RT-PCR with Lef-1 primers. Preimplantation embryos and ES cells were treated with LiCl (lanes 4,5,9,10,11) or untreated (lanes 3,4,7,8) before RNA extraction. PCR amplification of 2-cell (lane 13) or blastocyst (lane 14) untreated extracts without the addition of RT controlled for genomic contamination. Lane 1 contains DNA size markers and lane 2 contains PCR control (no template). The asterisk indicates the expected Lef-1 product.
D2 cyclin is an early marker of neural differentiation and is down regulated in msd mutant mice.
5.1 Abstract

During vertebrate gastrulation the neuroectoderm is formed in response to signals emitted from the underlying mesoderm (reviewed in Bronner-Fraser and Fraser, 1997). The early neural tube consists of a zone of proliferating cells (ventricular zone) and an outer layer of cells (mantle) which have migrated from the ventricular zone and have begun to undergo differentiation. We isolated the D2 cyclin gene and demonstrate that it is a marker for early neural differentiation. D2 cyclin first appears in the ectoderm overlying the expanding mesoderm wings followed by a restricted expression in the neural tube and the presumptive cerebellum.
5.2 Introduction

Mouse embryo development progresses in a manner different from that of non-mammalian vertebrate embryos. Immediately after blastocyst formation and implantation the embryo increases in size due to extensive cell proliferation of the extraembryonic ectoderm and the ectoplacental cone. During this time the newly formed mesoderm induces the overlying ectoderm to adopt a neural fate. During gastrulation and early neural development cell proliferation continues to play an important role. For example the epiblast must divide rapidly to ensure proper primitive streak formation. Proliferation is the driving force underlying morphogenetic movements which result in proper neural tube closure (Bellomo et al., 1996, Poelmann, 1980, Copp et al., 1988).

Development of different regions of the mouse central nervous system (CNS) proceeds in a similar manner. The formation of two distinct cell layers occurs, the ventricular zone and the mantle. The ventricular zone is an inner layer of proliferating cells. The mantle consists of cells that have exited the cell cycle, and migrated to the outer edge of the neural tube where they become competent to respond to differentiation signals. In the mouse the proliferation of neural tube precursor cells starts at d7.75 and proceeds to about d10 (Kauffman, 1968, Poelmann, 1980), when they begin to exit the cell cycle and migrate to the lateral edge of the developing neural tube and commence differentiation (Lee et al., 1994). In the cerebellum differentiation begins later with cells exiting from the cell cycle and commencing differentiation at d9.5-13.5 (Ben-Aire et al., 1997, Steindler and Trosko, 1989).
Growth factors are involved either directly or indirectly in the proliferation, induction and patterning of the mesoderm, ectoderm and neuroectoderm. Cell proliferation is controlled by extracellular signals during G1 phase of the cell cycle. The cells respond to these signals, both stimulatory and inhibitory, by way of a distinct set of serine/threonine kinases, termed cdk for cyclin dependent kinases due to their association with short lived regulatory proteins referred to as cyclins. Four mammalian G1 cyclins have been characterized-D1, D2, D3, and E. Each of the D cyclins is able to associate with one or more of kinases-cdk2, cdk4 and cdk 6 (Sicinski et al., 1995). Furthermore, the D cyclins seem unique as they respond directly to growth factor stimulation and less to normal endogenous cell cycle signals (Matsushime et al, 1991, Kato and Sherr 1993). The critical response period for the D cyclins is in G1, at START, as defined in yeast. Past this point the cell is no longer dependent on growth factors to continue the cell cycle (Merill et al., 1992).

We isolated the D2 cyclin gene during our screen to obtain mesoderm dependent genes. To better understand the role of D2 cyclin during gastrulation we investigated mRNA expression patterns from embryonic d4.5 to embryonic d17.5. We are able to demonstrate that D2 cyclin is an early marker of the CNS. At later stages of development, D2 cyclin down regulation correlates with the commencement of differentiation. Thus our data suggest that D2 cyclin is under tissue specific and developmental specific regulation.
5.3 Material and Methods

Production of cDNA probes and Library Screen:

The library screen and the production of the cDNA probes were carried out according to the method of Varmuza and Tate (1992). cDNA from either wild type CD-1 embryos at d7.5 or msd (Holdener et al., 1994) embryos at the same stage was made. This method results in short (300-600 bp) cDNA that represent the total mRNA population. These probes were hybridized to duplicate plaque lifts from a CD-1 d7.5 embryonic ectoderm library. Positive clones were carried through three rounds of screening.

Northern Blot analysis.

Total RNA was isolated from embryos using the method of Chomczynski, and Sacchi, (1987). Embryos were dissected free of the decidua and separated into embryonic and extra embryonic tissue. Ten μg of RNA were electrophoresed on a 1.2% agarose formaldehyde denaturing gel in 1X MOPS buffer for 5-7 hours and transferred to nylon membrane (ICN) (Sambrook et al.1989). Hybridization in buffer (35% formamide/ 7% SDS/ 200 mM Sodium phosphate/ 1mM EDTA/ 10 mg/ml BSA) was carried out in a 58°C water bath overnight. Washes were 2XSSC/ 1%SDS twice for 5 min at RT, 1X 30 min at 58°C, followed by 2XSSC/0.2% SDS one time for 30 min at 58°C. In some cases a further wash was done, 0.2X SSC/0.2% SDS for 30 min at 58°C.
Whole Mount In Situ mRNA hybridization:

The method of Conlon and Rossant (1992) was followed with some modifications. Embryos were used immediately, as storage in 70% methanol led to loss of signal. Bleaching and the post hybridization RNAse treatments were omitted. The color reaction was complete in 20-30 min at RT. In some cases the reaction went for 90 minutes. The reaction was stopped by replacing the color reagent with PBS + 0.5 mM EDTA. Stained embryos that were used for sectioning were overstained, washed in PBS and fixed in 3:1 ethanol:acetic acid, and embedded in Paraffin wax. Sections of 5-10 μm were affixed to slides, and dewaxed in Xylene. Two different D2 cyclin cDNA clones were used in separate experiments.

PCR reactions and Southern blots.

