Identification of multiple roles for Wnt signaling during mouse development

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Abstract

Signaling molecules play essential roles in communication between cells. Wnt signaling molecules are critical for embryonic development of several organisms. I examined the involvement of Wnt signaling during two major developmental processes, namely embryo implantation and formation of the embryonic body axes. Using RT-PCR analysis, I showed that multiple Wnt genes are expressed in the blastocyst at the time of implantation. Moreover, expression of Wnt 11 requires both estrogen produced by the mother and the uterine environment. Using a transgenic approach, I showed that β-catenin-regulated transcriptional activity, which is a major transducer of Wnt signaling, is activated in the uterus specifically at the site of implantation in an embryo-dependent manner. These results introduce Wnts as candidate signaling factors that may mediate the communication between the embryo and uterus that initiates implantation.

Wnt/β-catenin signaling triggers axis formation in *Xenopus* and zebrafish embryos. I showed that, during embryonic development, β-catenin-regulated transcriptional activity is first detected in the prospective primitive streak region prior to gastrulation. This demarcates the posterior region of the embryo. This activity then becomes restricted to the elongating primitive streak and to the node. In *Xenopus* embryos, β-catenin participates in the formation of the organizer through the activation of the homeodomain transcription factors Siamois and Twin. I obtained evidence that a Siamois/Twin-like binding activity exists in mouse embryos and is localized in the node. These results strongly suggest that, as the case in *Xenopus* and zebrafish, the Wnt/ β-catenin pathway is involved in establishing embryonic body axes.

Furthermore, using the transgenic mouse line that I generated for these studies, I mapped the transcriptional activity of β-catenin during mouse embryonic development. These results revealed when and where this activity, and presumably Wnt signaling, is active during the development of several organs and embryonic structures.

Résumé

Les molécules de signalisation jouent un rôle crucial dans la communication intercellulaire. Les molécules de signalisation de la famille Wnt sont essentielles pour le développement embryonnaire de nombreux organismes. J'ai étudié le rôle de la voie de signalisation de Wnt au cours de deux processus développementaux majeurs : l'implantation embryonnaire et la formation des axes embryonnaires. Par analyse de type RT-PCR, j'ai établi que de multiples gènes Wnt sont exprimés par le blastocyste au cours de l'implantation. De plus, l'expression de Wnt11 requiert la production d'œstrogène par la mère et l'environnement utérin. Par une approche transgénique, j'ai montré que l'activité transcriptionnelle régulée par β-caténine, un médiateur majeur de la voie de signalisation de Wnt, est spécifiquement activée dans l'utérus au site d'implantation, de façon dépendante de la présence de l'embryon. Ces résultats présentent les Wnts comme des facteurs de signalisation candidats dans la communication entre l'embryon et l'utérus au cours du processus d'implantation.

La signalisation par Wnt/β-caténine élicite la formation des axes dans les embryons de Xénope et du poisson zèbre. J'ai montré que, durant le développement embryonnaire, l'activité transcriptionnelle régulée par β-caténine est détectée, en premier, au site présomptif de la ligne primitive, avant la gastrulation. Cette expression démarque la région postérieure de l'embryon. Ultérieurement, l'activité se restreint à la ligne primitive en élongation et au nœud. Dans l'embryon de Xénope, β-caténine participe à la formation du centre organisateur par l'activation des facteurs de transcription à homéodomaine : Siamois et Twin. Mes résultats indiquent qu'une activité de liaison similaire à

Siamois/Twin existe dans les embryons de souris et est localisée au niveau du nœud. Ceci suggère fortement que, comme chez le Xénope et le poisson zèbre, la voie de signalisation de Wnt/β-caténine est impliquée dans la mise en place des axes embryonnaires chez la souris.

Enfin, en utilisant les lignées transgéniques générées pour ces études, j'ai déterminé les sites d'activité transcriptionnelle de β-caténine au cours de l'embryogenèse murine. Ces résultats révèlent où et quand cette activité, et donc presumément la signalisation par Wnt, opère au cours du développement des organes et des structures embryonnaires.

Acknowledgments

My deepest and most sincere gratitude goes to my supervisor Dr. Hugh Clarke. Thank you so much Hugh for all what you have done for me. Thank you for taking the burden of accepting me into your lab when I knew almost nothing about science and research. Thank you for putting all the time and extra effort to ensure that I get the best training in your lab. You taught me the best skills every scientist should have, which is to carry out research sincerely and honestly and to do science for the love of science. Foremost, thank you for being a good friend during all the time I have known you.

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CHAPTER 6: GENERAL DISCUSSION

Abbreviations

ADE = Anterior Definitive Endoderm

AER = Apical Ectodermal Ridge

APC = Adenomatous Polyposis Coli

ARK = Arkadia

ARM = Armadillo

AVE = Anterior Visceral Endoderm

BMP = Bone Morphogenetic Protein

CBP = CREB Binding Protein

CKI = Casein Kinase I

CMV = Cytomegalovirus

DE = Distal Element

DKK = Dickkopf

DSH = Dishevelled

EGF = Epidermal Growth Factor

EOMES = Eomesodermin

FGF = Fibroblast Growth Factor

FZ = Frizzled

GSK = Glycogen Synthase Kinase

GTF = General Transcription Factor

HB-EGF = Heparin-Binding Epidermal Growth Factor-like growth Factor

HDAC = Histone Deacytelase

HH = Hedgehog

HMG = High Mobility Group

HPRT = Hypoxanthine-guanine Phospho Ribosyl Transferase

ICM = Inner Cell Mass

IGF = Insulin like Growth Factor

IHH = Indian Hedgehog

KRM = Kremen

LEF = Lymphocyte Enhancer Factor

LIF = Leukemia Inhibitory Factor

LRP = low-density lipoprotein receptor-related proteins

MAPK = Mitogen Activating Protein Kinase

MESD = Mesodermin

NKD = Naked Cuticle

PCP = Planar Cell Polarity

PE = Proximal Element

PG = Prostaglandin

PKC = Protein Kinase C

PLC = Phospho Lipase C

RT-PCR = Reverse-Transcriptase Polymerase Chain Reaction

SFRP = Secreted Frizzled Related Protein

SHH = Sonic Hedgehog

T = Brachyury

TCF = T-Cell Factor

TGF = Transforming Growth Factor

WBSCR11 = William-Beuren Syndrome Chromosomal Region 11

WIF = Win Inhibitory Factor

WRE = Wnt Responsive Element

Publications from the work of this thesis

1- Mohamed OA, Dufort D. and Clarke HJ. Expression and estradiol regulation of *Wnt* genes in the mouse blastocyst identify a candidate pathway for embryo-maternal signaling at implantation. ***Biology Of Reproduction:*** **In Press.**

2- Mohamed OA, Clarke H and Dufort D. β-catenin marks the prospective site of primitive streak formation in mouse embryos. ***Developmental Dynamics:*** **In Press.**

3- Mohamed OA, Clarke HJ and Dufort D. Blastocysts induce Wnt signaling in the uterus at the implantation site. ***Submitted to Science.***

4- Mohamed OA, Clarke HJ and Dufort D. Siamois/Twin-like binding activity is present in mouse embryos and is specifically localized in the node. ***To be submitted to Mechanisms Of Development.***

5- Liu H, **Mohamed O,** Dufort D, Wallace VA. Characterization of Wnt signaling components and activation of the Wnt canonical pathway in the murine retina. ***Developmental Dynamics:*** 2003 Jul;227(3):323-34.

Contributions of Authors

1- Mohamed OA, Dufort D. and Clarke HJ. Expression and estradiol regulation of Wnt genes in the mouse blastocyst identify a candidate pathway for embryo-maternal signaling at implantation. ***Biology Of Reproduction: In Press.***

Clarke HJ and Dufort D participated in planning of experiments, interpretation of results and editing of manuscript.

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5- Liu H, **Mohamed O,** Dufort D, Wallace VA. Characterization of Wnt signaling components and activation of the Wnt canonical pathway in the murine retina. ***Developmental Dynamics: 2003 Jul;227(3):323-34.***

This manuscript is not included in this thesis. I participated in the work of this manuscript by providing transgenic embryos that were used to generate figure 5.

PREFACE

"Then fashioned We from the drop a clot, then fashioned We from the clot a lump, then fashioned We from the lump bones, then clothed We the bones with flesh, and then produced it We as new creation. So blessed be Allah, the Greatest of creators!"

The Holy Quran 23:14.

Reproduction is the mean by which all species propagate; hence its efficiency determines the success of any species. Mammals, as do many other classes of organisms, utilize sexual reproduction for their propagation. In mammals, development begins during gametogenesis within the ovaries and testes of the parental animals. Within these organs the gametes, oocyte and sperm, are produced, which possess haploid genome of their parental origin.

Life begins with a kiss between wiggling sperm and ripe egg in a process termed fertilization. At fertilization, sperm and oocyte fuse and co-mingle their genetic material- the sperm introduces the paternal half of the genome and the oocyte contributes the maternal half, to form a zygote that has a copy of each gene from each parent. Subsequent to fertilization, the newly formed zygote undergoes a truly spectacular embryonic development, to say the least, which takes it from a one-cell embryo to a fully developed organism with hundreds of millions of specialized cells. The beauty of studying development is that it gives one the opportunity to witness some of these transformation events unfold in front of his/her eyes. In the mouse, for example, it is technically possible to take sperm and egg, fertilize them *in vitro*, watch the embryo divide for few cycles, transfer it to a foster mother and then obtain a fully developed mouse in about three weeks

time. During this developmental journey, one can dissect the embryo out at different times and catch the development of the different structures and organs in action.

How the embryo achieves such task and goes from one-cell, that is not visible to the naked eye, to an organism with so many complex structures such as brain, eyes, lungs, etc, is far from understood, but absolutely amazing. What we know for certain, however, is that the fertilized egg, which you can touch, turn around and manipulate, does contain all the genetic material required to achieve such task. This genetic material will work as a blueprint from which all the complex structures of the adult organism will be built. It is all a matter of turning on specific genes at specific times in response to specific signals and achieving certain cell specialization one step at a time. Dissecting this program of development and figuring out how it is executed is the challenging job developmental biologists are facing and will have to face for some time to come.

The period of embryonic development varies in length between species, ranging from few days, for some species, to several months or even years for others. Amazingly enough, despite the large differences in appearance between species, there are remarkable similarities in their embryonic development. This includes strategies adapted to execute certain developmental processes as well as cellular and, in some cases, even molecular events used to achieve such strategies. This makes it exciting and satisfying to study numerous developmental processes in one species and use the gathered knowledge to illuminate similar processes in another such as our own.

In our world, there are two different schools of thought that present totally different views of how all this complex life has came about. The first believes that everything we see is

the active action of mighty God, who has engineered, executed and is watching his plan translate into real life. The second school believes that everything we see is the end result of evolution by natural selection. The first school believes that everything is built for a reason and is fulfilling a certain purpose. The second school believes that nothing is built for any reason or to fulfill any purpose; rather everything is the end result of random selection that has yielded a stable and functional product. Neither school has been able to present impeccable evidence supporting its notion in order to convince the other one. Therefore, for a scientist, the first question perhaps should not be who is right and who is wrong; rather it is how are things done. For that, I believe that neither God, nor nature, minds you following its footsteps and having a sneak peek of how it created such astonishing life.

In this thesis, I investigated the involvement of one molecular signaling pathway, named the Wnt signaling pathway, during mouse development. The Wnt signaling pathway is now recognized as indispensable pathway for plethora of developmental and cellular processes and its misregulation has been documented in wide range of cancers. Scientists around the world are racing to reveal the mechanism that governs its action and its roles during development and cancer. Here, I present evidence that this pathway is activated during several vital developmental processes. These include embryo implantation, axis formation and gastrulation as well as during development of several body organs. In my research I used several standard molecular and cellular techniques. In addition, I employed the technology of transgenic animals to answer key questions. Specifically, I generated a transgenic mouse that provides simple readout for activity of the canonical Wnt signaling pathway. The power of transgenic technology is undeniable and the

information it provides is impressive. Basically it involves the generation of a designed animal with this simple instruction: "show me where in the body and when during development this gene or this pathway is active".

The *Tcf/Lef-lacZ* transgenic animal I generated for my studies is a very specific and sensitive reporter for the canonical Wnt signaling pathway. This transgenic animal is precious research tool that will provide valuable insights in the regulation of numerous developmental processes, which will allow us to understand better how development is achieved. It can also be used to investigate the role of Wnt pathway in several different types of cancers and test new therapeutic agents to treat these cancers.

CHAPTER 1: GENERAL INTRODUCTION

1.1: THE WNT SIGNALING PATHWAYS

The orchestration of complex biological processes from embryogenesis to senescence requires exchange of information between cells. A variety of signaling molecules are employed to execute this task. From hydra to man, secreted signaling proteins of the Wnt family have come to be recognized as key mediators of a plethora of fundamental developmental and cellular processes. These include axis formation, development of the central nervous system, axial specification in limb development, generation of cell polarity, specification of cell fate, cellular proliferation and migration, organ development, cell growth and cancer (reviewed in (Beddington and Robertson, 1999; Cadigan and Nusse, 1997; Giles et al., 2003; Korswagen, 2002; Pandur et al., 2002; Tada et al., 2002; Wodarz and Nusse, 1998). The involvement of Wnt signaling in so many developmental and cellular processes is overwhelming, and almost no day passes with out a new role for Wnts identified in development or cancer.

The first *Wnt* gene to be identified, the mouse gene encoding for int-1, was discovered in 1982 as a proto-oncogene activated by integration of mouse mammary tumor virus in mammary tumors (Nusse and Varmus, 1982). A few years later, the segment polarity gene *Wingless* (*Wg*) in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980) was found to be homologous to the mouse gene encoding int-1, and hence the gene acquired the name *Wnt1* (Cabrera et al., 1987; Rijsewijk et al., 1987). Since then, numerous *Wnt* genes have been identified in a wide range of organisms, including vertebrates and invertebrates, but they appear to be absent from plants, unicellular eukaryotes such as yeast, and prokaryotes (reviewed in (Miller, 2002). To date, among vertebrates, 19 *Wnt* genes have been

identified in human and mouse, 16 in *Xenopus*, 11 in the chick and 12 in zebrafish. Among invertebrates, 7 *Wnt* genes have been identified in *Drosophila*, 5 in *Caenorhabditis elegans* and at least one in *Hydra* (http://www.stanford.edu/~rnusse/wntwindow.html)

1.1.1: Wnt gene organization and protein structure

Wnt genes encode a large family of cysteine-rich secreted glycoproteins that are very similar in size, ranging from 39 kDa (Wnt7a) to 46 kDa (Wnt10a). In human and mouse, several *Wnt* genes are located very close to each other in the genome. For instance, *Wnt1* and *Wnt10b* are adjacent to each other. Similarly, *Wnt6* and *Wnt10a* are adjacent to each other, but transcribed in opposite direction. Furthermore, *Wnt2* and *Wnt16*, *Wnt3a* and *Wnt14*, *Wnt3* and *Wnt15* are all very close to each other in the genome of both human and mouse (reviewed in (Miller, 2002). Most *Wnt* genes contain four coding exons, with exon 1 containing the translation initiation codon, methionine. *Wnt* genes that differ from this pattern are *Wnt14* with three exons, *Wnt2*, *Wnt5b* and *Wnt11* with five exons and *Wnt8b* with six exons.

Most Wnt proteins are notoriously insoluble and tend to stick to the extracellular matrix; hence, they are difficult to obtain in a soluble, biologically active form (Smolich et al., 1993). Therefore, little is known about their protein structure. All Wnt proteins contain 23-24 cysteine residues and the spacing between these residues is highly conserved. Glycosylation appears to be a common modification for Wnt proteins and is believed to be important for folding, secretion and biological activity (Smolich et al., 1993). In *Drosophila* the gene *porc* encodes a multi-transmembrane protein predominantly found in

the endoplasmic reticulum and is involved in the glycosylation process of Wg (Kadowaki et al., 1996).

1.1.2: Wnt reception and activation of downstream pathways

Wnt proteins transmit their signals by binding to the Frizzled (Fz) receptors (Bhanot et al., 1996; He et al., 1997; Yang-Snyder et al., 1996). In mouse and man, 9 frizzled receptors have been identified (http://www.stanford.edu/~rnusse/wntwindow.html). Frizzled proteins are seven-transmembrane receptors that contain an N-terminal, cysteine-rich extracellular domain (CDR), which is thought to bind Wnts, seven putative transmembrane domains and a variable length cytoplasmic C-terminus. Members of the low-density lipoprotein receptor-related proteins (LRP-5 and LRP-6) act as co-receptors for Wnts, meaning that Wnt ligands must bind to both frizzled and either LRP-5 or LRP-6 in order to activate the signaling (Pinson et al., 2000b; Tamai et al., 2000; Wehrli et al., 2000).

Not every Wnt molecule can bind to every Frizzled receptor and Frizzleds can discriminate between different Wnt ligands. This selectivity appears to be important for determining which downstream pathway is activated in the cell. Once a Wnt has bound to a suitable Frizzled, a specific intracellular signaling pathway is activated influencing different biological processes. To date, four different Wnt signaling pathways have been identified; the canonical pathway of β-catenin, the planar cell polarity (PCP)/convergent extension pathway, the calcium pathway and the recently identified, yet poorly characterized, the spindle orientation pathway.

Based on an axis duplication assay on *Xenopus*, Wnt proteins are divided into two distinct classes. The Wnt1 family (including Wnt1, Wnt2, Wnt3a, Wnt8a and Wnt8b) is active in axis duplication and thought to signal through the canonical pathway of β-catenin. The Wnt5a family (including Wnt5a, Wnt4 and perhaps Wnt11) are not active in axis duplication, and function through the calcium pathway (reviewed in (Wodarz and Nusse, 1998). Similarly, using the induction of Wnt/ β-catenin target genes and activation of protein kinase C (PKC) in *Xenopus*, Frizzled receptors are classified into two distinct families. RFz1, MFz7, MRz8, XFz1, DFz and DFz2 activate the canonical pathway of β-catenin targets, but not PKC. On the other hand, RFz2, MFz3, MFz4 and MFz6 activate PKC, but not Wnt/ β-catenin targets (Sheldahl et al., 1999). Caution regarding these classifications should be exercised, however, because they are based primarily on one system and deviation from these roles in different systems perhaps exists.

1.1.2.1: The canonical pathway of β-catenin signaling

β-catenin was originally identified by its association with the cytoplasmic domain of cadherins and was found to play a crucial role in calcium-dependent cell adhesion (Gumbiner and McCrea, 1993; McCrea and Gumbiner, 1991; McCrea et al., 1991; Takeichi, 1991). β-catenin is the homologue of the Armadillo (Arm) protein in *Drosophila* (McCrea et al., 1991). In *Drosophila* Wg signaling leads to post-transcriptional stabilization of Arm in the cytoplasm of embryonic and cultured cells (Peifer et al., 1994; Riggleman et al., 1990). Similarly, β-catenin is stabilized in Wnt1-transfected mammalian cells (Hinck et al., 1994). Wg/Wnt signaling predominantly stabilizes a soluble, cytoplasmic form of Arm/ β-catenin that is not associated with cadherins (Papkoff et al., 1996).

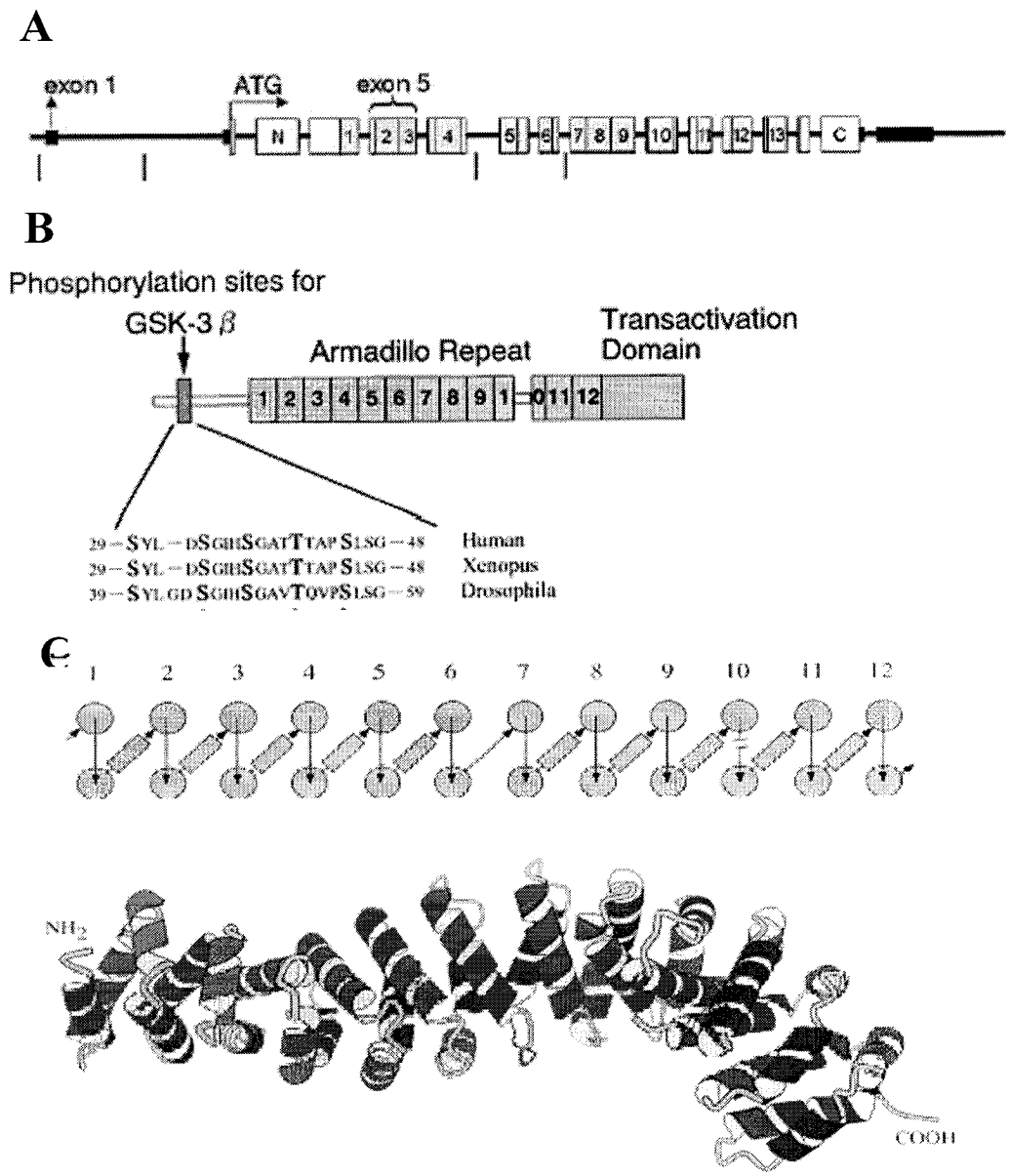
1.1.2.1.1: β-catenin gene and protein structure

The *β-catenin* gene is located on chromosome 3 in humans and chromosome 9 in mice (http://www.stanford.edu/~rnusse/wntwindow.html). The gene encodes a 92-kDa protein with three main domains: an N-terminal domain containing several putative serine/threonine phosphorylation sites; a central domain containing 12 imperfect sequence repeats of 42 amino acids known as the armadillo repeats; a C-terminal domain containing a potent transcriptional activation region (reviewed in (Barker and Clevers, 2000). At the N-terminus, β-catenin can be phosphorylated by casein kinase Iα (CKIα) at serine45 and by glycogen synthase kinase-3 β (GSK-3β) at threonine41, serine37 and serine33 (reviewed in (Kikuchi, 2003). Through the armadillo repeats region, β-catenin binds to several β-catenin-interacting proteins such as α-catenin, E-cadherins, adenomatous polyposis coli (APC), axin, T-cell factor/Lymphocyte enhancer factor (Tcf/Lef) family of transcription factors, Chibby and the histone acetyl transferase CBP (CREB binding protein)/p300 (Brantjes et al., 2002; Huber et al., 1997; Rubinfeld et al., 1993; Takemaru et al., 2003) (Figure 1).

1.1.2.1.2: Role of β-catenin in Wnt signaling

According to recent data, the mechanism of Wnt signaling through β-catenin is the following. In the absence of Wnt signal, β-catenin resides in a large cytoplasmic complex consisting of the negative regulators of Wnt signaling, the scaffold protein axin and the tumor suppressor APC, as well as the serine/threonine kinase GSK-3β and CKIα (Behrens et al., 1998; Kishida et al., 1998). In this complex, β-catenin is phosphorylated, ubiquitinated and degraded by the proteasome machinery. Following Wnt signaling, cytoplasmic β-catenin becomes stabilized and is detectable in the nucleus where it binds

Figure 1: β-catenin gene and protein structure

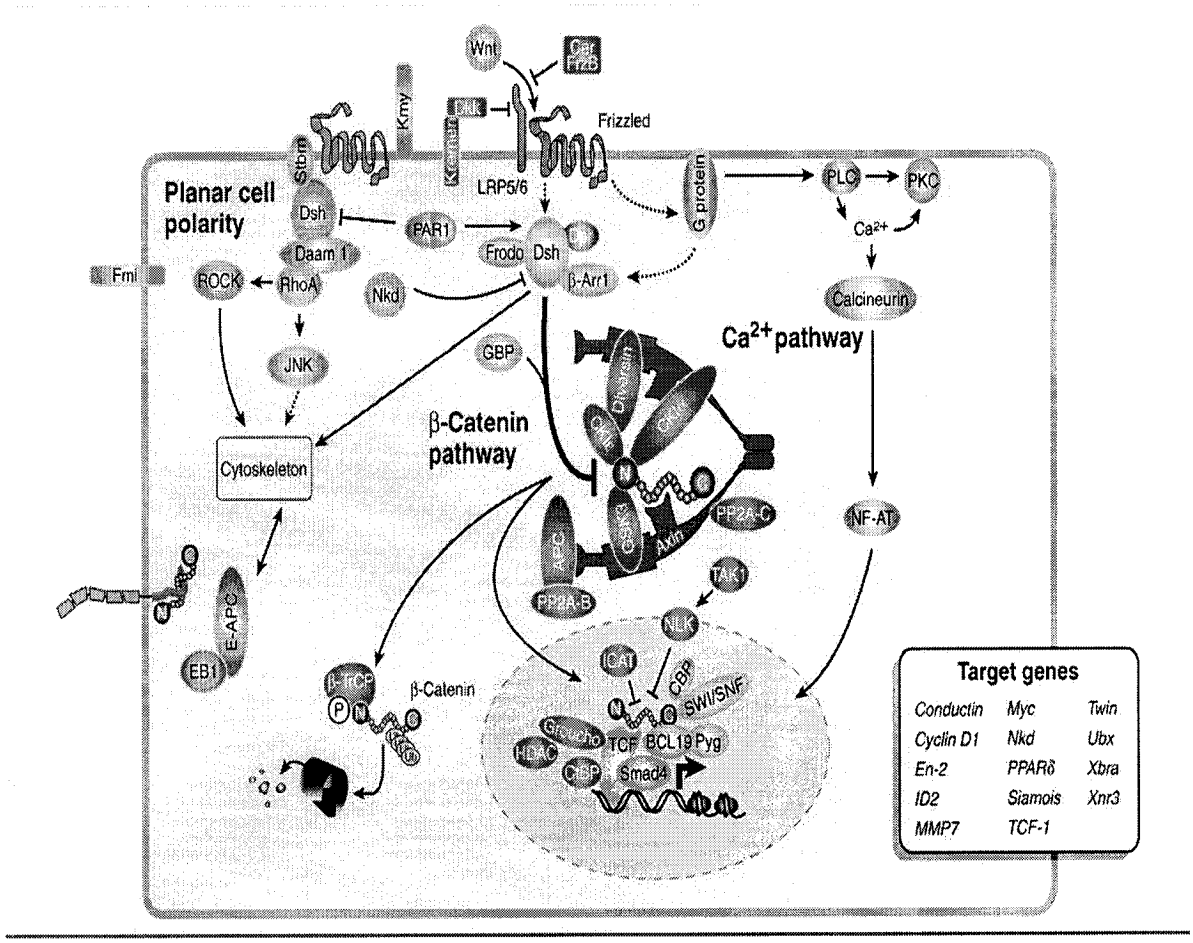


(A) Schematic diagram of *β-catenin* gene. Adapted from (Huelsken et al., 2000).

(B) Schematic diagram of β-catenin protein. Adapted from (Akiyama, 2000).

(C) Three dimensional structure of the armadello-repeat domain of β-catenin. Adapted from (Huber et al., 1997).

Figure 2: Wnt signaling pathways



Schematic diagram of the major Wnt signaling pathways (the Planar cell polarity pathway, the canonical pathway of β-catenin and the calcium pathway).

Figure is adapted from (Huelsken and Behrens, 2002).

to Tcf/Lef family of transcription factors and co-activates the transcription of Wnt target genes. β-catenin activates the expression of key cell-cycle (such as *c-myc* and *cyclin D*) and development regulating (such as *Siamois*, *Goosecoid* and *Xenopus nodal related 3*) genes (Figure 2).

1.1.2.1.2.1: β-catenin ubiquitination and degradation

Ubiquitination of proteins is a cellular regulatory process that involves sequential activation and conjugation of ubiquitin peptide to target proteins in order to initiate their degradation. Ubiquitin is a highly conserved, 76-amino-acid protein that can be covalently conjugated to the lysine residues on a wide assortment of proteins and thus regulate their function (reviewed in (Weissman, 2001). Ubiquitination can serve to target proteins either to proteasomes or to lysosomes for degradation (reviewed in (Lipkowitz, 2003). Ubiquitin-mediated proteolysis by proteasomes plays an essential role in the rapid elimination of short-lived key regulatory proteins such as cell cycle proteins (cyclins), rate-limiting enzymes, or transcriptional activators (β-catenin, IκB-NFκB complex, c-Jun, p53) (reviewed in (Coux et al., 1996). The ubiquitination of specific protein is carried by the ubiquitin-protein ligases known as (E3s), which act at the last step of three-enzyme cascade involving the ubiquitin-activating (E1) and ubiquitin conjugating (E2) enzymes.

In most cases, targeted proteins are recognized and tagged for proteolysis by the ligation of multiple ubiquitin molecules in a multimeric chain. Ligation of ubiquitin molecules to target proteins involves a series of enzymatic reactions. First, the ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-dependent reaction. Second, E1 transfers the ATP-activated ubiquitin onto the ubiquitin conjugating enzyme (E2) through the formation of a thioester bond between the E2 active site cysteine and the ubiquitin C

terminus. Third, E2 carries the ATP-activated ubiquitin and transfers onto the ubiquitin ligase (E3), which binds both a cognate E2 and the substrate and is responsible for catalyzing the covalent modification of lysine residues of the target protein with the ATP-activated ubiquitin. In subsequent cycles, additional ubiquitin molecules are added to the substrate. Multi-ubiquitinated proteins are then recognized by the 19S regulatory subunit of the proteasome and rapidly degraded into short peptides (reviewed in (Jentsch, 1992; Pickart, 2001).

E3s are a diverse family of proteins and protein complexes, which mediate ubiquitination in at least two distinct ways. The first family of E3s is called HECT-type E3s. These form an E3-ubiquitin thioester conjugate and then transfer the ubiquitin to the substrate. The second family is called RING-type E3s, which do not form an E3 ubiquitin conjugate and are thought to promote the ubiquitination of substrate directly by the E2s (reviewed in (Pickart, 2001). SCF belongs to the RING-type E3 family and is composed of four subunits: the scaffold protein Cul1, the RING-domain protein Rbx1/Roc1, the adaptor protein Skp1, and an F box protein that binds to the substrate. In this complex, Rbx1 associates with Cul1 and the E2, while Skp1 interacts simultaneously with Cul1 and the F box protein (reviewed in (Deshaies, 1999).

F box proteins represent the largest group amongst the SCF subunits. The mouse and human genomes each contains more than 70 F box encoding genes and the *C. elegans* genome contains 326 predicted F box proteins (Winston et al., 1999). F box proteins interact with Skp1 through the F box motif of Skp1 and with the substrate through C-terminal protein-protein interaction domains, including WD40 repeat (FBW subfamily) and leucine-rich repeats (LRRs; FBI subfamily). β-TrCP1 is an F box protein that is

highly conserved, from *C. elegans* to humans, and contains WD40 repeats. All known SCF/ β-TrCP1 substrates, such as β-catenin and IκB, contain what is called the destruction motif, which is the amino acid sequence DSGØXS (where Ø represents a hydrophobic and X any amino acid). Phosphorylation of both serines of the destruction motif is pre-requisite for β-TrCP1 binding, linking it for ubiquitination and destruction (Deshaies, 1999).

In the absence of Wnt signaling, β-catenin is first phosphorylated by CKIα at S45, which primes it for phosphorylation by GSK-3β, on T41, S37 and S33 in this sequential order. Phosphorylation of β-catenin on these sites by GSK-3β allows it to be recognized by the SCF/ β-TrCP1 E3 ubiquitination enzymes, which leads to its degradation by the proteasome machinery (Hart et al., 1999; Latres et al., 1999).

Upon Wnt binding to the Frizzled receptor in a complex with LRP-5/-6, Dishevelled (Dsh), which is cytoplasmic adaptor protein required for assembly of a signaling complex analogous to Grb-2 in the Ras pathway, becomes hyperphosphorylated and activated (Yanagawa et al., 1995). Active Dishevelled dephosphorylates axin and disrupts the complex of axin/ CKIα /GSK-3β/APC/ β-catenin. This leads to the inhibition of β-catenin phosphorylation by CKIα and GSK-3β. In vertebrates, inhibition of GSK-3β also involves the activity of GSK-3β binding protein (GBP/Frat), which binds to both Dishevelled and GSK-3β and promotes the dissociation of GSK-3β from the destruction complex (Miller, 2002). When β-catenin is not phosphorylated, it is no longer ubiquitinated and therefore not degraded by the proteosome machinery.

1.1.2.1.2.2: β-catenin nuclear translocation and activation of gene expression

There is overwhelming evidence that β-catenin translocates to the nucleus upon Wnt signaling in most organisms, including *Xenopus*, zebrafish, *Drosophila*, sea urchin, mice and humans (Ahmed et al., 2002; Hsu et al., 1998; Larabell et al., 1997; Willert et al., 1999; Yost et al., 1996). In the nucleus, β-catenin doesn't itself bind to DNA; instead it interacts with members of the Tcf/Lef family of transcription factors via its armadillo repeats. The mammalian genome harbors four Tcf/Lef family members, *Tcf-1, Lef-1, Tcf-3 and Tcf-4*, which share homology with the High Mobility Group (HMG) box transcription factor family. All Tcf/Lef family members recognize and bind to specific DNA sequence (AGATCAAAGGG) via the HMG box DNA-binding domain (Giese et al., 1992; Korinek et al., 1997; van Beest et al., 2000; van de Wetering et al., 1991). Tcf/Lef do not function as classical transcription factors, in that DNA binding alone is not sufficient to cause transcriptional activation. Promoter activation is only achieved after they complex with β-catenin. Within this complex, Tcf/Lef provide the DNA binding moiety and β-catenin provides the transcription activation domain (reviewed in (Barker and Clevers, 2000) (Figure 2).

Several mechanisms are known through which β-catenin can stimulate transcription. In the first mechanism, β-catenin, after interacting with Tcf/Lef, interacts with TATA box binding proteins either directly (Hecht et al., 2000) or indirectly via Pontin52 (Bauer et al., 1998). This would provide a direct interaction between the Tcf/β-catenin complex and the basal transcriptional machinery, resulting in activation of target genes (reviewed in (Brantjes et al., 2002). Another mechanism is through the interaction of β-catenin with the histone acetyl transferase CBP (CREB binding protein)/p300, which binds to a region C-

terminal of the armadillo repeat 10 of β-catenin. Through this interaction, CBP/p300 is tethered to DNA and may locally acetylate nucleosomal histones, resulting in more accessible chromatin, which promotes the binding of other transcription factors, allowing the general transcription machinery to gain access to the promotor and activate transcription (reviewed in (Goodman and Smolik, 2000). A third mechanism by which β-catenin is thought to activate transcription involves functional interaction with Brg-1, which is a core component of the SWI/SNF chromatin remodeling complex (Barker et al., 2001). SWI/SNF complex has been shown to participate in the displacement of histone octamers from DNA of promoter regions, which permits accessibility of DNA to the transcription machinery and initiates transcription (reviewed in (Whitehouse et al., 1999).

Tcf/Lef proteins do not act only as transcriptional activators, however. In the absence of β-catenin they repress the transcription of Wnt target genes. Examples of this were shown for the expression of the *Drosophila* genes *Ultrabithorax* (Yu et al., 1998) and *decapentaplegic* (Yang et al., 2000), the *Xenopus* gene *Siamois* (Brannon et al., 1997) and the mouse *Foxa2* gene (Merrill et al., 2004). Tcf/Lef proteins interact specifically with the *Drosophila* Groucho transcriptional co-repressor protein, or its mammalian homologs, to mediate their repression of Wnt target genes (Cavallo et al., 1998; Levanon et al., 1998; Roose et al., 1998). Groucho proteins are a family of proteins that function as co-repressors for a multitude of DNA-binding transcription factors of distinct families. The mammalian genome harbors four full-length *Groucho* homologue genes and one gene that encodes a truncated Groucho protein. The human full-length *Groucho* homologs are termed transducin-like enhancer of split (TLE-1, -2, -3, -4) and the truncated variant is named, amino-terminal enhancer of split (hAES). The mouse *Groucho* homologs are

termed *Groucho-related* genes, *Grg-1, -2, -3, -4* and the shorter variant *Grg-5* (reviewed in (Brantjes et al., 2002). Groucho proteins exert their repression function by interacting with histone deacetylases (HDAC), which act to promote modulation of local chromatin structure resulting in a compacted chromatin that is associated with a repressed transcriptional state (Chen et al., 1999a).

Besides interacting with Groucho to repress transcription, Tcf proteins also interact with the transcriptional co-repressor CtBP (Brannon et al., 1999). CtBP is thought to mediate short-range repression through a number of unrelated transcription factors, acting over distances of approximately 100 bp (Nibu et al., 1998). Groucho proteins, on the other hand, are classified as long-range repressors capable of silencing transcription over a distance of 1kb (Zhang and Levine, 1999). Therefore, Tcf proteins are capable of interacting with both short- and long-range co-repressors to exert their repression activity.

1.1.2.2: The Planar Cell Polarity/Convergent extension Pathway

The second Wnt signaling pathway is termed the planar cell polarity/convergent extension pathway because it was shown to be involved in the establishment of epithelial planar cell polarity (PCP) in *Drosophila* and the regulation of convergent extension taking place during vertebrate gastrulation (Heisenberg et al., 2000; Moon et al., 1997; Yamanaka et al., 2002). This pathway is Frizzled-regulated and does not involve the co-receptors LRP-5/-6 (Wehrli et al., 2000). It is thought that members of the Wnt5a family of Wnt ligands activate this pathway by binding to the Frizzled receptor, an interaction possibly facilitated by the GPI-anchored proteoglycan Kny (Tada et al., 2002). Downstream of the receptor, Dishevelled directly controls the routing decision of this pathway. In this regard, Dishevelled PDZ and DEP domains are responsible for the specific activation of the

PCP/convergent extension pathway, but not of the canonical pathway, through mediation of the activity of members of the small GTPases (Rho and Rac) (Korswagen, 2002). RhoA, which is linked by Daam1 with Dishevelled, activates an effector Rho-associated kinase (Rok) that in turn directly regulates the actin cytoskeleton. Alternatively or additonally, Dishevelled-mediated activation of Cdc42 signals to the c-Jun-N-terminal-kinase (JNK) that in turn regulates transcription of target genes such as c-jun (Adler and Lee, 2001; Mlodzik, 2000; Sokol, 2000; Tada et al., 2002) (Figure 2).

1.1.2.3: The Wnt/Ca^{++} pathway

The Wnt/Ca^{++} pathway involves activation of a heterotrimeric G protein (Gα0, Gαt and Gβ2) that leads to activation of phospholipase Cδ (PLCδ) enzyme resulting in an increase in the intracellular calcium and activation of the calcium/calmodulin-regulated kinase II (CamKII) and protein kinase C (PKC) (Kuhl et al:, 2000; Liu et al., 1999b; Sheldahl et al., 1999) (Figure 2). As the case in Wnt/PCP pathway, the Wnt/Ca++ pathway does not appear to require recruitment of LRPs into the membrane complex. The downstream targets of CamKII and PKC are currently unknown, but in *Xenopus*, it has been shown that activation of this pathway antagonizes the Wnt/ β-catenin canonical pathway (Torres et al., 1996). In *Drosophila*, this pathway, through the activation of PKC and CamKII, regulates cell adhesion and motility, which modulate asymmetric cell division (Lu et al., 2001a).