PCR reactions were carried out as described in Varmuza and Tate (1992). Five d 8.5-9.5 embryos were lysed in 50 μl Trizol (BRL). RNA was isolated and the pellet was resuspended in 10 μl water. 2-4 μl were used as template for reverse transcription reactions followed by PCR reactions to generate a representative cDNA population of the mRNA present in the embryos. The cDNA was electrophoresed on 1.2 % agarose gels and transferred to nylon membrane (ICN) and Southern blots were carried out using the D2 cyclin cDNA as a probe.
5.4 Results

Isolation of the D2 Cyclin gene

A differential screen of a d7.5 mouse embryonic ectoderm cDNA library was carried out using PCR probes derived from normal d7.5 mouse embryos (+) and mesoderm deficient (msd) (-) embryos. The msd mutation was selected as these embryos fail to gastrulate and do not produce any mesoderm. The screen produced several differentially expressed clones that maintained a positive signal with the wild type probe and a negative signal with the msd probe through repeated rounds of plaque purification. We report results of further analysis with two of these clones which are independent isolates of the same gene, D2 cyclin.

Wholemount in situ hybridization

In order to establish a more detailed analysis of D2 cyclin expression in early post implantation development was monitored by mRNA whole mount in situ hybridization. Embryos from d 7.5 to 15.5 were hybridized with D2 cyclin cDNA clones. Figure 1 shows the pattern of gene expression. The most striking observation is that D2 cyclin is ectoderm positive. The expanding expression of D2 cyclin at d7.5 of development seems to follow the expanding mesoderm wings (Figure 1A). This expression pattern correlates with the isolation of a gene that may be dependent directly or indirectly on mesoderm for its expression.
Neural tube expression

The neural tube expression observed in d 8.0 to d 9.5 embryos and the hind brain expression seen up to d 13.5 correlates with the expression expected from a gene that is active during the early differentiation stages of neural development. The expression of D2 cyclin coalesces in the neural tube at the one somite stage (Figure 1B) and remains strong along the length of the developing neural tube and into the hindbrain region with the most anterior expression terminating at rhombomere 5. At the 9 somite stage the initial expression diminishes along the mid portion of the neural tube but remains strong at the most anterior and posterior portion (Figure 1C). The expression is strong at the point of contact of the developing somites suggesting that mesoderm is still needed for maintenance of D2 cyclin expression. Cross-sections reveal that the expression of D2 cyclin is present throughout the lateral portion of the neural tube at d8 but is absent in both the dorsal and ventral portion (Figure 1D). By the 25 somite stage as the somites are reaching maturity, expression begins to disappear in the neural tube. Cross sections illustrate the different expression patterns for the regions of the neural tube that are at different stages of maturity. Figure 1 E, F (25 somite stage) show that the expression in the more posterior region moves from the ventricular zone to the outer dorsal lateral region. In the more mature anterior neural tube, expression is limited to the outer lateral area.

Brain expression

The expression of D2 cyclin in the presumptive brain is first visualized at the 6-8 somite stage with strong expression apparent by the 9 somite stage. Three bands are observed; the most
anterior band is in the presumptive midbrain. The two posterior bands are in rhombomeres 3 and 5. Expression of D2 cyclin in rhombomere 5 is continuous with the neural tube expression. As expression in the neural tube is down regulated, rhombomere 5 continues to express D2 cyclin and forms a distinct band of expressing cells (Figure 1C).

Early d9 embryos show expression at the midbrain-hindbrain junction (Figure 1G). As development continues the midbrain expression decreases as well as that of the forebrain. As discussed above, northern blot analysis show that D2 cyclin expression declines abruptly between d13.5 and d15.5. The mRNA whole mount in situ hybridizations provide insight to this observation. Although mRNA expression levels remain high, the expression becomes greatly localized between d9.5 and d11.5 as a sharp band in the presumptive cerebellum. The developing cerebellum first produces the upper and lower component of the rhombic lip. Cell migration continues and gives rise to the external germinal layer (EGL). The EGL is elaborated between d12.5 and 15.5, and produces two populations of cells, the deep neurons and an outer cortex. Upon close examination of d13.0 brain, D2 cyclin expression coincides with the production of the rhombic lip (Figure 1H). Once these regions undergo differentiation, D2 cyclin expression is down-regulated. (Ben-Arie et al., 1997). Embryos were dissected and prepared for wholemount in situ hybridization at d13.0, d13.25, and d13.5, showing that D2 cyclin is abruptly down regulated over a period of 6 hours in the cerebellum starting at d13.0. By d13.5 D2 cyclin was absent.
Expression of D2 cyclin in the limb bud

Overstaining of embryos in whole mount in situ hybridization experiments revealed that D2 cyclin expression occurs in the apical tips of the developing digits (Figure 1I). Studies on limb formation suggest that the expression localization of D2 cyclin coincides with a region in the process of exiting from the cell cycle and about to undergo differentiation (Johnson and Tabin, 1997).

Cyclin D1 expression

We also investigated the expression pattern of D1 cyclin (obtained from C.Sherr) to see if its expression in the neural tube complemented D2 cyclin expression. D1 also has a transient, highly localized expression in the developing rhombomeres. Six bands are apparent in the floor plate at stages when the neural tube is open (Figure 1J). As the anterior neural tube closes, the bands of expression lying below the closing neural tube disappear. Dissection of the embryos showed that the bands are not masked by the overlying tissue but are actually not present. Although both D1 and D2 are expressed in rhombomeres their expression seems to be independent of each other.
Figure 1

Whole mount RNA in situ hybridization of D2 cyclin to mouse embryos.

A) D7.0-7.75 embryos show D2 cyclin expression overlying the emerging mesodermal wings (arrow). B) One to eight somite stage embryos have strong midline expression adjacent to the immature somites, and (C) at the 9 somite stage, bands of D2 cyclin expression are observed in the midbrain and hindbrain (arrow). D) Cross section of a nine somite embryo. Expression is lacking in the dorsal and ventral areas of the neural tube but is present in the ventricular zone and the mantle (arrows). E) Cross section of the posterior neural tube (25 somites) and the anterior neural tube (F), D2 cyclin expressing cells are found in the outer mantle area (arrows). G) Strong expression at 25 somite stage occurs in the hindbrain, posterior midbrain near the hindbrain-midbrain junction and the anterior midbrain (arrows). H) Expression is a narrow band within the rhombic lip of the emerging cerebellum (arrow) and the telencephalon (arrowhead). I) At d15.5 in over stained embryos weak expression is observed in the limb bud (arrow). J) Mouse embryos of 12-15 somites were stained with Cyclin D1 as a comparison to D2 cyclin. Expression is found in 6 bands in the floor plate (arrows). At this embryonic stage the expression of the two cyclins is distinct from each other. D1 cyclin is in the rhombomeres and expression is lost as the neural tube closes.
RNA expression

The two independent isolates of D2 cyclin, Ai59 and Ai171, were sequenced and subjected to a BLAST search. Identity to human D2 cyclin was revealed to be 85% with the sequences we submitted, which were derived from the 3’ UTR. Initially, the human D2 cyclin cDNA was listed in the database as anonymous. Independent verification of our clones as isolates of the murine D2 cyclin was provided by E. Ross (pers. comm. and diagram 1).

Diagram 1  **D2 cyclin cDNA published map** (Ross and Riskin, 1994)

Expression of D2 cyclin was monitored by RNA blot hybridization (Figure 2A). Developmental northern blots using tissue from d7.5 to 17.5 embryo or placenta, and adult tissues (6 weeks post natal) were used. D2 cyclin gave a mRNA band of approximately 6.5 Kb. Expression was restricted to the embryonic ectoderm starting at d7.5, increased to a peak at d11-d13, and then decreased abruptly at d15.5. We tested for D2 cyclin expression at the blastocyst (d4.5) and at d6.5 by PCR. D2 cyclin was negative in both cases (data not shown).
D2 cyclin expression during gastrulation and neurulation

Expression in mesoderm deficient embryos

A representative population of mRNA was amplified by RT-PCR from mesoderm deficient (msd) embryos (Holdener et al., 1994), embryonic mesoderm deficient (eed) embryos (Faust et al., 1995) and their normal (heterozygous and wild type) littermates. The msd samples consisted of four msd homozygous embryos and one heterozygote. Extensive analyses of these embryos by Holdener et al., (1994) have shown that msd homozygous embryos lack all mesoderm. All msd embryos are negative for D2 cyclin expression and the heterozygote, which develops normally, is positive for D2 cyclin expression (Figure 2B). This result is expected as the D2 cyclin clone was isolated in a differential screen using msd embryo RNA as the minus probe.

We carried out a similar experiment with eed mutants. Eed is the mouse homologue of the Drosophila gene, extraxsexcombs, a suppresser of homeobox containing genes (Schumacher et al., 1996). Eed mouse embryos have normal extraembryonic mesoderm and will form a primitive streak but have reduced embryonic mesoderm due to the lack of outward migration of the cells. All eed embryos are positive for D2 cyclin. Thus the mesoderm present in eed embryos is sufficient to induce the expression of D2 cyclin (Figure 2B).
Figure 2A

Northern analysis of D2 cyclin.

A Northern blot containing RNA from embryos and placenta of different ages was hybridized with the D2 cyclin clone, D2 cyclin cDNA. Total RNA was isolated from embryonic (E) or extraembryonic tissues (P) from d7.5, 9.5, 11.5, 13.5, 15.5, 17.5 embryos. Loadings are standardized using a murine β-actin probe. D2 cyclin RNA is first detected in d7.5 embryo with expression increasing to d13.5 where it then abruptly decreases. No detectable expression is observed in extraembryonic tissues.

Figure 2B

D2 cyclin expression in msd and eed mutant embryos.

To test for possible mesoderm dependency of Ai59 expression we used RT-PCR to isolate cDNA from normal and homozygous msd or eed embryos. Msd mutants lack all mesoderm, whereas eed mutants only have extraembryonic and posterior mesoderm. The RT-PCR cDNA was hybridized with D2 cyclin cDNA. Msd homozygous embryos are negative for D2 cyclin and the heterozygous embryo is positive. All eed embryos were positive.
5.5 Discussion

Embryonic organizing centers provide for the initiation of the patterning of the vertebrate body axis. In the mouse the embryonic ectoderm migrates through the primitive streak and becomes mesoderm, which is patterned along the anterior-posterior and dorsal-ventral axes. During migration through the primitive streak the mesoderm is capable of signaling the overlying ectoderm to become neuroectoderm, which itself is patterned along the same axes. The subsequent differentiation requires that cells gain inducing properties as well as the competence to respond to the inducing signals. Studies by Ang and Rossant (1993) and Foley et al., (1997) suggest that a defined window exists where the ectoderm is competent to respond to neural inducing signals. The timing of the loss of competence and the onset of differentiation corresponds to the lengthening of the cell cycle in the mouse CNS (Kauffman 1968, Wilson and Centre 1974). In this study we suggest that D2 cyclin is a marker of neural competent cells, and that the down-regulation of D2 cyclin may mark a cell cycle transition from a proliferative state to a differentiated state.

Differentiation and the cell cycle

For differentiation to occur it is important for cells to exit the cell cycle. The importance of cell cycle length and differentiation has been illustrated in studies where the ectopic expression of cyclins or artificial alteration of the cell cycle has prevented proper differentiation (Kato and Sherr 1993, Oshugi et al., 1997, Gao and Zelenka, 1997). In normal mice the transition in the neural tube from a mainly proliferating population to a differentiating
population occurs between d10 and d11. The cell cycle lengthens and this signals the beginning of differentiation (Kauffman, 1968). A similar process occurs in both the cerebellum and the tectum (Ben-Arie et al 1997, Wilson and Center, 1974). The precursor neurons stop dividing and migrate to the outer layer where they undergo differentiation.

The relationship between growth and patterning implicates the cell cycle as a point of control of differentiation. Continuous S and M phases are not favorable for the transcription of genes. Since differentiation requires new gene expression it is mandatory that the cell exit the cell cycle and allow the chromatin to take on a configuration that encourages proper transcription. For example the cells of the chick anterior limb bud have a shorter cell cycle length then the posterior cells and they express different genes. When the cell cycle of anterior cells was lengthened to reflect the longer cell cycle length of the posterior cells, Ohsugi et al., (1997) found that anterior cells began to transcribe posterior specific genes, for example Bmp-2, Hoxd-11 and Fgf-4. In Drosophila, hedgehog, through its interaction with strings, a Drosophila homolog of yeast cdc25, is also capable of regulating the cell cycle as well as patterning mesoderm (Follette and O'Farrell, 1997).