1.1.2.4: The spindle orientation pathway

This pathway has been recently identified and therefore information is still lacking concerning its regulation and the molecules involved in its activation. It was initially discovered in *C.elegans* (Schlesinger et al., 1999), where it was observed that the Wnt

pathway is involved in directing the orientation of mitotic spindles and the plane of cell division. Interestingly, orientation of the mitotic spindle does not require gene transcription, suggesting that Wnt signaling may directly target the cytoskeleton in a responding cell. Involvement of Wnt signaling in vertebrate spindle orientation has not been established yet.

1.1.3: Regulation of Wnt signaling

Given the prominent involvement of Wnt signaling pathways in phenomena of developmental processes and cancer, rigid regulation of their activity is expected and indeed is the case. To restrain the impact of improper activation of these pathways, a wide range of strategies is deployed, including secretion of modulatory or antagonistic factors, translocation of downstream components, multi-complex formation, phosphorylation, ubiquitination and nuclear trafficking. Numerous molecules that target the key players of the different Wnt signaling pathways are involved in the regulation of these pathways at the extracellular level, the cytoplasmic level as well as at the nuclear level. Among the several Wnt pathways, the canonical pathway of β-catenin is the best understood in terms of its regulation.

1.1.3.1: Regulation of Wnt/β-catenin pathway at the extracellular level

To antagonize the Wnt signaling pathways, a variety of secreted Wnt inhibitors have been described that either directly bind to Wnt proteins or compete for binding to their receptors and thus prevent them from activating their pathways. Accordingly, Wnt antagonists are divided into two functional classes, the secreted Frizzled-related proteins (sFRP) class and the Dickkopf (Dkk) class. The sFRP class, which includes the sFRP family (sFRP1, 2, 3, 4 and 5), the Wnt inhibitory factor (WIF-1) and Cerberus, bind

directly to Wnts, thereby altering their ability to bind to the Wnt receptor complex. Like the Wnts, sFRPs are secreted glycoproteins, but structurally they resemble the Frizzled receptors although lacking the transmembrane domains (reviewed in (Jones and Jomary, 2002). Similarly, WIF-1 (Hsieh et al., 1999) and Cerberus (Piccolo et al., 1999) are related to the extracellular domains of Frizzled receptors and bind to Wnt proteins and antagonize their action. Members of the Dickkopf class inhibit Wnt signaling by binding to the LRP-5/LRP-6 component of the Wnt receptor complex and compete for Wnt binding to these co-receptors (reviewed in (Kawano and Kypta, 2003). Thus, in theory, antagonists of the sFRP class will inhibit both canonical and non-canonical pathways whereas those of the Dickkopf class specifically inhibit only the canonical pathway.

Activation of Wnt pathways does not only depend on the expression of the ligand or its receptor. One level of the activational control of Wnt/ β-catenin at the extracellular level is to control the expression of the co-receptors LRP-5/-6 proteins. These proteins are widely expressed during embryonic development and have been shown to be indispensable for embryonic survival (Pinson et al., 2000b). Recently, the *Mesd* gene was discovered to encode an LRP-5/-6 chaperone that is essential for membrane localization of these proteins and *Mesd* mutation antagonizes Wnt signaling (Hsieh et al., 2003). In addition to LRP5/6, Dkk-1 interacts with another class of receptors, the single-pass transmembrane proteins Kremen1 (Krm1) and Kremen2 (Krm2) (Mao et al., 2002). Krm, Dkk-1 and LRP6 form a ternary complex that disrupts Wnt/LRP6 signaling by promoting endocytosis and removal of the Wnt receptor from the plasma membrane (Mao et al., 2002).

1.1.3.2: Regulation of Wnt/β-catenin pathway at the cytoplasmic level

Wnt signaling is also regulated at the cytoplasmic level, and numerous molecules have been shown to be involved in this level of regulation. Downstream of the receptor, the multi-module protein Dishevelled interacts with many cytoplasmic regulatory molecules. An example of this is Frodo, which positively regulates Wnt signaling in *Xenopus* (Gloy et al., 2002). Similarly, Naked cuticle (Nkd), which is another Dishevelled-interacting protein, was shown to inhibit the Wnt canonical pathway and promote the PCP/convergent extension pathway (Yan et al., 2001). Therefore, Nkd could act as a molecular switch to direct the activity of common molecule toward a specific Wnt pathway. On the other hand, Dapper (Dpr) (which is required for notochord formation) has been shown to function as a Dishevelled- associated antagonist of both canonical and PCP Wnt signaling (Cheyette et al., 2002).

During inhibition of the canonical Wnt signaling, formation of the GSK3β/APC/Axin/β-catenin complex is essential for phosphorylation and subsequent degradation of β-catenin. Many intracellular molecules have been shown to be important for formation and regulation of this complex. The ser/thr kinases of the CKI family, CKIα and CKIε, phosphorylate β-catenin at ser 45, creating a classical GSK3β recognition motif to initiate β-catenin phosphorylation and subsequent degradation (Amit et al., 2002; Liu et al., 2002). In addition, the ankyrin repeat protein Diversin has been found to be important for the recruitment of CKIε to the β-catenin degradation complex (Schwarz-Romond et al., 2002).

1.1.3.3: Regulation of Wnt/β-catenin pathway at the nuclear level

The mechanism by which β-catenin translocates to the nucleus remains unknown. β-catenin itself does not contain a nuclear localization signal and therefore other proteins must be involved in its cellular trafficking. Regulation of such proteins offers another level of control over Wnt signaling. Once in the nucleus, β-catenin associates with Tcf/Lef family members of transcription factors and activates the expression of target genes. Similarly, regulation of *Tcf/Lef* expression is essential and provides yet another level of control over this pathway. Recently, a new, highly conserved nuclear protein named Chibby was discovered to antagonize Wnt signaling by competing with Tcf/Lef members of transcription factors in binding to β-catenin (Takemaru et al., 2003). In addition, Tcf/Lef family members associate with numerous other regulatory proteins such as the co-repressors Groucho and CtBP proteins and therefore the regulation of these proteins is also crucial for Wnt signaling.

β-catenin doesn't only interact with Tcf/Lef in the nucleus, but also with other transcription activating proteins such as CBP/300 and Brg-1 and the regulation of these proteins must be also important for Wnt signaling. Wnt signals were also shown to cooperate with other extracellular signals, such as members of the TGF family, during development (Crease et al., 1998). Therefore, it is likely that different combinations of nuclear factors activated by these various extracellular signals may cooperate to modulate expression of different target genes and the regulation of these transcription factors is similarly expected to be crucial.

1.1.4: Conclusion

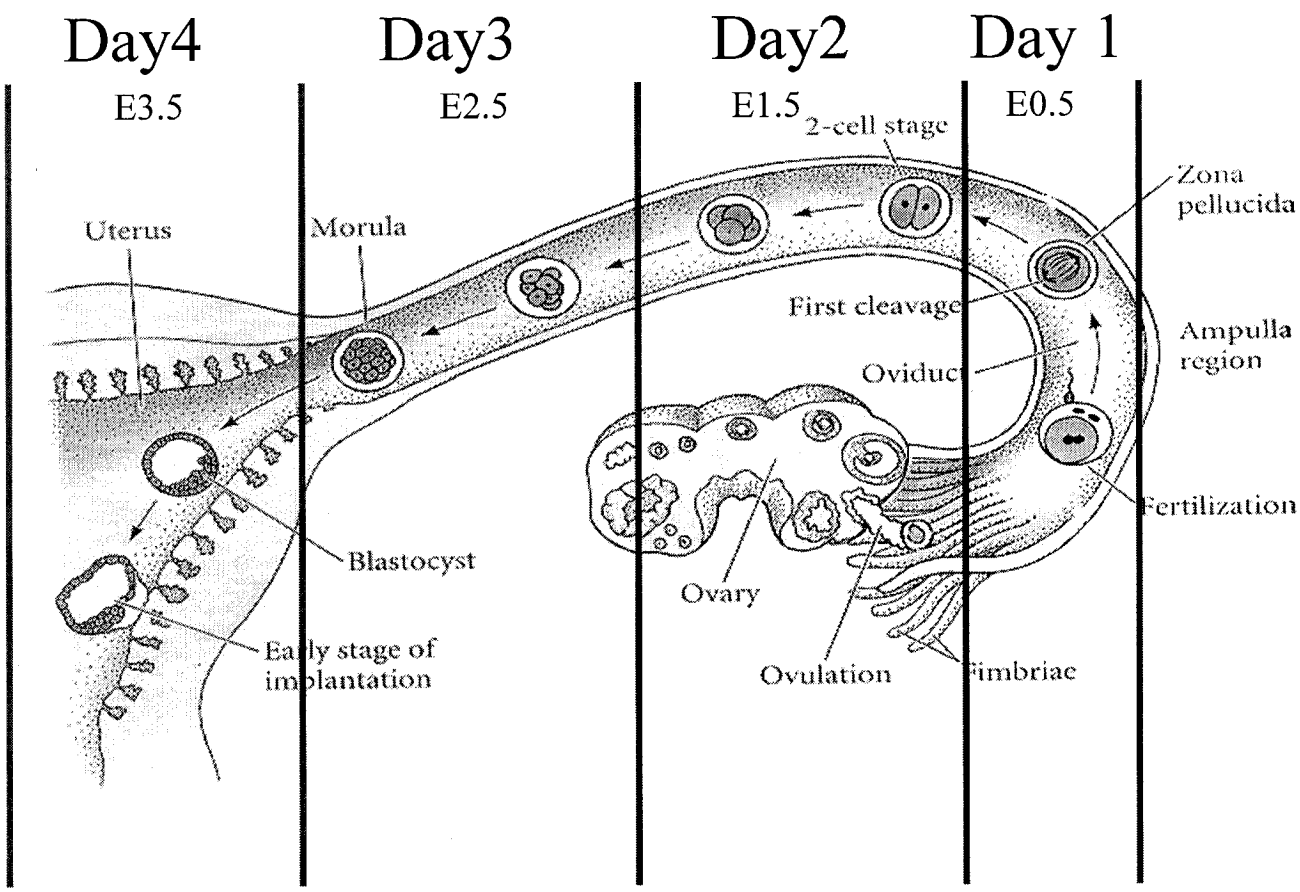
Wnt signaling pathways are crucial in animal development. Activity of these pathways is governed firmly by several mechanisms to ensure proper outcomes. The involvement of Wnt pathways in phenomena of cellular and developmental processes is overwhelming. A substantial amount of research is being conducted to reveal their participation in a variety of other developmental and cellular processes. In this thesis, I investigated the involvement of Wnt signaling in two fundamental developmental processes, embryo implantation and formation of embryonic body axes.

1.2: EMBRYO IMPLANTATION

Following fertilization, the mammalian embryo undergoes a series of cleavage divisions that divides the ooplasm into smaller cells called blastomeres and eventually forms an embryonic structure called the blastocyst. The blastocyst is composed of an outer epithelial cell layer called the trophectoderm, a fluid-filled cavity and a small group of cells called the inner cell mass (Watson, 1992; Wiley et al., 1990). All these events take place while the embryo is living freely in the oviduct and is moving towards the uterus. At the blastocyst stage, the free-living period of mammalian development ends and the embryo must implant in the maternal uterus and establish pregnancy in order to develop further (McLaren and Mitchie, 1956).

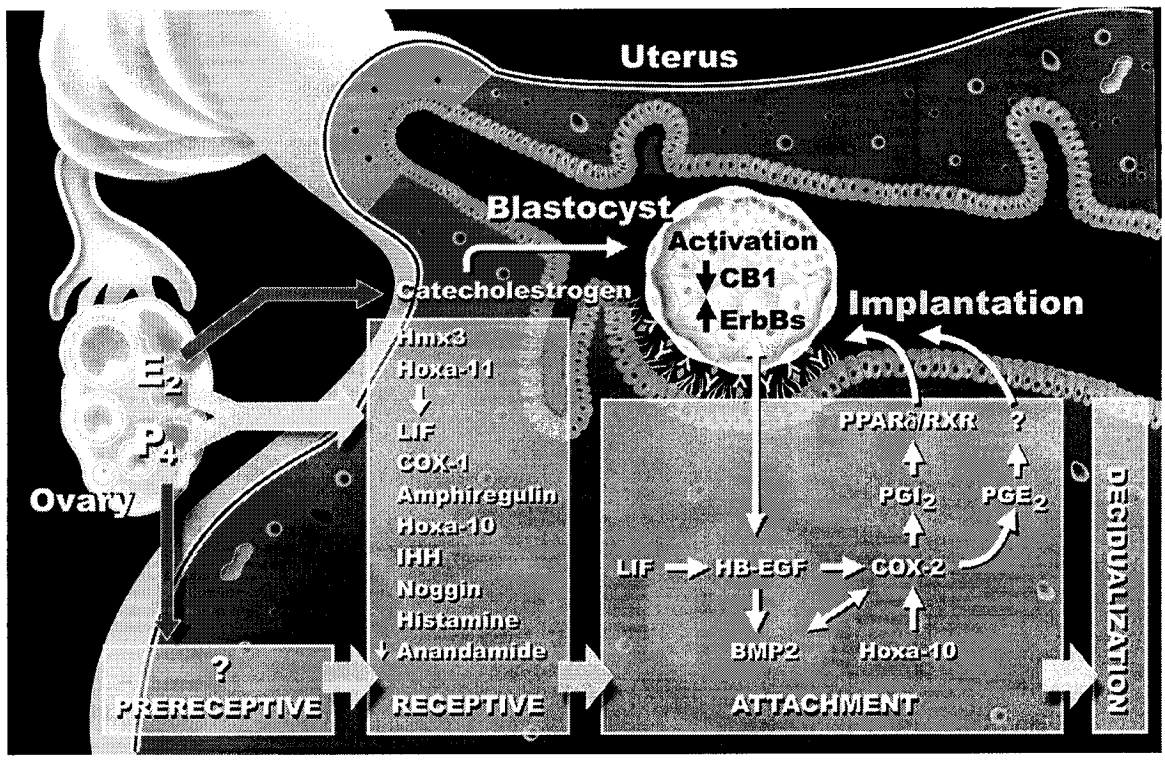
In the mouse, on day 4 of pregnancy, the uterine endometrium becomes receptive for embryo implantation. During this day, the embryo has reached the blastocyst stage and is within the uterine cavity looking for implantation site (Figure 3). One intriguing question is; how does the blastocyst identify the uterine implantation site? This is still an enigma, but an incomplete picture of some of the molecules involved has started to emerge. To achieve successful implantation, a highly coordinated dialogue between the embryo and the uterus is established at the time of implantation to regulate the production of numerous molecules that are required to execute this process (Paria et al., 2002) (Figure 4). In mice, the initial attachment of blastocyst trophectoderm to the uterine epithelia happens at 2200-2300 hr on day 4 of pregnancy and continues on day 5 (Paria et al., 2001).

Figure 3: Preimplantation stages of mouse development



Schematic diagram of human female reproductive tract used here to represent early mouse development. Figure is adapted from (Gilbert, 1997).

Figure 4: The mechanism of blastocyst implantation



Schematic diagram showing the intimate cross talk between the implanting blastocyst and the receptive uterus. Figure is adapted from (Paria et al., 2002).

For unknown reasons, it always takes place on the anti-mesometrial (ventral) half of the uterus. Attachment is initiated by a process called apposition, in which the blastocyst comes into close proximity with the uterus that is promoted by closure of the uterine lumen (Finn and Bredl, 1977). Subsequently, a paracrine communication between the blastocyst and the uterine luminal epithelia is initiated (Figure 4). Current research has focused on a hypothetical signaling molecule(s) that is released by the blastocyst, which in turn triggers a uterine response and facilitates attachment. The identity of such molecule(s), however, remains unknown. What makes the blastocyst release such molecule(s) is also unknown, but it is known that the blastocyst has to be activated by some uterine factor(s) in order to become implantation-competent (Paria et al., 1993b).

Implantation takes place only during a restricted time (18- 24 hours in mice), termed the implantation window, during which the uterus is permissive for implantation (Psychoyos, 1973b). If early events of implantation do not take place during this period, pregnancy failure occurs. Therefore a precisely timed synchrony of development between the implanting embryo and the receptive uterus is required for successful implantation. Hence, the onset of implantation can be seen as a successful meeting between two separate processes, embryo development and uterine differentiation.

1.2.1: Preimplantation development and formation of implantation-competent blastocyst

Development of the embryo from a fertilized egg to a competent blastocyst that is capable of implanting in the mother's uterus requires several preimplantation events to take place properly. The embryonic genome must be activated at the right time and in the appropriate stage of development (Schultz, 1993). Cell fates must be decided to establish specific cell

types such as the inner cell mass and the trophectoderm cell layer. Cell division must be carefully programmed in order for embryo cleavage to follow a relatively strict timing schedule to reach the blastocyst stage (Mohamed et al., 2001). This is particularly important so the embryo reaches the blastocyst stage within a defined period of time to target the uterus's window of receptivity. In addition, successful implantation requires a complex cell-to-cell interaction between the blastocyst and the receptive uterus; thus it is crucial for the embryo to develop an extraembryonic cell population that will mediate this interaction. The trophectoderm cells, which constitute an epithelial monolayer, is the part of the blastocyst that initiates uterine contact and invasion (Carson et al., 2000). It is also due to the ion and fluid-transporting ability of the trophectoderm, that the blastocoel is formed (cavitation), which is an essential event that prepares the blastocyst for implantation (MacPhee et al., 2000).

1.2.1.1: Compaction and Blastocyst formation

Compaction represents the onset of differentiation in mammalian development, in which the outer cells of the conceptus begin to polarize as a prelude to trophectoderm differentiation (Pratt et al., 1982; Wiley et al., 1990). In the mouse, after the third division, the embryo undergoes an increase in contact between its inner blastomeres that obscures the cell boundaries and continues until the embryos appears as a uniform cell mass called morula (Wiley et al., 1990). The outer cell layers develop a polarity, in which their free (apical) plasma membrane becomes distinguished from the opposed (basolateral) membrane (reviewed in (Watson and Barcroft, 2001). The apical membrane surface develops, Na-dependent amino acid transport systems (Miller and Schultz, 1985), Na-glucose co-transporter (Wiley et al., 1991) and Na-channels (Manejwala et al., 1989).

The basolateral surface accumulates tight junction ZO-1 polypeptide (Fleming et al., 1989), gap junctions and E-cadherin (Kemler, 1993; Vestweber et al., 1987). E-cadherin is crucial and its role in blastocyst formation is well established. Its removal by blocking antisera (Watson et al., 1990) or null mutants (Larue et al., 1994; Riethmacher et al., 1995) produces embryos that fail to develop into normal blastocysts. It is believed that E-cadherin contributes to the establishment of the epithelial junctional complex and the insertion of the sodium pump Na/K-ATPase enzyme into the basolateral membrane (reviewed in (Watson and Barcroft, 2001). Expansion of the blastocoel is caused by the transport of fluid (mainly water) across the trophectoderm, which is mediated by the Na/K-ATPase enzyme (Betts et al., 1997; Manejwala et al., 1989).

At the blastocyst stage, the embryo is composed of two cell types: the inner-cell mass (ICM) and the trophectoderm. The inner cell mass gives rise to the embryo proper, while the trophectoderm gives rise to the placenta. Blastocyst formation requires the pre-implanting embryo to orchestrate three important cellular events; cell division, cell differentiation and apoptosis (Fleming and Johnson, 1988). To achieve this, some sort of communication has to take place between the newly formed blastomeres, to decide their fate and assign their future shape, function and location. Communication between cells is executed through the employment of variety of signaling molecules such as hormones, growth factors, cytokines, Hedgehogs, Wnts.

1.2.1.1.1: Role of steroid hormones in the formation of implantation-competent blastocyst

The requirement of ovarian steroid hormones, progesterone (P_4) and estrogen (E_2), for preimplantation development is not absolute since embryos can develop normally *in vitro*

up to the blastocyst stage in the absence of these hormones. It is known, however, at least in the mouse, that the action of ovarian steroid hormones is needed to render the blastocysts competent for implantation (Paria et al., 1993b). Whether this action is direct or indirect is still a controversial issue. The actions of estrogen and progesterone are mainly mediated by their nuclear receptors, estrogen receptor (ER) and progesterone receptor (PR), which are ligand inducible transcription factors. They modulate gene transcription by binding to specific DNA sequences on the promotor of target genes (Beato et al., 1995; Tsai and O'Malley, 1994). There are two estrogen receptors (ERα and Erβ), which are encoded by two different genes and two progesterone receptors (PRA and PRB), which are produced from a single gene by transcription at two distinct promoters and by translation initiation at two alternative AUG sites (Kastner et al., 1990). In mouse, mRNA for both estrogen and progesterone receptors are detected in the blastocyst stage, but not in earlier preimplantation stages (Hou and Gorski, 1993). Estrogen receptor protein was also detected using immunohistochemistry in both the inner cell mass and trophectoderm cells (Hou et al., 1996).

Null mutation of the *ERα* leads to infertility, whereas implantation takes place in *ERβ* mutant mice albeit with reduced ovulation (Couse and Korach, 1999). Mice lacking the *PR* gene display numerous reproductive abnormalities including inability to ovulate and are sterile (Lydon et al., 1995). Selective ablation of *PRA*, however, allows ovulation but fails to rescue implantation (Mulac-Jericevic et al., 2000) indicating that PRA and PRB are functionally distinct. Recent findings, however, indicate that the actions of steroid hormones seem not to be mediated only through their receptors and alternative pathways have been demonstrated. In many cells, estrogenic effects occur within seconds or

minutes and do not require RNA or protein synthesis, and are considered to be mediated by a plasma membrane estrogen receptor (Couse and Korach, 1999; Das et al., 2000; Paria et al., 1999b). For instance, the rapid increase in intracellular cAMP, calcium influx, inositol triphosphate and the release of prolactin after estrogen treatment, are all attributed to a plasma membrane estrogen receptor (Watson and Gametchu, 1999).

Despite the detection of estrogen receptor in the blastocysts, incubation of blastocysts with estrogen doesn't activate them and make them implantation-competent. In this regard, blastocysts that are cultured in the presence of estrogen cannot implant in foster mothers that have been ovariectomized and maintained in progesterone and 2-fluro-estradiol, which has a potent estrogenic effect on the uterus, but cannot be metabolized to catecholestrogens. On the other hand, blastocyst that are cultured in the presence of the uterine estrogen metabolite, the catecholestrogen 4-hydoxy-estradiol (4-OH-E2), implant normally when transferred into similar foster mothers (Paria et al., 1998). These results suggest that estrogen is metabolized to 4-OH-E2 *in utero*, which then activates the blastocyst. Whether the action of 4-OH-E2 is mediated through the estrogen receptor in the blastocyst is not known.

1.2.1.1.2: Role of Growth factors and cytokines in the formation of implantation-competent blastocyst

Growth factors play a major role in cell proliferation and differentiation (reviewed in (Diaz-Cueto and Gerton, 2001). The preimplantation embryo produces autocrine growth factors to regulate its own development (Paria and Dey, 1990). Members of the epidermal growth factor (EGF) family ligands, such as TGF-α and amphiregulin, as well as the EGF receptors ErbB1 and ErbB4, are produced by both human and mouse preimplantation

embryos, as early as the 4-cell stage (Adamson, 1993; Chia et al., 1995; Dardik et al., 1992; Werb, 1990). Addition of TGF-α to the culture media significantly stimulated blastocyst formation (Dardik et al., 1992). Similarly, addition of amphiregulin accelerates blastocyst formation and increases embryo cell number (Tsark et al., 1997). Addition of EGF to the culture medium increases the outgrowth of the trophoblast *in vitro* (Muzikova and Clark, 1995) and improves implantation rates after transfer to recipient mothers (Morita et al., 1994). On the other hand, neutralizing the effect of TGF-α using antibodies (Dardik and Schultz, 1991) or depletion of the EGF receptor using antisense approach, delays the onset of blastocyst formation (Brice et al., 1993). These studies strongly suggest that the EGF family of growth factors play a significant role in the preparation of implantation-competent blastocyst.

Another member of the EGF family of growth factors is heparin-binding epidermal growth factor-like growth factor (HB-EGF), a 22 kDa glycosylated protein with binding affinity for both EGF-receptor and heparin sulfate proteoglycan (Raab and Klagsbrun, 1997). This growth factor exists in two forms; a cell membrane-anchored precursor form and a soluble mature form and both are capable of inducing the autophosphorylation of EGF-receptor by juxtracrine and paracrine mechanisms, respectively (Raab and Klagsbrun, 1997). HB-EGF is produced by the uterine epithelium at the time of implantation and is highly relevant to the implantation process. Addition of HB-EGF to the culture medium stimulates blastocyst proliferation, zona hatching and trophoblast outgrowth in the mouse (Raab et al., 1996). In human, HB-EGF is a potent growth factor for improving the development of *in vitro* fertilized embryo to the blastocyst stage and zona hatching (Martin et al., 1998).

The cytokine Leukemia inhibitory factor (LIF) is a member of the interleukin-6 (IL-6) family and functions through heterodimerization of LIF receptor (LIFR) and gp130 (Robinson et al., 1994). This receptor complex activates the Jak/Tyk kinases, which then phosphorylate the latent cytoplasmic STAT transcription factors, particularly STAT3, which then dimerize and translocate to the nucleus where they activate/repress target genes (Cheng et al., 2002). The transcripts for LIF and its receptor are detected in the blastocyst stage of both mouse and man (Chen et al., 1999b; Conquet and Brulet, 1990). The effect of LIF on embryo culture, however, is controversial. On one hand, co-culturing the embryo with cells that produce LIF enhances the onset of blastocyst formation (Kauma and Matt, 1995). On the other hand, including LIF itself in the media shows no effect on blastocyst formation (Fedorcsak and Storeng, 2003; Jurisicova et al., 1995).

1.2.1.1.3: Role of Wnt proteins in the formation of implantation-competent blastocyst

The role of Wnt proteins in development is mainly understood from knockout studies. All *Wnt* genes that have been knocked out so far showed only postimplantation defects (Greco et al., 1996; Liu et al., 1999a; Majumdar et al., 2003; McMahon and Bradley, 1990; Monkley et al., 1996; Parr et al., 2001; Parr and McMahon, 1995; Stark et al., 1994; Yamaguchi et al., 1999). Surprisingly, despite the critical roles *Wnt* genes play in a diverse of developmental processes, their role in pre-and peri-implantation stages has not been explored. Apart from a recent brief report showing the expression of *Wnt3a* and *Wnt4* during preimplantation stages of mouse embryos (Lloyd et al., 2003), no studies have been reported. Whether Wnt proteins are important and play a role in blastocyst formation and the process of implantation remains unclear.

1.2.2: Uterine events and development of implantation-receptive uterus

In terms of its ability to support implantation, the uterus is classified into three stages: pre-receptive, receptive and non-receptive stages. The pre-receptive stage is the period between ovulation and the implantation day (day 4 of pregnancy in mice) and is directed by the action of progesterone that is released by the corpus lutea after ovulation (reviewed in (Carson et al., 2000; Rinkenberger et al., 1997). The receptive stage is the period when the uterus is receptive for blastocyst implantation (the implantation window). It is regulated by the sequential action of progesterone followed by a small peak of estrogen that happens in the morning of day 4 (9:00-12:00 noon) (McCormack and Greenwald, 1974). At the end of the implantation window, the uterus becomes non-receptive and enters the refractory stage where transferred embryos cannot implant (Dickmann and Noyes, 1960; McLaren and Mitchie, 1956). Uterine sensitivity for embryo implantation is believed to be controlled at the luminal epithelium level, since the restriction on attachment and invasion is removed when this layer of cells is broken or absent as well as in the case of ectopic implantation (Cowell, 1969; Denker, 1993).

1.2.2.1: Role of steroid hormones in preparation of receptive uterus

The involvement of steroid hormones in regulating the implantation process is well documented (Carson et al., 2000). The precise mechanism by which they act, however, remains unclear, and is apparently species-specific (Wang et al., 2002). In all species, progesterone priming of the uterus appears to be essential for successful implantation, while the source and the need for estrogen varies between species. Ovulation is coordinated by the effects of pituitary gonadotropins that mediate follicular development, oocyte maturation, and rupture of antral follicles (Tsafriri et al., 1996).

The postovulatory follicles transform into corpora lutea, which start producing progesterone. Progesterone, along with preovulatory estrogen, work to stimulate the uterus to develop to a prereceptive phase (Psychoyos, 1973b). In rodents, ovarian estrogen secretion on the morning of day 4 (in the mouse) (McCormack and Greenwald, 1974) converts the progesterone primed uterus from a prereceptive to a receptive phase and is absolutely required for blastocyst implantation. This has been clearly demonstrated, since ovariectomy before the estrogen secretion on day 4, prevents implantation and results in blastocyst dormancy, a condition termed delayed implantation (Paria et al., 1993b). This condition can be maintained, up to 30 days, by daily injection of progesterone, in which case the uterus remains in a neutral phase (analogous to prereceptive phase) and the blastocyst remains dormant. Dormant blastocysts remain closely opposed to the uterine luminal epithelium, but do not initiate the attachment reaction (Paria et al., 2002). This condition can be terminated by a single dose of estrogen, after which the uterus enters the receptive stage and the blastocyst becomes activated (Paria et al., 1993b).

In contrast to rodents, where estrogen is maternally supplied, in pigs and rabbits, estrogen is derived by the embryo itself and is also required for implantation (Geisert et al., 1990; George and Wilson, 1978). In hamsters, maternal estrogen is not absolute requirement for embryo implantation and there is no molecular evidence indicating that the embryo itself is the source of estrogen (Wang et al., 2002). This also seems to be the case in man, where there is no clear evidence for the requirement of maternal estrogen in implantation or the existence of embryonic supply of estrogen (de Ziegler, 1995).

In mouse, the major uterine cell types, the uterine epithelial cells and stroma cells, respond differently to progesterone and estrogen. On days 1 and 2 of pregnancy,

preovulatory ovarian estrogen stimulates the proliferation of uterine luminal epithelial cells. On day 3, increasing levels of progesterone secreted by the newly formed corpora lutea induces the proliferation of stromal cells which is further potentiated by ovarian estrogen secretion that happens in the morning of day 4 of pregnancy (Carson et al., 2000). In contrast, the luminal epithelium becomes differentiated on day 4 for interactions with the blastocyst during the attachment reaction, which begins at 2200–2300 hr of this day (Das et al., 1994). A spatiotemporal expression of various growth factors, cytokines, lipid mediators and transcription factors, in the uterus, is regulated by steroid hormones at the time and site of implantation (Carson et al., 2000).

1.2.2.2: Expression of growth factors, cytokines, and their receptors in the uterus at the time of implantation

Several members of the epidermal growth factor (EGF) family are expressed in the peri-implantation uterus and embryo suggesting that these growth factors serve as local mediators to facilitate embryo-uterine interaction during implantation (Das et al., 1997a). In mice, transforming growth factor-α, heparin-binding epidermal growth factor-like growth factor, amphiregulin, β-cellulin, epiregulin, heregulins/neu-differentiating factor-1, which are members of the EGF family, are all expressed in the uterus at the time of implantation (Das et al., 1997a). These molecules are synthesized as plasma membrane proteins, which can be proteolytically cleaved to produce soluble forms. Both anchored and soluble forms of EGF proteins are biologically active (Massague and Pandiella, 1993). These ligands interact with the ErbB (ErbB1, ErbB2, ErbB3 and ErbB4) family of tyrosine kinase receptors. ErbB1 and ErbB4 are expressed in the blastocyst at the time of

implantation and at least the ErbB1 is also expressed in the uterus (Paria et al., 1999a; Paria et al., 1993b).

TGF-α does not appear to be required for implantation, since TGF-α null mutant mice are fertile (Luetteke et al., 1993). The amphiregulin gene is induced in the uterine epithelium on the day of implantation, but seems to interact more specifically with the ErbB1 receptor in the uterus rather than the ErbB1 in the blastocyst, suggesting a role for this growth factor in intra-uterine signaling (Das et al., 1995). The genes for epiregulin, β-cellulin and heregulins/neu-differentiating factor-1 are expressed in the luminal epithelium and stroma at the site of implantation (Das et al., 1997a; Reese et al., 1998). The role of epiregulin and heregulins/neu-differentiating factor-1 in implantation remains to be determined. Mice null mutant for of β-cellulin are viable and fertile (Jackson et al., 2003). Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is induced in the uterine luminal epithelium solely surrounding the blastocyst, at least 7 hours prior to attachment, in an estrogen dependent manner (Das et al., 1994). Mice null mutant for HB-EGF are viable and fertile (Jackson et al., 2003).

Thus, although the expression pattern of these growth factors seems to be very specific and relevant to the implantation process, their function appears to be dispensable. One possible explanation for this discrepancy is the existence of considerable redundancy between the EGF ligands that compensation among them is taking place. Similarly, null mutations of the EGF receptors ErbB1 or ErbB4 show no obvious implantation defects (Gassmann et al., 1995). The double knock out for both receptors has, surprisingly, not been done. It would be intriguing to know whether the blastocyst is capable of implantation when it lacks the only two EGF receptors it expresses.

The expression pattern of the cytokine Leukemia Inhibitory Factor (LIF) in the mouse uterus is very dynamic. It is expressed in the endometrial glands on the first day of pregnancy, declines in day 3, comes back strongly in day 4 (the implantation day) and declines again in day 5 and remains at low level throughout the rest of pregnancy (Bhatt et al., 1991). LIF is also produced in pseudopregnant females and its production is regulated by estrogen (Bhatt et al., 1991; Chen et al., 2000). LIF can substitute for estrogen in inducing implantation suggesting that the role of estrogen is perhaps only to stimulate the uterus to produce LIF (Chen et al., 2000). Uterine LIF is indispensable for implantation, but blastocysts lacking LIF implant normally (Stewart et al., 1992). Inactivation of gp130, a signaling partner for the LIF receptor, also results in a uterus that fails to support implantation, but the gp130 mutant blastocysts implant normally in wild type uterus (Ernst et al., 2001). These results suggest that the action of LIF to facilitate implantation is to be on the uterus rather than on the embryo. The uterine sites of LIF action, however, remain unknown, but the LIF receptor (LIFrα) and its partner gp130 are expressed in the luminal and glandular epithelium (Cheng et al., 2002). An increase in LIF expression at the time of implantation has also been described in other mammals, such as humans, rhesus monkey, marmoset, pig, sheep and rabbit (reviewed in (Cheng et al., 2002).

1.2.3: Embryo-Uterus cross talk

The current scenario of the language of communication between the uterus and the blastocyst to facilitate the implantation process is the following. The ovarian estrogen surge that occurs in the morning of day 4 induces LIF production in the progesterone-primed uterus. LIF then induces, very rapidly, a transient factor(s) that in turn either directly or indirectly activates the blastocyst *in utero*. Once the blastocyst is activated, it

produces a factor(s) that in turn activate gene expression in the uterus and facilitate the initial attachment events. The identity of the uterine factor(s) that is produced after the estrogen surge, which activates the blastocyst, is not very clear yet. The estrogen uterine metabolite, the catecholestrogen (4-OH-E2), however, is shown to be rapidly formed in the uterus after estrogen treatment and is able to activate the blastocyst *in vitro* (Paria et al., 1998). On the other hand, the identity of the signaling molecule(s) produced by the activated blastocyst that trigger uterine gene expression remains totally unknown. Implanting beads soaked with several signaling proteins, including those belonging to the Epidermal Growth factor (EGF), Bone morphogenetic proteins (BMPs) and Hedgehog (HH) pathways indicated that HB-EGF or IGF-1 induce many of the responses elicited normally by the blastocyst including local vascular permeability and decidualization. (Paria et al., 2001).

The effect of the blastocyst on gene expression in the uterus at the site of implantation is not understood yet. Gene microarray comparison of implantation and inter-implantation sites of the uterus showed that 36 up-regulated genes and 27 down-regulated genes at the implantation site (Reese et al., 2001). Thus far, no particular signaling pathway is shown to be specifically activated at the time and site of implantation.

1.2.4: Adhesion and attachment

Embryo attachment takes place through the apposition of the mural trophectoderm of the blastocyst (The trophectoderm opposite of the inner cell mass) to the uterine epithelium. Apposition and attachment of the embryo to the endometrium involves interaction between the apical surfaces of the blastocyst trophectoderm cells and the luminal epithelium. Prior to the time of attachment, both the blastocyst and uterine epithelium

become adhesive (Carson et al., 2000). The blastocyst first hatchs from the zona pelluicid, which is enveloping the embryo. Blastocyst hatching is achieved by the production of a trypsin-like protease, called strypsin, by the mural trophectoderm as well as by endometrium glands at the time of implantation, which work to dissolve the zona pellucida (O'Sullivan et al., 2002; Perona and Wassarman, 1986). The zona, however, is not the only factor since zona removal is not sufficient to convert the preimplantation embryo to an adhesive state (Carson et al., 2000).

In rodents, prior to implantation, the blastocyst trophectoderm change to become trophoblast cells that are capable of invasive phenotype (Kimber and Spanswick, 2000). On the uterine side, the uterus down regulates the expression of anti-adhesion molecules. Normally, the uterine epithelium cells, under most conditions, produce high molecular weight glycoproteins, such as MUC1 at their apical surface, which provides a physical barrier to enzymatic attack and infection (Hilkens et al., 1992). MUC1 is an anti-adhesion protein and was shown to inhibit cell-cell and cell-extracellular matrix adhesion (Wesseling et al., 1995). MUC-1 was also shown to greatly impair access to the surface of uterine epithelia, while its removal increase both enzymatic attack and blastocyst attachment (DeSouza et al., 1999). During the receptive phase of the uterus, MUC1 expression is severely reduced and thus enhancing access for blastocyst attachment (Surveyor et al., 1995).

Initial attachment of the blastocyst into the uterus wall is thought to be mediated by heparin-sulphate (HS) proteoglycans. HS-proteoglycans are proteins bearing one or more high molecular weight linear, highly negatively charged glycosaminoglycan chains of the HS variety. HS proteoglycans participate in cell adhesion processes in a variety of systems

and some, such as syndecan and perlecan are detected in the blastocyst stage at the time of implantation (Dziadek et al., 1985; Farach et al., 1987). Several proteins, including HB-EGF, can bind to the HS-proteoglycans to facilitate adhesion between cells. It is thought that the anchored form of HB-EGF expressed in the uterine epithelium binds to the HS-proteoglycans found on the blastocyst and this facilitates the initial attachment of the blastocyst to the uterine wall (Kimber and Spanswick, 2000). The soluble form of HB-EGF, on the other hand, is involved in promoting blastocyst hatching (Martin et al., 1998). Further firmer attachment and anchoring of the blastocyst into the uterine wall is believed to be mediated by several other adhesion molecules such as cadherins, integrins, selectins and galectins family of proteins (reviewed in (Aplin, 1997; Kimber, 2000).

1.2.5: Vascularisation, trophoblast invasion and decidualization

In rodents, the first conspicuous sign that the implantation process has been initiated, is an increased endometrial vascular permeability at the sites of blastocyst apposition, which can be visualized by discrete blue bands along the uterus after injection of a blue dye (Psychoyos, 1973b). Several vasoactive agents including histamine, platelet-activating factor and vascular endothelial growth factor have been implicated in implantation (Paria et al., 2002). Recent evidence implicates the involvement of prostaglandins in this process. Prostaglandins are lipid mediators that are generated via the cyclo-oxygenase (COX) pathway (Smith and Dewitt, 1996). In mice, COX-2 is expressed in the uterus specifically at the site of implantation and animals that lack the COX-2 gene have implantation and decidualization failures (Chakraborty et al., 1996; Lim et al., 1999). Recently, prostaglandin I2 (PGI2) was shown to be generated by COX-2 and to be the key mediator in these processes via its activation of the nuclear hormone receptor, peroxisome

proliferator-activated receptor-δ (PPARδ), which heterodimerizes with retinoid X receptor (RXRs) and activate specific genes (Lim et al., 1999).

After the embryo adheres to the uterine wall, the uterine epithelium undergoes cell death and decidulaization begins (Welsh and Enders, 1991). Decidualization involves transformation of the stromal fibroblasts to decidual cells, starting closest to the embryo and moving outwards (Fazleabas and Strakova, 2002). During decidualization, cells acquire extensive cell-cell contacts and swell up to 10 times their stromal cell volume and become polyploid. They lay down a basement membrane-like extracellular matrix around each cell and become very cohesive (El-Shershaby and Hinchliffe, 1975). Each implantation site becomes a separate mass of cells with out an epithelium-lined lumen, but with an embryo at the core. Meanwhile, the extraembryonic tissues from the developing embryo ingress into the stromal/decidual mass and keep dividing and differentiating. At E6.5 days post coitum, the embryo begins gastrulation where it forms its three germ layers and continues to develop inside the mother until term.