D cyclins, acting via growth factors, control the cell cycle at the point of G1 to S transition (Sherr, 1993). FGF and Activin are both potent growth factors, which can alter D cyclin levels as well induce differentiation (Halaban, 1996, Rao and Kohtz, 1995). In addition, colony stimulating factor (CSF) and epidermal growth factor (EGF) are both known to alter the levels of D cyclins (Adachi, 1997). D2 cyclin is first observed in the proliferating ectoderm of d7 embryos but not in the population that migrates through the primitive streak. The initial
expression of D2 cyclin and the fact that it only remains positive in areas of the neural tube in contact with the immature somites suggests that mesoderm might be involved in the maintenance of D2 cyclin. The importance of the somites in neural tube patterning is evident in a study by Gould et al., (1998). Explant studies using mice that are transgenic for a reporter gene regulated by either the early or late Hox 4b gene neural enhancer show that proper activity of the early enhancer requires a signal of retinoic acid from the neighboring somites. The abrupt down-regulation of D2 cyclin in the neural tube coincides with the mitotic index data showing the point of transition from proliferation to differentiation (Kauffman, 1968). Since D2 cyclin seems to respond to signals from the mesoderm, it appears to act as a link between growth factor stimulation, cell division and differentiation.
Chapter 6 Discussion

6.1 Early cell division and zygotic gene activation; Differences in process and timing between *Xenopus* and mouse

Mouse and *Xenopus* differ in many ways with regard to their mode of embryogenesis. Despite obvious differences that arise between mouse and frog, in the field of developmental biology, *Xenopus* has become the model organism of choice due to the fact that it offers several experimental advantages over the mouse. These include ease of obtaining a high number of embryos for study, the ability to follow all stages of embryo development *in vitro*, and a time frame for development consisting of hours not days.

In *Xenopus* a series of unequal and rapid cell divisions consisting of only S and M phases of the cell cycle, characterize the early stages of development (Kirschner et al., 1985). The purpose of these rapid cell divisions may be to segregate cytoplasmic determinants, maternally derived proteins and mRNA’s, that have been asymmetrically distributed during the cytoplasmic rotation that occurs immediately after fertilization (Gerhart et al., 1989). At the end of the twelfth division 4000 cells make up the blastula and the cell cycle expands to include G1 and G2 phases. Two more cell divisions will occur before gastrulation commences. During this period, termed the Mid Blastula Transition (MBT), the chromatin is extensively remodeled, zygotic gene activation (ZGA) commences, and Spemann organizer specific genes such as *gsc* are expressed (Cho et al., 1991).
In contrast to *Xenopus*, the first cleavages in the mouse are long with pronounced G1 and G2 phases. A minor activation of the zygotic genome occurs in the G2 phase of the first cell cycle followed by a major activation during G1 of the second cell cycle corresponding to the rapid degradation of maternal mRNA (Aoki et al., 1997). The onset of ZGA, in part, is due to extensive remodeling that the chromatin undertakes during the first few cell divisions (Latham et al. 1991a, Clarke et al., 1998).

In *Xenopus*, the dorsal axis is determined by the cortical rotation assisted redistribution of maternally stored factors (Gerhart et al., 1986). In mouse there is no evidence of an equivalent rotation or asymmetric distribution of molecules. Although there are morphological landmarks that suggest that asymmetries occur, and they may correlate with axis formation, no molecular factors are associated with these asymmetries (Gardner, 1997). In the mouse the earliest molecular asymmetry that marks the future A-P axis is in the endoderm with the appearance of Hex-1 on d5.5. Hex-1 marks the head organizer and the future anterior component of the embryo. Twenty-four hours later, on the posterior side of the embryo, brachury expression commences and delineates the beginning of the primitive streak and the mouse trunk organizer (Thomas et al., 1998, Herrmann, 1987). This is in sharp contrast to *Xenopus* where maternal, not zygotic genes are the conveyors of axial determinants.

6.2 Prepattern in *Xenopus* and Mouse

Although it is accepted that *Xenopus* embryos contain a prepattern and mouse embryos do not, the data leave room for re-interpretation of some of the conclusions. First, there is
abundant evidence for the localization of maternal mRNA's in the *Xenopus* oocyte. Disaggregated cell studies in *Xenopus* show that prior to MBT gsc does not require cell interactions to be expressed (Lemaire and Gurdon 1994). In contrast, single cell transplantation studies suggest that early blastula cells are pluripotent. Heasman et al., (1984) transplanted cells from the vegetal pole of mid blastula cells, which contributed to all three germ layers. Overall Heasman argues that a prepattern does exist but it can be altered. One must be careful in the interpretation of the data concerning transplantation studies. In the case of transplanting a dorsal vegetal blastomere from a 32-cell embryo into the ventral vegetal side of an equivalent embryo, one gets a second axis. Thus the fate of the dorsal cells (donor) is determined but the fate of the ventral (recipient) cells is not. Either way, cell fate is determined early and may or may not be irreversible. Since the actual induction and patterning of mesoderm does not occur until the onset of gastrulation, it is possible that flexibility in cell fate exists and may allow for the fine tuning of the pattern.

In mouse a similar paradox arises. Cell marking studies by Lawson et al., (1991), have shown that epiblast cells of the early streak are limited in potency and that cells will reproducibly contribute to specific tissues, such that a mouse fate map can be made. This suggests some sort of predetermined event. In contrast, cell transplantation studies have shown that the cells take on the fate of the host tissue (Parameswaran and Tam, 1995). Studies using late streak cells illustrate that the totipotency is lost with time and with ingestion through the primitive streak. This paradox is explained by the fact that the fate map is not a result of a cell autonomous prepattern as in the frog, but the timing of the passage of the pluripotent cell through the primitive streak. Extensive analysis has shown that the first cells through will be
allocated to the anterior and lateral mesoderm while late ingressing cells take on a posterior fate (Lawson et al., 1991, Tam and Beddington, 1987).

With differences in embryogenesis between *Xenopus* and mouse evident at cleavage stages, blastocyst formation and gastrulation, it is expected that the underlying mechanisms will also be different. In contrast to this expectation, strong molecular homology exists between molecules expressed in the early embryo of both *Xenopus* and mouse (*Gsc, Activin, Fgf, BMP's*) (Lemaire and Kodjabachian, 1996), but does this mean functional homology? Homology with regard to living things can be thought of at two levels- phenotype homology, referring to structures like limbs, or genotype homology referring to the similarity of genes. The most obvious question arising from the above statement is: Does genotype homology lead to phenotype homology? According to Goodwin, (1994), the answer is no. The evidence put forth is that some genes have the ability to produce more than one phenotype. On the other side of the argument there are examples of many different genes being able to give rise to a single phenotype. The best illustration of this comes from knockout mice (null mice) that do not have any abnormalities. It is thought that this is due to the fact that two or more genes are able to produce the same phenotype and compensate for the lost gene.

Does phenotype homology take precedence over genotype homology? Natural selection exerts selective pressure leading to a functional form. Reinvention of a functional structure is redundant and wasteful, thus once a complex structure has evolved, its general form will be maintained. For example, the eye of a human, mouse, or shark is fundamentally the same. Since the basic structure is sound, there is no need to produce a distinct eye structure for similar
species. If homologous structures are based on functional efficiency, and the genetic origins of
the structures are irrelevant, than phenotype homology should not imply genotype homology,
and attempts to draw comparisons between biological function based on genetic similarity may
be fruitless. However, the rules regulating structural homology (efficiency and conservation),
can be applied to gene homology. If a DNA sequence can give rise to a useful structure that can
be used by other organisms, then that gene sequence should be maintained throughout evolution
(Wagner, 1994).

Evidence from the animal world does not clearly indicate whether phenotype and
genotype homology are linked. A homologous gene sequence is the basis for the success of gene
screens resulting in mouse genes being isolated using *Xenopus* cDNA, for example, cerberus
and the wnts. On the other hand, β-catenin is involved in defining the *Xenopus* dorso-anterior
axis, but current evidence suggests that β-catenin fails to perform a similar function in the
mouse (Haegel et al., 1995, Rogers, Chapter 4).

A third group of genes which perform similar molecular functions in different tissues is
exemplified by the *Drosophila engrailed* gene. *Engrailed* is important in maintaining borders
between larval segments in the fly. In the mouse, *engrailed* is also important in maintaining
borders, but does so in the brain. Thus *engrailed* in the fly and mouse performs homologous
functions but in non homologous tissues.

Even within a single organism, a pathway will be conserved, but it will serve a different
function at different times throughout development. The best example in *Xenopus* and mouse
are the BMP's (bone morphogenetic proteins). These molecules play a strong role both in the active ventralization of the early embryo as well as the active neuralization of the ectoderm (Furuta et al., 1997, Winnier et al., 1995). Wnts are another large family whose members have very divergent roles. Wnt-1 in mouse is responsible for the formation of the cerebellum (McMahon et al., 1992), wnt-3A is required for posterior mesoderm and neural development in the mouse (Yoshikawa et al., 1997) and Xwnt-8b in *Xenopus* can induce dorsal fate in ventral cells. Therefore molecular homology does not mean functional homology in this case.

The importance placed on morphological similarities and molecular similarities is a result of the experimental process. As mentioned above, the accessibility and ease of using *Drosophila* and *Xenopus* for the study of embryology has somewhat skewed the field of mouse embryo development. There is no doubt that there is an abundance of experiments and information derived from frog studies that can be applied to the mouse, and these same studies would be impossible to carry out in the mouse embryo at this point in time. But as mentioned above fundamental differences, both morphological and molecular exist. We took two approaches to study mouse embryo development. One approach involved using LiCl as a tool to study early patterning events in the mouse embryo and to compare these results with those obtained from similar experiments using *Xenopus* embryos.

6.3 β-catenin is not used by the mouse to initiate primary axis formation

Studies using either teratogenic compounds like lithium or dominant negative and gain of function molecules, revealed that the active pathway in the early *Xenopus* dorsalizing center
is the wnt-β-catenin pathway. As discussed in chapter 5 recent studies have shown that an asymmetrical distribution of β-catenin occurs as early as the 4-cell embryo and localization to the nucleus can be observed as early as the 8-cell embryo (Larabell et al., 1997).

Lithium treatment of the early cleavage stage frog embryo results in the overspecification of mesoderm. The phenotype induced by lithium phenocopies mutational studies of the wnt pathway. For example, injection of GSK dominant negative constructs (DN-GSK) (Wylie et al., 1996) and injection of wnt into the vegetal ventral cells (Smith et al., 1991) also produced dorsalized embryos. On the other hand the lithium phenotype can be rescued by the addition of myo-inositol, thus implicating the PI cycle. The DN-GSK effect can also be rescued by the addition of inositol (Hedgepeth et al., 1997). This further strengthens the link between the two signaling pathways. Studies show that β-catenin undergoes nuclear localization in the cells of the presumptive dorsal side, and that lithium treatment broadens this range, which leads to the expansion of dorsal mesoderm. The discovery that GSK-3β is specifically inhibited by lithium reinforces the concept that the dorsalizing activity is dependent on both the phosphinositol (PI) cycle and the GSK/β-catenin pathway (Klein and Melton, 1996).

We targeted cleavage stage mouse embryos (2-cell to blastocyst) because treatment with lithium of the equivalent stages in Xenopus led to alterations in the dorso-anterior axis. Our goal was to elucidate, using lithium as a tool, whether Xenopus and mouse use similar mechanisms to establish the initial body plan. The problem with attempting to draw developmental parallels between mouse and frog is that the timing of events is quite different as noted above. In frog because of the short developmental time, the β-catenin signaling pathway is active just a few
hours before gastrulation. Is the important point here that it is a maternal pathway or that it is activated just before gastrulation? In the former situation the equivalent mouse stage would be the 1 cell embryo, before major zygotic gene activation. In the latter case the mouse equivalent would be peri-implantation embryo, around d5.0, just prior to Hex-1 and brachyury expression. The problem with trying to define equivalent stages in mouse and frog embryos may embody part of the answer, namely that there is no equivalence at these stages and that differences will be observed at the morphological and molecular level.