1.3: AXIS FORMATION AND GASTRULATION

1.3.1: Axis formation

Polarity is essential for development, meaning that the early embryo has to break symmetry at one stage in order to provide cues that are needed to determine the body plan of the whole animal. In all animals the body plan is arranged along three axes: the anteroposterior (AP) axis, the dorsoventral (DV) axis and the left-right (LR) axis. Different species manifest different strategies and timing when establishing polarity. In this regard, polarity can already exist in the oocyte for some species or be acquired with the events of fertilization and development for others. During development of several species, such as *Drosophila melanogaster*, *Caenorhabditis elegans*, zebrafish and *Xenopus laevis*, the polarity of the embryo takes its roots from the spatial patterning of the egg (reviewed in (Goldstein and Hird, 1996; Gotta and Ahringer, 2001; Moon and Kimelman, 1998; Riechmann and Ephrussi, 2001; Schier, 2001; van Eeden and St Johnston, 1999).

1.3.1.1: Axis formation in the mouse

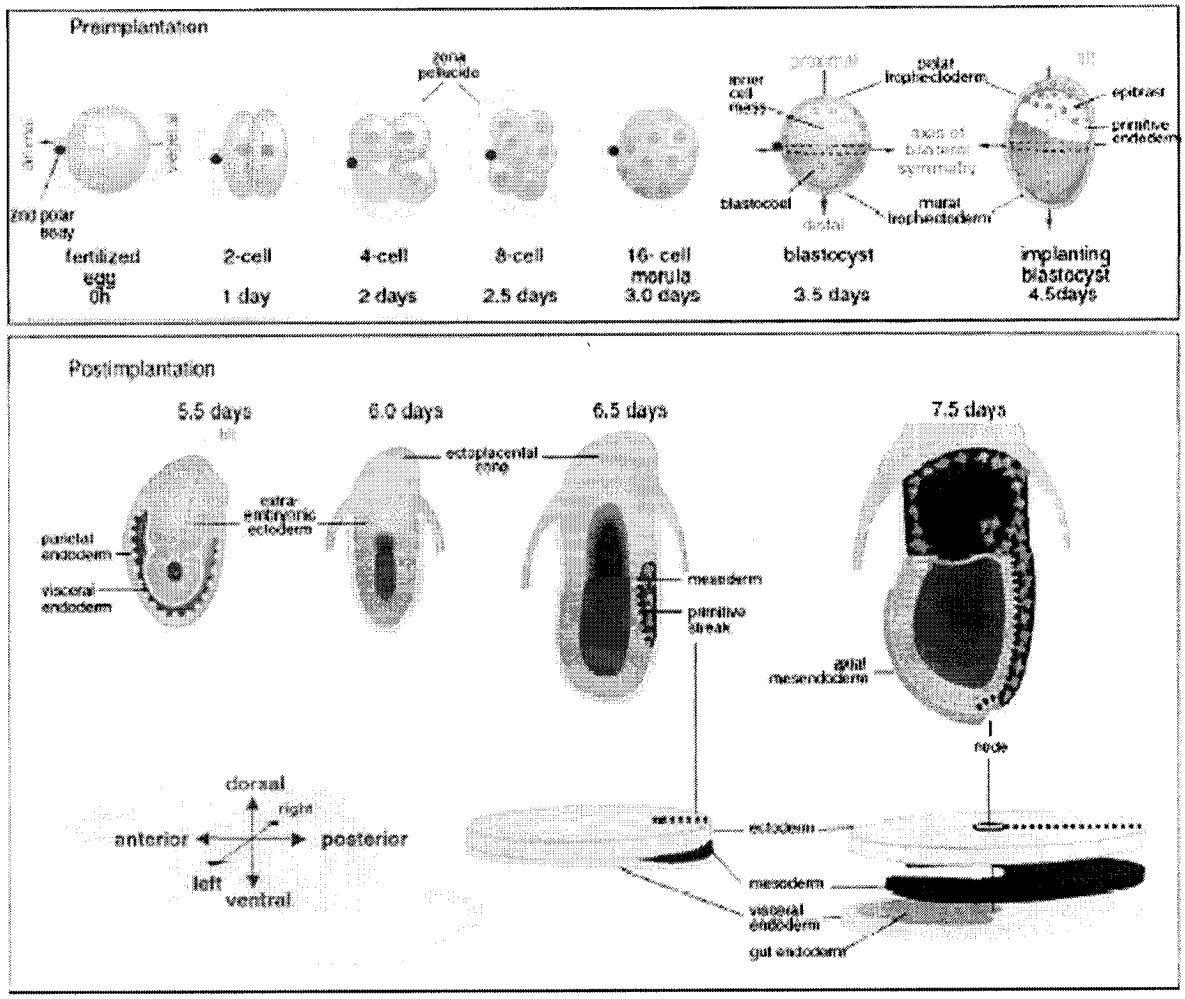
On day 4 of development the mouse embryo reaches the blastocyst stage, which, as discussed earlier, is a ball-shaped structure composed of inner cell mass (ICM), fluid cavity (blastocoel), and a single layer of epithelial tissue called the trophectoderm that surrounds both. The trophectoderm is divided into two regions, the polar trophectoderm, which surrounds the ICM, and mural trophectoderm, which surrounds the blastocoel. Furthermore, the blastocyst is divided into two poles; the embryonic pole which contains

the ICM and the abembryonic pole which contains the blastocoel (Gardner, 1997). By the end of embryonic day 4 (E4.0), the blastocyst implants into the uterine wall.

On day E4.5, a third tissue called the primitive endoderm differentiates on the blastocoelic surface of the ICM. The remainder of the ICM is now called the epiblast and is referred to as embryonic tissue, because it is the progenitor tissue of the whole future embryo as well as of the extraembryonic mesoderm and endoderm (Gardner, 1983). The trophectoderm and primitive endoderm are referred to as extraembryonic tissues, because they do not contribute any descendants to the future body. The trophectoderm contributes only to the trophoblast and the extraembryonic ectoderm (ectoplacental cone). The primitive endoderm is destined to generate only the extraembryonic parietal and visceral endoderm (the tissue that surrounds both, the epiblast and the extraembryonic ectoderm) (Gardner, 1983) (Figure 5).

Between E4.5 and E5.5, the embryo changes its shape and size rather dramatically. The conceptus tissue volume increases almost 40-fold largely due to trophectoderm proliferation, which yields the extraembryonic ectoderm (Snow, 1977). Because of the physical block imposed by the uterine wall, the proximal proliferating polar trophectoderm pushes the proliferating ICM (epiblast) and the enveloping visceral (primitive) endoderm into the space available in the blastocoel cavity towards the distal pole of the conceptus (Copp, 1979). Subsequently, a cavity forms in the center of the epiblast as a result of apoptotic and survival signals from the visceral endoderm (Coucouvanis and Martin, 1995). As a result, the epiblast becomes a cup-shaped epithelial tissue (embryonic ectoderm) apposed on its open end to the extraembryonic

Figure 5: Preimplantation and early postimplantation stages of mouse embryo



Schematic diagram of early mouse development. Figure is adapted from (Beddington and Robertson, 1999).

ectoderm and both are covered with visceral endoderm cells (Figure 5). Due to its shape, the embryo at this time is referred to as an egg-cylinder.

Embryonic body axes become morphologically evident only on day E6.5 when the anterior/posterior axis is specified at the time of gastrulation. Gastrulation is the process by which the embryo is converted from one with two germ layers (ectoderm and visceral endoderm) into one with three germ layers, namely the ectoderm, mesoderm and endoderm. It begins when a group of cells at the proximal region of the epiblast undergo an epithelial to mesenchymal transition after ceasing the expression of E-cadherin (Burdsal et al., 1993; Takeichi, 1988). These cells then ingress between the epiblast (embryonic ectoderm) and the visceral endoderm and form the transient site of ingression known as the primitive streak (Hashimoto and Nakatsuji, 1989). The primitive streak region demarcates the posterior side of the embryo, whereas the region opposite from the streak becomes the anterior side of the embryo. The streak then elongates until it reaches the distal tip of the embryo where another organizer center called the Node forms (Beddington, 1994). At this stage the dorsal/ventral and left/right axes of the embryo are specified (Beddington and Robertson, 1999; Hamada et al., 2002).

The time and stage at which the mammalian embryo breaks symmetry and provides cues for the overt body axes that are specified at the time of gastrulation is still an unresolved issue. Mammalian axis formation has long been considered to be a late developmental event that is established only at or shortly before gastrulation. Recent studies, however, suggest that directional cues for axis formation in mammalian embryo perhaps, as the case in lower animals, are present within the egg.

1.3.1.1.1: Polarity during oocyte development within the follicle

During the period of its growth within the follicle, the oocyte receives signals from the follicle cells for growth and meiotic maturation (reviewed in (Eppig, 1991). Some proteins become asymmetrically localized within the oocyte during this time. Examples of this are STAT3, which is involved in signal transduction and activation of transcription (Darnell et al., 1994) and leptin, a cytokine that is involved in the activation of STAT3 (Matsuoka et al., 1999). STAT3 and leptin localize to the pole of the oocyte that coincides with the area in which the first and second polar bodies will be extruded, and there is evidence that follicle cells are involved in this localization (Antczak and Van Blerkom, 1997). The region where the first body is located is traditionally known as the animal pole, whereas the vegetal pole is opposite to the polar body. During cleavage, STAT3 and leptin preferentially segregate to the trophectoderm rather than the ICM lineage (Antczak and Van Blerkom, 1997). Thus, at least conceptually, STAT3 and leptin could be considered to demarcate the animal pole of the oocyte, which could be correlated to blastocyst polarity later on development.

1.3.1.1.2: Polarity during fertilization

In the mouse, sperm entry normally occurs away from the region where the polar body is located. This is believed to happen because the oocyte surface close to the polar body is a microvilli-free zone and sperm can not attach to this zone (reviewed in (Scott, 2000). After fertilization, the oocyte extrudes the second polar body at the animal pole, close to the first polar body. The second polar body is found to have a pronounced tendency to be located near the boundary between the embryonic and abembryonic poles of the blastocyst (Gardner, 1997). Thus, the association of the polar body with a certain region of the

blastocyst provides a link between a region in the oocyte animal pole and its location later at the blastocyst stage.

Tracing the lineage of early blastomeres indicates that the animal cytoplasmic contents of the zygote are conveyed to the region that lies adjacent to the polar body in the blastocyst. On the other hand, cytoplasmic contents of the vegetal pole are conveyed to the region opposite the polar body in the blastocyst (Ciemerych et al., 2000). Similarly, the sperm entry position (SEP) is found to be located close to the cleavage furrow of the 2-cell stage in most cases. At later stages, the SEP is found to be located close to the boundary between the embryonic and abembryonic regions of the blastocyst (Piotrowska and Zernicka-Goetz, 2001). Consequently, these results suggest that sperm entry demarcates the location of the first cleavage and separates the embryonic-abembryonic regions of the blastocyst.

1.3.1.1.3: Polarity at the blastocyst stage and during implantation

Before implantation, the blastocyst is bilaterally symmetrical, that is, it is polarized along two intersecting axes. One is the embryonic-abembryonic axis, which is evident by formation of the blastocyst cavity (blastocoel) and the inner cell mass (ICM). In this axis, the ICM is located in the embryonic region and the blastocoel is located in the abembryonic region. This represents the first overt manifestation of morphological asymmetry in mammalian embryos. The second axis is within the embryonic region itself, in which the polar body is located on one side of the ICM (Gardner, 1997). Cell labeling studies have indicated that cells located close to the polar body tend to become the distal visceral endoderm of the conceptus. On the other hand, cells that are located away from the polar body tend to become the proximal visceral endoderm (Weber et al., 1999).

In rodents, the blastocyst appears not to initiate implantation symmetrically, rather it adheres to the uterine wall through the mural trophectoderm at one side of its embryonic-abembryonic axis (reviewed in(Zernicka-Goetz, 2002). Previous studies have indicated that, just prior to implantation, the ICM and polar trophectoderm complex is seen to be slightly tilted with respect to the embryonic-abembryonic axis (Smith, 1980).

1.3.1.1.4: Arguments against the existence of graded information in the mammalian egg that is essential for dictating subsequent embryonic pattern

The notion that the overt body axes established postimplantation is based on directional cues present within the preimplantation embryo is still very fragile. The asymmetrical localization of STAT3 and leptin within the oocyte and early embryo is intriguing, but whether it has any significant function in axis formation is not known. Similarly, the frequent localization of the polar body to the area that separates the embryonic-abembryonic regions of the blastocyst was not shown to exert any influence on axis formation. Misallocation of the polar body that occurs normally in some embryos is not associated with any defects in axis formation. Removal of the polar body does not impair development or disturb body axes. Normal offspring are produced when most or all the animal or vegetal pole of the egg has been mechanically removed (Zernicka-Goetz, 1998). This strongly suggests that there cannot be axis formation essential determinants that are localized to one pole of the egg or the other.

In addition, unlike other organisms, the mammalian preimplantation embryos display a high degree of developmental plasticity. For example, when cells of early mouse embryos are removed or re-positioned, normal development can still occur (reviewed in (Papaioannou, 1986). The strongest argument against the existence of cues within the egg

that dictate the orientation of axes comes from embryo aggregation studies. When two intact 8-cell stage embryos are placed in contact with each other, they aggregate and form a single chimeric blastocyst with out cells from the two 8-cell embryos being intermixed or sorted out (Garner and McLaren, 1974). If each 8-cell stage embryo contains its own array of axial determinants, the outcome of these experiments would then be expected to be two embryos, rather than a single chimeric one.

1.3.1.1.5: Polarity at the postimplantation stages

Postimplantation and prior to gastrulation, the embryo contains two regions, the extraembryonic ectoderm and the anterior visceral endoderm, which play an essential role in axis formation. The first tissue in the conceptus that is polarized and plays an important role in axis formation is the extraembryonic ectoderm, which is positioned just above the epiblast. Extraembryonic ectoderm is polarized because several genes that are essential for establishing embryonic body axes are expressed asymmetrically within this region (reviewed in (Lu et al., 2001b). The ability of the extraembryonic ectoderm to pattern epiblast cells has been clearly demonstrated. When cells from the distal tip of the epiblast, which are destined to form the neuroectoderm, are grafted to the proximal side of the epiblast, they adopt new fate and give rise to primordial germ cells and extraembryonic mesoderm (Tam and Zhou, 1996). This strongly suggests that extraembryonic ectoderm actively pattern adjacent epiblast cells to adopt posterior fate. This activity is not a result of physical location, since explants of extraembryonic ectoderm can induce distal epiblast cells to become primordial germ cells (Yoshimizu et al., 2001).

1.3.1.1.5.1: Genes expressed in the extraembryonic ectoderm that are essential for axis formation

At the egg-cylinder stage, there is proximal-distal polarity in the extraembryonic ectoderm as indicated by the expression pattern of *Bmp4*, a member of the TGF-β super family of signaling proteins (Hogan, 1996; Waldrip et al., 1998). On day (E5.5), *Bmp4* is initially detected throughout the uncavitated extraembryonic ectoderm. Shortly before gastrulation (E6.0), *Bmp4* expression continues in the extraembryonic ectoderm, but is localized in cells that abut the epiblast (Lawson et al., 1999). Embryos null mutant for *Bmp4* die around gastrulation (E6.5) and fail to express the mesoderm inducing gene *Brachyury* (*T*) (Winnier et al., 1995). The T-box gene *Eomesodermin* (*Eomes*) is another gene that is expressed in the extraembryonic ectoderm and is essential for development. After implantation (E5.5) *Eomes* is expressed only in the extraembryonic ectoderm. Shortly before gastrulation (E6.0), *Eomes* continues to be expressed in the extraembryonic ectoderm, but is also detected in the epiblast cells where the primitive streak will form (Russ et al., 2000).

Wnt3 is also expressed prior to gastrulation in a ring of extraembryonic ectoderm cells that are adjacent to the epiblast. Shortly before gastrulation (E6.0), *Wnt3* is detected only in the posterior epiblast and adjacent visceral endoderm cells in the area where the primitive streak will form. Embryos lacking *Wnt3* fail to initiate gastrulation and subsequently lack any embryonic axes (Liu et al., 1999a). *Mesd* is expressed in both embryonic and extraembryonic tissues prior to and during gastrulation and embryos mutant for *Mesd* locus fail to initiate gastrulation (Holdener et al., 1994).

Interestingly, a reciprocal interaction between the extraembryonic ectoderm and the epiblast is apparently needed to pattern the epiblast. This is evident from studies done with the TGF-β-related protein Nodal. *Nodal* expression during early postimplantation stages is highly dynamic. The onset of *Nodal* expression occurs at day E5.0 in the visceral endoderm and throughout the epiblast (Varlet et al., 1997). Prior to gastrulation (E5.5-E6.26), *Nodal* expression is gradually down-regulated in the distal tip of the epiblast and becomes confined to cells within the prospective posterior proximal epiblast marking the site of primitive streak formation (Collignon et al., 1996; Varlet et al., 1997).

Shortly after the onset of gastrulation and elongation of the primitive streak, *Nodal* expression becomes restricted to cells of the developing node (Collignon et al., 1996). Embryos that are null mutant for *Nodal* fail to gastrulate and do not show any sign of axis formation (Conlon et al., 1994). In *Nodal* null mutants, *BMP4* and *Eomes* expression in the extraembryonic ectoderm is not maintained and expression of *Wnt3, T and Fgf8* in the proximal posterior epiblast is abolished (Brennan et al., 2001). This indicates that a signal from the epiblast (*Nodal*) is required to maintain the expression of a signal in the extraembryonic ectoderm (*BMP4 and Eomes*) in order to initiate the expression of posterior determination signals in the epiblast (*Wnt3, T and Fgf8*).

1.3.1.1.5.2: Role of Anterior Visceral Endoderm (AVE) in establishing embryonic axes

Recent evidence indicates that anterior patterning of the embryo precedes primitive streak formation and hence posterior patterning of the embryo. An increasing number of genes have been shown to be expressed in the anterior visceral endoderm cells, before or coincident with the onset of gastrulation. The anterior visceral endoderm is a strip of visceral endoderm cells located on the anterior side of the embryo opposite the primitive

streak. Before primitive streak formation, a subpopulation of visceral endoderm cells located at the distal tip of the egg cylinder move proximally to mark the prospective anterior side of the embryo and become known as anterior visceral endoderm cells (Thomas and Beddington, 1996). Physical removal of anterior visceral endoderm cells from the conceptus results in embryos that lack anterior structures (Thomas and Beddington, 1996), indicating the involvement of these cells in patterning the anterior side of the embryo.

Coincident with their anterior movement, several genes are expressed in the anterior visceral endoderm cells. These include; VE-1 (Rosenquist and Martin, 1995), *Otx2* (Acampora et al., 1995; Ang and Rossant, 1994), *Lim1*, *Gossecoid*, *Cerberus-related 1* (Belo et al., 1997), *Dickkopf (Dkk)*, *Lefty1* (Lu et al., 2001b) and *Hex (Thomas et al., 1998)*. *Hex* represents the earliest gene known to be expressed asymmetrically along the anterior-posterior axis of the embryo. On day E4.5, *Hex* is expressed throughout the primitive endoderm as it forms on the blastocoelic surface of the implanting blastocyst. On day E5.5, *Hex* expression is restricted to only a few visceral endoderm cells at the distal tip of the epiblast that will give rise to the anterior visceral endoderm (Thomas et al., 1998). Embryos that lack *Hex*, however, still gastrulate and form embryonic axes, but show defects in the development of the forebrain, liver and thyroid gland (Martinez Barbera et al., 2000).

A model for the mechanism by which anterior visceral endoderm specifies anterior embryonic identity has been suggested. In this model, the anterior visceral endoderm cells inhibit posterior structures, such as primitive streak, from forming in the anterior side of the embryo. To accomplish this, the anterior visceral endoderm cells express Wnt and

TGF-β inhibitors, such as *Dkk1*, *Cer1* and *lefty1*, which are thought to protect the adjacent ectoderm from caudalizing or mesoderm-inducing influences (Beddington and Robertson, 1999).

Molecular evidence for this model has been provided. On one hand, inhibiting the formation of anterior visceral endoderm not only leads to the absence of anterior structures, but also causes the epiblast to adopt total posterior identity. This is evident in chimeric embryos that lack *Smad2* (an essential transducer for TGF-β signaling) (Brennan et al., 2001; Waldrip et al., 1998) or both *Foxa2* and *Lim1* (Perea-Gomez et al., 1999) in the visceral endoderm. In these embryos, the anterior visceral endoderm fails to form and there is widespread expression of *T*, *Fgf8* and *Nodal* (normally expressed only in the posterior epiblast). Consequently, the epiblast adopts completely a proximal-posterior identity.

On the other hand, inhibiting anterior movement of the visceral endoderm, and thus forcing the anterior visceral endoderm to form at the distal tip of the embryo causes all anterior structures to shift posteriorly. This is evident in embryos that are null mutant for *Otx2* (Acampora et al., 1998; Rhinn et al., 1998) and *Crpito* (Ding et al., 1998). In these embryos, the visceral endoderm fails to move proximally (anteriorly) and the anterior visceral endoderm forms at the distal tip of the embryo. In such case, distal epiblast adopts anterior fates and the expression of posterior genes such as *T* is shifted more posteriorly where it becomes restricted to the most proximal posterior part of the epiblast. These results suggest the primary role of the anterior visceral endoderm is to restrict expression of posterior genes involved in mesoderm induction, thereby allowing the anterior epiblast to remain receptive for later anterior patterning.

The mechanism that causes the visceral endoderm cells at the distal tip of the conceptus to move proximally/anteriorly and become the anterior visceral endoderm is not known. Cell-labeling experiments suggest that their movement is part of a global anteriorward rotation of the visceral endoderm before streak formation (Weber et al., 1999). This, however, is disputed, because recent ultrastructural analysis has shown that visceral endoderm cells, which will give rise to the anterior visceral endoderm cells acquire distinct morphology from other visceral endoderm cells and detach from the epithelial visceral endoderm sheet before their anteriorward movement (Kimura et al., 2000). The mechanism by which the anterior visceral endoderm cells are induced to express the genes that antagonize the formation of posterior structures is not known. Nodal, however, seems to play an important role, where embryos lacking Nodal fail to express any of these genes in the anterior visceral endoderm (Brennan et al., 2001).

1.3.2: Gastrulation

Gastrulation in mice begins around day E6.5 with the formation of the primitive streak at the proximal posterior region of the epiblast. Embryonic ectodermal cells begin ingression through the streak. As gastrulation proceeds, more embryonic ectoderm cells are recruited from the epiblast, which results in the extension of the streak along the posterior side of the epiblast. Epiblast cells are recruited for ingression when the primitive streak reaches into their vicinity. When ectoderm cells ingress through the streak, they leave it as specified cells that incorporate into the mesoderm or the definitive endoderm germ layers (Bellairs, 1986; Tam and Behringer, 1997).

The first mesoderm cells to be specified are the extraembryonic mesoderm that ingress through the streak when it first forms at the proximal posterior region of the epiblast (referred to as early-gastrula organizer) (Parameswaran and Tam, 1995). When the streak lengthens and reaches midway of the epiblast, embryonic mesoderm (precursor cells of the heart and cranial mesenchyme) and definitive endoderm (foregut definitive endoderm) are found in the distal region of the mesodermal layer near the anterior extremity of the streak (Lawson et al., 1991; Parameswaran and Tam, 1995). This anterior region of the primitive streak is referred to as mid-gastrula organizer and is identified by the expression of some genes such as *Foxa2*, *Gossecoid* and *Chordin* (Ang et al., 1993; Kinder et al., 2001). The mid-gastrula organizer contains the axial mesendoderm cells, which are the precursor cells that give rise to the anterior definitive endoderm (ADE) and the prechordal plate mesoderm, as well as the progenitors of the node and its derivatives, the notochord and floor plate (Vincent et al., 2003). The primitive streak continues to elongate and the cells localized in the anterior primitive streak of the mid-streak stage embryo reach the distal tip of the embryo, by E7.5 (late streak stage), and form another organizer called the node.

Fate mapping studies indicate that cells that ingress through different regions of the streak at the late-streak stage become incorporated into different tissues of the body. Cells that emerge from the node differentiate into axial mesoendoderm (such as prechordal mesoderm and the notochord), the neuroectoderm (floor plate), midgut endoderm and somites (Beddington, 1994; Smith et al., 1994; Sulik et al., 1994). Cells that emerge from the peri-nodal region (the anterior segment of the streak closest to the node) differentiate into paraxial mesoderm. Cells that emerge from mid-segment of the streak differentiate

into lateral mesoderm and cells that emerge from the posterior segment of the streak differentiate into extraembryonic mesoderm (Lawson et al., 1991; Smith et al., 1994; Tam, 1987; Wilson and Beddington, 1996). Therefore, there seem to be a correlation between the A/P regions of the streak and the prospective ventral-dorsal mesoderm pattern (Tam and Behringer, 1997).

The precise nature of the signals within the different segments of the streak that lead to different mesodermal lineages is still not known. Mice mutant in FGF, TGF-β and Wnt signaling pathways, however, indicate that these pathways are involved. Mutations in the FGF receptor1 gene results in mice that are deficient in the formation of paraxial mesoderm (Deng et al., 1994; Yamaguchi et al., 1994). Mutations in *BMP4* activity (TGF-β) produce embryos that are deficient in ventral mesoderm derivatives (Winnier et al., 1995). Mutation of the *Wnt3a* gene results in a deficiency of trunk paraxial mesoderm (Takada et al., 1994; Yoshikawa et al., 1997).

At later stages (E7.5-9.5), as anterior structures are forming, caudal extension of the embryonic axis takes place to form the embryonic trunk. Although at early stages a large proportion of the epiblast ingress through the streak and form the mesoderm and definitive endoderm, at later stages only a small strip of epiblast cells (that are adjacent to the streak) ingress though the streak and form mesoderm (Tam, 1989). Yet, a large number of mesoderm cells are generated at these stages, which participate significantly in the caudal extension of the embryonic axis. This has led to the suggestion that a permanent pool of stem cells forms in the primitive streaks, which serve as the source of mesodermal cells that constitute the embryonic trunk (Nicolas et al., 1996; Wilson and Beddington, 1996).

Gastrulation ends when the primitive streak disappears and ingression of superficial ectoderm ceases, the posterior neuropore closes and the 30 somites that constitute the trunk mesoderm have formed (Wilson and Beddington, 1996). At these later stages, extension of anterior-posterior axis is accomplished by the addition of cells from the tailbud (remaining of the primitive streak) to the caudal end of the embryo. The precise molecular mechanism governing trunk formation is still unknown, but Wnt3a seems to play an essential role. *Wnt3a* is expressed extensively in cells fated to give rise to embryonic mesoderm, at the egg cylinder stage and in the dorsal region of the neural tube of day 9.0 embryos (Takada et al., 1994). Null mutation for *Wnt3a* produces embryos that show a complete absence of tailbud development and are truncated rostral to the hindlimbs (Greco et al., 1996). The downstream signaling effectors of Wnt3a, however, remain unknown.

1.3.2.1: Primitive streak specification

Despite the crucial importance of the primitive streak for embryonic patterning and morphogensis, very little is known about the molecular mechanism that induces its formation. Mutations in several genes, such as *Nodal*, *BMP4*, *Eomes*, *Mesd* and *Wnt3*, lead to the failure of primitive streak formation, but the hierarchy of the signaling cascade is still unclear. It is clear, however, that the extraembryonic tissues (extraembryonic ectoderm and visceral endoderm) are required for the induction of the primitive streak. [1]Chimera studies, in which extraembryonic tissue is wild type and epiblast cells are *Bmp4*

[1] Chimera studies are powerful strategies to study the role of genes expressed in extraembryonic or embryonic lineages in establishment of the body plan. In this regard, one can generate embryo that is chimera of two different backgrounds, the embryonic lineage being from one background and the extraembryonic lineage is from another. This is accomplished when embryonic stem (ES) cells are aggregated with 8-cell stage embryos. The ICM of the formed blastocyst will be entirely derived form ES

mutant, express proximal-posterior genes such as *Brachyury* in the epiblast and form epiblast-derived primordial germ cells, indicating that *Bmp4* is required in the extraembryonic ectoderm for specification of epiblast posterior fate (Lawson et al., 1999). Similarly, chimeric embryos, in which the extraembryonic tissue is wild type and the epiblast lacks *Eomes*, are rescued from early lethality and express *Brachyury* (Russ et al., 2000). On the other hand, embryos that have wild-type epiblast cells, but extraembryonic tissue mutant for *Mesd* fail to initiate gastrulation (Hsieh et al., 2003). These results clearly indicate that a signal from the extraembryonic tissue is required for the initiation of gastrulation and primitive streak formation.

The preliminary picture of primitive streak induction is the following. Prior to gastrulation, *Nodal* expression becomes restricted to the proximal posterior region of the epiblast where the prospective streak will form. This localization is perhaps a result of global cell movement of visceral endoderm that results in physical accumulation of Nodal in this region (Lu et al., 2001b). Nodal then works to maintain the expression of *BMP4* and *Eomes* in the extraembryonic ectoderm and activates *Wnt3* in the posterior epiblast ectodermal cells and the adjacent visceral endoderm. Selective depletion of *Nodal* in the posterior epiblast, however, still allows gastrulation to be initiated, indicating that other factors are involved (Vincent et al., 2003).

The specific role of posterior visceral endoderm in induction of the primitive streak is not known. Transgenic explant culture systems, however, show that posterior visceral

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cells and will give rise to the epiblast of the conceptus (one background). The trophectoderm and the future primitive endoderm, which will give rise to the extraembryonic ectoderm and the visceral endoderm, respectively, will be derived from the 8-cell embryo (second background) Nagy, A., and Rossant, J. (1993).

endoderm are able to induce mesoderm (Belaoussoff et al., 1998). The role of posterior visceral endoderm in the elongation of the primitive streak and formation of the mid-gastrula organizer, on the other hand, has been demonstrated. *Foxa2* is required for primitive streak elongation (Ang et al., 1993). Chimeric embryos, in which the visceral endoderm is wild type, but the epiblast is *Foxa2*-mutant show elongation of the primitive streak (Dufort et al., 1998). Similarly, deletion of the RING-domain protein Arkadia (Ark), a putative intracellular protein that interacts with Nodal (Niederlander et al., 2001), results in loss of *FoxA2* and *Goosecoid* expression in the anterior primitive streak region and thus defective formation of the mid-gastrula organizer. Chimera experiments have shown that Arkadia is required in the visceral endoderm for induction of *Foxa2* and *Goosecoid* in the epiblast and for formation of mid-gastrula organizer (Episkopou et al., 2001). Furthermore, Amnionless, a novel type I transmembrane protein, is expressed throughout the visceral endoderm and is required for the formation of middle streak derivatives (Kalantry et al., 2001).

1.3.2.2: Node specification

The node forms from precursor cells that are located in the anterior primitive streak (Mid-gastrula organizer). The molecular mechanism that controls node specification is not known. The TGF-β signaling pathway, however, is essential for node specification. Knockout of *Foxa2*, *FAST* and *Arkadia* all lead to defects in the formation of mid-gastrula organizer and consequently lack nodes (Ang et al., 1993). Interestingly, defects in the anterior primitive streak formation do not necessarily lead to defects in node formation.

Production of completely ES cell-derived fetuses. *In* "Gene targeting; A technical approach" (A. L. Joyner, Ed.), pp. 147-179. IRL Press, Oxford, UK..

Conditional knockout of *Smad2* in the epiblast and *Nodal* in the anterior primitive streak region, both result in defects in the formation of the mid-gastrula organizer, but showed no effect on node formation (Vincent et al., 2003). In these embryos, the anterior definitive endoderm and the prechordal plate were not specified correctly, but the node and its midline derivatives, the notochord and the floor plate developed normally. On the other hand, *Foxa2* is required cell autonomously in node precursors for node formation (Dufort et al., 1998). Interestingly, *Arkadia* expression in the visceral endoderm is essential for node formation providing the first evidence for a signal from the extraembryonic cells (visceral endoderm) that is required for the specification of the mammalian organizer (Episkopou et al., 2001).

1.3.2.3: Role of Wnt signaling in primitive streak and node formation

The Wnt pathway signaling through β-catenin is implicated in specification of the organizer in *Xenopus* and zebrafish (reviewed in (Moon and Kimelman, 1998; Schier, 2001). If an analogy can be drawn between *Xenopus* and the mouse, then the equivalent of *Xenopus* Nieuwkoop center would be the extraembryonic tissue (extraembryonic ectoderm and posterior visceral endoderm). The Nieuwkoop center in *Xenopus*, which is located in the dorsal vegetal cells, induces the formation of the organizer in the adjacent dorsal marginal cells in the animal hemisphere (Nieuwkoop, 1973). In mouse, the extraembryonic ectoderm and posterior visceral endoderm are adjacent to and essential for the induction of the primitive streak (Hsieh et al., 2003; Lawson et al., 1999; Russ et al., 2000; Winnier et al., 1995; Yoshimizu et al., 2001). In addition, the posterior visceral endoderm was shown to be essential for elongation of the primitive streak and for specification of the node (Dufort et al., 1998; Episkopou et al., 2001).

In mouse, there is increasing evidence that the Wnt/β-catenin signaling pathway plays a similar role in specification of the primitive streak and of the node. Transgenic overexpression of *Wnt8c* leads to posterior axis duplication (Popperl et al., 1997). Mutations in negative regulators of the Wnt signaling pathway such as *axin, APC*, the *LIM domain-binding protein 1* or *Tcf3*, which result in an increase of Wnt signaling, lead to axis duplications and formation of ectopic nodes (Ishikawa et al., 2003; Merrill et al., 2004; Mukhopadhyay et al., 2003; Zeng et al., 1997). In addition, Mesd, which is LRP-5/-6 chaperone, and Wnt3 are essential for primitive streak formation (Hsieh et al., 2003; Liu et al., 1999a). Furthermore, targeted deletion of *β-catenin*, the downstream effector of Wnt signaling, results in failure of primitive streak formation and initiation of gastrulation (Haegel et al., 1995; Huelsken et al., 2000). Chimera studies have shown that β-catenin function is required in the epiblast cells (Huelsken et al., 2000). Finally specific deletion of *β-catenin* within the primitive streak prevents node formation (Lickert et al., 2002).

In *Xenopus*, β-catenin, through its association with Tcf-3, activates the expression of *Siamois* and *Twin* in the Nieuwkoop center, which in turn activate the expression of organizer specific genes such as *Goosecoid* through the Wnt responsive element in the *Goosecoid* promotor (Brannon et al., 1997; Laurent et al., 1997; Lemaire et al., 1995; Watabe et al., 1995). The mammalian homologue of *Siamois* has not been identified yet; hence the notion that a similar mechanism of β-catenin/Siamois exists in the mouse remains elusive.

1.3.2.4: Mouse embryos contain two organizers: an anterior organizer and a posterior organizer

The mouse node is considered to be the equivalent of *Xenopus* organizer (Beddington, 1994; Tam and Behringer, 1997). Transplantation experiments, however, have indicated that, unlike the *Xenopus* organizer that can generate a complete secondary axis, the mouse node generates only a secondary trunk axis when grafted into a host embryo (Beddington, 1994). Furthermore, node ablation experiments have indicated that node removal doesn't abolish formation of anterior structures (Davidson et al., 1999; Klingensmith et al., 1999). These results have led to the conclusion that mammalian embryo contains two organizers; trunk organizer (the node) and a head organizer (potentially the anterior visceral endoderm) (Beddington and Robertson, 1999).

Recent experiments have indicated that the anterior visceral endoderm is not sufficient to pattern anterior structures, such as neuroectoderm or cause formation of anterior embryonic structures (Lu et al., 2001b). The anterior definitive endoderm, which arises from the anterior region of the streak before node formation and displaces the anterior visceral endoderm (Lawson and Pedersen, 1987) was shown to play an important role in anterior neuroectoderm specification. The anterior definitive endoderm was shown to express many of the same genes as the anterior visceral endoderm, such as *Hex* and *Cer1* (Martinez Barbera et al., 2000). Removal of anterior definitive endoderm results in truncation of the anterior neuroectoderm (Camus et al., 2000). This has lead to the suggestion of a model for anterior specification, in which the anterior visceral endoderm primes the epiblast for anterior patterning, while the anterior definitive endoderm and other anterior streak derivatives pattern the anterior neuroectoderm (Lu et al., 2001b).

Molecular evidence supporting this model has been provided. Deletion of the forkhead DNA-binding protein *FAST* (*FoxH1*), which is thought to bind to Smad2 in response to *Nodal* signaling, produces embryos that lack anterior definitive endoderm, but the anterior visceral endoderm is specified (Hoodless et al., 2001). In these embryos the anterior central nervous system structures are missing. Similarly, embryos lacking Arkadia, fail to form anterior definitive endoderm, but the anterior visceral endoderm is still specified (Episkopou et al., 2001). These embryos also lack anterior structures, further supporting the need for ADE to pattern the anterior structures correctly.

1.3.3: Conclusion

In mammalian embryos, clear evidence that links the overt axes established postimplantation to the early preimplantation stages is still missing. Unlike the case in *Xenopus* and zebrafish, the initial molecular trigger that leads to the induction of the primitve streak and node is unknown. Members of the Wnt and TGF-β signaling proteins seem to cooperate and are involved in the induction of the mammalian organizers.

CHAPTER 2: MANUSCRIPT I

Expression and estradiol regulation of Wnt genes in the mouse blastocyst identifies a candidate pathway for embryo-maternal signaling at implantation

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PREFACE

Wnt genes play crucial roles during embryonic development. Most of the studies available have focused on the role of these signaling proteins at postimplantation stages. The objective of this study was to investigate Wnt genes expression in mouse embryos during pre/peri-implantation stages.

In the following manuscript, I describe the expression of numerous *Wnt* genes in the embryo at the time of pre/peri-implantation stages and study the regulation of expression of these genes in relevance to the implantation process.

ABSTRACT

Implantation of the mammalian embryo depends on differentiation of the blastocyst to a competent state and of the uterine endometrium to a receptive state. Communication between the blastocyst and uterus ensures that these changes are temporally coordinated. Although considerable evidence indicates that the blastocyst induces expression of numerous genes in uterine tissue, potential signaling mechanisms have yet to be identified. Moreover, whereas a surge of maternal estradiol occurring on day 4 of pregnancy in the mouse is critically required for many of the peri-implantation uterine changes, whether this surge also affects blastocyst gene expression has not been established. I show here that mouse morulae express genes encoding several members of the Wnt family of signaling molecules. Additional *Wnt* genes are newly expressed following development to blastocyst. Unexpectedly, *Wnt5a* and *Wnt11* are expressed in embryos that undergo the morula-to-blastocyst transition in vivo, but only weakly or not at all in embryos that do so in vitro. Up regulation of *Wnt11* is temporally coordinated with the surge of maternal estradiol on day 4. *Wnt11* fails to be upregulated in blastocysts obtained from mice ovariectomized early on day 4 or from mice treated with the estradiol antagonist, ICI 182,780. Administration of estradiol-17β or its metabolite, 4-OH-estradiol, to ovariectomized mice restores *Wnt11* expression. Moreover, *Wnt11* expression is not upregulated when blastocysts are trapped in the oviduct following ligation of the utero-tubal junction, nor when estradiol-17β or 4-OH-estradiol are administered to blastocysts in vitro. These results establish a comprehensive profile of *Wnt* gene expression during late preimplantation development, demonstrate that estradiol regulates gene expression in

the blastocyst via uterine factors, and identify Wnts as potential mediators of embryo-uterine communication during implantation.

INTRODUCTION

Successful implantation depends on two temporally coordinated developmental processes. On one hand, the embryo must become a blastocyst that is competent for implantation. On the other hand, the uterus must develop to a receptive condition where it is capable of supporting implantation. Early on day 4 (day 1 = plug day) the uterine lumen narrows so that the blastocysts become closely apposed to the epithelium, and between 1400 hr and 1600 hr on day 4, the luminal epithelium adjacent to the blastocysts shows increased expression of heparin-binding epidermal-like growth factor (HB-EGF) (Das et al., 1994). Subsequently, the vascular permeability of the stroma increases at these sites (Psychoyos, 1973a). At about 2200 h, the mural trophoblast of the blastocyst becomes attached to the anti-mesometrial side of the luminal epithelium and begins the invasive process of implantation, initially characterized by apoptosis of the uterine epithelium and proliferation and decidualization of the stroma (Enders, 1976; Parr et al., 1987; Psychoyos, 1973a). Accompanying implantation are changes in the expression of many genes both at and between implantation sites, and the encoded products presumably mediate early post-implantation events (reviewed in (Carson et al., 2000; Paria et al., 2000; Reese et al., 2001; Rinkenberger et al., 1997)). In the mouse, many of the uterine changes depend on a transient rise in estradiol that occurs between 900 hr and 1200 hr on day 4 and persists for about 24 hrs (McCormack and Greenwald, 1974). The central role of estradiol in triggering these events is indicated by their failure to occur in ovariectomized animals and their subsequent induction when such animals are given an injection of estradiol (Ma et al., 2003; Paria et al., 1998; Paria et al., 2002). Nonetheless,

the pathway linking estradiol to these events is in most cases not well defined, although its ability to regulate expression of many growth factors and cytokines is well established (Chen et al., 2000; Cheng et al., 2002; Das et al., 2000; Smith et al., 1997).