Our lithium studies initially suggested that the mouse two-cell embryo may contain axial determinants, as treatment disrupted axes formation. Although treatment at the two-cell stage led to severe axial defects and these results suggest that an organizer may be present in the early mouse embryo, this interpretation is inconsistent with results of other experiments. For example, it is well documented that both mouse cleavage stage blastomeres and ES cells are totipotent and it is unlikely that the cells are prepatterned (Vinh and Rossant, 1980, Joyner, 1994). As mentioned in Chapter 3, the increase in both cell number and the volume of the embryo between the time of treatment and the first evidence of effect suggests that lithium will be diluted to below the effective concentration by the time the first organizer activity is observed in the mouse embryo. Although we did disrupt axial development with lithium, the type of disruption we observed was similar to that observed with post MBT treatment of *Xenopus* with lithium, that of a reduction, not enhancement, of dorso-anterior structures (Yamaguchi and Shinagawa, 1989). We concluded that lithium treatment of mouse 2 cell embryos alters the competence of the blastomeres to respond to the specific signals they will receive during gastrulation. Lithium treatment of early mouse embryos results in epigenetic changes that are inherited throughout
subsequent cell divisions and result in gastrulation specific defects. The result of brachyury gene expression suggests that the primary defect is a reduced primitive streak. Treated embryos autopsied at d7.5 do not exhibit many gross morphological alterations. The similar size to the control embryos and the similar thickness of the embryonic ectoderm suggests that cell growth is not affected. The ectoderm cells of the lithium treated embryos must migrate normally as no thickening of the proximal posterior ectoderm is observed as occurs in the migration defective msd mutant mouse (Holdener et al., 1994). Also, the brachyury staining was present in the distal epiblast, although in fewer cells. This suggests that the competence of the ectoderm to respond to inducing signals is lost, resulting in a reduced mesoderm which leads to defects such as fused somites, a feature indicative of missing notochord. This has been previously shown by Masui (1961) and Kao and Elinson, (1998) who clearly illustrated that the loss of anterior neural structures of Triturus pyrrhogastor due to lithium treatment is the result of changes in cellular competence of the ectoderm and not due to alterations of the mesoderm signal.

6.4 Possible mechanisms of lithium in mouse axial disruption

Position effect variegation (PEV), X-chromosome inactivation, imprinting and mating type silencing in yeast are all examples of inherited chromosomal changes. X-chromosome inactivation and imprinting in mammals suggests that a molecular modification such as methylation is the epigenetic tag that that ensures reproducibility of allele silencing through multiple cell divisions. The epigenetic changes we suggested in chapter 3 (Rogers and Varmuza, 1996), were based upon lithium targeting the inositol pathway. The fact that lithium is known to also target GSK3β led to the work in chapter 5. The result of exploring a possible role for
GSK3β in mouse axial development has brought us full circle, back to our original hypothesis that lithium is causing an inheritable change to the chromatin of the two-cell embryo.

The results from Klein and Melton (1996) showing that the main target of lithium induced axial disruption in *Xenopus* is GSK3β allowed for more direct hypothesis testing in mouse for lithium teratogenesis. As outlined previously, lithium blocks the activity of GSK3β, which normally phosphorylates β-catenin, thereby targeting it for destruction. In the absence of an active GSK3β, β-catenin accumulates and translocates to the nucleus along with Lef-1/Xtcf. The results of our study to test for lithium induced changes in β-catenin protein levels and changes in nuclear translocation clearly indicated that the GSK/β-catenin pathway is not used in lithium induced teratogenesis in the mouse. A viable hypothesis as to the mechanism of lithium teratogenesis is that lithium compromises Protein Kinase-C (PK-C) phosphorylation of chromatin proteins resulting in changes in gene expression.

Lithium treatment aside, we would expect that the presence in mouse of a homologous, maternally inherited organizer would result in nuclear β-catenin localization during the time points observed, between zero and 120 hours post treatment. Our negative results suggest that no maternal organizer is present in mouse oocytes. This result would suggest that lithium in mouse is disrupting another pathway besides the wnt pathway. This does not mean that mouse GSK is not susceptible to lithium action, just that another unknown pathway is affected first. The search for a mouse *siamois* homolog has been unsuccessful to date (Beddington and Robertson, 1998).
It is important to address the observation that lithium treatment of two-cell embryos results in the first observable defect occurring at gastrulation, five days after treatment. As the delay is too long to be explained by the presence of maternal factors or the retention of lithium, alterations in the chromatin as a result of lithium treatment may provide the answer. Recent studies in *Xenopus*, have shown that changes in histone binding can alter cell competence for mesoderm induction. *Xenopus* animal caps are competent to form mesoderm for a defined period of time. Induction of mesoderm by activin at appropriate times causes specific gene expression. The loss of competence is cell autonomous and is dependent on the change over from histone B4 to histone H1. *Xenopus* embryos prior to MBT contain an oocyte specific histone, B4. This histone is replaced by histone H1 variants between MBT and late neurula stage. In a recent study, Steinbach et al., (1997) found that prevention of B4 to H1 transition resulted in an extended competence of the animal cap cells to produce mesoderm. As expected, an increase of H1 expression resulted in the premature loss of competence. All four mesoderm genes tested underwent histone H1 mediated changes in expression. Increased histone H1 levels did not interfere with the transcription rates of non-developmentally regulated genes.

Is the early mouse embryo also in a state of dynamic change? Is the chromatin susceptible to modifications by lithium? The mouse 2-cell embryo undergoes extensive changes in protein expression patterns. This strongly implies changes at the chromatin level are occurring at this time and that the alterations in chromatin structure make the two-cell embryo susceptible to insults which can affect DNA binding proteins. Linker histone transition as described for *Xenopus* also occurs in the mouse (Clarke et al., 1998). The process begins at pre-fertilization and continues until the 8-cell stage. Replacement of histones is only one part of a very dynamic
process of genome transformation.

Transgene studies have revealed a role for chromatin in the activation of the zygotic genome. Transgenes composed of enhancers and a reporter are unable to promote transcription until the 2-cell to 8-cell stage, coincident with the commencement of the major ZGA (Forlani et al., 1998). The ability of long-range enhancers was also dependent on DNA replication thus implying a regulatory role for chromatin structure. Although they do not suggest a mechanism for the role of DNA replication in the activation of the zygotic genome, histone subclass changes, as found in *Xenopus* (Steinbach et al., 1997) or alterations of chromatin structure by non-histone binding proteins is a good possibility. Other changes occurring in the embryo which contribute to genome remodeling are the redistribution of acetylated histones beginning at the 2-cell stage (Worrad et al., 1995), and the ability of the scaffold attachment region to enhance gene transcription, which begins at the 8-cell stage (Thompson et al., 1995).