The blastocyst also appears to play a key role in regulating at least some of the events required for implantation. Transcription of the genes encoding integrins α_2, α_{6A} and α_7, as well as activity of the receptors for HB-EGF (encoded by the ErbB gene family), are upregulated in blastocysts, and these could in a paracrine manner modulate gene expression in the surrounding uterine tissue (Paria et al., 1998; Sutherland et al., 1993). Indeed, several genes including HB-EGF, epiregulin and beta-cellulin become transcriptionally activated at implantation specifically in the region of the uterine luminal epithelium and underlying stroma that is adjacent to the implanting blastocyst (Das et al., 1997a; Das et al., 1994). Moreover, these genes are not expressed in ovariectomized mice, but are induced at the site of implantation following injection of estradiol. This strongly suggests that their expression requires estradiol-dependent signals emanating from the blastocyst. Further support for a role for the blastocyst derives from the following observations. When blastocysts have been exposed to the estradiol surge in vivo, they are able to implant in ovariectomized mice up to 16 hr after the hosts have received an injection of estradiol. In contrast, blastocysts not exposed to the estradiol surge are able to implant into such recipients only within the first hour after estradiol injection (Ma et al., 2003). Thus, exposure to estradiol in the uterine environment appears to induce changes in the blastocyst that are required for implantation. Taken together, these results suggest that implantation requires factors secreted by the blastocyst that modulate gene expression in uterine cells and that expression of at least some of these factors is dependent on

estradiol (or its metabolite 4-OH-estradiol). However, the identity of the blastocyst-derived factors is unknown.

Wnt genes encode a large family of cysteine-rich secreted glycoproteins that function as signaling molecules and play key roles in a wide variety of cellular and developmental processes, (reviewed in (Cadigan, 2002; Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). To date, 19 *Wnt* genes have been identified in human and mouse. Wnt proteins act by binding to members of the Frizzled protein family (He et al., 1997; Miller, 2002; Yang-Snyder et al., 1996). These transmembrane receptors contain an N-terminal, cysteine-rich extracellular domain that is thought to bind Wnts, a putative seven-transmembrane domain, and a variable length cytoplasmic C-terminus (Orsulic and Peifer, 1996; Wodarz and Nusse, 1998). Nine Frizzled proteins have been identified in human and mouse. Within the cell, multiple pathways have been identified through which Wnt signals may be transduced. In the best known, so-called canonical pathway, Wnt signaling leads to stabilization and nuclear translocation of β-catenin, which then acts as a transcriptional co-activator of target genes (reviewed in (Cadigan, 2002; Wodarz and Nusse, 1998). More recent work has shown that Wnts also signal through pathways that do not involve β-catenin, including the planar cell polarity pathway and a pathway involving Ca^{2+} signaling and activation of protein kinase C (Veeman et al., 2003). Current evidence suggests that the Wnts may be grouped into families that preferentially signal through different pathways. Thus, the Wnt1 family (including Wnt1, Wnt2, Wnt3a, Wnt8a and Wnt8b) is thought to signal through the canonical pathway, whereas the Wnt5a family (including Wnt5a, Wnt4 and perhaps Wnt11) appear to signal through the Ca^{2+} pathway (reviewed in (Wodarz and Nusse, 1998)).

Despite their widespread role in many developmental processes, the potential role of *Wnt* genes in the implantation process has not been extensively explored. A recent report of *Wnt3a* and *Wnt4* expression at the 8-cell stage in the mouse (Lloyd et al., 2003) is to my knowledge the sole report concerning *Wnt* gene expression in preimplantation embryos. Here, I have investigated the expression of *Wnt* family members in morulae and blastocysts, and have studied the role of uterine factors including estradiol in regulating expression of certain of these *Wnt* genes.

METHODS

Collection and culture of embryos

Embryos were obtained as described (Clarke et al., 1992). Briefly, CD-1 females (Charles River Canada) were super ovulated by an injection of 7.5 IU of pregnant mares' serum gonadotropin (Sigma) followed 44-48 h later by 5 IU of human chorionic gonadotropin (hCG, Sigma) and caged individually with CD-1 males overnight. Mating was indicated by the presence of a vaginal plug the following morning. Morula stage embryos were flushed using Hepes-buffered KSOM medium from the oviducts of day 3 pregnant females (day 1 is plug day), 72 hrs after hCG injection. They were either lysed immediately in a 0.5-ml microfuge tube containing 100 µl of lysing buffer (Trizol, Invitrogen) and stored at -80°C until the time of RNA extraction or placed in 10 µl droplets of bicarbonate-buffered KSOM medium under mineral oil (Sigma) at 37°C in a humidified atmosphere of 5% CO2 in air. After 24 hrs of incubation, blastocyst stage embryos (as judged by the formation of blastocoelic cavity) were selected and lysed and stored as above. Blastocysts that developed in vivo were flushed from uteri of day 4 pregnant females at the times indicated in the Results, and lysed and stored as above.

RNA extraction

Tubes containing embryos in Trizol were removed from the –80°C freezer and were allowed to stand for 5 minutes at room temperature to thaw. Ten µg of glycogen was added to the tubes, which were mixed and allowed to stand for another 5 minutes. Thirty µl of chloroform was then added and the tubes were vigorously shaken, then allowed to

stand for 3 minutes. Following centrifugation at 4°C for 15 minutes at 13,000 rpm, the aqueous phase was transferred to a clean tube, to which was added 100 µl of isopropanol. After 10 minutes incubation at room temperature, the tubes were centrifuged at 4°C for 15 minutes at 13,000 rpm and the supernatant was withdrawn. The RNA pellet was washed with 70% ethanol, allowed to dry for 10-15 minutes, and dissolved in 10 µl of DEPC-treated water.

cDNA synthesis

cDNA synthesis was carried out using standard procedures. Briefly, each reaction mixture contained 10 µl of RNA solution, 27 units of RNase inhibitor (Pharmacia), 3 µl of a dNTP mix of 10 mM concentration, 6 µl of 5X reverse buffer (Invitrogen), 3 µl of 10 mM DTT, 1 µl of dimethyl sulfoxide (DMSO) (Sigma, D-8779), 500 ng of oligo-$d(T)_{12-18}$ (Invitrogen) and 200 units of MMLV reverse transcriptase (Invitrogen). cDNA synthesis was allowed to proceed for 3 hrs at 37°C, after which the mixture was heated at 85°C for 10 minutes.

PCR amplification

The primers used are shown in Table 1. Amplification was carried out using cDNA from 5 embryo-equivalents in a buffer consisting of 10 mM Tris (pH 8.3), 50 mM KCl, 200 mM dNTPs, 60-100 pmoles of each primer and 2.5 units of *Taq* polymerase (Invitrogen) in a total volume of 50 µl. The concentration of $MgCl_2$ was as indicated for each set of primers. Each cycle consisted of 1 minute at 94°C, 1 minute at a gene-specific temperature (as indicated for each set of primers) and 1 minute at 72°C. Forty cycles were

run for each reaction. To visualize the amplified products, 15 μl of each PCR reaction was run through a 2% (w/v) agarose gel containing 0.025% ethidium bromide.

Semi-quantitative analysis of RT-PCR products

The signal intensity of each PCR product in ethidium bromide-stained gels was measured using a FluorChem 8800 imaging system (Alpha Innotech Incorporation, San Leandro,CA). The size of the area selected for measurement was the same for each band in a gel, and values obtained were subtracted from a background value obtained from an area of the same size in the same gel. The value obtained for each product was then normalized to a designated control product for each PCR reaction, as indicated in the figure legends. Means and standard deviations were calculated. Statistical tests were carried out as indicated in the figure legends.

Ovariectomy and drug treatments

Mice were ovariectomized on the morning of day 4 of pregnancy between 700 and 900 hr. Immediately after surgery, mice received subcutaneous injection of progesterone (2 mg/mouse) only or progesterone with estradiol-17β (50 ng/mouse), or progesterone with 4-hydroxy-estradiol (4-OH-estradiol) (50 ng/mouse). Mock-treated animals were anesthetized and surgically opened, and the ovaries were exposed outside and then returned in place. Animals treated with ICI 182,780 received a subcutaneous injection (0.5 mg/mouse or 1 mg/mouse) of the drug at 700 hr on day 4 of pregnancy. Animals used for the study of trapping blastocysts in the oviduct were exposed to surgery in the afternoon of day 3 of pregnancy. The junction between the oviduct and the uterus was

tightly tied using 6-0 silk thread (Ethicon, Somerville, NJ). Animals were sacrificed on day 4 at 2000 hr and the blastocysts were recovered from the oviducts.

Drugs

Progesterone (4-pregnen-3, 20-dione; Steraloids, Newport, RI; Q2600-000) was dissolved in sesame oil at a concentration of 20 mg/ml. Estrogen (1,3,5[10]-estratriene-3, 17β-diol) was purchased from Sigma (E-8875) and was dissolved in sesame oil at a concentration of 500 ng/ml. 4-OH-estradiol (Steraloids; E2500,000) was dissolved in sesame oil at a concentration of 500 ng/ml. ICI 182,780 (Zeneca) was dissolved in sesame oil at a concentration of 10 mg/ml. For in vitro studies, embryos were cultured in 10 μl micro drops of KSOM containing either estradiol-17β or 4-OH-estradiol at a concentration of 2 ng/ml.

RESULTS

Expression of several *Wnt* genes is upregulated at the blastocyst stage

To investigate the potential involvement of Wnts in the implantation process, I examined the expression of *Wnt* genes in developing embryos. Morulae were flushed from the oviducts of pregnant females on the afternoon of day 3 and processed for RT-PCR using specific primers for hypoxanthine guanine phosphoribosyl transferase (*HPRT*) and fifteen *Wnt* genes (Table 1). As shown in Figure 1A, products corresponding to 6 of the 15 *Wnt* genes tested were observed. Next, I flushed blastocysts, which were now in the uteri, between 1900 hr and 2000 hr on day 4 of pregnancy and processed them for RT-PCR. All of the *Wnt* genes expressed in morulae were also expressed in blastocysts (Figure 1B), certain of them at a higher level (e.g., *Wnts 4, 5b, 7b, 10b*). In addition, several *Wnt* genes (*1, 5a, 7a, 11, 13*) were newly detectable in the blastocysts. These results establish that expression of a large number of *Wnt* genes is upregulated in blastocysts near the time of implantation.

To investigate the mechanism by which the expression of these *Wnt* genes was upregulated, I collected morulae from the oviducts of pregnant females on day 3. These were cultured in vitro until the evening of day 4 and those that reached the blastocyst stage were processed for RT-PCR as previously described. Several of the *Wnt* genes (7a, 7b, 10b, 13) that were upregulated in the blastocysts that developed in vivo were similarly upregulated in blastocysts that developed in vitro (Figure 1C). In contrast, *Wnt5a* and *Wnt11* were expressed at relatively low levels in the in vitro blastocysts. Figure 1D

represents the expression of each *Wnt* gene in blastocysts that developed in vivo compared those that developed in vitro. These results imply that upregulated expression of certain *Wnt* genes in blastocysts required exposure to the uterine environment during the morula-to-blastocyst transition.

Up regulation of *Wnt11* expression by the blastocyst requires an ovarian factor that can be replaced by administration of estradiol-17β

Based on these results, I examined the timing of *Wnt11* gene up regulation in vivo. Since implantation is preceded by an obligatory surge in estradiol (Ma et al., 2003; Paria et al., 1993b; Psychoyos, 1973b), I focused on this event. Blastocysts were flushed from uteri of pregnant females on day 4 in the morning (before the estradiol surge), at midday (just after the estradiol surge) and in the early evening, and processed for RT-PCR using primers for *Wnt6* and *Wnt11*, and *HPRT* as a control. As shown in Figure 2, *Wnt11* expression was modestly higher at midday as compared to the morning and by the evening was considerably higher. In contrast, *Wnt6* expression did not change substantially during this period. These results establish a correlation between the onset of the estradiol surge and the up regulation of *Wnt11* expression. A similar time course was observed for *Wnt5a* expression (not shown).

To test whether up regulation of *Wnt11* expression depended on the estradiol surge, I performed the following experiments. First, I ovariectomized pregnant females between 700 hr and 900 hr of day 4- i.e., before the estrogen surge. Control animals were mock-ovariectomized or left untreated. Blastocysts were collected between 2000 hr and 2200 hr on day 4 from all groups and processed for RT-PCR. Figure 3 show that ovariectomy did

not detectably affect the expression of *Wnt1, Wnt5a, Wnt5b and Wnt6*. This suggests that up regulation of *Wnt5a* expression, although it occurs only in embryos that developed from the morula to blastocyst stage in vivo, does not require ovarian factors produced during this time. In contrast, *Wnt11* expression was much lower in blastocysts collected from ovariectomized animals as compared to those obtained from control mock-ovariectomized and intact animals. Thus, up regulation of *Wnt11* expression requires ovarian factor(s) that are secreted during the period just preceding implantation.

To study the potential role of estradiol more directly, we tested the effect on *Wnt11* expression of administering ICI 182,780, an estradiol antagonist that competes for binding to nuclear estrogen receptors (Howell et al., 2000; Wakeling et al., 1991). Pregnant females received an injection of ICI 182,780 on the morning of day 4, before the estrogen surge, and blastocysts were collected in the evening of that day and processed for RT-PCR. Up regulation of *Wnt11* expression was blocked in blastocysts collected from animals injected with ICI 182,780 whereas expression of *Wnt5a* was unaffected. Next, we ovariectomized pregnant females before the estrogen surge and then administered either estradiol-17β or a metabolite, 4-OH-estradiol, that is a component of uterine fluid (Paria et al., 1998) to these females. Blastocysts were collected on the evening of day 4 and *Wnt* expression was assayed. Both estradiol-17β and 4-OH-estradiol restored *Wnt11* expression to near control levels (Figure 4C). These results indicate that up regulation of *Wnt11* expression is estradiol-dependent and that the effect of estradiol is mediated through an ICI-sensitive receptor.

Up regulation of *Wnt11* expression by the blastocyst requires the uterine environment

To determine whether estradiol was the sole component of the in vivo environment required for up regulation of *Wnt11* expression by blastocysts, I recovered morulae from the oviducts of day 3 pregnant females and cultured them in the presence of estradiol-17β or 4-OH-estradiol. At 2000 hr of day 4 the embryos (which were now at the blastocyst stage) were collected and processed for RT-PCR. Figure 5 shows that *Wnt11* was not upregulated under these conditions.

The failure of estradiol to up regulate *Wnt11* expression in blastocysts cultured in vitro suggested that this activity of estradiol might require the uterine environment. To test this idea, we ligated the utero-tubal junction of pregnant females on day 3, thus trapping the embryos in the oviduct. I reasoned that the embryos in the oviduct would witness the systemic estradiol surge, but would be isolated from the uterine environment.

On the evening of day 4, blastocysts were collected from the oviducts and processed for RT-PCR. *Wnt11* expression was not upregulated in the blastocysts trapped in the oviduct. Rather, it was similar to that observed in blastocysts following ovariectomy (Fig 4A, lower; Fig 4B). These results imply that up regulation of *Wnt11* expression by the blastocyst requires both estradiol and the uterine environment.

Unexpectedly, expression of *Wnt5a* also was significantly reduced in blastocysts that were trapped in the oviduct (Fig 4A, middle; Fig 4B), although expression of *Wnt5b* (Fig 4A,

upper) and *Wnt6* (not shown) were unaffected. This suggests that up regulation of *Wnt5a* expression, although not estrogen-dependent, also requires a uterine factor(s).

DISCUSSION

Implantation requires changes both in the blastocyst that bring it to a condition where it is competent to implant and in the uterine tissue that bring it to a condition where it is receptive to the blastocyst (Carson et al., 2000). Previous work has clearly established that expression of numerous genes in the uterine epithelium and mesenchyme depends on the estradiol surge on the morning of day 4 (Das et al., 1997a; Das et al., 1994). In contrast, although several gene products potentially involved in implantation are upregulated in blastocysts, the role of the estradiol surge in their expression has not been precisely defined. For example, mRNAs encoding integrins α_2, α_{6A} and α_7 become more abundant in late blastocysts; however, this increase also occurs in embryos that develop to blastocysts in vitro and thus in the absence of estradiol (Sutherland et al., 1993). Perlecan is a heparin sulfate proteoglycan present in extracellular matrix. Although the encoding mRNA is upregulated in blastocysts, it is also present in 'delayed' blastocysts recovered from ovariectomized animals (Smith et al., 1997). The protein, however, is induced in delayed blastocysts following estradiol injection into the mother, suggesting that estradiol may act post-transcriptionally in this case (Smith et al., 1997). Blastocysts also express members of the ErbB family of EGF receptors. Moreover, addition of EGF to embryos cultured in vitro increases both the blastocyst cell number and the fraction of embryos that hatch from the zona pellucida (Das et al., 1994; Paria and Dey, 1990; Paria et al., 1991). This implies that functional EGF receptors are expressed in the absence of estradiol. However, estradiol may maintain ErbB expression, as ErbB mRNA levels fall in delayed blastocysts and are restored by estradiol injection (Paria et al., 1993a). The results

reported here provide direct evidence that the uterine environment, and estradiol in particular, regulate expression of specific genes in the blastocyst.

Several observations in this study suggest that estradiol does not act directly on the blastocyst, but rather indirectly via uterine cells. First, neither estradiol-17β nor its metabolite 4-OH-estradiol could up regulate *Wnt11* expression when applied to blastocysts in vitro under our conditions. Second, *Wnt11* expression was not upregulated in blastocysts that were prevented from migrating from the oviduct to the uterus. Although we cannot be certain that these blastocysts were exposed to estradiol, this observation suggests that *Wnt11* up regulation requires an additional factor specific to the uterine environment. Third, *Wnt11* expression in blastocysts was not upregulated when the mothers received ICI 182,780, implying that this effect is mediated through a nuclear estradiol receptor. Therefore, the effect of injected estradiol-17β and 4-OH-estradiol on *Wnt11* expression is likely mediated through these receptors. In contrast, the previously documented activity of 4-OH-estradiol towards blastocysts is not inhibited by ICI 182,780 (Paria et al., 1998). Thus, the estradiol effect described here and the previously reported effect of 4-OH-estradiol on the blastocyst (Paria et al., 1998) appear to be mediated through different signaling pathways. Consistent with the notion that estradiol does not act directly on the blastocyst to up regulate *Wnt* expression, mouse embryos lacking the estrogen receptor-α gene are viable, although persistence of maternal gene product to the blastocyst stage cannot be formally excluded (Krege et al., 1998; Lubahn et al., 1993). Estradiol is known to trigger expression of leukemia inhibitory factor (LIF) from uterine glandular cells and LIF can largely or completely replace the nidatory function of estradiol

surge (Chen et al., 2000). LIF did not up regulate *Wnt11* expression in vitro in our hands (unpublished data), which could reflect the absence of necessary co-factors.

The function of the *Wnts* expressed by the blastocyst remains to be identified. Expression of numerous genes, including HB-EGF, epiregulin and betacellulin becomes upregulated only in the region of the uterine epithelium adjacent to the blastocyst (Das et al., 1997a; Das et al., 1994). This strongly suggests that up regulation depends on signals emanating from the blastocyst. The molecular nature of these signals, however, remains unknown. Although formal demonstration that the *Wnt* mRNAs expressed in blastocysts are translated to produce Wnt proteins awaits the production of effective antibodies, this is a reasonable inference. Thus, the Wnts are attractive candidates to be signaling molecules that mediate the blastocyst effect on uterine gene expression. We propose that estradiol acts on uterine cells to trigger the expression of diffusible molecules that in turn act on the blastocyst to up regulate *Wnt11* expression (Figure 6). Secreted Wnts may modulate uterine differentiation or, in an autocrine manner, blastocyst differentiation. This model reinforces the concept (Paria et al., 2002) that implantation depends on reciprocal and mutually dependent interactions between the blastocyst and uterine environment.

Finally, it may be noted that mice lacking individual *Wnt* genes, including *Wnt11* and *Wnt5a*, do not show implantation defects (Greco et al., 1996; Liu et al., 1999a; Majumdar et al., 2003; McMahon and Bradley, 1990; Monkley et al., 1996; Parr, 2001; Parr and McMahon, 1995; Stark et al., 1994; Yamaguchi et al., 1999). As defined by the absence of an implantation defect in 'knockout' animals, other genes that are expressed specifically in the uterus at the site of the implanting blastocyst, such as HB-EGF and betacellulin, do not seem to be essential for implantation (Jackson et al., 2003). Given the

tightly regulated pattern of expression of these genes, however, it would be surprising if they played no role in this process. In this case of the Wnts, we observed that both *Wnt5a* and *Wnt11* are upregulated in blastocysts that develop in vivo. It may be speculated that the expression of several genes whose products serve the same biochemical function means that absence of a single gene product has no detectable effect under laboratory conditions.

ACKNOWLEDGEMENTS

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Table 1: Primers and conditions used to detect expression of Wnt genes

Gene	Primer	Sequence	[$MgCl_2$] mM	Annealing temp. (°C)	Size
Wnt1	5'	AAATCGCCCAACTTCTGCA	1.50	53.2	599
	3'	AATACCCAAAGAGGTCACAGC			
Wnt2	5'	CGGCCTTTGTTTACGCCATC	2.25	55.3	513
	3'	TGAATACAGTAGTCTGGAGAA			
Wnt3	5'	GCCGACTTCGGGGTGCTGGT	2.00	61.4	317
	3'	CTTGAAGAGCGCGTACTTAG			
Wnt3a	5'	ATTGAATTTGGAGGAATGGT	1.50	57.5	317
	3'	CTTGAAGTACGTGTAACGTG			
Wnt4	5'	TGTACCTGGCCAAGCTGTCAT	2.25	60.0	344
	3'	TCCGGTCACAGCCACACTT			
Wnt5a	5'	TCCTATGAGAGCGCACGCAT	2.25	60.0	224
	3'	CAGCTTGCCCCGGCTGTTGA			
Wnt5b	5'	TCGGAGGAGCAGGGCCGAGC	2.25	65.0	225
	3'	CAGCTTGCCCTGGCGGGTGA			
Wnt6	5'	GCACCGAGTGTAAGTGCCAT	1.50	56.2	377
	3'	GAAGCGGCACAGACAGTTCT			
Wnt7a	5'	CAAGGCCAGTACCACTGGGA	1.75	56.5	307
	3'	GGCTCCACGTGGACGGCCTC			
Wnt7b	5'	ACCAAAACTTGCTGGACCAC	1.50	56.2	385
	3'	ACGTGTTGCACTTGACGAAG			
Wnt8b	5'	AACGTGGGCTTCGGAGAGGC	1.50	62.9	272
	3'	GCCCGCGCCTTGCAGCAGGT			
Wnt10a	5'	AAAGTCCCCTACGAGAGCCC	1.00	52.3	179
	3'	CAGCTTCCGACGGAAAGCTT			
Wnt10b	5'	CGGCTGCCGCACCACAGCGC	1.00	59.3	179
	3'	CAGCTTGGCTCTAAGCCGGT			
Wnt11 (set-1)	5'	GCCATGAAGGCCTGCCGTAG	2.00	62.9	152
	3'	GATGGTGTGACTGATGGTGG			
Wnt11 (set-2)	5'	CGTGTGCTATGGCATCAAGT	1.50	54.6	200
	3'	GCTCAATGGAGGAGCAGTTC			
Wnt13 (Wnt2b)	5'	TGTACTCTGCGCACCTGCT	2.25	60.0	318
	3'	TGCACTCACACTGGGTGAC			
HPRT	5'	GCTGGTGAAAAGGACCTCT	1.50	60.0	249
	3'	CACAGGACTAGAACACCTGC			

Figure 1: Expression of Wnt genes in pre-implantation embryos

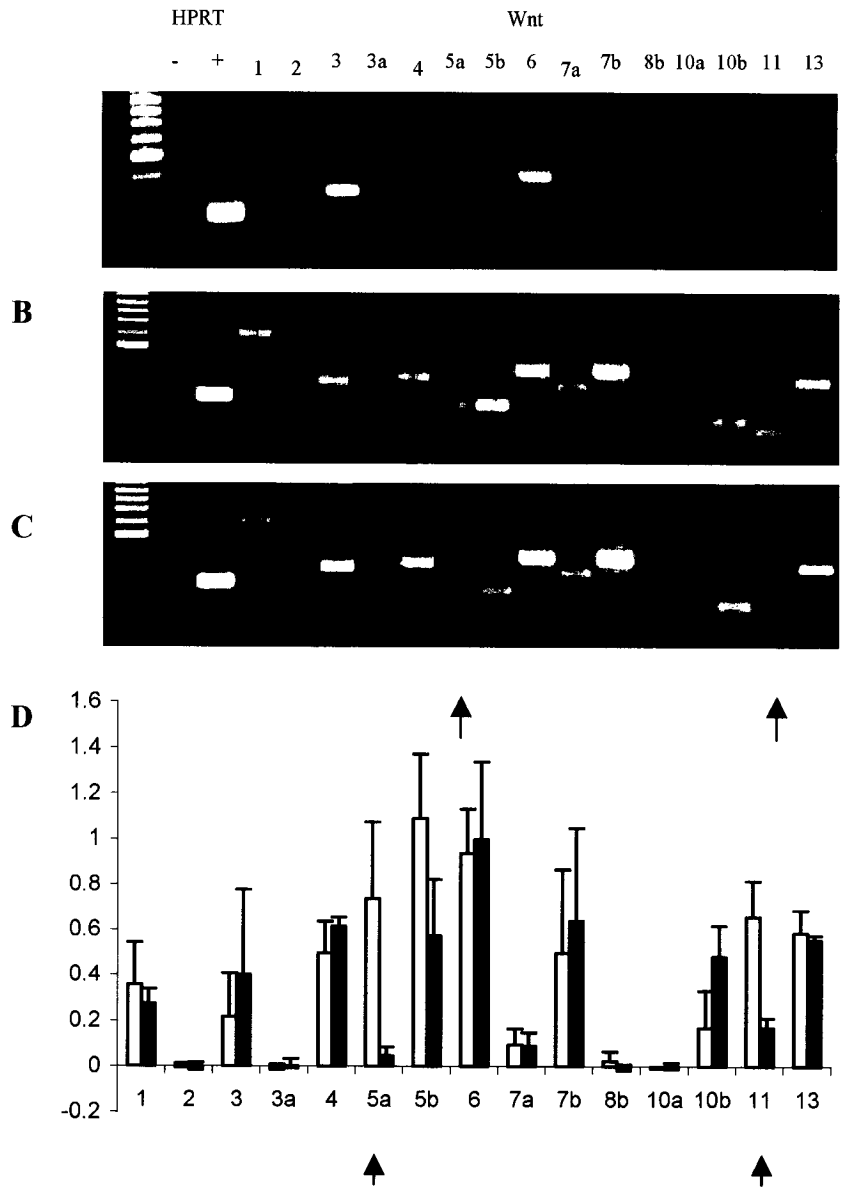

Figure 1 legend

(A) Morula stage embryos were flushed from oviducts on day 3 of pregnancy, and cDNA was prepared from pooled embryos. Separate aliquots obtained from the same pool were used to analyze expression of *Wnt* genes and *HPRT* by RT-PCR using the primers shown in Table 1 and five blastocyst-equivalents per reaction. (B) Blastocysts were flushed from uteri between 1900 hr and 2000 hr of day 4. Gene expression was analyzed by RT-PCR as above. (C) Morulae were flushed from oviducts on day 3 and cultured until 2000 hr on day 4. Blastocysts containing an expanded blastocoel were collected for RT-PCR analysis as above. For each panel, (M) 100-bp ladder; (HPRT -) *HPRT* primers, no reverse transcriptase during cDNA synthesis; (HPRT +) *HPRT* primers, with reverse transcriptase during cDNA synthesis. *Wnt* gene analyzed is indicated at the top of the other lanes. Arrows indicate *Wnt5a* and *Wnt11*. (D) Three independent experiments were performed in which the intensity of ethidium bromide staining of the band corresponding to each gene product was measured as described in the Methods for blastocysts that developed in vivo (white bars) or in vitro (black bars). In each experiment, the value obtained for each *Wnt* gene was expressed relative to the value obtained for *HPRT* using the same pool of cDNA. The mean and standard deviation were calculated. The in vitro values were significantly lower than the in vivo values (t-test, $p < 0.05$) for *Wnt5a* and *Wnt11*, as indicated by arrows.

Figure 2: Upregulation of Wnt11 expression during day 4 of pregnancy

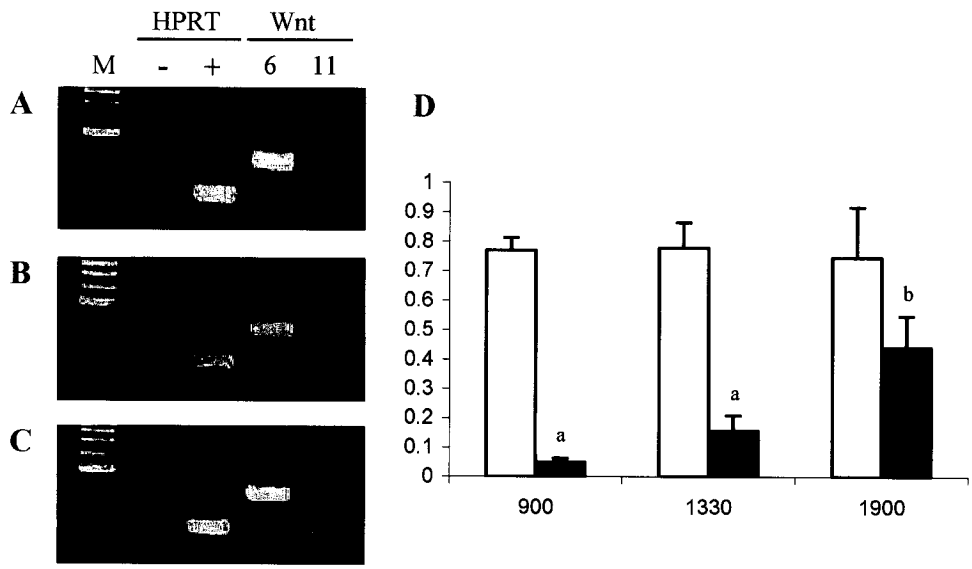

Figure 2 legend

Blastocysts were flushed from the uteri on day 4 at 900 hr (A), 1300 hr (B), or 1900 hr (C). cDNA was prepared from pooled blastocysts at each time point. Separate aliquots obtained from the same pool were used to analyze expression of Wnt genes and HPRT by RT-PCR, using five blastocyst-equivalents per reaction. For each panel, (M) 100-bp ladder; (HPRT -) HPRT primers, no reverse transcriptase during cDNA synthesis; (HPRT +) HPRT primers, with reverse transcriptase during cDNA synthesis. Wnt gene analyzed is indicated at the top of the other lanes. (D) Three independent experiments were performed in which the intensity of ethidium bromide staining of the band corresponding to each gene product was measured as described in the Methods. In each experiment, the value obtained for Wnt6 (white bars) and Wnt11 (black bars) gene was expressed relative to the value obtained for HPRT using the same pool of cDNA. The mean and standard deviation were calculated. Different letters over bars indicate statistically different values (t-test, $p < 0.05$).

Figure 3: Absence of Wnt11 upregulation following ovariectomy

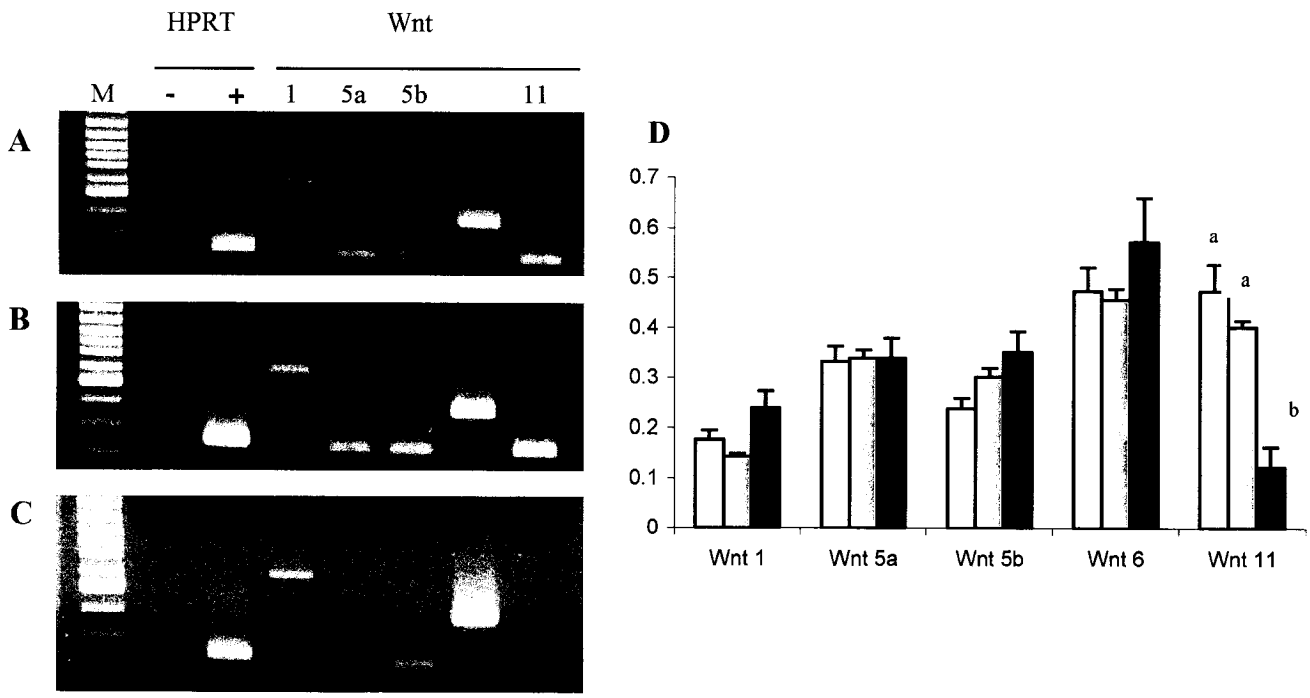

Figure 3 legend

Day 4 pregnant mice were divided into 3 groups. (A) Mice were not operated on. (B) Mice were sham-operated between 700 hr and 900 hr. (C) Mice were ovariectomized between 700 hr and 900 hr. For each group, blastocysts were flushed from the uteri between 2000 hr and 2200 hr, and cDNA was prepared from pooled embryos. Aliquots obtained from the same pool were used to analyze expression of HPRT and Wnt genes by RT-PCR using five blastocyst-equivalents per reaction. For each panel, (M) 100-bp ladder; (HPRT -) HPRT primers, no reverse transcriptase during cDNA synthesis; (HPRT +) HPRT primers, with reverse transcriptase during cDNA synthesis. Wnt gene analyzed is indicated at the top of the other lanes. (D) Two (Wnt1, Wnt5a, Wnt5b) or three (Wnt6, Wnt11) independent experiments were performed, using three or four females per treatment-group in each, in which the intensity of ethidium bromide staining of the band corresponding to each gene product was measured as described in the Methods. In each experiment, the value obtained for each Wnt gene was expressed relative to the value obtained for HPRT using the same pool of cDNA. The mean and standard deviation were calculated. White bars: not operated. Grey bars: sham-operated. Black bars: ovariectomized. Different letters over the bars indicate statistically different values (t-test, $p < 0.05$).

Figure 4: Dependence of *Wnt11* upregulation on estradiol

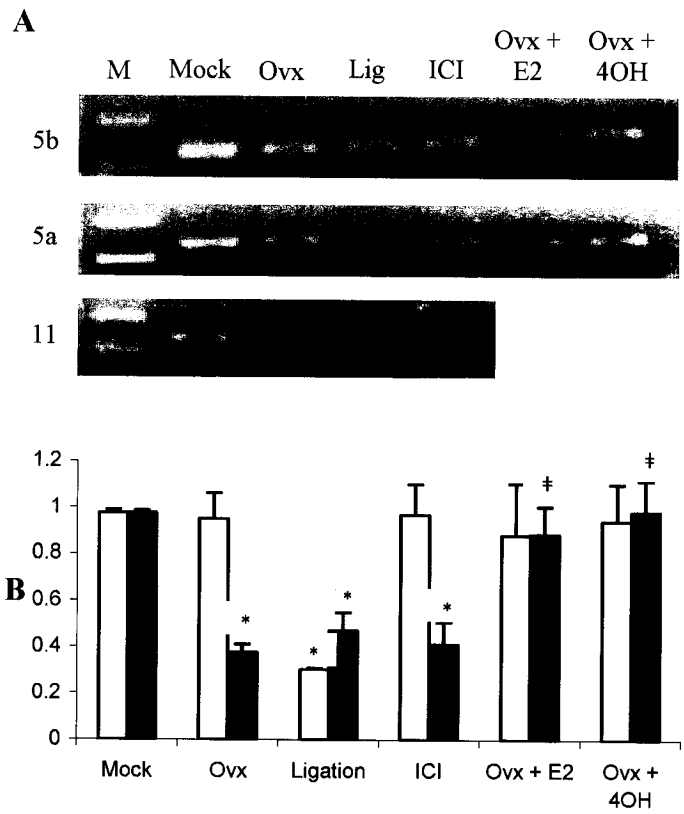

Figure 4 legend

Pregnant females were treated as described below and blastocysts were flushed between 2000 hr and 2200 hr on day 4. For each group, blastocysts were flushed from the uteri between 2000 hr and 2200 hr, and cDNA was prepared from pooled embryos. Aliquots obtained from the same pool were used to analyze expression of *Wnt5b, Wnt5a* and *Wnt11* by RT-PCR, using five blastocyst-equivalents per reaction. Lanes: (M) 100-bp ladder; (Mock) sham-operated; (Ovx) ovariectomized between 700 hr and 900 hr on day 4; (Lig) oviduct ligated on day 3; (ICI) injected with 0.5 or 1 mg of ICI 842,780 at 700 hr on day 4; (Ovx+E2) ovariectomized between 700 hr and 900 hr on day 4 and injected after surgery with 50 ng of estradiol-17β; (Ovx+4-OH) ovariectomized between 700 hr and 900 hr of day 4 and injected after surgery with 50 ng of 4-OH-estradiol. (D) Two (*Wnt5a*, white bars) or three (*Wnt11*, black bars) independent experiments were performed, using between two and four females per treatment-group in each, in which the intensity of ethidium bromide staining of the band corresponding to each gene product was measured as described in the Methods. In each experiment, the value obtained for each *Wnt* gene was expressed relative to the value obtained for *Wnt5b* using the same pool of cDNA. The mean and standard deviation were calculated. Asterisk over a bar indicates the value differs statistically compared to mock group (t-test, $p < 0.05$). Double-cross over a bar indicates the value differs statistically compared to ovariectomized group (t-test, $p < 0.05$).

Figure 5: Effect of estrogen on Wnt genes expression in vitro

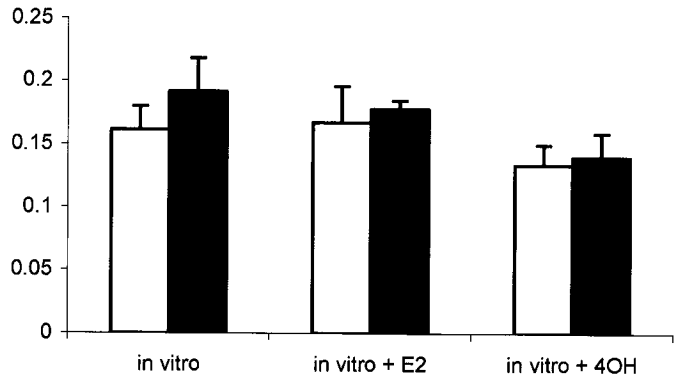

Figure 5 legend

Morula stage embryos were flushed from oviducts of day 3 pregnant females and cultured in unsupplemented medium or in medium supplemented with 2 ng/ml estradiol-17β (E2) or with 2 ng/ml 4-OH-estradiol (4OH) until 2000 hr on day 4. Blastocysts were collected and expression of Wnt5b, Wnt 6 and Wnt11 were analyzed by RT-PCR, using five blastocyst-equivalents per reaction. Three independent experiments were performed in which the intensity of ethidium bromide staining of the band corresponding to each gene product was measured as described in the Methods. In each experiment, the value obtained for Wnt5a (white bars) and Wnt11 (black bars) was expressed relative to the value obtained for Wnt5b using the same pool of cDNA. The mean ratio and standard deviation were calculated.

Figure 6: Uterine-embryonic communication at implantation

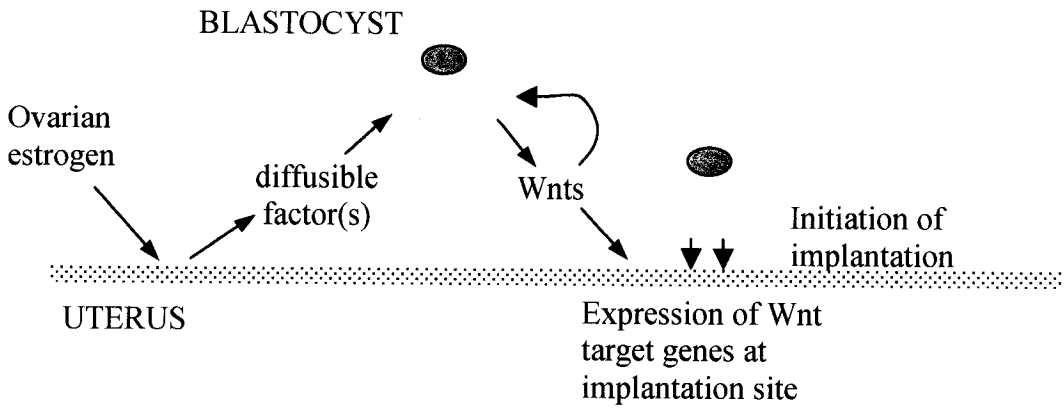

Figure 6 legend

Estradiol is proposed to act on uterine cells to induce synthesis of a secreted molecule that acts on the blastocyst to trigger expression of estradiol-regulated genes encoding factors such as Wnt11. These may induce expression of target genes in the uterus or in the blastocyst that facilitate implantation.

CHAPTER 3: MANUSCRIPT II

Blastocysts induce uterine Wnt signaling at the site of implantation

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PREFACE

Having established that *Wnt* genes are expressed in the blastocyst at the time of implantation, I wished to investigate whether these proteins work as mediators for the embryo-uterus cross talk established during the implantation process. To investigate this, I examined the expression of Wnt target genes in the uterus at the time of implantation.

Instead of looking for a particular Wnt target gene, I generated a Wnt reporter transgenic mouse that faithfully provides readout of the Wnt canonical pathway of β-catenin. In the following manuscript, I examined the activation of the reporter gene in the uterus at the time of implantation.

ABSTRACT

Successful implantation requires a receptive uterus and a competent blastocyst. The mechanisms controlling interaction between the uterus and blastocyst remain poorly understood. *Wnt* genes encode secreted molecules that regulate gene expression in target tissues, mainly by activating β-catenin. I generated a transgenic mouse that faithfully provides readout of the canonical Wnt/β-catenin signaling pathway. Using this transgenic mouse as a tool, I examined the timing and control of β-catenin transcriptional activity in the uterus during the peri-implantation period. I found that β-catenin transcriptional activity was transiently active in the uterus at the time of implantation. Activity was localized in transverse bands that corresponded to implantation sites. Activity was initially detected in the circular smooth muscle of the myometrium at the antimesometrial side and then becomes restricted to the uterine epithelium opposing the implanting embryo. No activity was observed in the uteri of pseudopregnant animals or in the uterine horn of animals that had been ovariectomized on that side, indicating that activation of this pathway required the embryo. Additionally, I show that β-catenin transcriptional activity in the uterus is regulated by ovarian estrogen. I propose that β-catenin-regulated gene expression in the uterus may be involved in implantation and their expression requires signaling molecules secreted by the blastocyst.

INTRODUCTION

Successful implantation requires synchronized development between the implanting embryo and the receptive uterus. The embryo develops to an implantation-competent blastocyst, while the uterus becomes receptive for implantation (Paria et al., 1993b; Psychoyos, 1973a). In mice, by the end of day 4 of pregnancy (day 1= plug day) the zona free blastocyst establishes the first physical connection between mother and the conceptus. This is preceded by embryo spacing, uterine edema and luminal closure leading to an intimate apposition of the blastocyst trophectoderm with the uterine epithelium (Das et al., 1994; Enders and Schlafke, 1967; Yang et al., 1997).

In rodents, the first sign that the implantation process has begun is the increase in vascular permeability of the endometrium at the sites of blastocysts apposition (Enders and Schlafke, 1967). This event can be easily detected as discrete blue bands that appear after intravenous injection of a blue dye solution (Psychoyos, 1973a). Attachment is followed by endometrial decidualization of the stroma at the site of implantation, luminal epithelium apoptosis and subsequent invasion of trophoblast cells into the stroma (Abrahamsohn and Zorn, 1993; Enders, 1976; Parr et al., 1987). One intriguing question is how the blastocyst identifies its uterine implantation site? Several studies have indicated that an intimate cross-talk between the blastocyst and the receptive uterus is set into action to facilitate the implantation process (Paria et al., 2002). The molecular details of this cross talk, however, remain unclear.

In mice, ovarian steroid hormones, progesterone (P_4) and estrogen (E_2), influence the uterus to cycle between three phases; prereceptive, receptive and nonreceptive or refractory phases (Psychoyos, 1973a). Progesterone, produced by the newly formed corpora lutea, along with preovulatory estrogen stimulate the uterus to develop to a prereceptive phase (Paria et al., 1993b; Psychoyos, 1973b). The ovarian estrogen rise that takes place in the morning of day 4 of pregnancy or pseudopregnancy (McCormack and Greenwald, 1974) induces the P_4 primed uterus to become receptive. The uterus remains receptive for 18-24 hrs and then enters the refractory phase (Psychoyos, 1973a; Psychoyos, 1986). The mechanism by which estrogen renders the progesterone-primed uterus receptive, is not fully understood. A spatiotemporal expression of various growth factors, cytokines, lipid mediators and transcription factors, in the uterus, was shown to be regulated by steroid hormones at the time and site of implantation (Carson et al., 2000).

Several members of the epidermal growth factor (EGF) family and their receptors are expressed in the peri-implantation uterus (Das et al., 1997b). The EGF receptors, ErbB1 and ErbB4 are also expressed in the blastocyst at the time of implantation (Dardik et al., 1992; Paria et al., 1999a). These results suggest that these growth factors perhaps serve as local mediators to facilitate embryo-uterine interaction during implantation. HB-EGF pattern of expression is particularly relevant to the implantation process. HB-EGF is expressed solely in the luminal epithelium at the sites of blastocyst apposition 7 hours before the attachment reaction and its expression is dependent on the presence of active blastocyst (Das et al., 1994). A series of studies in mice and human indicate that HB-EGF mediates embryo attachment to the uterine epithelium by acting as an adhesive ligand (Paria et al., 2001; Raab et al., 1996; Song et al., 2000; Yoo et al., 1997). Mice double

null for betacellulin and HB-EGF, however, are viable and fertile (Jackson et al., 2003). These results suggest that redundancy between the EGF ligands perhaps exist, so that compensation between them is happening.

The cytokine Leukemia Inhibitory Factor (LIF) is essential for implantation (Chen et al., 2000; Stewart et al., 1992). The maternal LIF is the one that is required, since LIF-/- blastocysts can implant in wild type uterus, while wild type blastocysts fail to implant in LIF-/- uteri (Stewart et al., 1992). The precise action of LIF is not known, but in the mouse, both LIFR and its co-receptor, gp130, are expressed in the luminal epithelium (Cheng et al., 2002). Mice deficient for gp130 display a similar phenotype to LIF mutants, indicating that LIF action is primarily on the uterus (Ernst et al., 2001). LIF expression is estrogen dependent and can substitute for estrogen in initiation of implantation indicating that the principal function of nidatory estrogen is to induce LIF expression, which in turn initiates the cascade of events that mediate implantation (Chen et al., 2000; Song et al., 2000).

The Wnt proteins are a large family of cysteine-rich secreted glycoproteins that function as signaling molecules and play decisive roles in a wide variety of cellular and developmental processes, (reviewed in (Cadigan, 2002; Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). In human and mouse, 19 *Wnt* genes have been identified to date. Wnt proteins transmit their signal by binding to their transmembrane receptors which belong to the Frizzled protein family (He et al., 1997; Miller, 2002; Yang-Snyder et al., 1996). Upon binding to their receptors, Wnts can activate several downstream cellular pathways, including the canonical pathway of β-catenin (reviewed in (Cadigan, 2002; Wodarz and Nusse, 1998).

β-catenin plays a central role in activating the Wnt canonical signaling pathway. In the absence of Wnt signaling, β-catenin is associated with a large cytoplasmic protein complex containing glycogen synthase kinase 3β (GSK3 β), the tumor suppressor adenomatous polyposis coli (APC) and axin (Behrens et al., 1998; Kishida et al., 1998). In this complex, GSK3 β phosphorylates sites within the N-terminal domain of β-catenin, which targets it for degradation by the ubiquitin-dependent proteasome pathway (Hart et al., 1999; Kitagawa et al., 1999; Latres et al., 1999). Upon binding of Wnt ligands to their receptors and co-receptors, the cytoplasmic protein Dishevelled is activated, which then inhibits GSK-3β activity, resulting in the stabilization and cytoplasmic accumulation of hypophosphorylated form of β-catenin. (Pinson et al., 2000a; Willert and Nusse, 1998; Yost et al., 1996). β-catenin then translocates to the nucleus and interacts with members of the Tcf/Lef family of transcription factors and activates downstream target genes (reviewed in (Barker and Clevers, 2000; Sharpe et al., 2001). In the absence of β-catenin, Tcf/Lef proteins bind to their target DNA and act as transcriptional repressors (Brannon et al., 1997; Merrill et al., 2004; Riese et al., 1997) by interacting with Groucho-related co-repressors (Brantjes et al., 2001).

Despite their crucial role in development, the role of this family of signaling proteins has not been characterized during the periimplantation stages. Aside from recent report describing the expression of *Wnt4* in the stroma cells and the Wnt inhibitor sFRP4 in the circular muscle of the myometrium, little is known about the involvement of this family of proteins in the implantation process. In this study I investigated the potential involvement of this family of signaling proteins as mediators of embryo-uterine cross talk

at the time of embryo implantation. I generated a transgenic mouse that provides readout of Wnt signaling through the canonical pathway of β-catenin.

I show that activation of this pathway is very dynamic during the periimplantation stages. First, it is transiently activated in the circular muscle of the myometrium at the anti-mesometrial side 7-8 hours before attachment time. Activation of this pathway is then restricted to the lumen epithelium solely at the embryo implantation site. In addition, I show that activation of this pathway requires the presence of the embryo and appears to be estrogen dependent. These results strongly suggest involvement of this pathway as a mediator of embryo-uterus communication to facilitate the implantation process.

METHODS

Generation of reporter construct and transgenic animals

To generate the β-catenin responsive reporter construct, 6 copies of the Tcf/Lef response element (CCTTTGATC) (Korinek et al., 1997), were cloned in the XhoI site of plasmid 1-11 that contains the hsp68 minimum promoter driving a LacZ reporter gene (Rossant et al., 1991). Transgenic mice were produced using standard protocols. Approximately 10 pl of 10 ng/μl linear DNA was injected into the male pronucleus of 1-cell stage embryos. Following microinjection, embryos were incubated overnight and the following day, those that had cleaved to the 2-cell stage were transferred into the oviducts of pseudopregnant females that were plugged in the day of transfer. Between 20 and 30 embryos were transferred per mouse.

Transgenic animals were genotyped using the polymerase chain reaction technique. Briefly, 1 cm of tail was cut and digested in 300 μl of lysis buffer (100 mM Tris.HCL(pH 8.0), 10 mM EDTA, 0.5% Tween 20, 0.5% NP-40, 0.4 mg/ml proteinase K) overnight at 55°C. The following day, DNA was phenol/chloroform extracted and 1μl of DNA was used for each PCR reaction. Primers used for the PCR reaction were LacZ-F1 5'CAGTGGCGTCTGGCGGAAAACCTC 3' and LacZ-B1 5'AAACAGGCGGCAGTAAGGCGGTCGG 3'. The PCR conditions were: 94°C for 1 min, 62°C for 1 min, 72°C for 1 min for 28 cycles.

Detection of β-galactosidase activity

Uteri were dissected in PBS (pH 7.3), rinsed in 100 mM sodium phosphate pH 7.3, and then fixed in 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 100 mM sodium phosphate pH 7.3 for 10 minutes at room temperature. Uteri were then washed 3 times in wash buffer (0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$, 100 mM sodium phosphate pH 7.3) for 15 minutes each at room temperature. To reveal β-galactosidase activity, they were incubated in 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$, 100 mM sodium phosphate pH 7.3 overnight at 37°C. They were then rinsed with wash buffer and PBS, and post fixed overnight in 4% paraformaldehyde at 4°C.

Surgical procedures

Tcf/Lef-lacZ transgenic females used to study the requirement of the embryo to activate the Wnt pathway in the uterus were ovariectomized on one side only or the junction between the oviduct and the uterus on one side was tightly tied using 6-0 silk thread (Ethicon Inc. Somerville, New Jersey, USA). Animals were allowed to recover for 2-3 weeks, then mated with fertile males. Uteri were recovered as described in the Results and stained for β-galactosidase activity.

Tcf/Lef-lacZ females to be used for embryo transfer experiment were mated with vasectomized males. In the morning of day 4 (day 1 is plug day), 5-10 blastocysts were transferred into each horn. Uteri were recovered as indicated in the Results and stained for β-galactosidase activity. *Tcf/Lef-lacZ* females used for artificial decidualization were mated with vasectomized males and on the morning of day 4, 10 μl of sesame oil was

then injected into each horn. Uteri were recovered from these animals as indicated in the Results and stained for β-galactosidase activity.

Tcf/Lef-lacZ transgenic females to be used for the estrogen dependency study were ovariectomized in the morning of day 4 of pregnancy between 7:00-9:00 AM. Immediately after surgery, mice received subcutaneous injection of progesterone (2mg/mouse) only or subcutaneous injection of progesterone with β-estradiol (50ng/mouse). Uteri were recovered as indicated in the Results and stained for β-galactosidase activity

Drug preparation

Progesterone (4-Pregnen-3, 20-Dione) was purchased from Steraloids Inc. Newport RI, USA (Q2600-000) and was dissolved in sesame oil at a concentration of 20 mg/ml. Each mouse received a subcutaneous dose of 100 μl (2mg/mouse). Estrogen (1,3,5[10]-Estratriene-3, 17β-diol) was purchased from Sigma (E-8875) and was dissolved in sesame oil at a concentration of 500 ng/ml. Each mouse received a subcutaneous dose of 50 ng.

RESULTS

Wnt reporter gene is first activated in the circular muscle of the myometrium in the implantation site at least 8 hrs before embryo attachment time

To investigate whether the canonical Wnt signalling pathway is involved in the implantation process, I generated a transgenic mouse (*Tcf/Lef-lacZ*) that faithfully provides readout of Wnt signaling through the canonical signaling pathway of β-catenin (Mohamed et al., 2004a). I examined activation of this reporter gene in uteri of pregnant females during implantation. Mature *Tcf/Lef-lacZ* transgenic females were mated with wild type males and uteri from pregnant females were collected on day 4 of pregnancy (day 1 = plug day) and stained for β-galactosidase activity. Figure 1 shows the uterine staining pattern of non-pregnant female (A), day 4 pregnant female at 1500 hr (B) and day 4 pregnant female at 2300 hr (C). As can be seen from these micrographs, when females were not pregnant (Figure 1A), there was no activation of the reporter gene in the uterus, while there is strong β-galactosidase activity in the oviduct (red arrow). We consistently detected β-galactosidase activity in the oviduct region only in the *Tcf/Lef-lacZ* transgenic animals, but not in wild type females, which served as control for specificity of the staining reaction.

In the afternoon of day 4, at 1500 h (7 to 8 hours before embryo attachment) (Das et al., 1994), β-galactosidase activity is detected in the uterus in a form of discrete bands in both horns (Figure 1B, blue arrows). Several hours later, at 2300 hr, when most embryos have begun the implantation process, more of these discrete bands are detected (Figure 1C).

Sectioning and histological examination of these uteri indicate that β-galactosidase activity is restricted to the myometrium circular muscle layer (Figure 1D & E, black arrows).

For unknown reasons, embryo implantation always takes place on the lumen epithelium of the anti-mesometrial (ventral) half of the uterus (Paria et al., 2001). Interestingly, activation of the reporter in the myometrium region is detected only in the anti-mesometrial half of the uterus, thus apparently demarcating the uterine side where the embryo is about to implant.

Wnt reporter gene is exclusively activated in the uterine luminal epithelium surrounding the implanting blastocyst

Attachment reaction of the trophectoderm to the luminal epithelium occurs between 2300 hr of day 4 and 12000 of day 5 of pregnancy (Das et al., 1994). To dissect activation of the Wnt canonical pathway during this period, I closely examined activation of the Wnt reporter in *Tcf/Lef-lacZ* transgenic animals during day 5 of pregnancy. As shown in Figure 2A, whole mount β-galactosidase staining of day 5 uterus at 12:00 noon indicates that the reporter gene is activated in very specific sites, resembling beads, within the uterus.

To gain better insights on the type of cells where this reporter has been activated, I examined histological sections of the uterus throughout day 5 of pregnancy. As shown in Figure 2B, C & D (12:00 noon), activation is restricted to the uterine lumen epithelium apposing the implanting embryo (red arrow). No activation is detected in adjacent sections where there is no embryo (Figure 2E & F). This finding indicates that β-galactosidase activity in the lumen epithelium is specific to the site where the embryo is

about to implant. Several hours later, (4:00 PM), β-galactosidase activity has expanded into the entire luminal epithelium surrounding the implanting embryo (Figure 2G, H & I). Similarly, adjacent areas where there is no embryo do not show activation of the reporter (Figure 2J & K).

Interestingly, the earlier activation of the reporter observed in day 4 in the myometrium region is no longer detected. By the end of day 5 of pregnancy, however, β-galactosidase activity is not detected in the uteri of these females indicating that its activation is transient and is associated with the initial stages of implantation (data not shown).

Activation of the Wnt reporter gene in the uterus during pregnancy requires the presence of the embryo

The previous results indicated that a Wnt reporter gene is activated in the uterus at the periimplantation stages in a very dynamic and specific manner that is highly relevant to embryo implantation. To investigate the mechanism by which this pathway is activated in the uterus and whether its activation is dependent on the presence of the embryo, I performed the following experiments. First, I mated the *Tcf/Lef-lacZ* females with vasectomized males and then examined activation of the reporter in their uteri at day 4 and 5 of pseudopregnancy. As shown in Figure 3B, β-galactosidase activity was not detected in the uteri of these females either in day 4 or in day 5 of pregnancy (Table 1). Second, I transferred blastocysts into the uteri of *Tcf/Lef-lacZ* pseudopregnant females in the morning of day 4 and then examined activation of the reporter gene in the uteri of these females in the evening of day 4 and in the morning of day 5. As shown in Table 1,

in most cases (5/7), specific β-galactosidase activity, in the same fashion of discrete banding as in the wild type, was restored in the uteri of these females.

Third, to further link activation of the reporter gene to the presence of the embryo, I deprived one horn of the uterus of embryos and then examined β-galactosidase activity in these uteri. I achieved this by two means. In one group (8 females), one ovary was surgically removed from these animals, while the other ovary was kept intact. In a second group (9 females) the junction between the oviduct and the uterus was tightly tied in one side, while the other side was not manipulated. Two to three weeks after surgery, these females were mated with fertile males and in the morning of day 5 their uteri were examined for reporter gene activation. Specific activation of the reporter gene was detected only in the side where the ovary was kept intact or the oviduct-uterus junction was not ligated (Fig 3C & Table1).

Fourth, I artificially induced decidualization in *Tcf/Lef-lacZ* pseudopregnant females by injection of sesame oil into their uteri in the morning of day 4 and then examining activation of this pathway in subsequent days. Using this method, I was not able to observe specific activation of this pathway neither in the evening of day 4 nor through out day 5 of pseudopregnancy although by day 6 clear deciduas were easily observed (Table 1 and data not shown).

Collectively, these results clearly indicate that activation of a Wnt reporter gene in the uterus is dependent on the presence of the embryo, suggesting that a signal emanating from the embryo is what induces its activation in the uterus.

Activation of the Wnt reporter gene in the uterus during pregnancy is regulated by estrogen

To investigate whether activation of the Wnt reporter gene is regulated by the ovarian estrogen surge, I performed the following experiment. I mated *Tcf/Lef-lacZ* females with fertile males and then removed both ovaries from pregnant females in the morning of day 4 (between 7:00-8:00 AM), before the onset of the estrogen surge. After surgery, animals were administered with a dose of progesterone to support pregnancy and their uteri were then examined for β-galactosidase activity in the morning of day 5. After this treatment, I was not able to detect any specific β-galactosidase activity in the uteri of these females (Figure 5A & Table1). Bands of β-galactosidase activity similar to that of untreated females were restored, however, in the uteri of ovariectomized females when the animals were administered with a dose containing a mixture of progesterone and estrogen after surgery (Figure 4B & Table1). These results demonstrate that activation of the Wnt reporter gene in the uterus is regulated by ovarian estrogen.

DISCUSSION

Embryo implantation is a complex process that requires the action and coordination of a network of regulatory factors on both the embryo and uterine side. Despite the absolute requirement for implantation for reproduction of all mammals, very little is known about the molecular mechanisms involved in the regulation of this process in any species. Understanding the communication language between the implanting embryo and the receptive uterus is, no doubt, crucial for reproductive technology.

Although several molecules have been implicated in this process (Paria et al., 2002), no specific signaling pathway has been shown to be involved in the implantation process. In this study I present evidence that a reporter gene, reflecting the Wnt canonical signaling pathway of β-catenin, is activated at the periimplantation stages in a very dynamic and specific manner that is highly relevant to the implantation process. I show that this pathway becomes first activated in the smooth circular muscle of the myometrium at least 8 hours before attachment time. The smooth circular muscle of the myometrium is thought to play an essential role in embryo spacing and was shown to specifically express some prostaglandin (PG) receptors such as EP3 (PGE2 receptor) and FP (PGF2alpha receptor) (Yang et al., 1997). Other factors implicated in embryo spacing belong to the BMP (Bone Morphogenetic Proteins) family of proteins (Paria et al., 2001; Pfendler et al., 2000). Activation of this pathway specifically in the circular smooth muscles of the myometrium at the time when embryo spacing decisions are being committed provides another possible mechanism by which this process is executed.

Interestingly, β-catenin signaling activity is detected mainly at the anti-mesometrial (ventral) side of the uterus, which is the side where the embryo always implants (Paria et al., 2001). Blastocyst apposition to the luminal epithelium is initiated by the creation of an implantation chamber surrounding the blastocyst at the anti-mesometrial pole of the uterus. This is followed by decidualization of stromal cells and apoptosis of luminal epithelia at the implantation site (Abrahamsohn and Zorn, 1993). Decidualization begins at the anti-mesometrial pole of the uterus and then spreads to the mesometrial pole (the presumptive site of placentation) and thus orienting the uterus shape in an anti-mesometrial-mesometrial direction (Daikoku et al., 2004).

The mechanism by which the embryo implantation chamber is always oriented in the anti-mesometrial-mesometrial direction, however, remains unknown. Several genes are differentially expressed along the mesometrial-antimesometrial axis at the time of attachment. For example, BMP2 and FGF2 are expressed only in the anti-mesometrial pole of the uterus, while FGF10 is expressed in the mesometrial pole (Paria et al., 2001). Indian hedgehog (Ihh) is expressed at a higher level in the luminal epithelium of the anti-mesometrial pole as compared to the mesometrial pole (Paria et al., 2001). The observation that the Wnt canonical pathway is activated in the circular muscle and underlying stroma only in the side where the embryo is going to implant, is very intriguing. It leads to the suggestion that, by activating this pathway only in this side, perhaps the uterus creates conditions that favor embryo implantation only in this side.

In the morning of day 5, activation of this Wnt/β-catenin signaling pathway in the myometrium region is no longer detected. Interestingly, sFRP4, which is Wnt antagonist, becomes activated at this time specifically in this region (Daikoku et al., 2004). I

hypothesize that it functions to suppress the β-catenin signaling pathway in this area at this particular time. Concurrently, signaling through β-catenin becomes detected in the uterine luminal epithelium, initially in the anti-mesometrial pole of the lumen epithelium and few hours later in the uterine epithelium surrounding the embryo (Figure 2G, H & I). Activation of this pathway specifically in the uterine epithelium where the embryo is about to implant strongly suggests that it is activated by a signal that is secreted by the embryo. Indeed, using several approaches I showed that β-catenin signaling activity in the uterus is dependent on the presence of the embryo (Figure 3).

Furthermore, I have previously shown that the blastocyst expresses numerous Wnt genes at the time of implantation, which could serve as the ligand that activates the β-catenin signaling pathway in the uterus (Mohamed et al., 2004b). By the end of day 5, no specific β-catenin signaling activity is detected in the uterus (data not shown). It is interesting to note that although Wnt4 mRNA is detected in the stroma surrounding the implanting embryo (Daikoku et al., 2004; Paria et al., 2001), I did not detect any activation of the Wnt/β-catenin signaling pathway in the stroma surrounding the embryo at any time. This agrees with the observation that Wnt4 does not activate the Wnt canonical signaling pathway of β-catenin, rather it activates the calcium pathway (Wodarz and Nusse, 1998), and suggests that Wnts participate in the implantation process through several pathways.

Ovarian steroid hormones have long been known to orchestrate the action of the different regulatory factors that are essential for implantation (Psychoyos, 1973a; Psychoyos, 1973b). In mice, ovarian estrogen surge, which takes place in the morning of day 4 of pregnancy, is essential for blastocyst implantation (McCormack and Greenwald, 1974). Although the precise molecular mechanism by which estrogen induces implantation is not

understood, delayed implantation studies have indicated that nidatory estrogen is essential for activation of genes encoding several growth factors solely at the site of implantation (Das et al., 1997a; Das et al., 1994; Ma et al., 2003). In this study I showed that activation of the Wnt/β-catenin signaling pathway in the implantation sites is also estrogen dependent (Figure 4). This leads to the interesting suggestion that these growth factors perhaps are downstream targets of the Wnt canonical pathway. Indeed, sequence analysis of the promoter region of the gene encoding HB-EGF, which is activated specifically in the uterine epithelium surrounding the implanting embryo (Das et al., 1994), show that there are 3 Tcf/Lef binding sites in the promoter region, which makes it an ideal downstream target of this pathway.

In mice, expression of the cytokine LIF in the uterus was shown to be estrogen dependent and, remarkably, can substitute for estrogen in the induction of embryo implantation (Chen et al., 2000). On the other hand, uteri from LIF mutant mice can not support implantation even in the presence of estrogen (Stewart et al., 1992). These results strongly suggest that estrogen perhaps only functions to activate LIF expression, which in turn initiates a cascade of events that facilitates implantation. It would be very interesting to investigate whether activation of the Wnt/β-catenin signaling pathway functions downstream or upstream of LIF. This could be tested by placing the transgene in a LIF mutant background and then investigate whether this pathway is activated in the absence of LIF.

In conclusion, previous research has indicated that signaling pathway(s) must be operating to facilitate the communication between the implanting embryo and the receptive uterus. Although several molecules have been identified to be specifically expressed at the time

of implantation, no particular signaling pathway had been implicated in this process thus far. In this study I presented compelling evidence that an important signaling pathway, such as the Wnt/β-catenin signaling pathway, is activated in the uterus in a very specific manner that is highly relevant to the implantation process, making it an attractive pathway to facilitate this complex process.

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Table 1: Activation of reporter gene in the uteri of *Tcf/Lef-lacZ* transgenic pregnant females

Treatment	Number of animals used	Activation of the reporter gene in the oviduct region		Specific activation of the reporter gene in the uterus*	
		Day 4	Day 5	Day 4	Day 5
None	**32**	**14**	**18**	**9**	**14**
Mated with Vas males	**13**	**5**	**8**	**0**	**0**
Mated with Vas males + blastocyst transfer	**7**	**3**	**4**	**2**	**3**
Ovariectomy on one side	**8**	**ND**	**8**	**ND**	**5**
Oviduct-uterus junction ligation	**9**	**ND**	**9**	**ND**	**6**
Artificial Decidualization	**17**	**4**	**13**	**0**	**0**
Ovariectomy + Progesterone	**7**	**ND**	**7**	**ND**	**0**
Ovariectomy + Progesterone + Estrogen	**8**	**ND**	**8**	**ND**	**5**

* Specific activation means observing discrete bands throughout the uterine horns. In the case where specific bands were not observed, we usually observe diffused staining throughout the uterus, perhaps owing to over staining. ND = Not determined

Figure 1: Wnt reporter gene is activated in the uterus during implantation day at least 7 hours before the onset of embryo attachment

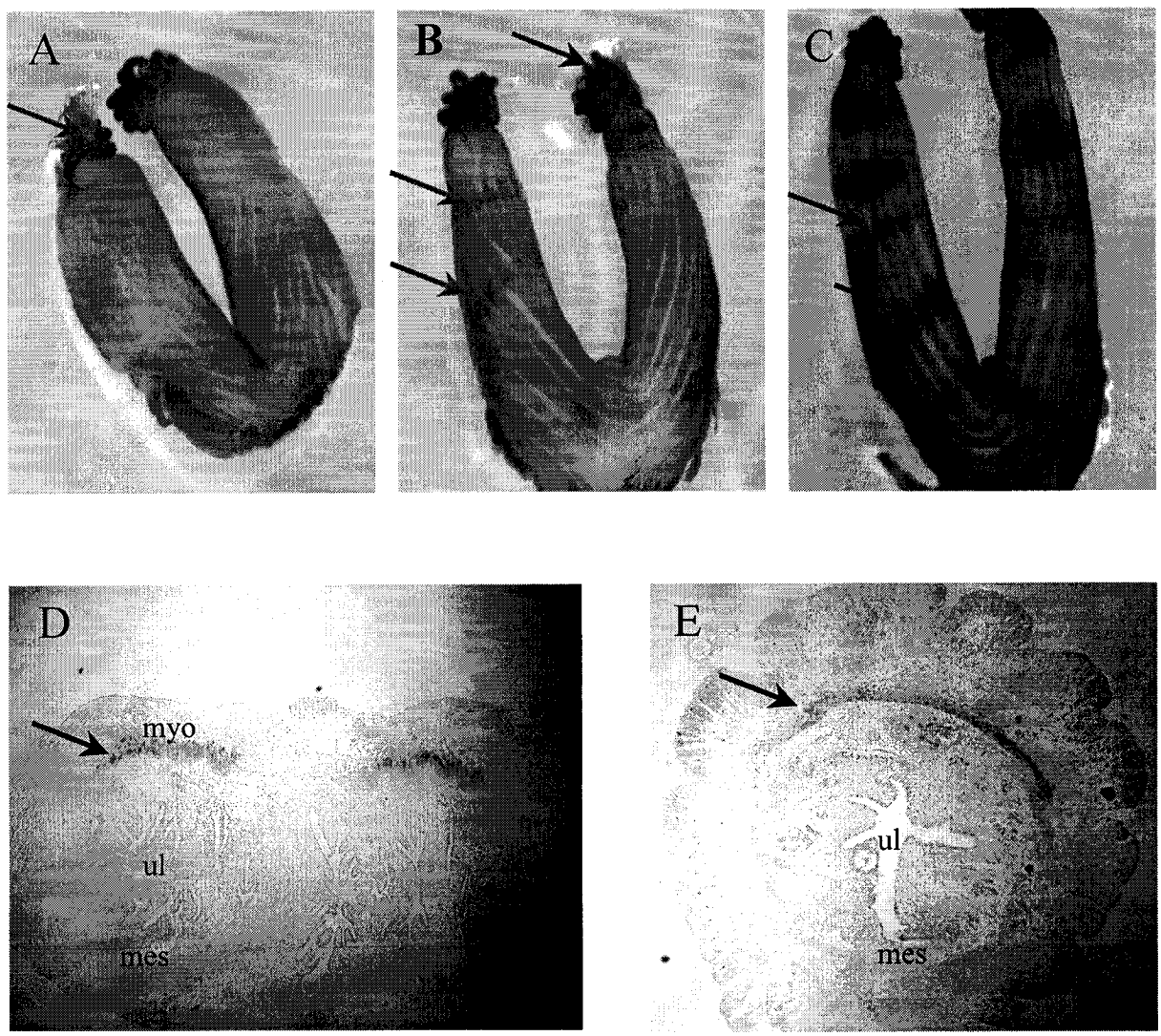

Figure 1 legend

A: Whole mount β-galactosidase staining of uterus dissected from non-pregnant *Tcf/Lef-lacZ* female (red arrow points to the oviduct region)

B: Whole mount β-galactosidase staining of uterus dissected from day 4 pregnant *Tcf/Lef-lacZ* female at 1500 hr (Blue arrows points to staining in the uterus region)

C: Whole mount β-galactosidase staining of uterus dissected from day 4 pregnant *Tcf/Lef-lacZ* female at 2300 hr (Blue arrows points to Wnt canonical pathway activation in the uterus region)

D: Sagital section of the uterus of day 4 pregnant female showing specific activation of the Wnt canonical pathway in the circular muscle of the myometrium (black arrow)

E: Transverse section of day 4 pregnant uterus showing specific activation of the Wnt canonical pathway in the circular muscle of the myometrium (black arrow)

(myo = myometrium, mes = mesometrial, ul = uterine lumen)

Figure 2: Wnt reporter gene is activated in the uterine epithelium opposing the implanting embryo

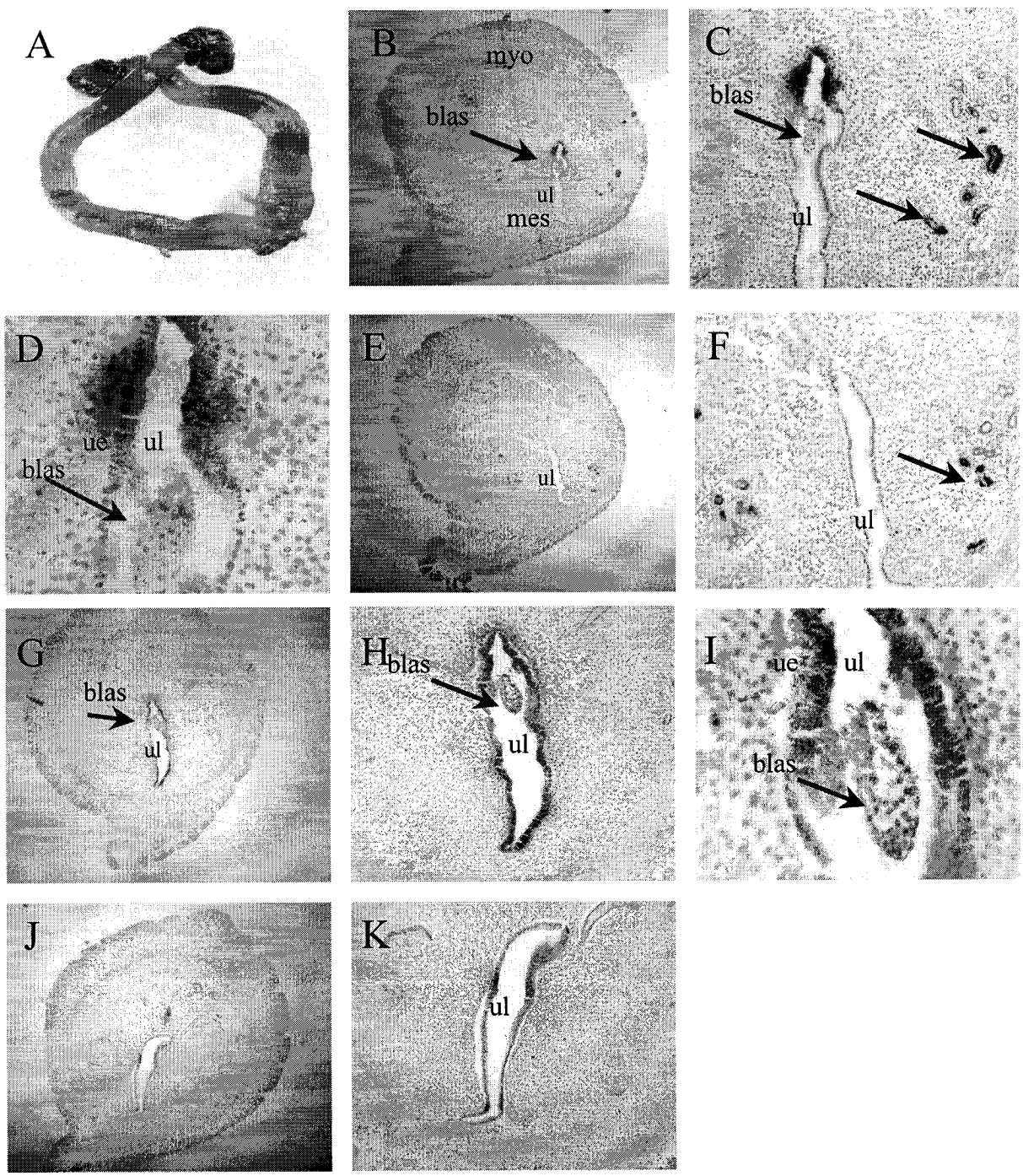

Figure 2 legend

A: Whole mount β-galactosidase staining of uterus dissected from day 5 pregnant *Tcf/Lef-lacZ* female at 16:00 hr.

B: Transverse section of uterus dissected from day 5 pregnant *Tcf/Lef-lacZ* female at 12:00 hr (magnification = 10X, red arrow points to the blastocyst).

C: 20X magnification of the same section in B, red arrow points to the blastocyst).

D: 40X magnification of the same section in B, red arrow points to the blastocyst).

E: Adjacent section to the section shown in B, where no embryo is detected, magnification is 10X.

F: 20X magnification of the same section in E.

G: Transverse section of uterus dissected from day 5 pregnant *Tcf/Lef-lacZ* female at 16:00 hr (magnification = 10X, red arrow points to the blastocyst).

H: 20X magnification of the same section in G, red arrow points to the blastocyst).

I: 40X magnification of the same section in G, red arrow points to the blastocyst).

J: Adjacent section to the section shown in G, where no embryo is detected, magnification is 10X.

K: 20X magnification of the same section in J.

(myo = myometrium, mes = mesometrial, ul = uterine lumen, ue = uterine epithelium, blas = blastocyst)

Figure 3: Activation of Wnt reporter gene activation in the uterus is dependent on the presence of the embryo

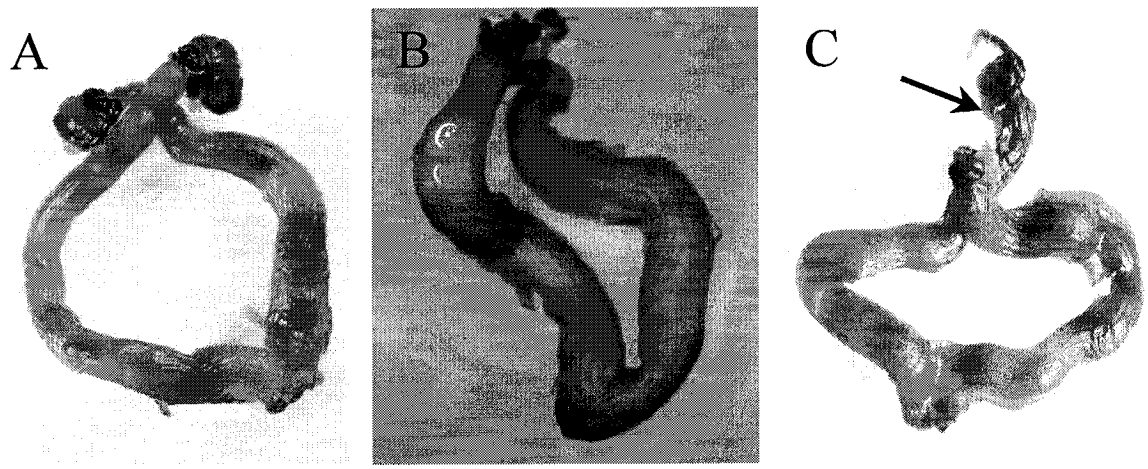

Figure 3 legend

A: Whole mount β-galactosidase staining of uterus dissected from day 5 pregnant (mated with fertile male) *Tcf/Lef-lacZ* female at 16:00 hr.