Having the chromatin of early cleavage stage mouse embryos in a state of oscillation provides for an abundance of opportunities for lithium to disrupt chromatin structure. Since the phosphoinositol pathway regulates PK-C and lithium is able to disrupt this pathway, there is the possibility that lithium could act by causing the premature phosphorylation of a chromatin protein which would lead to its ectopic binding. The inositol pathway could provide an explanation for my observations in Chapter 3. Studies by Latham et al. (1991a) which describe the changes of expression levels of over 500 proteins in the mouse 2-cell embryo, demonstrate that 45% of the proteins studied undergo changes in protein expression, with a subset undergoing changes in phosphorylation. Lithium treatment, through its action on the inositol
pathway and PK-C, may disrupt some of these preprogrammed changes in protein expression patterns and phosphorylation states, possibly leading to heritable chromatin changes.

Lithium treatment of 2-cell embryos phenocopies the HNF3β mutation. Cirillo et al., (1998), recently demonstrated that HNF3β is able to displace histone H1 from the nucleosome, whereas other transcription factors could not. Their study suggests a unique relationship between histone H1 and HNF3β. Lithium may interrupt the normal H1-HNF3β balance and prevent HNF3β induced transcription. Although HNF3β has not been detected in the two-cell embryo, its specific regulation of histone H1 provides an example of a lithium sensitive molecule (HNF3β-like) that may occur in cleavage stage embryos and interact with chromatin.

Disruption of late stage development by treatment of early cleavage stage embryos is not unique to lithium. Generoso (1980) has treated pregnant mice during the early stages of development with EtoH for example, and was able to elicit late stage perturbations. Roemer et al., (1997) observed that transplantation of pronuclei into enucleated oocytes of genotypically different mice resulted in changes in protein expression patterns in adult mice. One such affected protein is the major urinary protein required for the initiation of puberty. Thus the cytoplasm of the foreign egg was able to elicit a change in gene expression in the adult. Surprisingly these changes in gene expression could be passed on to the offspring.
6.5 Why do mice lack a prepattern?

Lithium experiments raise questions concerning the apparent lack of maternal positional information in mouse axial development. Unlike *Xenopus*, *Zebrafish* or *Drosophila*, the initial differentiation event is not the establishment of the embryonic axis, but the separation of the trophectoderm from the embryonic ectoderm lineages. This suggests that the mouse places placental development in a position of higher priority than embryo development. The importance of proper placental formation is emphasised by the observation that many natural and induced mutations in mice cause embryo death through improper placental formation and fetal circulation. Defects of other major organ systems like the central nervous system, result in postnatal death. (Copp, 1995). Thus mouse cleavage stage totipotency, a characteristic quite different from that of *Drosophila* or *Xenopus*, may have its roots not in embryo development but in delaying placenta development.

6.6 Differentiation and the cell cycle

The lithium studies described in this thesis suggest that in the mouse, lithium has the ability to alter chromatin structure. During the 2-cell embryo stage the chromatin is in a transitional state as it develops a transcriptionally active configuration. This makes the 2-cell embryo very susceptible to treatments that may target DNA binding proteins, thus resulting in a heritable change to the chromatin. The importance of chromatin structure to gene expression is observed from both *in vivo* and *in vitro* studies looking at cell differentiation, cell cycle changes and transcriptional activation of transgenes or enhancers (Forlani et al. 1998). These studies
have shown that cells must develop longer gap phases, which allows for a rearrangement of the chromatin, thereby creating a transcriptionally active state.

During embryo development this relationship between differentiation and cell cycle is observed in *Xenopus*. For example the competence of the ectoderm to respond to neural inducing signals is lost abruptly and this loss of competence is associated with changes in the cell cycle (Kauffman, 1968). In the mouse down regulation of D2 cyclin coincides with a cell cycle change. The transition from a proliferative state to a differentiated state is evident by the migration of D2 expressing cells from the ventricular zone out towards the mantle and the subsequent loss of D2 cyclin expression.

A link between the cell cycle and morphogenesis can be provided by the fact that cell cycle length exerts control over gene expression. This is illustrated in the fruitfly. During the 13 rapid cell divisions consisting of S and M, the phosphatase cdc\textsuperscript{25}\textsubscript{string} becomes limiting in some cells (Follette and O'Farrell, 1997). The function of cdc\textsuperscript{25}\textsubscript{string} is to allow cdc2 to bind to its partner cyclin and promote passage through the cell cycle. Therefore by limiting the amount of cdc\textsuperscript{25}\textsubscript{string} available, the cell cycle slows down and a G2 phase develops. This allows for the activation of the zygotic genome in those cells with no cdc\textsuperscript{25}\textsubscript{string}. Since cdc\textsuperscript{25}\textsubscript{string} is differentially lost it provides a connection between the cell cycle and positional information.

In higher eukaryotes the role of the cell cycle in the regulation of gene expression has been studied in chick limb bud. Anterior cells, which cycle faster can be made to mimic posterior cells by artificially slowing down the cell cycle. When this is done the anterior cells
express posterior limb bud specific genes and develop mirror image duplications (Ohsugi et al, 1997).

The D2 cyclin gene provides an intriguing insight into the possible relationship between cell cycle and morphogenesis. Previous work by Sherr (1993), has suggested that the D cyclins may act as growth factor sensors as they are induced by specific growth factors and can both positively and negatively regulate the cell cycle. Early studies done on the mitotic indices of the early mouse embryo show that the D2 cyclin expression coincides with areas of the embryo exhibiting a decrease in the cell cycle rate (Poelmann, 1980, 1981, Miller 1982, Kauffman, 1969). In the neural tube the transition from a mainly proliferating population begins at d10. The cell cycle lengthens and this signals the beginning of differentiation (Kauffman, 1968). A similar process occurs in both the cerebellum and the cerebral cortex (Wilson and Center, 1974 and Ben-Arie et al 1997). In all cases the precursor neurons stop dividing and migrate from the proliferating ventricular zone to the mantle where they undergo differentiation.