B: Whole mount β-galactosidase staining of uterus dissected from day 5 pseudopregnant (mated with Vas male) *Tcf/Lef-lacZ* female at 16:00 hr.

C: Whole mount β-galactosidase staining of uterus dissected from day 5 pregnant (mated with fertile male) *Tcf/Lef-lacZ* female at 16:00 hr. The junction between the oviduct and uterus of this female was ligated 2-3 weeks earlier from one side only. Blue arrow points to the uterus-oviduct junction where the ligation was performed.

Figure 4: Activation of Wnt reporter gene in the uterus is regulated by estrogen

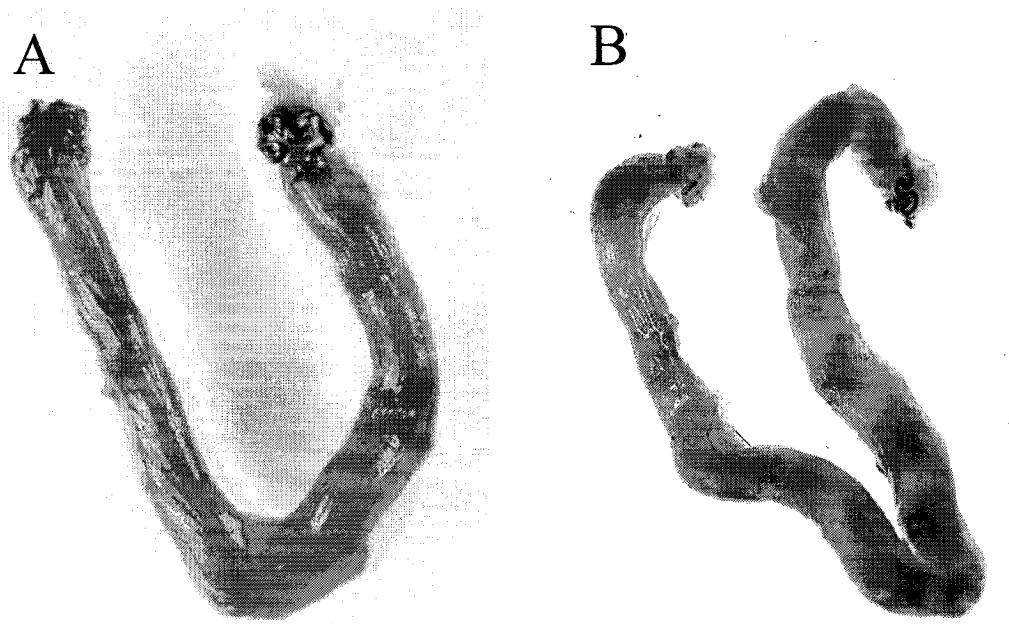

Figure 4 legend

A: Whole mount β-galactosidase staining of uterus dissected from day 5 pregnant (mated with fertile male) *Tcf/Lef-lacZ* female at 16:00 hr. This female was ovariectomized from both sides in the morning (7:00-8:00 AM) of day 4 of pregnancy and administered with a 2 mg dose of progesterone.

B: Whole mount β-galactosidase staining of uterus dissected from day 5 pregnant (mated with fertile male) *Tcf/Lef-lacZ* female at 16:00 hr. This female was ovariectomized from both sides in the morning (7:00-8:00 AM) of day 4 of pregnancy and administered with a 2 mg dose of progesterone plus 100 ng of estrogen.

CHAPTER 4: MANUSCRIPT III

β-catenin signaling marks the prospective site of primitive streak formation in the mouse embryo

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PREFACE

Wnt/ β-catenin signaling pathway plays an essential role in axis formation and organizer specification in *Xenopus* and zebrafish embryos. The mechanism by which mammalian embryonic axes are determined, and in particular whether the Wnt/ β-catenin signaling pathway plays a similar role, is not known. In the following manuscript, I determined when and where the canonical Wnt/β-catenin signaling pathway first becomes activated in the mammalian embryo. Using immunofluorescence and transgenic techniques, I examined the distribution and transcriptional activity of β-catenin within the mouse embryo from the time of fertilization until the onset of axis formation.

ABSTRACT

β-catenin signaling has been shown to be involved in triggering axis formation in a number of organisms including *Xenopus* and zebrafish. Genetic analysis has demonstrated that the Wnt/β-catenin signaling pathway is also involved in axis formation in the mouse since a targeted deletion of *β-catenin* results in embryos that have a block in anterior-posterior axis formation, fail to initiate gastrulation and do not form mesoderm. However, since β-catenin is ubiquitously expressed, the precise time and cell types in which this signaling pathway is active during early embryonic development remain unknown. Thus, to better understand the role of the Wnt/β-catenin signaling pathway in axis formation and mesoderm specification, I have examined both the distribution and signaling activity of β-catenin during early embryonic development in the mouse. I show that the N-terminally non-phosphorylated form of β-catenin as well as β-catenin signaling is first detectable in the extraembryonic visceral endoderm in day E5.5 embryos. Prior to the initiation of gastrulation at day E6.0, β-catenin signaling is asymmetrically distributed within the epiblast and is localized to a small group of cells adjacent to the embryonic-extraembryonic junction. At day E6.5 and onwards, β-catenin, signaling was detected in the primitive streak and mature node. Thus, β-catenin signaling precedes primitive streak formation and is present in epiblast cells that will go on to form the primitive streak. These results support a critical role for the Wnt/β-catenin pathway in specifying cells to form the primitive streak and node in the mammalian embryo as well as identify a novel domain of Wnt/ β-catenin signaling activity during early embryogenesis.

INTRODUCTION

β-catenin is a multifunctional protein that plays at least two roles in cells. One is in cell adhesion, where its role is mediated through interactions with cadherins (Gumbiner and McCrea, 1993; McCrea and Gumbiner, 1991; McCrea et al., 1991). The second is as a downstream effector of the canonical Wnt signaling pathway (reviewed in (Cadigan and Nusse, 1997)). In the absence of Wnt signaling, β-catenin associates with a large cytoplasmic protein complex containing glycogen synthase kinase 3β (GSK3β, the tumor suppressor adenomatous polyposis coli (APC) and axin (Behrens et al., 1998; Kishida et al., 1998). In this complex GSK3β phosphorylates sites within the N-terminal domain of β-*catenin* and subsequently targets it for degradation by the ubiquitin-dependent proteasome pathway (Hart et al., 1999; Kitagawa et al., 1999; Latres, 1999). Binding of Wnt ligands to their Frizzled receptors and the low-density lipoprotein receptor-related protein (LRP) co-receptors, LRP-5 and LRP-6, results in the activation of Dishevelled protein, which inhibits GSK-3β activity thus leading to the stabilization and accumulation of the hypophosphorylated form of β-catenin. (Pinson et al., 2000a; Willert and Nusse, 1998; Yost et al., 1996). β-catenin then translocates to the nucleus, where it interacts with members of the Tcf/Lef family of transcription factors and activates downstream target genes (reviewed in (Barker and Clevers, 2000; Sharpe et al., 2001). In the absence of β-catenin, Tcf/Lef proteins bind to their target DNA and act as transcriptional repressors (Brannon et al., 1997; Merrill et al., 2004; Riese et al., 1997) by interacting with Groucho-related co-repressors (Brantjes, 2002; Brantjes et al., 2001; Roose et al., 1998).

In both *Xenopus* and zebrafish, β-catenin signaling plays an important role in specifying the embryonic axis (reviewed in (Moon and Kimelman, 1998; Schier, 2001; Sokol, 1999). In *Xenopus*, the dorsal-ventral axis is specified upon fertilization, when sperm entry initiates a cortical rotation relative to the cytoplasm of the fertilized egg leading to the stabilization and nuclear accumulation of β-catenin in the prospective dorsal side of the embryo. Upon embryonic genome activation, β-catenin interacts with Tcf/Lef proteins to activate the expression of genes, such as *Siamois* and *Twin,* that participate in the formation of the organizer, which is essential for axis specification (Brannon et al., 1997; Carnac et al., 1996; Lemaire et al., 1995). Overexpression of several components of this pathway leads to axis duplication (reviewed in (Moon and Kimelman, 1998). Similarly, in zebrafish, nuclear β-catenin accumulates in nuclei on the dorsal side of blastulae (Schneider et al., 1996) and overexpression of β-catenin in early zebrafish embryos induces the formation of a complete secondary axis (Kelly et al., 1995).

In the mouse, the first morphological sign of axis formation occurs at day E6.5 of embryogenesis with the formation of the primitive streak. The region where the primitive streak forms becomes the posterior end of the embryo (reviewed in (Beddington and Robertson, 1999)). Several lines of evidence demonstrate that, as in *Xenopus* and zebrafish, the Wnt/β-catenin signaling pathway plays an essential role in axis specification in the mouse. Most dramatically, a targeted deletion of β-catenin produces embryos which have a block in anterior-posterior axis formation at E6.0, fail to initiate gastrulation and do not form mesoderm (Haegel et al., 1995; Huelsken et al., 2000). Moreover, overexpression of *Wnt8c* (Popperl et al., 1997) or, conversely, deletion of genes encoding proteins involved in the degradation of cytoplasmic β-catenin, including Axin, APC and

the LIM domain-binding protein 1 all produce embryos with at least partially duplicated axes (Ishikawa et al., 2003; Merrill et al., 2004; Mukhopadhyay et al., 2003; Zeng et al., 1997). β-catenin is required at the time of axis formation for correct positioning of the anterior visceral endoderm. Although some anterior visceral endoderm genes are expressed in embryos lacking β-catenin, the expressing cells remain at the distal tip of the embryo and fail to migrate to the future anterior region of the embryo (Huelsken et al., 2000).

Although these results clearly indicate an obligatory role for Wnt/β-catenin signaling during formation of the primitive streak and gastrulation, the precise time and cell types in which the signaling pathway is active remain unknown. Furthermore, some of the phenotypic effects observed in *β-catenin* mutant embryos may reflect its role in cell adhesion. Thus, to better understand the role of Wnt/β-catenin signaling in axis formation and mesoderm specification, it is essential to identify when and where this pathway is active during these processes. Although several transgenic lines that can report Wnt/β-catenin signaling have been generated (DasGupta and Fuchs, 1999; Maretto et al., 2003), the domains of active Wnt/β-catenin signaling during the pre- and early post-implantation period have not been reported. I have examined both the distribution and signaling activity of β-catenin during early embryonic development. As recent evidence indicates that N-terminally non-phosphorylated β-catenin interacts with Tcf/Lef (van Noort et al., 2002), I used immunohistochemistry to examine the distribution of this non-phosphorylated isoform of β-catenin. I have also used a transgenic reporter approach to identify where β-catenin cooperates with Tcf/Lef to activate gene expression during early embryonic development.

METHODS

Immunofluorescence

Embryos were obtained from CD-1 female mice (Charles River Canada). The day on which the plug was observed was designated day E0.5. Pre-implantation embryos were collected by flushing the oviducts of pregnant females. Post-implantation embryos were dissected from the uteri of pregnant females. Embryos were fixed in freshly prepared 4% para-formaldehyde in phosphate-buffered saline (PBS) either for 15 min at room temperature for pre-implantation embryos or overnight at 4°C for post-implantation embryos. They were permeabilized in blocking solution consisting of PBS, 3% bovine serum albumin (BSA), 0.1% Triton X-100, for at least 30 min at room temperature and either stored at 4°C in blocking solution or processed immediately for immunofluorescent staining. Immunostaining was carried out in a blocking solution containing either a monoclonal mouse antibody against total β-*catenin* (Transduction Laboratory) diluted 1:500 in blocking solution or a monoclonal mouse antibody against non-phosphorylated β-catenin (Upstate Biotechnology) diluted 1:500. Following overnight incubation at 4°C with agitation, the embryos were washed 3 times for 15 minutes each in blocking solution, then incubated for 1 hr at room temperature with agitation in the presence of a fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (Jackson Immunoresearch) diluted 1:100 in blocking buffer. The embryos were then washed and mounted on a microscope slide in the presence of 1 μg/ml of DAPI to stain the DNA. Specimens were examined using either a Leitz Laborlux S microscope or a Zeiss confocal laser scanning microscope.

Generation of reporter construct and transgenic animals

To generate the β-catenin responsive reporter construct, 6 copies of the Tcf/Lef response element (CCTTTGATC) (Korinek et al., 1997), were cloned in the XhoI site of plasmid 1-11 that contains the hsp68 minimum promoter driving a LacZ reporter gene (Rossant et al., 1991). Transgenic mice were produced using standard protocols. Approximately 10 pl of 10 ng/μl linear DNA was injected into the male pronucleus of 1-cell stage embryos. Following microinjection, embryos were incubated overnight and the following day, those that had cleaved to the 2-cell stage were transferred into the oviducts of pseudo-pregnant females that were plugged in the day of transfer. Between 20 and 30 embryos were transferred per mouse. Transgenic animals were genotyped using the polymerase chain reaction technique. Briefly, 1 cm of tail was cut and digested in 300 μl of lysis buffer (100 mM Tris.HCL (pH 8.0), 10 mM EDTA, 0.5% Tween 20, 0.5% NP-40, 0.4 mg/ml proteinase K) overnight at 55°C. The following day, DNA was phenol/chloroform extracted and 1ul of DNA was used for each PCR reaction. Primers used for the PCR reaction were LacZ-F1 5'CAGTGGCGTCTGGCGGAAAACCTC 3' and LacZ-B1 5'AAACAGGCGGCAGTAAGGCGGTCGG 3'. The PCR conditions were: 94°C for 1 min, 62°C for 1 min, 72°C for 1 min for 28 cycles. Homozygous *Vestigial tail* mice were generously provided by Dr. Lohnes (University of Montreal).

Detection of lacZ activity

Embryos were dissected in PBS (pH 7.3), rinsed in 100 mM sodium phosphate pH 7.3, and then fixed in 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 100 mM sodium phosphate pH 7.3 for 5 minutes at room temperature. Embryos were then washed 3 times

in wash buffer (0.02% NP-40, 0.01%deoxycholate, 2 mM $MgCl_2$, 100 mM sodium phosphate pH 7.3) for 15 minutes each at room temperature. To reveal lacZ activity, they were incubated in 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$, 100 mM sodium phosphate pH 7.3 overnight at 37°C. They were then rinsed with wash buffer and PBS, and post fixed overnight in 4% paraformaldehyde at 4°C.

Transfection of embryonic fibroblasts

Embryonic fibroblasts were isolated from E12.5-day embryos following standard protocols. Transfections were carried out using Lipofectamine (Invitrogen) according to the manufacture's recommendations. Plasmids used for this study, were: S37A (coding for a mutated form of β-*catenin* in which serine-37 has been changed to alanine, a gift from Dr. S. W. Byers, Georgetown University School of Medicine, Washington, D. C.), pCMV5B-Lef1-HA (coding for mouse lef-1, a gift from Dr. D. Lohnes, University of Montreal), pCMVβ (coding for β-galactosidase, Clontech Inc.).

RESULTS

Non-phosphorylated β-catenin accumulates in extraembryonic visceral endoderm and in epiblast cells located where the primitive streak will form.

To investigate in which cell types of the early mouse embryo β-catenin was N-terminally non-phosphorylated and therefore could be translocated to the nucleus to regulate gene expression, I stained embryos at different stages of development using two different antibodies. The first is an antibody that recognizes β-catenin independently of its phosphorylation state, which I refer to as total β-catenin. The second is an antibody that recognizes only the N-terminally non-phosphorylated form of the protein, which I refer to as non-phosphorylated (van Noort et al., 2002). Preimplantation embryos from the 2-cell to blastocyst stage stained for total β-catenin revealed strong immunoreactivity localized at the cell surfaces, but no nuclear staining was detected, consistent with previous reports (Figure 1a-e) (Pauken and Capco, 1999; Rogers and Varmuza, 2000). Furthermore, non-phosphorylated β-catenin was not detectable in these embryos (Figure 1f-j). These results imply that the Wnt/β-catenin signaling pathway is not active in embryos up to the blastocyst stage.

The primitive streak first develops at the future posterior region of the embryo, near the junction of the embryonic and extra-embryonic ectoderm, and is morphologically detectable beginning at day E6.5 (Beddington and Robertson, 1999; Lu et al., 2001b). Accordingly, I analyzed the distribution of β-catenin in early post-implantation stage embryos, ranging in age from E5.5 to E6.75 days. At each stage examined, total β-catenin

was detected throughout the embryos and showed no evidence of asymmetrical distribution (Figures 2a and 2c). In contrast to the apparently uniform distribution of total β-catenin, the non-phosphorylated form of the protein showed a tissue-restricted and dynamic pattern of expression during this period of embryogenesis. In E5.5 day embryos, non-phosphorylated β-catenin was readily detected in the extra-embryonic visceral endoderm. However, no immunostaining could be detected in other tissues, notably including those of the embryo proper (Figure 2b). At day E6.0, although staining remained largely restricted to the extra-embryonic visceral endoderm, a small group of immunoreactive cells could be identified that projected down towards the embryo proper (Figure 2d). Moreover, by day E6.75, this group of cells had expanded both laterally and along the proximal-distal axis (Figure 2e, red arrow).

To examine this transition in more detail, E6.25 day embryos were stained for non-phosphorylated β-catenin and examined using confocal microscopy. As illustrated in Figures 2f and 2g, non-phosphorylated β-catenin could be detected in extraembryonic visceral endoderm cells. In addition, no immunoreactivity could be detected in the extra-embryonic ectoderm or in the majority of the embryonic ectoderm cells. However, a small group of embryonic ectodermal cells adjacent to the embryonic-extraembryonic junction were clearly stained (Figure 2g, arrow). Furthermore, accumulation of non-phosphorylated β-catenin was also detected in the visceral endoderm overlying this region of the epiblast. Although non-phosphorylated β-catenin was detected in the epiblast starting at E6.25, staining in the visceral endoderm could be detected slightly earlier, at E5.75- E6.0 (data not shown). These results indicate that, several hours prior to the

formation of a morphologically identifiable primitive streak, non-phosphorylated β-catenin accumulates in a subset of cells within the region where this structure will form.

A β-catenin responsive reporter gene is first expressed in extraembryonic visceral endoderm cells and then in cells located where the primitive streak will form

β-catenin activates transcription of target genes through association with members of the Tcf/Lef protein family, which bind to DNA at a well-characterized consensus sequence (Korinek et al., 1997; van Beest et al., 2000). The presence of β-catenin converts the complex, which in its absence represses transcription, to a transcriptional activator. To map sites in the embryo of transcription regulated by β-catenin, I generated lines of transgenic mice that carried a lacZ reporter gene linked to six copies of the Tcf/Lef binding site together with a minimal promoter element (Rossant et al., 1991). Six independent *Tcf/Lef-lacZ* transgenic lines that were tested for expression all gave similar expression patterns, as described below.

To verify that the lacZ transgene could be activated by β-catenin, I isolated embryonic fibroblast cells from hemizygous embryos derived from one transgenic line. β-galactosidase activity was measured following transfection with different constructs and normalized against the activity measured in cells transfected with a CMV-lacZ under the control of the strongly active cytomegalovirus promoter (Figure 3, column 1). Very low activity was detected in mock-transfected transgenic fibroblasts (Figure 3, column 2) or in those transfected with a construct encoding Lef-1 (Figure 3, column 3). In contrast, high β-galactosidase activity was measured following transfection of a construct encoding a mutant form of β-catenin in which serine-37 has been replaced by alanine (S37A) (Zorn et

al., 1999) rendering the protein non-phosphorylatable at this site and therefore constitutively active (Figure 3, column 4). Co-transfection of Lef-1 and S37A did not further increase β-galactosidase activity (Figure 3, column 5). These results confirmed that in these transgenic lines non-phosphorylatable form of β-catenin induced endogenous lacZ expression, strongly suggesting that the activity of the *Tcf/Lef-lacZ* transgene accurately reflects endogenous activity of nuclear β-catenin.

Next, hemizygous transgenic embryos were stained for β-galactosidase activity at different stages of development. First, I examined whether β-galactosidase activity could be detected in tissues where *Wnt* expression, which lies upstream of β-catenin activity, has previously been reported. Activity was detected in the midbrain-hindbrain region of day E8.25 embryos, (Figure 4a) and at day E8.75 and E9.5, high levels of activity were detected in the dorsal region of the neural tube and somites (Figure 4b-d). This activity is consistent with expression of *Wnt1* and *Wnt3a* in the roof plate and their essential role in neuronal specification of the dorsal neural tube (Muroyama et al., 2002). β-galactosidase activity was also present in the apical ectodermal ridge of developing limb buds (Figure 4e and f). This is consistent with the recent demonstration that ectodermal Wnt3/β-catenin signaling is required for both the establishment and maintenance of this structure (Barrow et al., 2003).

To further test whether the *Tcf/Lef-lacZ* transgenic line reflects endogenous Wnt signaling, I placed the transgene onto a homozygous *vestigial tail* (*vt*) mutant background, which is hypomorphic allele of *Wnt3a* (Greco et al., 1996). *Wnt3a* is normally expressed in the dorsal region of the neural tube of day E9.0 embryos (Takada et al., 1994). Thus, if the transgene responds to Wnt signaling, reduced β-galactosidase activity on the *vt*

background would be expected in the dorsal region of the neural tube, but not in more anterior regions of the embryo where other Wnt family members other than *Wnt3a* are expressed. As shown in Figure 4g, significantly less β-galactosidase activity was detectable in the dorsal neural tube of day E9.0 *vt/vt* embryos as compared to +/*vt*. In more anterior regions, where *Wnt3a* is not expressed, β-galactosidase activity was equal between embryos of the two genotypes. Taken together, these results demonstrate that the *Tcf/Lef-lacZ* transgene is expressed when the Wnt/ β-catenin signaling pathway is activated.

Based on these results, I then stained pre-implantation and post-implantation transgenic embryos for β-galactosidase activity. No activity was detected at any pre-implantation stage (data not shown), consistent with the absence of detectable non-phosphorylated β-catenin in these embryos. β-galactosidase activity was first detected at day E5.5, where it was restricted to the extra-embryonic visceral endoderm cells (Figure 5a and data not shown). This activity was transient and at day E6.0 had been lost from these cells (Figure 5b). At this stage of embryogenesis, however, β-galactosidase activity was detected in a subset of cells located within the embryo proper near the embryonic-extraembryonic junction. Moreover, the intensity of staining became considerably stronger as development progressed to day E6.25, suggesting that more cells were expressing the transgene (Figure 5c).

To identify the cells expressing lacZ, E6.25 day embryos were stained for β-galactosidase activity then paraffin-embedded and sectioned. As shown in Figure 5d, β-galactosidase activity was present in a portion of the epiblast. Importantly, the morphology of these cells indicates that they were ectodermal (epithelial) rather than mesenchymal in nature, and

the absence of mesenchymal cells confirms that gastrulation had not yet begun. Unexpectedly, no β-galactosidase activity was detected in the overlying visceral endoderm although non-phosphorylated β-catenin was present in these cells (Fig 2f and g).

β-galactosidase activity remained present in the posterior epiblast at day E6.5 (Figure 5e) and no activity was detected in the posterior visceral endoderm at this stage (Figure 5d). By day E6.75 the domain of β-galactosidase in the epiblast began to expand towards the distal tip of the embryo (Figure 5f). Between days E7.5 and E7.75, which correspond to the headfold stage, β-galactosidase activity was restricted to the region of the primitive streak and node (Figure 5g and h). Sectioning of headfold stage embryos demonstrated staining in the posterior embryonic ectoderm, the nascent mesoderm and mesodermal wings, as well as the posterior visceral endoderm (Figure 5i).

Thus, prior to morphological manifestation of the primitive streak, activity of a β-catenin-responsive transgene becomes detectable first in extraembryonic visceral endoderm and subsequently in a subset of epiblast cells near the border with the extra-embryonic tissue. When the primitive streak is present, β-catenin/Tcf transcriptional activity is found in cells of this structure as well as the overlying visceral endoderm.

DISCUSSION

Previous work using targeted gene deletion established an essential role for β-catenin in the formation of the primitive streak and embryonic mesoderm as well as specification of the anterior visceral endoderm during early post-implantation embryogenesis in the mouse. However, this approach could not identify the specific cell types in which β-catenin activity was required. Moreover, as β-catenin has an extracellular function in mediating cell adhesion in addition to its nuclear function in regulating transcription from Tcf/Lef-binding sites, the nature of the β-catenin requirement was not established. To identify when and where β-catenin exerts its nuclear function during this period of embryogenesis I mapped the distribution of the non- phosphorylated isoform and the activity of a β-catenin-regulated transgene in pre- and early post-implantation embryos. I found that neither non-phosphorylated β-catenin nor β-catenin signaling through Tcf/Lef were detectable in pre-implantation stage embryos. Near the time of primitive streak formation, I identified two regions of β-*catenin* activity: the extraembryonic visceral endoderm and the future posterior portion of the embryonic ectoderm.

Non-phosphorylated β-catenin was first detected in the extraembryonic visceral endoderm in day E5.5 embryos. Moreover, β-catenin transcriptional activity was also detected in this tissue at day E5.5. This provides the first evidence that β-catenin signaling is active in the extraembryonic visceral endoderm of early post-implantation embryos. Studies using chimeric embryos have suggested that β-catenin may not be required in the extraembryonic tissues during early post-implantation development (Huelsken et al.,

2000). However, a detailed marker analysis of these chimeric embryos has not been done to determine if β-catenin signaling in the extraembryonic tissues is required for early embryonic development. It is also possible that genes activated through β-catenin at day E5.5 are required in later development or that other gene products can compensate for the absence of β-catenin. My results also indicate that β-catenin signaling is only transiently activated in the extraembryonic visceral endoderm. By day E6.0, although non-phosphorylated β-catenin was still present, β-galactosidase activity was no longer detectable. Transient expression may be due to the loss of other factors required for β-catenin-regulated gene expression. Indeed many studies have demonstrated that the Wnt signaling pathway is subject to numerous levels of regulatory control, both at the extracellular and intracellular levels (Brantjes, 2002; Yamaguchi, 2001).

Non-phosphorylated β-catenin became asymmetrically distributed within the epiblast at day E6.25, prior to the formation of the primitive streak. At this stage, non-phosphorylated β-catenin was detected in a small group of epiblast cells located adjacent to the embryonic-extraembryonic junction. As development proceeded, the presence of non-phosphorylated β-catenin in the epiblast expanded both laterally and along the proximal-distal axis in a manner similar to primitive streak elongation. Consistent with the presence of non-phosphorylated β-catenin in the epiblast, β-catenin transcriptional activity was detected in a small group of epiblast cells prior to the formation of the primitive streak. At day E6.5 and onwards, β-catenin transcriptional activity was detected in the primitive streak and mature node. These results demonstrate that β-catenin signaling precedes primitive streak formation and is present in epiblast cells that will go on to form the primitive streak.

The factor most likely responsible for activating β-catenin signaling in the epiblast is Wnt3. *Wnt3* is expressed before primitive streak formation in the proximal epiblast adjacent to the embryonic-extraembryonic junction, and at the time of gastrulation in the posterior epiblast and associated visceral endoderm (Liu et al., 1999a). Thus, in the epiblast there is a strong temporal and spatial correlation between *Wnt3* expression and β-catenin signaling activity. Moreover, Wnt3 is known to signal through β-catenin (Shimizu et al., 1997) and targeted deletion of *Wnt3* results in embryos that fail to form a primitive streak, similar to *β-catenin* mutant embryos (Liu et al., 1999a). However, despite the expression of *Wnt3* in the posterior visceral endoderm, nuclear β-catenin signaling is not detected in these cells at this time. This may be due to the absence of functional Wnt3 protein in the visceral endoderm cells or to the presence of signaling inhibitors in this region. Although it is possible that the transgene is unable to report Wnt/β-catenin signaling in the visceral endoderm, this possibility seems unlikely in view of the fact that activity of the transgene is detected in the extraembryonic visceral endoderm cells at earlier stage as well as in the posterior visceral endoderm at the late primitive streak stage. Although *Wnt3* is initially expressed in the proximal epiblast, I have neither detected accumulation of non-phosphorylated β-catenin or β-catenin signaling in this region of the embryo. These results suggest that although *Wnt3* is initially expressed in this region it does not activate the Wnt/β-catenin signaling pathway. Since we have detected both the accumulation of non-phosphorylated β-catenin and β-catenin signaling only in posterior epibalst cells prior to the initiation of gastrulation, our results suggest that Wnt3 signaling is only active in this region of the embryo.

β-catenin has been shown to be required at the time of axis formation for correct positioning of the anterior visceral endoderm. Although some anterior visceral endoderm genes are expressed in embryos lacking *β-catenin*, the expressing cells remain at the distal tip of the embryo and fail to migrate to the future anterior region of the embryo (Huelsken et al., 2000). However, in *Wnt3* mutant embryos, the anterior visceral endoderm is correctly positioned suggesting that β-catenin signaling is either required in a domain other than the domain of *Wnt3* expression or that this process does not require β-catenin signaling but rather the cell adhesion function of β-catenin. I have identified two domains where non-phosphorylated β-catenin accumulates at the time of axis specification. The first is in the extraembryonic visceral endoderm and the second in the posterior visceral endoderm. Whether β-catenin is required in these domains for correct positioning of the anterior visceral endoderm still needs to be determined.

I found that β-catenin transcriptional activity was maintained throughout the primitive streak at later stages of embryonic development. Several *Wnt* family members such as *Wnt3a, Wnt2b and Wnt5a* are expressed in the primitive streak (Gavin et al., 1990; Takada et al., 1994; Zakin et al., 1998) and may contribute to β-catenin signaling in the primitive streak. Consistent with the β-catenin/Tcf signaling activity we observed in the primitive streak, Tcf/Lef binding sites have been identified in the promoter region of at least two genes that are expressed in the primitive streak, *Brachyury* and *Cdx1* (Arnold et al., 2000; Lickert and Kemler, 2002; Prinos et al., 2001). In addition, both of these genes appear to be downstream target of Wnt3a (Prinos et al., 2001; Yamaguchi et al., 1999). I detected no signaling through Tcf/Lef in anterior structures until the late headfold stage.

Inhibition of Wnt signaling in the future anterior side of the embryo is thought to be important for proper axis formation (Beddington and Robertson, 1999). Expression of Wnt inhibitors such as Dickkopf-1 (Dkk1) (Glinka et al., 1998; Mukhopadhyay et al., 2001) and secreted Frizzled-related protein 5 (sFRP5) (Finley et al., 2003) in the anterior visceral endoderm cells are thought to protect adjacent ectoderm from caudalizing or mesoderm-inducing influences (Beddington and Robertson, 1999).

In this study, I focused my analysis on the domains of β-catenin/Tcf transcriptional activity during the pre- and early post-implantation stages of mouse embryos. Although I have not performed a complete analysis of this pathway at later stages of development, I have found that this pathway is active in multiple sites within the embryo that correlate with domains where Wnt signaling is known to be required (O.A.M. et al, unpublished). This *Tcf/Lef-lacZ* transgenic line will be useful in identifying cells where the Wnt/β-catenin signaling pathway is active during later embryonic development and organogenesis as well as in adult mice.

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Figure 1: Distribution of total and non-phosphorylated β-catenin in preimplantation stage embryos

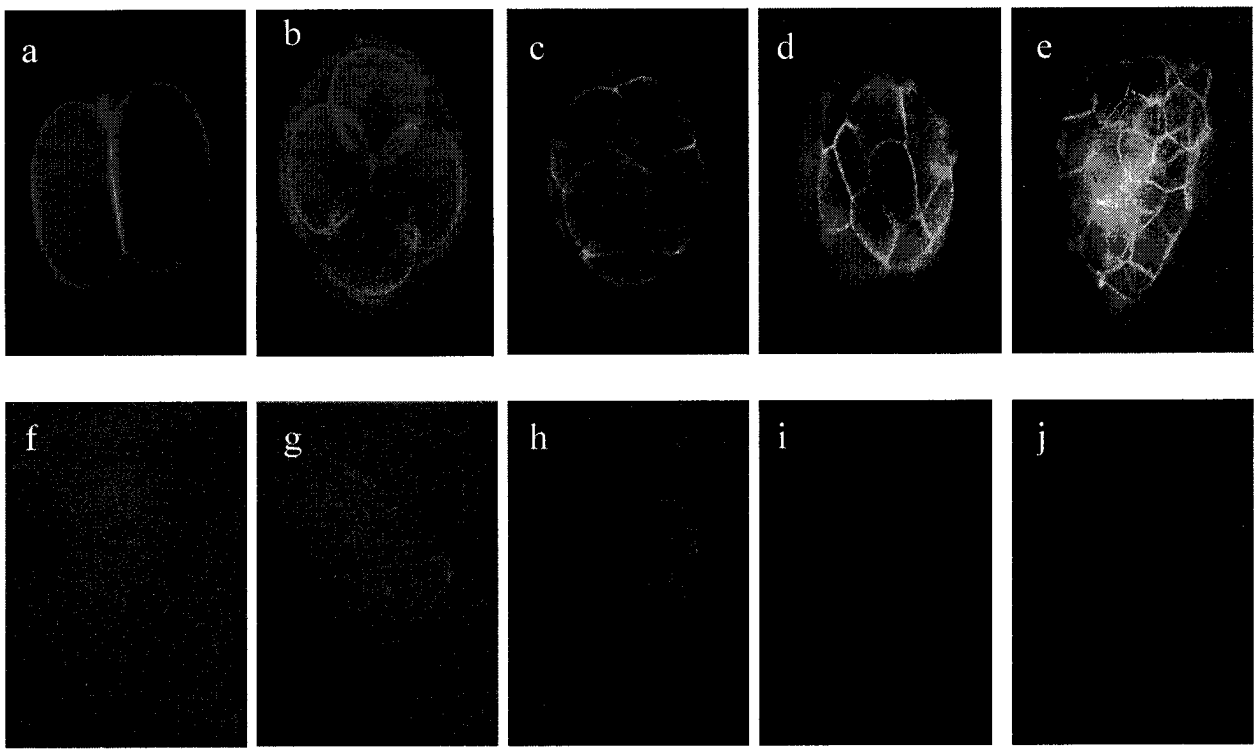

Figure 1 Legend

Immunohistochemistry was performed using either an antibody that detects all forms of β-catenin (a-e) or an antibody that recognizes only the non-phosphorylated form of β-catenin (f-j). Immunohistochemistry was performed on 2-cell stage (a and f), 4-cell stage (b and g), 8-cell stage (c and h), morula stage (d and i) and blastocyst stage (e and j).

Figure 2: Distribution of total and non-phosphorylated β-catenin in postimplantation stage embryos

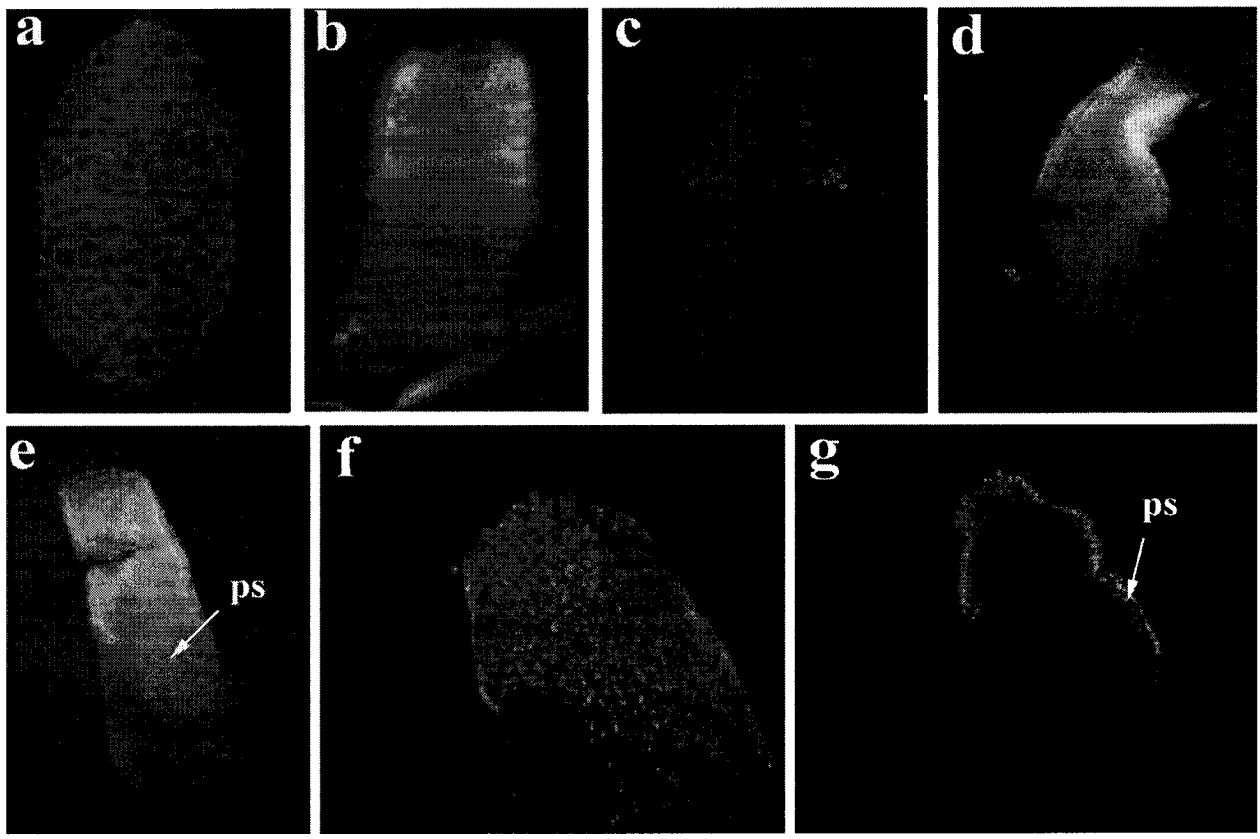

Figure 2 legend

(a, c) Immunohistochemistry using an antibody that detects all forms of β-catenin was performed on day E5.5 (a) and day E6.0 (c) embryos. (b, d, e) Immunohistochemistry using an antibody that specifically detects only non-phosphorylated β-catenin was performed on day E5.5 embryos (b), day E6.0 embryos (d) and day E6.75 embryos (e). (f,g) Confocal microscopy of day E6.25 embryos showing the surface of an embryo (f) or a longitudinal section through a different embryo (g). β-catenin is shown in green, DAPI-stained nuclei are shown in red.

Figure 3: β-catenin dependent activation of the endogenous *Tcf/Lef-lacZ* transgene in transgenic mouse embryonic fibroblasts

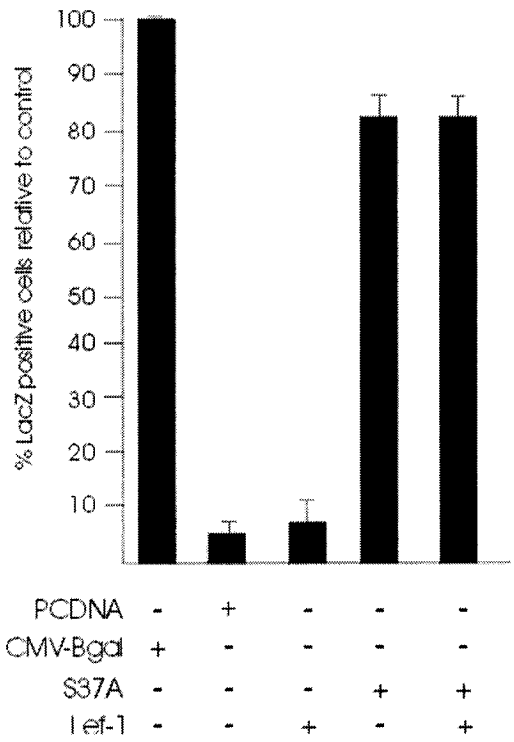

Figure 3 legend

Mouse embryonic fibroblast cells were isolated from hemizygous day E12.5 transgenic embryos and transfected using the indicated constructs. Transfections were performed in triplicate and the average value is indicated. The lacZ gene driven by the CMV promoter was used as a positive control whereas the empty pCDNA vector (mock transfection) was used as a negative control. (S37A) Constitutively active form of β-catenin cloned in pCDNA. (Lef-1) Lef-1 cDNA cloned in pCDNA.

Figure 4: Expression of the endogenous *Tcf/Lef-lacZ* transgene in day 8.25 to day 10.5 embryos

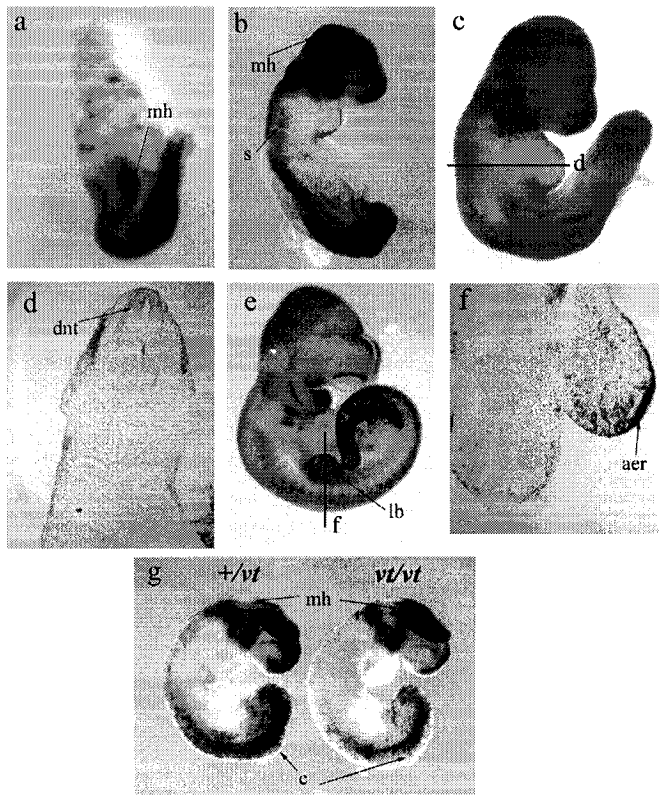

Figure 4 legend

Transgenic embryos were isolated at the indicated stages and stained for β-galactosidase activity. (a) day E8.25 (b) day E8.75 (c) day E9.5 (d) section through a day E9.5 embryo as indicated in c. (e) day E10.5 (f) section through the limb bud of a day E10.5 day embryo as indicated in e, showing staining in the apical ectodermal ridge (aer). (g) Day E9.0 heterozygous (+/vt) and homozygous(vt/vt) vestigial tail, a Wnt3a hypomorphic allele stained for lacZ activity. (mh) midbrain-hindbrain region, (s) somite, (dnt) dorsal neural tube, (c) caudal region.

Figure 5: Expression of the endogenous *Tcf/Lef-lacZ* transgene in gastrulation stage embryos

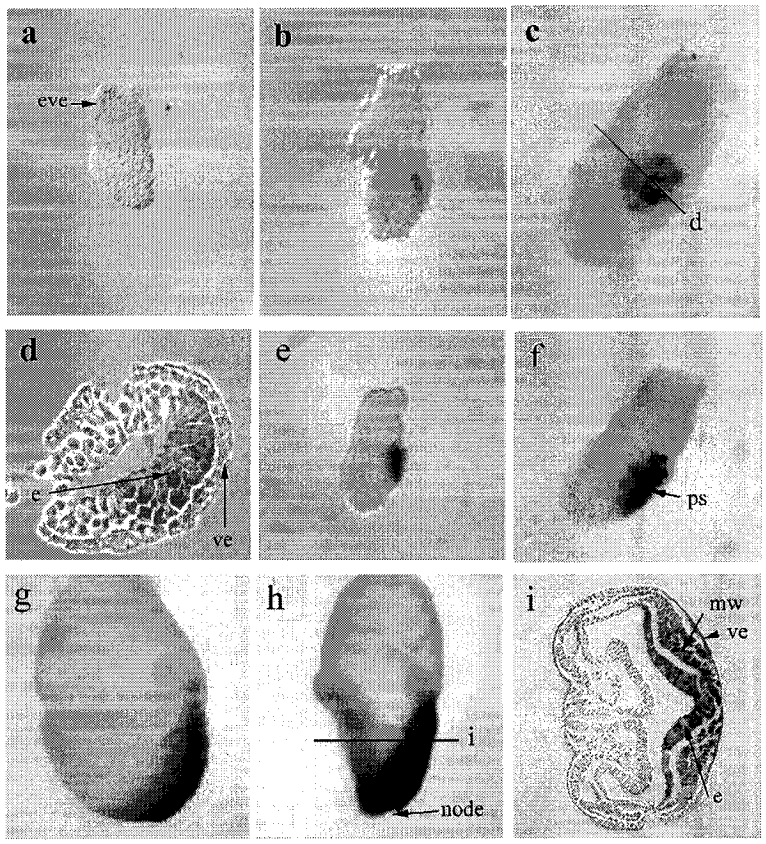

Figure 5 legend

Transgenic embryos were isolated at the indicated stages and stained for β-galactosidase activity. (a) day E5.5 (b) day E6.0 (c) day E6.25 (d) section through a day E6.25 embryo as illustrated in c, showing β-galactosidase activity in the epiblast. (e) day E6.5 (f) day E6.75 (g) day E7.5 (h) day E7.75 (i) Section though a day E7.75 embryo as illustrated in h. (eve) extra-embryonic visceral endoderm, (ve) visceral endoderm. (ps) primitive streak, (e) ectoderm, (mw) mesodermal wing.

CHAPTER 5: MANUSCRIPT IV

Siamois/Twin-like binding activity is present in mouse embryos and is specifically localized in the node

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PREFACE

In chapter 4 (manuscript III), I showed that signaling through β-catenin is active in the epiblast prior to gastrulation in the region where the primitive streak will form as well as in the node. In *Xenopus*, β-catenin activates the expression of the homeodomain transcription factors Siamois and Twin, which in turn activate organizer specific genes such as *Goosecoid*, *Cordin* and *Noggin*. Whether a similar pathway operates in mammalian embryos is not known. No *Siamois* or *Twin* homologs have been identified in mammalian genomes. The objective of this study was to investigate the potential existence of similar β-catenin/Siamois/Goosecoid pathway in mammalian embryos.

ABSTRACT

In *Xenopus* embryos, sperm entry initiates a cortical rotation that leads to the accumulation of β-catenin in the nuclei of the future dorsal side (opposite of sperm entry side). At the onset of embryonic genome activation, β-catenin activates the expression of homeodomain transcription factors Siamois and Twin, which in turn induce the formation of Spemann organizer at the dorsal equator of the embryo. The molecular cascade that leads to the specification of the mammalian organizer is not understood. Here, I examined the potential existence of a similar mechanism in mammalian embryos. Using a transgenic approach, I show that a promoter element (Siamois binding site) from the mouse *Goosecoid* promoter is able to drive reporter gene expression asymmetriacally in the blastocyst stage. At postimplantation stages, this element drives reporter gene expression specifically in the node. Furthermore, using the yeast-one hybrid screen, I identified a candiate factor (mWBSCR11, also known as General Transcription Factor II) as potentially regulating activity of this element in vivo. These results strongly suggest that a similar β-catenin/Siamois pathway exists in mammalian embryos and perhaps is involved in node specification.

INTRODUCTION

A central issue in developmental biology is to understand how the embryonic body plan is established. Different organisms employ different strategies to break the initial egg symmetry and provide cues needed to determine the embryonic body plan. In *Drosophila*, *C. elegans*, Zebrafish and *Xenopus*, the polarity of the embryo takes its roots from the spatial patterning of the egg (reviewed in (Lyczak et al., 2002; Moon and Kimelman, 1998; Riechmann and Ephrussi, 2001; Schier, 2001). The classical transplantation experiments of Spemann and Mangold (Spemann and Mangold, 1924) in *Xenopus laevis* embryos led to the discovery of the dorsal signalling center "the Spemann organizer" and introduced the concept of organizing centers for the establishment of embryonic body axes (reviewed in (Moon and Kimelman, 1998)). Since then, equivalent "organizing" centers have also been identified in other organisms; including "Hensen's node" in avian embryos, the "embryonic shield" in zebrafish embryos and the "node" in mammalian embryos, (reviewed in (Beddington and Robertson, 1999; Boettger et al., 2001; Solnica-Krezel, 1999).

The molecular mechanism leading to the specification of these organizing centers is still under investigation, but is best understood in *Xenopus* embryos. In *Xenopus*, induction of the Spemann organizer depends on the activity of an earlier signaling center termed the Nieuwkoop center (Nieuwkoop, 1973) that depends on maternal factors. Sperm entry induces a 30^0 microtubule-dependent, rotation of the cortical cytoplasm relative to the deep endoplasm (Gerhart et al., 1989; Vincent and Gerhart, 1987). Cortical rotation is

believed to result in the transport of dorsal determinants from the vegetal pole to the dorsal side of the embryo, opposite to the sperm entry side, establishing the Nieuwkoop center in the dorsal vegetal side, which then induces the Spemann organizer at the dorsal equator of the embryo (Kageura, 1997; Kikkawa et al., 1996; Rowning et al., 1997; Sakai, 1996).

Induction of the Spemann organizer is dependent on the cooperative activity of at least two signalling pathways, the TGF-β and Wnt pathways, in the Nieuwkoop center. These two pathways involve maternally encoded proteins, the T-box family transcription factor VegT, which is required to convert cells of the equatorial zone into mesoderm (Zhang et al., 1998) and β-catenin, which is required for axis formation (Heasman et al., 1994; Wylie et al., 1996). Depletion of maternal RNA encoding VegT or β-catenin, inhibits Spemann Organizer formation resulting in absence of mesoderm or failure to develop axial structures, respectively (Heasman et al., 1994; Zhang et al., 1998). Depletion of VegT diminishes the expression of members of the TGF-β super family, such as Vg1, activin and nodal, which were shown to regulate mesoderm formation (Kofron et al., 1999). β-catenin is the downstream effector of the canonical Wnt signalling pathway (reviewed in (Cadigan, 2002).

Convincing experimental evidence indicates that formation of Nieuwkoop center depends on activation of the Wnt pathway in the dorsal vegetal region of the embryo before the mid-blastula transition. Ventral injection of mRNA encoding several Wnt family members or molecules acting along the Wnt pathway, including β-catenin, results in the formation of ectopic Nieuwkoop center and complete secondary axis (reviewed in (Moon and Kimelman, 1998). β-catenin accumulates in the nuclei of cells in the Nieuwkoop

center, where it interacts with members of HMG box transcription factors of the Tcf/Lef class and activates the transcription of target genes when the zygotic genome activation takes place after the mid-blastula transition (Larabell et al., 1997; Schneider et al., 1996; Yost et al., 1996).

Among the earliest embryonic genes to be transcribed in the Nieuwkoop center are the homeobox transcription factor *Siamois* (Brannon et al., 1997) and its closely related *Twin* (Laurent et al., 1997). Like the case of *β-catenin*, ectopic expression of *Siamois* or *Twin* in the ventral side of an early *Xenopus* embryo results in the formation of a complete secondary axis (Carnac et al., 1996; Funayama et al., 1995; Laurent et al., 1997; Lemaire et al., 1995). Siamois and Twin, in cooperation with TGF-β family members, activate organizer specific genes such as *Goosecoid* (Brannon and Kimelman, 1996; Fan and Sokol, 1997; Laurent et al., 1997).

Goosecoid is a homeobox gene and was the first organizer-specific gene to be discovered (Blum et al., 1992; Cho et al., 1991; Izpisua-Belmonte et al., 1993). Ectopic expression of *Goosecoid* in the ventral side of early *Xenopus* embryos leads to the formation of an almost complete secondary axis (Cho et al., 1991). Analysis of the *Goosecoid* promoter has identified two cis-acting elements, a distal element (DE) that is required for activin/nodal induction (TGF-β responsive element) and a proximal element (PE) that is required for Wnt induction (Wnt responsive element) (Watabe et al., 1995). XWBSCR11 (also known as general transcription factor II) binds to the DE and interacts with FoxH1 (downstream effectors of TGF-β signaling pathway) influencing the transcription of DE-mediated activin/nodal signaling (Ring et al., 2002). Twin, on the other hand, activates *Goosecoid* by binding to the Wnt responsive element (PE) (Laurent et al., 1997).

Zebrafish embryos utilize a similar mechanism, in which, as in frogs, cortical arrays of microtubules facilitate the cytoplasmic rearrangements required for the formation of the Yolk Syncytial Layer (YSL), the equivalent of Nieuwkoop center, in the dorsal side (Jesuthasan and Stahle, 1997). Transplantation of YSL to the animal pole region induces and patterns mesoderm (Ober and Schulte-Merker, 1999). Similarly, nuclear β-catenin accumulates in the dorsal side of zebrafish embryo and is first observed in the YSL (Schneider et al., 1996) and ectopic expression of β-catenin leads to the induction of a secondary axis (Kelly et al., 1995). In addition, the zebrafish homologue of Siamois, the homeodomain transcription factor Dharma/Nieuwkoid (encoded by *Bozozok*) is a downstream target of β-catenin/Tcf pathway in the YSL (Fekany et al., 1999; Yamanaka et al., 1998). Mutations of *Bozozok* leads to defects in embryonic body axis that are characterized by a ventralized phenotype (Solnica-Krezel et al., 1996). Furthermore, Dharma/Nieuwkoid and the TGF-β member, Nodal-related Znr2/Ndr1, act non-cell autonomously in the YSL to induce the Organizer marker gene *Goosecoid* (Erter et al., 1998; Koos and Ho, 1998; Yamanaka et al., 1998).

In mammals, the first morphological sign of axis formation is evident only at postimplantation stages when gastrulation begins. Although recent studies suggest that cues for axis determination can be traced back to preimplantation stages (Gardner et al., 1992; Scott, 2000; Zernicka-Goetz, 2002), no molecular evidence is available yet in support of this notion. Whether a similar β-catenin/Siamois pathway exists in mammalian embryos is not clear yet. Strong evidence indicates that TGF-β and Wnt signaling pathways are essential for the specification of mammalian node. Mice embryos lacking members of TGF-β signaling pathway, such as *Foxa2*, *FAST*, *FoxH1* or *Arkadia*, show

defects in the formation of mid-gastrula organizer and consequently lack nodes (Ang et al., 1993; Episkopou et al., 2001; Hoodless et al., 2001; Yamamoto et al., 2001).

The Wnt/β-catenin signaling pathway also plays an essential role in node formation and consequently axis formation of mouse embryos. Transgenic overexpression of *Wnt8c* leads to partial axis duplication (Popperl et al., 1997). Mutations in the negative regulators of this pathway, such as *Axin*, *APC*, the *LIM* domain-binding protein 1 or *Tcf3*, all result in axis duplication and formation of ectopic nodes (Ishikawa et al., 2003; Merrill et al., 2004; Mukhopadhyay et al., 2003; Zeng et al., 1997). Targeted deletions of *Wnt3* or *β-catenin* result in embryonic lethality and mutant embryos fail to form mesoderm or establish proper body axes (Haegel et al., 1995; Huelsken et al., 2000; Liu et al., 1999a). The role of downstream targets of Wnt/β-catenin in specification of mouse node, however, is not known. So far, no mammalian homologues of *Siamois* or other equivalent factors have been identified in the mouse. Surprisingly, deletion of the mouse *Goosecoid* gene showed no overt effects on gastrulation or axis formation, suggesting that other related genes are compensating its function in early mammalian embryos (Rivera-Perez et al., 1995; Yamada et al., 1995).

In this study I investigated whether a Siamois/Twin like activity exists in mouse embryos. We used the Wnt responsive element (WRE), known as the proximal element, from the mouse *Goosecoid* promoter, which was shown to have a high homology to the *Xenopus* proximal element and to activate a reporter gene in the animal cap of *Xenopus* embryos (Watabe et al., 1995). Using a transgenic approach, I first show that a reporter gene driven by 2 copies of this element is asymmetrically expressed at the blastocyst stage providing the earliest molecular evidence of break of symmetry reported in mammalian embryos.

Second, I show that, at early postimplantation stages, this reporter gene is specifically expressed in the node. Mutating the Twin-binding site in this element abolishes both, asymmetrical expression in the blastocyst and node expression. Furthermore, using yeast-one hybrid screen, I identified the mouse WBSCR11 (mGTF-II), as a potential regulator of this element in vivo.

METHODS

Electrophoretic Mobility Shift Assay (EMSA)

Xenopus Siamois cDNA was cloned in expression vector pTriEx-2 following standard cloning procedure to generate Sia-TriEx3 plasmid. This plasmid was used to transfect embryonic fibroblast cells that were isolated from E12.5-day embryos following standard protocols. Transfections were carried out using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. 2-5 μg DNA of Trix-sia3 plasmid was used for each transfection reaction. Nuclear extracts were prepared as described by Andrews and Faller (Andrews and Faller, 1991). Briefly, transfected plates were rinsed twice with PBS and cells were then scraped into 1ml PBS. Cells were then harvested by centrifuging at 3,000 rpm for 5 minutes and resuspended in 400 μl (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, before use 0.5 μl of 1 M DTT, 2 μl of 100 mM PMSF and 10 μl of protease inhibitor cocktail were added per ml of buffer) and allowed to swell for 10 minutes on ice. Cells were then vortexed for 10 seconds, centrifuged at 1,000 rpm for 30 seconds and supernatant was removed carefully. Cells were then centrifuged again at 12,000 rpm for 5 minutes and supernatant was discarded. Nuclear pellet was then resuspended in 50 μl (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, before use 0.5 μl of 1 M DTT, 2 μl of 100 mM PMSF and 10 μl of protease inhibitor cocktail were added per ml of buffer).

EMSA was performed using 2 μl of nuclear extract incubated in 20 mM HEPES, pH 7.4, 80 mM KCl, 1 mM DTT, 0.1% NP-40, 10% glycerol in a final volume of 10 μl with 200

ng of poly dI-dC and 1-50 ng of specific competitor, as specified in Figure 1B. 40,000 c.p.m of end-labeled P3 probe (prepared as described below) were then added to each reaction. Tubes were then centrifuged briefly and incubated first on ice for 20 minutes and then at room temperature for 30 minutes. Samples were then loaded on a 5% polyacrylamide gel (30:1) and run at 100 volts for 3 hours in 50 mM Tris, 0.38 M glycine, and 1 mM EDTA, pH 8.5. Gels were dried and visualized by autoradiography.

P3 radiolabeled probe was prepared as follow: P3 Sense (5' AGCTTGATTAGGTTAATTTCATTAATTCTCAATCCACAA 3') and antisense (5' AGCTTTGTGGATTGAGAATTAATGAAATTAACCTAATCA 3) oligonucleotide sequences corresponding to the region that contains the highest homology between mouse and *Xenopus Goosecoid* promoters (P3 element underlined in Figure 1A) were labeled separately as follow. 1 µl of sense or antisense P3-oligo (200 ng/µl) was incubated for 1 hr at $37C^0$ in 20 µl solution containing 2 µl of 10 PNK buffer, 1 µl of T4 polynucleotide kinase, 1-3 µl of gamma-P32-ATP and 13-15 µl ddH2O. Sense and antisense labeled probes were then annealed together as follow: 20 µl of Sense labeled probe was mixed with the 20 µl of anti-sense labeled probe. 10 µl of 5X annealing buffer (100 µl of 1 M Tris, pH 8.0, 1 µl of 0.5 M EDTA, pH 8.0, 200 µl of 5M NaCl, 700 µl H2O) was then added and the solution was heated to $95C^0$ for 2-3 minutes and cooled slowly (over several hours) to below $45C^0$.

Mutant P3 element, used as cold specific competitor, was prepared as following. Twin-binding site (Figure 1A, Italic) within this sequence was mutated as indicated by (Laurent et al., 1997). mP3 Sense (5' AGCTTGATTAGGTTACGGCAGATCTTGAACAATCCACAA 3') and antisense (5'

AGCTTTGTGGATTGTTCAAGATCTGCCGTAACCTAATCA 3') were synthesized and annealed to each other as indicated above. 1-50 ng of cold P3 and mP3 were used as indicated in Figure 1.

Generation of reporter constructs and transgenic animals

To generate 2p3-lacZ and m2p3-lacZ reporter constructs, P3 & mP3 sense and antisense oligonucleotide sequences were synthesized as indicated above. HindIII sites were added 5' of each oligo and both oligos were then annealed to each other as described above. Annealed oligos were then self-ligated following standard protocols and inserted into HindIII site of plasmid 1-11 that contains the hsp68 minimum promoter driving a LacZ reporter gene (Rossant et al., 1991). Constructs were verified by sequencing and clones that contained 2 copies of P3 (2p3-lacZ) or two copies of mP3 (m2p3-lacZ) were used. Generation of transgenic animals, genotyping of transgenic animals and detection of β-galactosidase activity were performed as described in (Mohamed et al., 2004a).

Yeast-one hybrid screening

Yeast-one hybrid screening was performed using Clontech screening kit (Clontech Biosciences, 1020 East Meadow Circle Palo Alto, CA 94303-4230) following manufacturer's instructions. E9.5 mouse cDNA library (a generous gift from Dr. Featherstone, McGill Cancer Centre) was screened for transcription factors that bind to the P3 element. Plasmid construction, yeast and bacterial transformation and selection, DNA recovery, were carried out following standard protocols.

Two hundered initial yeast colonies were picked and were grown under stringent selection (increasing concentrations, 15, 30 and 45 mM of the specific competitor 3-AT).

Seventy-five clones survived the highest concentration, 45 mM of 3-AT. These clones were sequenced and their sequences were analyzed using the GeneBank. 13 clones that contained sequences that are relevant to this study (coding for transcription factors or DNA-binding proteins) were further subjected to a secondary screening using the LacZ screening procedure indicated in Clontech protocol. Clones chosen for secondary screen were the following: **(1)** (gene bank number 13569625), Mus musculus histone binding protein NASP (Nasp) gene, **(8)** (9966767), Mus musculus general transcription factor II, **(9)** (6754643), Mus musculus metastasis associated 1-like 1 (Mta111), **(10)** (23270978), Mus musculus, Similar to hypothetical protein FLJ10099, **(11)** (18044722), Mus musculus, ubiquitin B, **(14)** (25046986), Mus musculus cysteine rich protein 1 (Csrp1), **(19)** (27714924), Rattus norvegicus similar to hypothetical protein DKFZp434M1616.1, **(22)** (6671683), Mus musculus catenin beta (Catnb), **(34)** (17389250), Mus musculus, tuftelin-interacting protein, 39 kD, **(54)** (6754309), Mus musculus insulin-like growth factor 2 (Igf2), **(55)** (26340187), Mus musculus 7 days embryo whole body cDNA, RIKEN full-length enriched library, clone: C430014N20 product:hypothetical Ubiquitin-associated domain containing protein, **(59)** (18204152), Mus musculus, Similar to hypothetical protein, clone MGC:29235, **(63)** (27924101), Mus musculus, Similar to hypothetical protein FLJ25471.

Among those clones, clone number **8** (containing the DNA-binding domain of mouse general transcription factor II) gave the strongest activation of lacZ and thus was chosen for further investigations. This DNA-binding domain of GTF-II was subcloned in BlueScript vector (Invetrogen) to be used for the production of In Situ hybridization probe. cDNA coding for human general transcription factor II was generous gift from Dr.

Hoodless, U.B.C., BC Canada. The human GTF-II cDNA was subcloned in pTrix2 expression vector for the production of Tag labeled protein following standard protocols.

Embryo and Node cells collection

Embryos at different stages of preimplantation development were obtained as described (Clarke et al., 1992). Node cells were obtained by surgically dissecting the node region from E7.5 mouse embryos. Embryos or cells were lysed immediately in a 0.5-ml microfuge tube containing 100 µl of lysing buffer (Trizol, Invitrogen) and stored at -80°C until the time of RNA extraction

RNA extraction and cDNA synthesis

Tubes containing embryos or cells in Trizol were removed from the –80°C freezer and were subjected to RNA extraction and cDNA synthesis as described in (Mohamed et al., 2001).

PCR amplification

Primers used for mouse general transcription II amplification were (sense, 5' TCTGCAACAATGCCAAGGTG 3' and antisense, 5' TGGCGGCAGGAATATAGTGT 3'). Primers used for *HPRT* amplification were (sense, 5' GCTGGTGAAAAGGACCTCT 3' and antisense, 5' CACAGGACTAGAACACCTGC 3'). Amplification was carried out using cDNA from 5 embryo-equivalents in a buffer consisting of 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs, 60-100 pmoles of each primer and 2.5 units of *Taq* polymerase (Invitrogen) in a total volume of 50 µl. Each cycle consisted of 1 minute at 94°C, 1 minute at 58°C and 1 minute at 72°C. Thirty five cycles were run for each

reaction. To visualize the amplified products, 15 µl of each PCR reaction was run through a 2% (w/v) agarose gel containing 0.025% ethidium bromide.

RESULTS AND DISCUSSION

Xenopus Siamois binds to the proximal element (PE) of mouse Goosecoid promoter

In Xenopus, the Wnt/β-catenin signaling pathway, in cooperation with the TGF-β pathway, activates the organizer-specific gene, Goosecoid. Whether Goosecoid expression in mammalian embryos is regulated in a similar fashion is not known. Genebank search, however, revealed that human and mouse genomes do not contain any genes that are structurally similar to either Siamois or Twin. The pivotal role these proteins play in Xenopus axis formation and the conservation of the regulatory sequences of one of their target genes, however, suggest that a Siamois/Twin like activity perhaps exists in mouse embryos. To test this, we first investigated whether Xenopus Siamois protein is able to bind to the PE of mouse Goosecoid promoter in vitro. We synthesized an oligonucleotide sequence that corresponds to the region in the mouse PE that shares the most homology with the Xenopus PE, which we refer to as P3 (Figure 1A). This region contains the 14 bp Twin-binding site, in which 12 bp are absolutely conserved between Xenopus and mouse sequences (Figure 1A). As control, we synthesized a mutant version of this element (mP3), in which the Twin-binding site was altered by randomly rearranging the nucleotide sequence within this site.

As shown in Figure 1B, using an electrophoretic mobility shift assay (EMSA), *Xenopus* Siamois specifically binds to the mouse P3 element of the *Goosecoid* promoter. Mutating the Twin-binding site, however, abolished the ability of Siamois to bind to this element (Figure 1B and data not shown). These results indicate that Siamois is able to bind to

mouse *Goosecoid* PE, which prompted us to test whether this binding activity is also present in vivo.

Goosecoid promoter proximal element activates asymmetrical expression of a reporter gene at the blastocyst stage

In mice, the embryonic axes become evident only after one week of gestation when gastrulation begins. Experimental perturbations of the pre-implantation embryo, such as removal or re-positioning of blastomeres do not affect axis specification (reviewed in (Papaioannou, 1986). Due to the extensive developmental plasticity of the pre-implantation mouse embryo, it has long been assumed that the establishment of polarity, leading to axis specification, is generated only at or just prior to the initiation of gastrulation. Recently, there has been increasing evidence that polarity may be established earlier, during the pre-implantation stages of development (reviewed in (Zernicka-Goetz, 2002). To determine if polarity leading to axis specification is established at pre-implantation stages of development, asymmetrically localized activities need to be identified at these stages. Currently, no such asymmetrically distributed activities have been identified.

To investigate whether Siamois/Twin-like binding activity exists in mouse embryos, we cloned 2 copies of the Wnt responsive element (WRE) of the mouse Goosecoid promoter upstream of LacZ reporter gene (2P3-lacZ) and generated transgenic embryos using this construct. Transient expression experiments indicated that expression of the reporter gene is first detected at the blastocyst stage. Expression was detected asymmetrically at the blastocyst stage and was restricted to the trophectoderm cells (Figure 2, Table 1). The

observation that only 5 blastocysts expressed the reporter gene asymmetrically (out of 14 transgenic blastocysts generated) required further investigations. Since those transgenic blastocyst were produced by transient expression of the reporter gene, however, one explanation for the lack of clear asymmetrical expression in all embryos could be due to the time and developmental stage at which the reporter vector has integrated into the genome. This had led me to examine reporter activation in blastocysts that were produced from a stable transgenic line. Analysis of reporter gene expression in blastocysts collected from stable 2P3-lacZ transgenic lines showed asymmetrical expression that was similar to that observed in the transient expression experiments albeit at a lower level (data not shown).

The earliest molecular asymmetry detected in mouse embryos thus far is the asymmetrical expression of Hex gene in the anterior visceral endoderm cells at E5.5 (Thomas et al., 1998). My results provide the first identification of an asymmetrically distributed transcriptional activity in the preimplantation stages of mammalian embryos.

P3 proximal element of the mouse *Goosecoid* promoter activates node-specific expression of a reporter gene

In *Xenopus* embryos, *Goosecoid* is expressed in the dorsal marginal zone above the dorsal lip, which becomes known as the organizer (Cho et al., 1991). In mouse embryos, *Goosecoid* is transiently expressed in the anterior end of the developing primitive streak, in a region known as the mid-gastrula organizer (Blum et al., 1992). This region contains progenitors of the mouse organizer "the node" and its derivatives, the notochord and floor plate, and expresses genes, such as *Foxa2*, which are essential for node specification (Ang

et al., 1993; Kinder et al., 2001; Vincent et al., 2003). The role of Goosecoid in organizer specification in *Xenopus* is well established (Cho et al., 1991). Although the function of Goosecoid in node specification in the mouse is dispensable (Rivera-Perez et al., 1995; Yamada et al., 1995), transplantation of *Goosecoid* mutant nodes into chick embryos indicate that in the absence of *Goosecoid*, the neural-inducing ability of the mouse node is severely impaired (Zhu et al., 1999).

In *Xenopus*, Siamois/Twin activate organizer specific genes (Fan and Sokol, 1997; Laurent et al., 1997; Lemaire et al., 1995) and the mouse node is considered to be the equivalent of *Xenopus* organizer (Beddington, 1994). Based on this, I reasoned that if a Siamois/Twin-like activity is present in mouse embryos and plays a role in node specification, one would expect this activity to be present in the node. To test this, I analyzed the expression of the reporter gene at postimplantation stages. In 8 independent 2p3-lacZ transgenic animals, 6 showed the same highly temporally and spatially restricted pattern of LacZ activity (Table 2). At day E7.75, activity was detected specifically in the node of transgenic embryos (Figure 3a- c). No expression was detected in the derivative of the node, the axial mesendoderm (Figure 3b). Expression was also detected in the extra-embryonic mesoderm (Figure 3a and b), and weak expression could be seen in a punctuate pattern in the visceral endoderm. By E8.0, node expression was no longer detected (data not shown). At E9.5, expression of the reporter gene was detected in the hindbrain, the ventral aspect of the neural tube, progenitors of the liver, and the distal tip of the tail (Figure 4a). Two stripes of expressing cells could be seen in the neural tube (Figure 4b). Transverse sections within the neural tube showed that the transgene was expressed within or close to the region where motor neurons will form (figure 4c).

Thus, a regulatory sequence from the *Goosecoid* promoter that was shown in *Xenopus* to mediate Siamois/Twin activation of the *Goosecoid* gene and to activate a reporter gene in the organizer (Laurent et al., 1997; Watabe et al., 1995) is also able to activate a reporter gene in mouse node.

In *Xenopus*, mutations have been defined that abolish the ability of Twin to bind to the PE (Laurent et al., 1997). As indicated above, these mutations also abolished the ability of Siamois to bind to this element in the mouse PE (Figure 1b). To test whether introduction of these mutations would affect the ability of the PE (mPE) to drive node-specific reporter gene expression in the mouse, six independent transgenic lines were generated carrying two copies of the mPE driving lacZ reporter gene. No node-specific expression was detected in any of these transgenic lines, while weak staining could be seen in the extra-embryonic mesoderm (data not shown). Thus, mutations that abolish the ability of Twin and Siamois to bind to the PE also prevented this element from activating node-specific expression of a reporter gene in mouse embryos. Together, these results strongly suggest that a Siamois/Twin like activity is present in mouse embryos and is located in the node during gastrulation.

mGTF-II (mWBSCR11) potentially activates the PE of *Goosecoid* promoter in mouse embryos

Since no *Siamois/Twin* homologs is present in mouse genome, we wished to identify factors that potentially compensate their function in regulating genes that contain their highly conserved binding site. I employed the yeast one-hybrid system using the proximal element (PE) from the mouse *Goosecoid* promoter as a bait and E9.5 day mouse cDNA

library. Using this system, I identified mouse general transcription factor II (mGTF-II), also known as (mWBSCR11), as an endogenous factor that potentially regulates activation through this element. Interestingly, in X*enopus* embryos this factor (XWBSCR11) was identified, using the yeast one-hybrid system, to bind to the distal element of the *Goosecoid* promoter and to regulate activation through the proximal element (Ring et al., 2002). Using RT-PCR analysis, I found that mGTF-II was expressed in the preimplantation stages and in node cells of mouse embryos (Figure 5).

Since mouse genome lacks structural homologs of *Siamois/Twin* and based on my results that identified Siamois/Twin-like binding activity in mouse embryos, I suggest that mGTF-II is compensating for the Siamois/Twin function. Further functional studies for mGTF-II, such as gene knockout experiments, will reveal whether the function of this factor is needed for node specification and establishment of mammalian body axes.

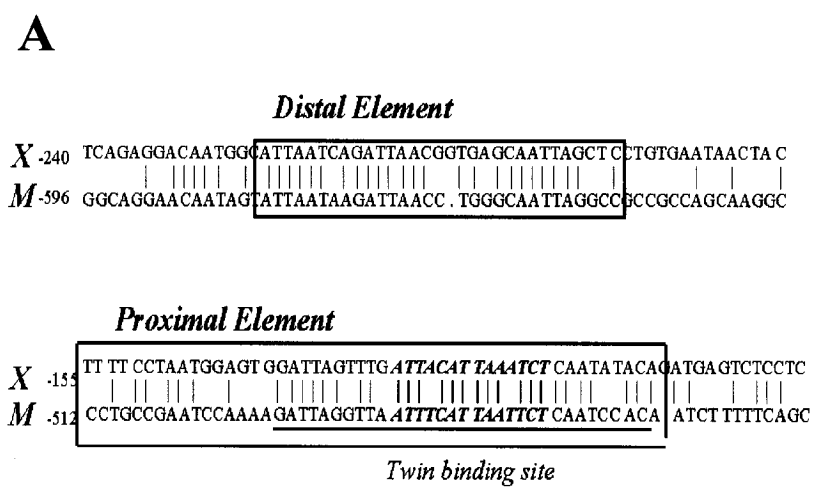
Table 1: Expression of 2p3-lacZ reporter gene at the blastocyst stage embryos

# of embryos microinjected	# of embryos reached the blastocyst stage	# of blastocyst stage embryos that are transgenic	# of transgenic blastocysts that express the transgene asymmetrically
360	74	14	5

Table 2: Expression of *2p3-lacZ* and *m2p3-lacZ* reporter genes at E7.5

Transgenic line	# of transgenic lines generated	# of transgenic lines showed node specific expression
2p3-lacZ	8	6
m2p3	6	0

Figure 1: *Xenopus* Siamois protein binds to the mouse *Goosecoid* P3 promoter element



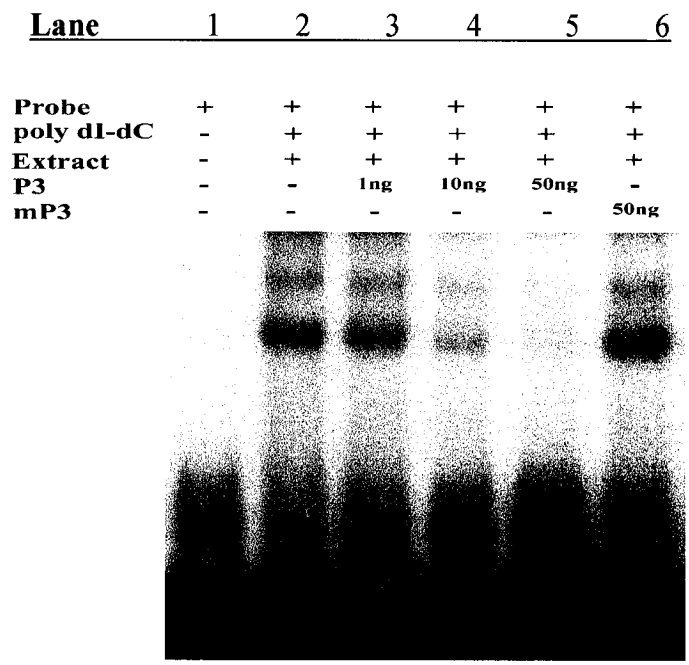


Figure 1 legend

A: Sequence comparison of mouse and *Xenopus Goosecoid* promoters. Boxed sequences include the distal and proximal elements identified by (Watabe et al., 1995). Underlined sequence in the proximal element is what we refer to as the P3 element. Sequence highlighted in Italic is the Twin-binding site identified by (Laurent et al., 1997).

B: Electrophoretic Mobility Shift Assay (EMSA) using extracts prepared from mouse embryonic fibroblast cells transfected with a Xenopus Siamois expression vector, and labeled P3 element as a probe. Where indicated, 200 ng of non-specific competitor, poly dI-dC, was added to the reaction. Specific competition was performed by adding unlabeled P3 or mutant P3 (mP3) as indicated. Arrow points to the shifted band.

Figure 2: Asymmetrical expression of the *2p3-LacZ* reporter construct in the blastocyst stage embryo

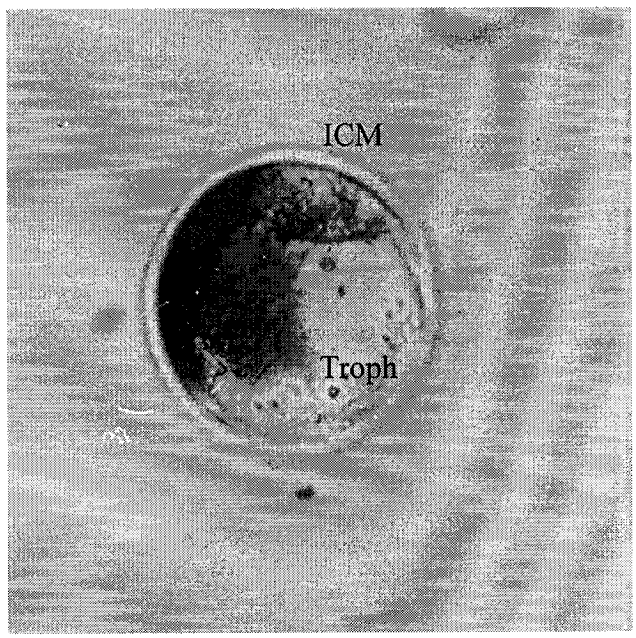


Figure 2 Legend

Blastocyst stage embryo stained for β-galactosidase activity. This embryo was microinjected with 2p3-lacZ plasmid in the male pronucleus at the 1-cell stage.

Abbreviations (ICM = Inner Cell Mass, Troph. = Trophectoderm)

Figure 3: Expression of the *2p3-LacZ* reporter construct in E7.75 stage embryos

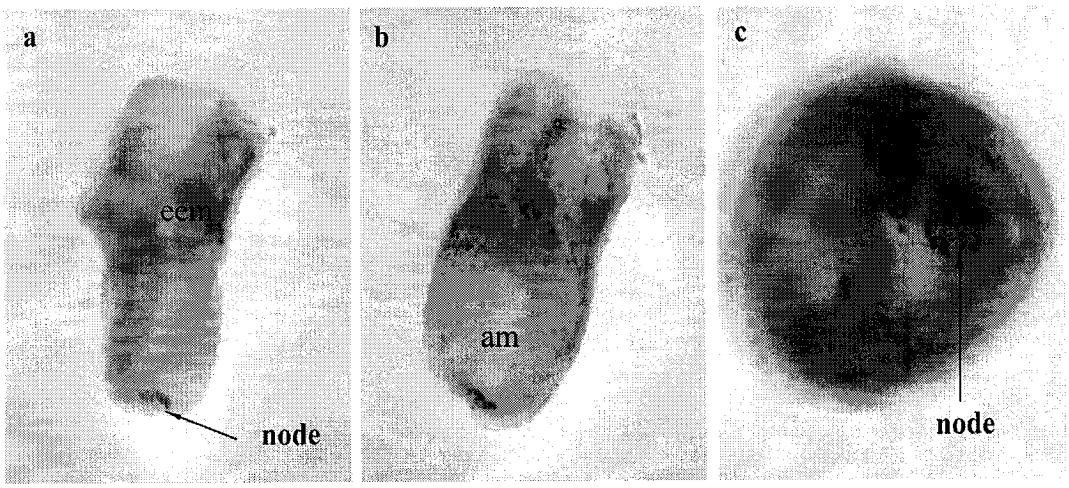


Figure 3 legend

a- Lateral view of a late headfold stage embryo showing staining in the node and extraembryonic mesoderm.

b- Anterior view showing staining in the node but no staining in the axial mesendoderm.

c- View of the distal tip of the embryo showing specific staining in the node.