The major difficulty in the establishment of specific functions for the cyclins is the complex regulation that accompanies them. The cyclins have multiple cdk (cyclin dependent kinase) partners and inhibitors. Thus the redundancy of the system ensures that the cell cycle will continue under a fair amount of control even with the lack of specific proteins, but this makes it much more difficult to decipher a role for each of the components. This is evident in the phenotype of the D2 cyclin null mouse (Sicinski et al., 1995). The only defect observed is that females lack granulosa cells which interferes with the maturation of the oocytes, and leads to sterility. The expression patterns of D2 cyclin reported above would suggest a neural defect,
but redundancy in the system may act to rescue the cell cycle in neural cells.

Cell proliferation plays a secondary key role in mouse gastrulation. Besides regulating gene expression, proliferation causes important morphological changes. Post implantation proliferation accounts for a large increase in the volume of the embryo (Rugh, 1968). Also at the beginning of primitive streak formation the cells of the epiblast migrate towards the primitive streak driven by rapid cell divisions of the ectoderm. During gastrulation the body axis elongates due to proliferation of the epiblast cells (Poelmann 1980, 1981).

6.7 Summary

The use of a teratogen for the study of mouse gastrulation and neurulation suggests that the mouse uses novel pathways to establish the primary body axis. Lithium acts to disrupt the formation the *Xenopus* body axes by interfering with the activity of GSK3β. This results in the ectopic nuclear translocation of β-catenin, which causes a change in the competence of affected cells leading to an increased domain of anterior mesoderm. We hypothesized that the treatment of mouse cleavage stage embryos with lithium chloride would either disrupt axes development in a similar manner to that of *Xenopus*, or that lithium might have a unique effect in the mouse. In the former case, lithium studies would allow us to show that the GSK3β/β-catenin dorso-anterior determining pathway is conserved in mouse and *Xenopus*. In the latter case, lithium studies would lead us to explore novel axis determining pathways. As illustrated in the thesis, the latter case prevailed.
The results generated from the lithium studies are novel. The experiments indicate that the mouse does not use the GSK3β/β-catenin pathway during the two-cell to blastocyst stage to generate patterning information. Because of the inaccessibility of implanted blastocysts or d5.0 embryos, I cannot conclude that the GSK3β/β-catenin pathway is not used at this time to signal the initiation of the body axis.

A second significant discovery is that the treatment of the two-cell embryo for a short time (5 min) can give rise to a change in developmental potential that leads to the disruption of gastrulation 5 days after treatment. I hypothesize that lithium treatment is affecting chromatin proteins, resulting in heritable changes that manifest themselves at a later time; the sensitivity of the two-cell embryo to lithium coincides with a period of time when the zygotic genome is being activated and the chromatin is undergoing extensive remodeling. The state of the chromatin as the basis for the lithium effect in mouse, highlights the importance of chromatin in gene regulation. The chromatin is required to take on a loose or open conformation to promote gene activation, and a closed heterochromatin state to silence gene expression. This imposes a constraint on cells with respect to developmentally regulated gene expression. Another constraint placed on gene transcription is the requirement for gap phases (G-phases) in the cell cycle. Rapid cell division prevents gene transcription by limiting the time the DNA is accessible to transcription factors. Since cell differentiation requires novel gene transcription, cells must develop gap phases in order to reach their final differentiated state.

The molecular approach for the study of mouse gastrulation and neurulation presented in this thesis involves the characterization of the expression pattern of the D2 cyclin gene. Experiments enabled me to show previously unknown expression patterns for D2 cyclin. First
evident in wild type d7.0 embryos, D2 cyclin is localized to the ectoderm overlying the emerging lateral mesoderm. This expression may be dependent on the presence of a functional mesoderm.

Throughout the discussion I have suggested that the dynamic state of the chromatin in the early mouse embryo is a possible target for the epigenetic changes brought about by lithium treatment. Furthermore the fact that cells in general must exit the cell cycle in order to differentiate implicates chromatin structure (eg. Histones), and the cell cycle (eg. D2 cyclin) as having pivotal roles as regulatory components of gene expression and hence embryo development.

6.7 Future Experiments

Although treatment of the 2-cell mouse embryo with LiCl results in the loss of dorso-anterior structures, the mechanism by which lithium exerts its effect is not through the GSK pathway. This result raises two questions. First, what is the mechanism by which lithium acts to generate the observed phenotype? Interestingly the fact that the lithium effect occurs 5 days after treatment suggests a mechanism involving alterations in chromatin structure. Second, is GSK the initiator of the doro-anterior organizer, as observed in Xenopus, but at times not covered in our study? In order to answer some of these questions I propose the following studies.

A. As proposed in the thesis we speculate that if lithium is acting through the Phosphoinositol
pathway and protein kinase C then it is possible that lithium treatment will result in altered phosphorylation of an unknown target protein(s). It is also possible that lithium is directly affecting transcriptional or translational events. To detect changes in protein levels or changes in phosphorylation of a protein in cleavage stage mouse embryos I propose using 2-D gel electrophoresis. This method was used successfully by Latham (1991a) to follow changes in protein levels and phosphorylation patterns of early cleavage stage mouse embryos during the onset of zygotic gene activation. In my study I would compare lithium treated and untreated 2-cell embryos. The 2-cell embryos would be allowed to develop in vitro and then analyzed at various stages of development. Embryos will be labeled in vitro with $^{35}$S-Met to detect protein translation or $^{32}$P to detect changes in phosphorylation. The use of a phosphorimager would greatly expedite this type of study. Any protein undergoing lithium-induced changes could be isolated from the gel and indentified by microsequencing.

B. The question of whether lithium is altering the ability of cells to send a signal or to receive a signal is not addressed in the thesis. Recent works by others have indicated a role for both the endoderm and the ectoderm in axial patterning events (reviewed in Beddington, 1998). Thus we can attempt to test for changes of competence induced by lithium by treating two cell embryos with lithium and letting them grow in vivo to d7, then dissecting embryos into ectoderm, endoderm and mesoderm components and making tissue explant sandwiches (Ang and Rossant, 1993). The explants can then be tested for differentiation by carrying out in situ hybridization with tissue specific markers.
C. It is possible that β-catenin initiates the formation of a organizing center during stages not covered in my study. As discussed in the thesis it is possible that β-catenin may act at peri-implantation stages or late post implantation stages. Once the correct stage embryo was obtained immunocytochemistry with anti-β-catenin antibody would be done. With practice d5.5 embryos can be dissected from the uterus and these could also be used in immunohistochemistry studies with anti-β-catenin antibodies.
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