Abbreviations (eem = extraembryonic mesoderm, am = axial mesendoderm)

Figure 4: Expression of the *2p3-LacZ* reporter construct in E9.5 stage embryos

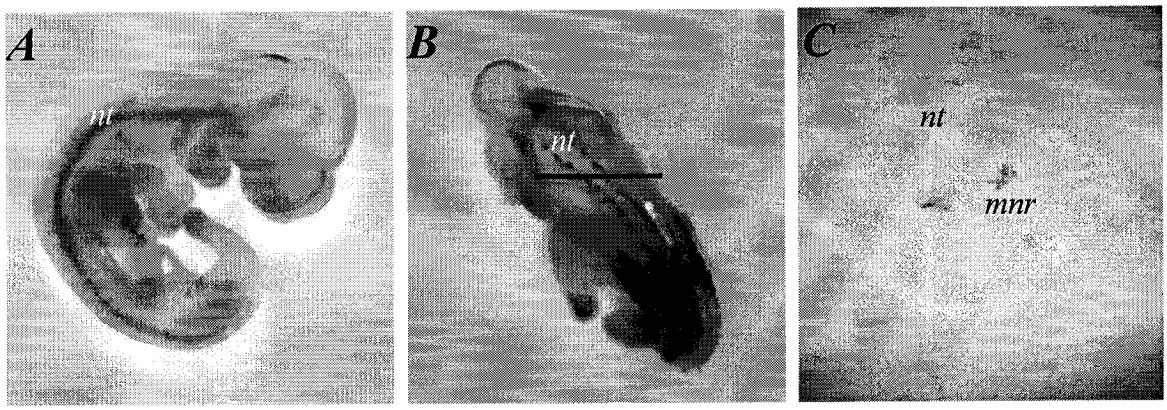


Figure 4 legend

A- Lateral view of the embryo. Note expression in the neural tube and tip of the tail.

B- Dorsal view of the embryo.

C- Cross-section showing expression of this element in the region where motor neurons will form adjacent to the neural tube.

Abbreviations (nt = neural tube, mnr = motor neurons region)

Figure 5: RT-PCR showing mouse *GTF-II* expression during pre-implantation stages and in cells of the node

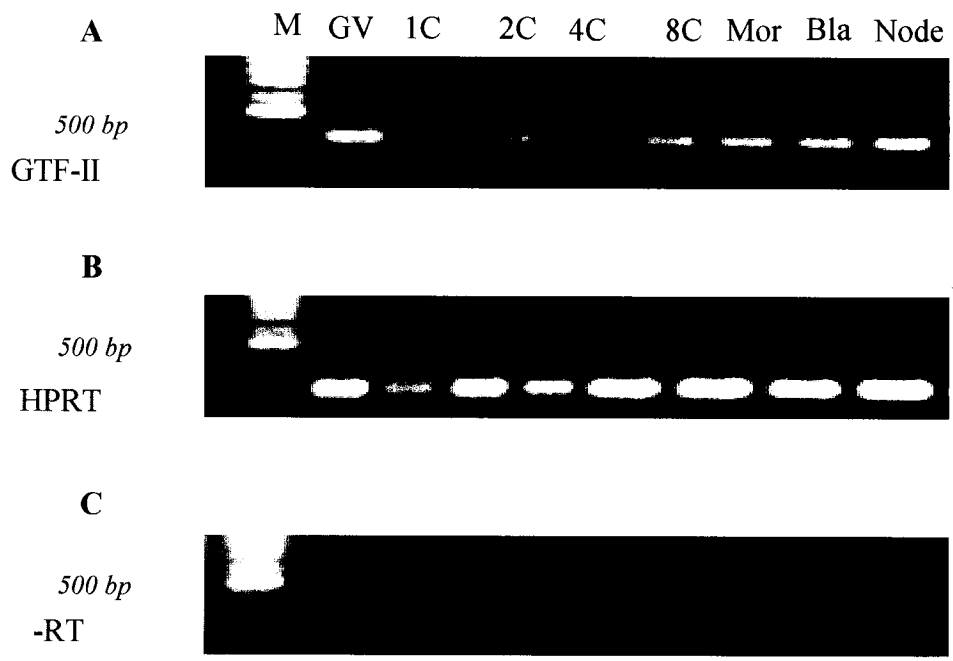


Figure 5 legend

30 embryos were collected from each stage and processed for RNA extraction. RNA equivalent of 10 embryos was then incubated with cDNA synthesis solution with out RT and the remaining RNA was incubated with cDNA solution containing RT. cDNA was then divided into two portions, one portion was used to amplify mGTF-II and the other portion was used to amplify *HPRT* mRNA. The RNA incubated with out RT was used to amplify *HPRT* mRNA. All PCR reactions were carried simultaneously.

A: Amplification of mouse GTF-II mRNA during the different stages of pre/post-implantation stages as indicated.

B: Amplification of PPRT mRNA during the same stages as a positive control.

C: No RT samples amplification with *HPRT* primers as a negative control.

Abbreviations (M = 100 bp DNA marker, GV = Germinal vesicle, MII = metaphase II arrested oocytes, 2C = 2 cell stage embryos, 4C = 4 cell stage embryos, 8C = 8 cell stage embryos, Mor. = Morula stage embryos, Bla. = Blastocyst stage embryo, Node = dissected node cells, GTF-II = general transcription II, HPRT = Hypoxanthine-guanine Phospho Ribosyl Transferase, RT = Reverse Transcriptase).

CHAPTER 6: GENERAL DISCUSSION

During embryonic development, the embryo progresses from a single cell to a multicellular organism that contains, in some species, billions of specialized cells that execute diverse functions to maintain the organism's life. This embryonic journey is a complex one that involves the action of many regulatory molecules that form a communication network to coordinate and successfully accomplish this mission. Communication between cells is carried out through signaling molecules. A variety of signaling proteins and pathways have been identified thus far and many more certainly wait to be discovered. Wnt proteins are major signaling proteins that play decisive roles during development. What we know about their functions so far perhaps is only the tip of the iceberg. In this thesis, I examined the involvement of Wnt signaling in two major developmental processes, namely embryo implantation and formation of embryonic body axes.

6.1: Involvement of Wnt signaling in the implantation process

Embryo implantation is a complex process that requires synchronized development of implantation-competent blastocyst and receptive uterus. Development of the embryo from a fertilized egg to a competent blastocyst that is capable of implanting in the mother's uterus is a challenging task the newly formed embryo has to accomplish in order to target the mother uterine implantation window and implant successfully. Blastocyst formation requires several preimplantation events to take place properly. One of these is the establishment of specific cell types such as the inner cell mass and the trophectoderm cell layer. Trophectoderm cells, which constitute an epithelial monolayer, comprise the portion of blastocyst that initiates uterine contact and invasion and gives rise to the

extraembryonic tissues (Carson et al., 2000). The inner cell mass is the part of the blastocyst that will give rise to the embryonic tissues.

Establishing specialized compartments within the embryo requires communication between its blastomeres in order to assign their fates. Preimplantation embryos produce many growth factors that may regulate their development (Diaz-Cueto and Gerton, 2001; Paria and Dey, 1990). The role of other signaling molecules, such as Wnts, in blastocyst formation has not been examined thus far.

In chapter 2 of this thesis, I show that genes for several members of the Wnt family of signaling proteins (namely Wnt3, Wnt4, Wnt5b, Wnt6, Wnt7b and Wnt10b) are expressed in the embryo at the morula stage. The time at which these genes are expressed suggest that they might be involved in blastocyst formation, a role that has not been explored for Wnts thus far. At the time of implantation, I show that the blastocyst express yet several other *Wnt* genes, some of which (*Wnt1, Wnt5a, Wnt5b and Wnt11*) are differentially expressed between blastocysts developed in vivo versus those developed in vitro. This differential gene expression between blastocyst developed in vivo versus those developed in vitro is a novel observation that sheds some light on gene regulation in the embryo. Furthermore, I show that *Wnt11* expression in the blastocyst is regulated by ovarian estrogen. This finding shows, for the first time, regulation of gene expression in the blastocyst by a maternal factor.

It is known that successful implantation requires communication between the implanting embryo and the receptive uterus (Paria et al., 2002). On one hand, uterine factors are required to render the blastocyst competent for implantation (Ma et al., 2003; Paria et al.,

1998). On the other hand, the blastocyst induces specific uterine gene expression, such as the HB-EGF, specifically at their prospective implantation site (Das et al., 1994). The nature of molecules involved in this communication, however, remains unknown. In chapter 3, I show that the implanting blastocyst activates the Wnt canonical pathway specifically in the uterine epithelium where it is about to implant, and that activation of this pathway is dependent on the mother's estrogen supply. These results clearly support the concept of a dialogue established between the embryo and the uterus at the time of implantation. They also provide, for the first time, evidence for the participation of an important signaling pathway- the Wnt signaling pathway- in this process.

The collective results of these studies allow me to present a model for the involvement of Wnts in the implantation process (Mohamed et al., 2004b). In this model, the blastocyst expresses multiple Wnts at the time of its implantation. The expression of some of these *Wnt* genes (such as *Wnt5a and Wnt11*) is regulated by uterine factors. These Wnt molecules then work as messengers from the blastocyst to induce gene expression, such as HB-EGF, in the uterus, which in turn facilitate the initial stages of implantation (Das et al., 1994; Raab et al., 1996). Preliminary evidence for the potential activation of HB-EGF by the Wnt/β-catenin comes from the sequence analysis of its promoter. The promoter of HB-EGF gene contains 3 putative Tcf/Lef (downstream effectors of canonical Wnt signaling) binding sites (positions, -1749, -1580 and –1169) (unpublished data). In addition, some of the Wnt molecules produced by the blastocyst may function in an autocrine manner and are involved in preparation of the blastocyst for invasiveness.

6.1.1: Experiments in progress

Understanding the molecular mechanism of implantation and deciphering the communication language between the embryo and the uterus is highly significant for reproductive medicine. On one hand, it would help increase the poor 20% implantation rate resulting from *in vitro* fertilization (IVF) in spite of the high rates of fertilization and blastocyst formation. On the other hand, it may lead to the development of novel contraceptive approaches targeting the post-fertilization period without disrupting the endocrine system. The results presented in this thesis provide novel scientific contributions in the field of implantation. They also pave the road for the scientific community to conduct further research in this area to better understand the molecular mechanism of implantation. To add more functional data to these studies, we are in the process of performing several experiments in the aim of answering the following questions:

Are the Wnts produced by the blastocyst essential for implantation? To try to answer this question, we are attempting to neutralize the function of Wnt molecules produced by the blastocyst using the Wnt antagonists; the secreted Frizzled related proteins (sFRP). sFRPs are secreted glycoproteins that structurally resemble the Frizzled receptors but lacking the transmembrane domains (reviewed in (Jones and Jomary, 2002). They work to antagonize Wnts by directly binding to them, thereby altering their ability to bind to their Wnt receptors. Five members of sFRP proteins have been identified, which include sFRP1, 2, 3, 4 and5. Three of these proteins (sFRP1, 2 and 3) are commercially available, which we have obtained in the lab. Recently, expression of sFRP2 was shown to be down-

regulated in the uterus at the time of implantation (Ho Hong et al., 2004), suggesting that these proteins perhaps are implicated in the implantation process.

To neutralize the function of Wnts produced by the blastocyst at the time of implantation, we are performing two types of experiments. The first one is to inject these sFRP proteins (individually or as a mixture) in one side of the uterus of pregnant females during the implantation day, while injecting the other side with control medium. Implantation will then be evaluated by decidual formation few days later and the effect of these proteins will be assessed. Alternatively or additionally, cells producing these antagonists will be grown on beads in culture and then transferred into one uterine horn of pregnant females during the implantation day, while control beads will be transferred into the other horn. This is expected to give better results, since they will provide an endogenous continues supply of these antagonists.

The second type of experiments we are performing is to incubate blastocysts for few hours with sFRP (individually or as a mixture) and then evaluate their implantation potential in pseudopregnant females as compared to blastocysts incubated in control medium.

Are Wnts the molecules that activate the β-catenin signaling pathway in the uterus? In chapter 3, I show that the embryo induces activation of the Wnt/β-catenin at the uterine implantation site. Whether activation of this pathway at this time is due to Wnt molecule(s) produced by the blastocyst is not known. In order to investigate this, we are performing the following experiment. Cells that are modified to produce different Wnts will be grown on beads in culture and then will be transferred into uteri of *Tcf/Lef-lacZ*

pseudopregnant females. Activation of the Wnt/β-catenin in the uterus will then be examined and compared to controls. These experiments will indicate whether these Wnts, which include those that are known to activate the canonical pathway of β-catenin (Wnt3a and Wnt7b) and those that are known to activate the calcium pathway (Wnt5a and Wnt5b), will activate the β-catenin pathway in the uterus at implantation. In chapter 2, I showed that three of these *Wnt* genes (*Wnt5a, Wnt5b and Wnt7b*) are expressed in the blastocyst at the time of implantation, while *Wnt3a* is not expressed (Mohamed et al., 2004b). Results from these experiments will shed light on the species of Wnt that activates β-catenin signaling in the uterus.

Is LIF activation in the uterus regulated by the Wnt/β-catenin? Uterine LIF is known to be essential for implantation and is regulated by estrogen (Chen et al., 2000; Stewart et al., 1992). In chapter 3 of this thesis, I showed that activation of the Wnt canonical pathway of β-catenin in the uterus is also regulated by estrogen. The cascade of these events, however, is not known. To investigate whether LIF expression in the uterus is regulated by estrogen through the Wnt/β-catenin pathway or that activation of the Wnt signaling pathway is regulated by estrogen through LIF, we are performing the following experiment. We are placing the *Tcf/Lef-lacZ* transgene on LIF mutant background and then we will examine the specific activation of the Wnt/β-catenin pathway in the uterus at the time of implantation. If activation of this pathway is detected in uteri that are mutant for *LIF*, then that would clearly demonstrate that this pathway is operating upstream of LIF. Conversely, if activation of this pathway is abolished in in uteri that are mutant for *LIF*, then that would indicate that this pathway is operating downstream of LIF.

6.1.2: Future directions

Several studies can be envisioned based on the results presented in this thesis. First, it would be interesting to test the potential involvement of Wnt signalling in blastocyst formation. This can be tested by trying to down-regulate *Wnt* gene expression at the preimplantation stages, either by using antisense technology or RNA interference, and then examining the effect of that on blastocyst formation and embryo implantation potential. Alternatively or additionally, Wnt signaling can be inhibited by incubating preimplantation embryos with sFRP and then examining the effect of that on the rate of blastocyst formation. Second, to better understand the effect of blastocyst on gene expression in the uterus, it would be interesting to investigate whether HB-EGF gene, and other growth factors encoding genes, which were shown to be specifically expressed during implantation (Das et al., 1997a), are downstream targets of the Wnt signaling pathway. This can be tested by cloning promoter regions from those genes upstream of a reporter gene and using that in cell transfection system with vectors encoding Wnts.

6.2: Involvement of Wnt/β-catenin signaling pathway in the formation of embryonic body axes

In several organisms, determinants for embryonic body axes are laid down in the egg (*Drosophila melanogaster*) or established at the time of fertilization (*Caenorhabditis elegans, Xenopus Laevis* and Zebrafish) (Lyczak et al., 2002; Moon and Kimelman, 1998; Riechmann and Ephrussi, 2001; Schier, 2001). In mice, the embryonic body axes become morphologically evident at E6.5 when the primitive streak is formed and gastrulation begins. The molecular mechanism of organizer formation in *Xenopus laevis* and zebrafish embryos are fairly well understood and a role for Wnt/β-catenin signaling is well

established (reviewed in (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). In contrast, the molecular mechanism underlying the induction of mammalian organizers (primitive streak and node) is still unknown. Increasing evidence, however, indicates that the Wnt/β-catenin is also involved in mammalian embryo axis formation (Huelsken et al., 2000; Liu et al., 1999a; Popperl et al., 1997).

Targeted deletion of *β-catenin* produces mutant embryos that lack mesoderm and fail to properly position the anterior visceral endoderm (Huelsken et al., 2000). The role of β-catenin in mesoderm formation and axis specification has been difficult to assess due to its ubiquitous expression starting at the two-cell stage as well as its dual role as a mediator of Wnt signaling and as a co-factor involved in cell adhesion.

In chapter 4 of this thesis, I examined both the distribution and signaling activity of β-catenin, during early embryonic development. I showed that neither non-phosphorylated β-catenin (the isoform of the protein that is presumed to be involved in transcription) nor β-catenin signaling through Tcf/Lef is present in pre-implantation stage embryos. These results imply that, unlike the case in *Xenopus* and zebrafish embryos, this pathway is not active at early stages of development. Accumulation of non-phosphorylated β-catenin and β-catenin transcriptional activity is first detected in the extra-embryonic visceral endoderm of day 5.5 embryos. This is the first demonstration that β-catenin signaling is active in the extra-embryonic visceral endoderm of early post-implantation embryos. Whether β-catenin plays a role in regulating gene expression in this tissue is not known. Chimera studies, however, have indicated that the extraembryonic tissue is essential for the induction of primitive streak and thus for axis formation (Holdener et al., 1994; Lawson et al., 1999; Russ et al., 2000). No Wnt ligand is reported to be expressed in the

extraembryonic visceral endoderm region. This leads to the interesting suggestion that the Wnt source that activates this pathway in this tissue may be maternal, emanating from uterine tissues and providing the initial cues for establishing the embryonic body axes. I also show that β-catenin transcriptional activity demarcates the prospective site of primitive streak formation within the embryo proper, which is the site where gastrulation begins and where the posterior side of the embryo is determined.

In *Xenopus* and zebrafish embryo, Wnt signaling activates the expression of homeodomain transcription factors genes, such as *Siamois* and *Twin*, which in turn induce the organizer, through the activation of organizer specific genes, such as *Goosecoid* and establish embryonic axes. In chapter 5 of this thesis, I presented evidence that Siamois/Twin-like binding activity is present in mouse embryos. First, I showed that Siamois/Twin binding site from the mouse *Goosecoid* promoter is able to drive asymmetrical expression of a reporter gene in the blastocyst stage. This is the first identification of an asymmetrically distributed activity in the blastocyst and represents the earliest molecular asymmetry reported in mammalian embryos. Second, I showed that this element drives node specific expression at later stages suggesting that the molecular mechanism of axis formation perhaps is conserved between species. Third, using the yeast-one hybrid system, I identified a candidate factor (WBSCRII, also known as mGTF-II) as a potential regulator of this element in vivo.

6.2.1: Future directions

The results presented in this thesis provide new evidence that the Wnt signaling pathway is involved in induction of the primitive streak and node. This has shed some light on the molecular mechanism of axis formation in mammals and provided a framework for future

studies in this field. Specific questions remained to be answered, which will add valuable information to this subject:

Does early asymmetry detected in the blastocyst stage correlates with future axis specification? Polarity in mammalian embryos has long been considered a late developmental event that occurs just prior or at gastrulation. Recent evidence suggest that the overt polarity established post implantation can be traced back to the preimplantation stages (Zernicka-Goetz, 2002). No molecular evidence for polarity in preimplantation stages, however, is available. The early asymmetrical transcriptional activity I detected for the Wnt responsive element in the blastocyst stage is very intriguing and stimulates further investigations. It will be very interesting to test whether this activity correlates with axis specification at postimplantation stages. Such investigations are in progress in Dr. Dufort's lab, which involve generation of transgenic animals that carry this element upstream of green fluorescent protein (GFP). Cells that express this reporter in the blastocyst stage will then be labeled, using cell-tracing dye, and the embryo will then be transferred into foster mothers. The fate of these cells will be examined at later stages, when body axes are established, and see if they are destined to participate in establishing embryonic axes.

Is WBSCRII (GTFII) essential for axis formation? In *Xenopus* embryos, the homeodomain transcription factors Siamois and Twin are essential for axis formation (Fan and Sokol, 1997; Laurent et al., 1997; Lemaire et al., 1995). Similarly, the zebrafish homolog Dharma/Nieuwkoid is essential for axis formation (Solnica-Krezel et al., 1996). Mouse and human genomes do not contain genes that share sequence homology to either Siamois or Twin. The pivotal roles these proteins play in establishing embryonic axes in

Xenopus, however, suggest that other proteins are compensating their function in mammalian embryos. Using Siamois/Twin binding site, I isolated a transcription factor, WBSCRII, which I hypothesize to potentially compensate for the function of Siamois/Twin in mammals. To test this, a gene knockout experiment for this factor will be valuable to examine whether its absence has any effect on axis formation.

6.3: *Tcf/Lef-lacZ* transgenic animal is a useful tool to study development

Wnt proteins play decisive roles in development. The information gathered about their involvement in different developmental processes came mainly from knockout studies. Despite their valuable information, these studies, however, are time consuming and usually provide specific information about specific Wnt molecule. For the studies I presented in this thesis, I developed a transgenic mouse (*Tcf/Lef-lacZ*) that provides a simple and very effective readout for the canonical pathway of β-catenin. I used this transgenic animal as a basis to tackle the involvement of this pathway in embryo implantation and formation of embryonic body axes. In this section, I will present examples of how this transgenic animal is useful to study the involvement of Wnt signaling in the development of several other organs and tissues.

6.3.1: Activation of the Tcf/Lef-lacZ reporter gene during brain development

In early mouse gastrula, inhibition of Wnt signaling in the anterior region is essential for head induction (Glinka et al., 1998; Mukhopadhyay et al., 2001). At later stages, however, Wnt signaling plays a major role in specification of the head region (Adams et al., 2000; Joyner, 1996). At the time when the neural tube closes, the brain region is divided into series of vesicles. The forebrain consists of two vesicles, the telencephalon and the diencephalon, whereas the midbrain develops to one vesicle, the mesencephalon. The

hindbrain is divided into rhombomeres with the most anterior (rhombomeres1 and 2) is called the metencephalon. Several transcription and secreted factors including, Wnt1, Fgf8, Pax2, Engrailed1, Otx2 and Gbx2 where shown to cooperate to define the brain architecture and function (Ye et al., 2001). The precise area in the brain where all Wnt signaling is active, however, is still not known.

Appendix I to Appendix IV in chapter 7, show activation of the canonical Wnt/β-catenin pathway, as assayed by the activation of *Tcf/Lef-lacZ* reporter gene, in the head region of embryos at E9.0 to E13.5. This in principle should reflect activity of all Wnt molecules that use the canonical pathway to transmit their signals. Activation of this pathway is detected in the neuroepithelium of the hind, mid and forebrain vesicles. Activity is detected in the dorsal midline of the telencephalon (Appendix I-b, g & h, Appendix III-b, c &d), which is consistent with *Wnt1* and *Wnt3a* expression (Megason and McMahon, 2002).

6.3.2: Activation of the Tcf/Lef-lacZ reporter gene during ear development

Development of the inner ear begins during late gastrulation when surface ectoderm areas on either side of the neural tube are specified to become the otic placodes, which are epithelial cells that subsequently proliferate extensively and invaginate to form the otocyt (the otic vesicle). At E11.0, the otic vesicle subsequently lengthens with an out pocketing of its ventral side forming the cochlea. The lateral and medial walls of the dorsal side begin to migrate towards each other and fuse in the middle to form the ear canals (reviewed in (Rinkwitz et al., 2001).

Strong activation of this pathway is detected in the otic region throughout the time of its development (Appendix I-IV). Interestingly, Wnt activity is not suspected to be involved in induction of the otic placode (Phillips et al., 2004). Therefore, expression of the *Tcf/Lef-lacZ* reporter in this region provides an example of how this model is useful to reveal unsuspected areas for Wnt signaling.

6.3.3: Activation of the Tcf/Lef-lacZ reporter gene during eye development

The vertebrate eye develops from apposed neural (optic vesicle) and surface (lens placode) ectoderm and its early development involves reciprocal inductive processes between these two tissues (Pichaud et al., 2001). Pax genes were shown to be essential for eye formation in human and mice (Glaser et al., 1994; Hill et al., 1991). Expression of the *Tcf/Lef-lacZ* reporter gene is detected in the inner and outer layer of optic cup at E11.0-13.5 (Appendix IV, c-g). These tissues give rise to the future nervous layer of the retina and the future pigment layer of the retina, respectively.

In a more extensive study, in collaboration with Dr. Wallace at the Ottawa Health Research Institute, we showed that activation of this pathway is detected in the ciliary margin in both the presumptive retina pigment epithelium (RPE) and non-pigmented inner layer, from E11.5 to E14.5. At E13.5, activation of this pathway is detected in cells located in the outer neuroblast layer of the neural retina and in the anterior lens epithelium. At E17.5, activity is detected exclusively in the future ciliary body and iris. The pattern of activation of this pathway in the ciliary margin overlaps with and is adjacent to *Wnt13 (Wnt2b)* expression domain (Liu et al., 2003).

6.3.4: Activation of the Tcf/Lef-lacZ reporter gene during limb development

The formation of the apical ectodermal ridge (AER) has long been known to play a critical role in the distal outgrowth and patterning of the vertebrate limb. Classical experiments performed by Saunders over half a century ago, demonstrated that surgical removal of the AER shortly after its formation results in severe truncations of the entire limb, whereas removal of this tissue at progressively later stages in development allows outgrowth of the more distal elements in a progressive fashion (Saunders, 1948). Several signaling pathways including Wnts and FGF signaling pathways are essential in establishing the AER (Kawakami et al., 2001).

Expression of the *Tcf/Lef-lacZ* reporter gene is detected specifically in the AER of the developing limb from day E9.5-E11.5 (Appendix V). This pattern of expression of the reporter gene corresponds well with a known function for β-catenin in the formation of this tissue. Recently, it was shown that Wnt3 signaling in the limb ectoderm is required for the formation of the AER and conditional removal of *β-catenin* in the limb ectoderm, the pre-AER ventral ectoderm, results in total absence of the limb (Barrow et al., 2003).

6.3.5: Activation of the Tcf/Lef-lacZ reporter gene during lung development

Lung development in the mouse begins around E9.5 with the budding of the primitive lung mass from the ventral gut epithelium (foregut endoderm). This early lung endoderm, which is surrounded by mesodermally derived mesenchyme, undergoes branching morphogensis, in which by E12.0, the lung bud forms the preliminary trachea and pulmonary bronchi, forming the single left lung lobe and the four lobes of the right lung. By E14.5-E16.5, the bronchial tree expands and the bronchi and bronchioles are formed. During this time, epithelial cells differentiate to begin to form the prealveolar saccules. By

E16.5-E17.5, the cell mass comprising the prealveolar sacs expands exponentially with septation into alveoli continuing through birth and until maturation of the adult lung (reviewed in (Minoo and King, 1994). During branching morphogenesis, the mesenchyme produces essential growth factors and signaling molecules required for airway epithelial development and branching including members of the fibroblast growth factor (FGF) family. In turn, the epithelium also produces signaling molecules important for mesenchymal differentiation and proliferation including Bmp4 and sonic hedgehog (shh) (reviewed in (Warburton et al., 2000).

Strong expression of the *Tcf/Lef-lacZ* reporter gene is detected in the developing lung bud at E9.5-E10.5 (Appendix VI, a-c). Expression is then restricted to the airway epithelium of the developing lung at E12.5-E14.5 (Appendix VI, d-f). *Wnt7b* was shown to be expressed in the airway epithelium and its deletion leads to early lethality due to respiratory failure (Shu et al., 2002).

6.3.6: Activation of the Tcf/Lef-lacZ reporter gene during hair follicle development

After gastrulation, a single layer of pluripotent ectoderm covers the embryo surface, which becomes the skin. The mesenchymal cells begin to populate the skin and soon after and the epidermis and its appendages begin to form. The skin epithelium is separated from its underlying mesenchyme by a basement membrane of extracellular matrix. The formation of hair follicles occurs during embryogenesis and relies on a series of signals sent between dermal cells and overlying surface epithelial cells that cause fate changes in both cell populations, ultimately resulting in differentiation of the hair shaft, root sheaths, and dermal papilla (reviewed in (Fuchs et al., 2001; Millar, 2002). Several signalling

pathways, including Wnt, Fgf, TGF-β and hedgehog signalling pathways, are thought to be involved in the development of hair follicles (Andl et al., 2002; Millar, 2002).

Strong expression of the *Tcf/Lef-lacZ* reporter gene is detected in the developing hair follicles as early as E12.5 (Appendix VII, a-d). In accordance with this, similar reporter gene (TOP-gal) was shown to be expressed in the developing hair follicle (Fuchs et al., 2001). Expression of *Wnt10b* and *Wnt10a* was found to be up regulated in this region at the onset of follicle morphogenesis and in postnatal hair follicles beginning a new cycle of hair growth (Reddy et al., 2001). It is interesting to note that the earliest expression of the TOP-gal reporter in hair follicles was detected at E16.5. On the other hand, expression of the *Tcf/Lef-lacZ* reporter in these tissues was detected few days earlier. This gives an example of how *Tcf/Lef-lacZ* reporter provides a more sensitive readout of the Wnt/β-catenin pathway than similar reporters available. This perhaps is due to the type of minimum promoter used to construct each reporter or due to the number of copies of the transgene and the location they integrated within the genome.

6.3.7: Activation of the Tcf/Lef-lacZ reporter gene during taste bud development

In rodents, four different types of tongue papillae can be found on the dorsal surface of the tongue: fungiform, circumvallate, foliate, and filiform papillae. In mice, morphogenesis of the tongue papillae requires coordinated interactions among the lingual epithelium and mesenchyme as well as nerve innervation of the tongue (reviewed in (Mbiene and Mistretta, 1997). Development of fungiform papillae is divided into two stages, one is morphogenesis, which produces the papillae structures and the other is nerve innervation, which develops the taste buds (Kim et al., 2003). Several signalling molecules, including

Shh, Bmp-2/4 and *Fgf-8,* are expressed within the epithelia of the primordial tongue and thought to play essential role during papillae morphogensis (reviewed in (Nosrat, 1998).

Strong expression of the *Tcf/Lef-lacZ* reporter is detected in the developing taste bud at E13.5 (Appendix VII, e). Interestingly, no Wnt molecule has been reported to be expressed in these cells thus far. This is another example of how this transgenic animal is useful in revealing unknown regions for Wnt activity.

6.3.8: Activation of the Tcf/Lef-lacZ reporter gene during female reproductive tract and mammary gland development

Development of the female reproductive tract, which in mammals includes the oviducts (fallopian tubes), uterus, cervix and vagina, begins soon after gastrulation, through the differentiation of the intermediate mesoderm. This embryonic tissue subsequently proliferates and some cells undergo the transition from the mesenchymal to the epithelial cell type to generate the tubules that compose the male and female reproductive tracts. Before sexual differentiation, mammalian embryos have two pairs of genital ducts: the Wolffian ducts (which differentiate into structures of the male reproductive tract, such as the epididymides, vas deferentia and seminal vesicles) and the Müllerian ducts (which differentiate into the oviducts, uterus, cervix and upper portion of the vagina of the female reproductive tract) (reviewed in (Kobayashi and Behringer, 2003).

Strong expression of the *Tcf/Lef-lacZ* reporter gene is detected in the forming Wolffian and Müllerian ducts beginning on day E11.5-E18.5 (Appendix VIII). At E15.5-E18.5, when the Müllerian duct becomes the uterus, expression is detected in the uterine epithelium (Appendix VIII, insets in d-f). Expression is also detected in the surface epithelium of the ovary at these stages. After birth, strong expression is maintained in the

uterine epithelium until day 21, after which it is no longer detected (Appendix VIII, g-i). Expression in the oviduct, however, is maintained throughout the life span of the animal. Subset of *Wnt* genes (*Wnt4*, *Wnt5a* and *Wnt7a*) is involved in the development of several female reproductive organs (Miller et al., 1998). *Wnt4*-mutant female mice lack a female reproductive tract (Vainio et al., 1999), *Wnt7a* mutant males don't show regression of the Müllerian duct (Miller and Sassoon, 1998) and Wnt5a is required for posterior growth of the female reproductive tract (Mericskay et al., 2004).

Strong expression of the reporter gene is also detected in the developing mammary glands as early as E11.5 (Appendix VIII, d). Signalling through β-catenin was shown to be involved in the development of mammary glands. For example, mice lacking Lef-1 (downstream effector of the β-catenin pathway) show an early arrest in mammary gland formation at stage E13.5 (van Genderen et al., 1994). Similarly, mice expressing the negative regulator of Wnt/β-catenin signaling (K14-Dkk), fail to form mammary buds (reviewed in (Hatsell et al., 2003).

6.3.9: Activation of the Tcf/Lef-lacZ reporter gene during kidney development

The kidney develops as a result of interactions between the ureteric bud epithelium and the nephrogenic mesenchyme. Kidney mesenchyme initiate organogenesis by inducing formation of a ureteric bud, which then signals reciprocally to the mesenchyme and initiates a sequence of events that lead to the formation of a mesenchymal cell condensate and of a pretubular cellular aggregate. These will then undergo morphogenesis via comma and s-shaped bodies to form the nephron. The mesenchyme regulates the branching of the ureteric bud, which in turn induces condensation and tubule formation in close proximity

to each tip, to generate the number of nephrons required in the adult (reviewed in (Vainio, 2003).

Strong *Tcf/Lef-lacZ* reporter expression is detected in the nephritic duct at E9.5-10.5 (Appendix IX, a-d). At E12.5 and beyond, strong expression is detected in the forming nephrons (Appendix IX, e-h). In collaboration with Dr. Goodyear (McGill University), we are examining in more details the expression of this reporter gene in kidney development during all embryonic stages.

Several Wnt molecules are expressed and play important role in kidney development. For example, *Wn-11* expression is up regulated at the ureteric tips of the ureteric bud at the initiation of kidney development around E10.0. *Wnt7b* gene is activated proximal of *Wnt11* expression domain in the ureteric bud around E13.5. *Wnt6* is also expressed in the ureteric bud, while *Wnt4* and *Wnt2b* genes are expressed in the kidney mesenchyme (reviewed in (Vainio, 2003). Null mutation of *Wnt4* results in lack of nephrons (Stark et al., 1994).

6.3.10: Activation of the Tcf/Lef-lacZ reporter gene during gut development

Development of embryonic gut begins by the formation of a simple tube called the primitive gut tube. Initially, through morphogenic movements, two invaginations in the ventral walls of the gut endoderm are created. One is at the rostal end of the embryo and the other is at the caudal end of the embryo. These invaginations then migrate toward each other and the endoderm cells behind them form tubes, which extend and fuse at the umbillicus. The primitive gut tube subsequently undergoes regional specialization followed by morphogenesis and differentiation to generate the different organs of the

digestive tract. The gut tube is subdivided into three regions: foregut, midgut, and hindgut (reviewed in (Theodosiou and Tabin, 2003). Signalling molecules, such as SHH was shown to be involved in the formation of the primitive gut tube and its subsequent regionalization (Roberts, 2000).

Expression of the *Tcf/Lef-lacZ* reporter is detected in the tracheal diverticulum where the foregut will form at E9.5-E11.0 (Appendix X, a-c). Expression is also detected in specific region of the forming stomach at E12.5 (Appendix X, d). Concurrent with these results, activity of the Wnt pathway is implicated in gut development (reviewed in (Theodosiou and Tabin, 2003).

6.3.11: Activation of the Tcf/Lef-lacZ reporter gene during somitogenesis

Through gastrulation, the paraxial mesoderm is produced at the level of the primitive streak, or the tail bud, at the caudal region of the embryo. The paraxial mesoderm subsequently becomes organised as two rods of mesenchymal cells termed the presomitic mesoderm (PSM). Somites are bilateral epithelial spheres formed sequentially from the presomitic mesoderm as the embryonic axis extends. Once formed, somites subsequently differentiate into skeletal muscle, cartilage and dermis, under the influence of the surrounding structures (reviewed in (Saga and Takeda, 2001). Several signaling pathways including Notch, Wnt, FGF and MAPK pathways have been implicated in somitogenesis (reviewed in (Rida et al., 2004)

Expression of the *Tcf/Lef-lacZ* reporter gene is detected in the forming somites (Appendix X, e-h). In support of this, Tcf-1/Lef1 double mutant mice lack somites (Galceran et al., 1999). This phenotype is also observed in mice lacking *Wnt3a* (Aulehla et al., 2003;

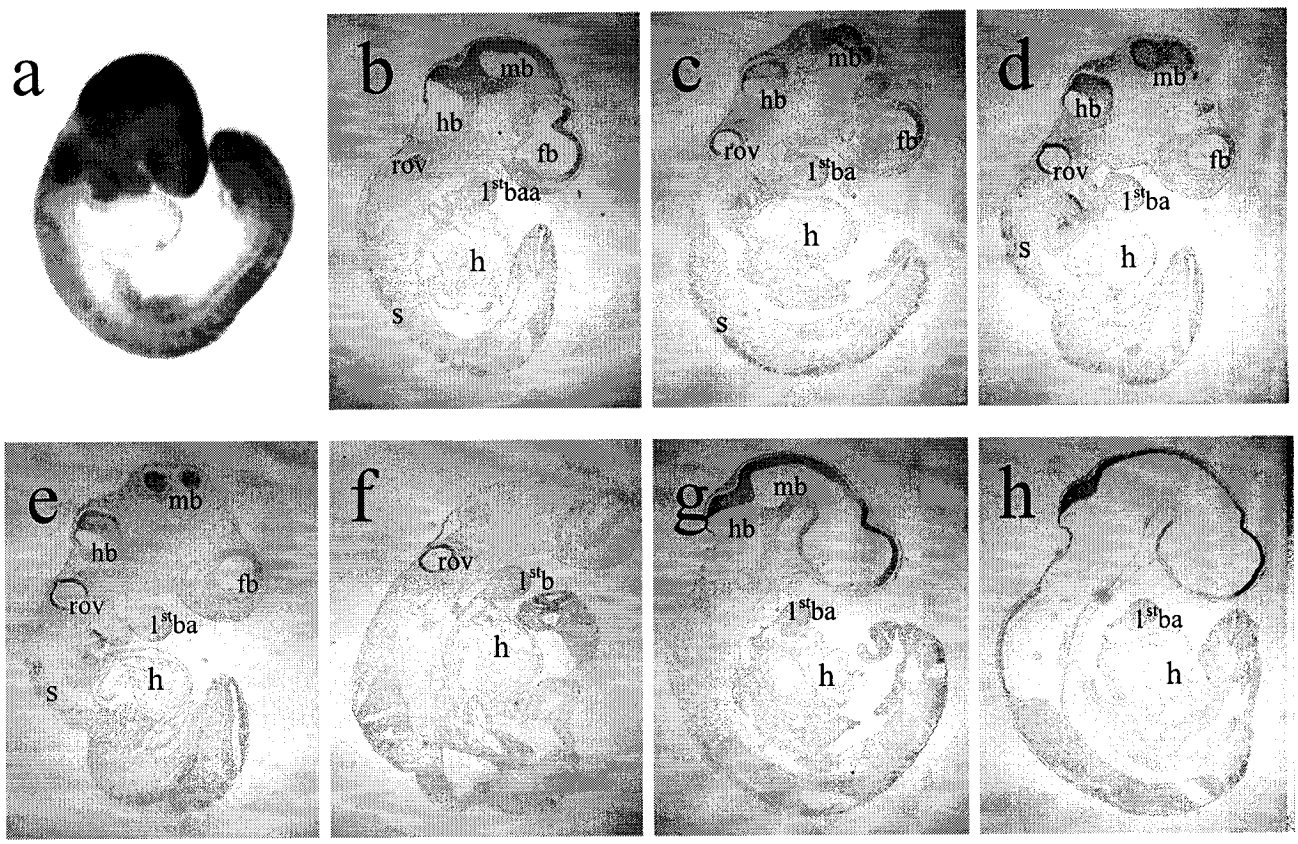
Takada et al., 1994) suggesting that Wnt3a transmits its signal through the canonical pathway of β-catenin in this region. Indeed, when I placed this transgene on *Wnt3a* hypomorphic background, substantial reduction in reporter activity was specifically observed in this region (Mohamed et al., 2004a).

6.4: Final remarks

It is evident that the Wnt signaling pathways, particularly the Wnt/β-catenin pathway, are involved in many developmental and cellular processes. Owing to their central role in regulating different cellular responses, the activity of these pathways is tightly controlled. The biological responses elicited by Wnt pathways do not depend only on which Wnt molecule is expressed or what receptor is present on the plasma membrane. Instead, it involves many of regulatory molecules that act at different levels to ensure the implementation of proper instructions. Therefore, it becomes imperative to develop new systems that can easily and efficiently monitor the activation of such pathways in vivo.

The *Tcf/Lef-lacZ* transgenic mouse I described in this thesis represents a simple and very effective reagent to monitor the activity of the Wnt canonical pathway of β-catenin. This transgenic animal could be thought of as a three dimensional atlas for the canonical Wnt/β-catenin signaling, which provides the final readout where this pathway is active in vivo. It is valuable research tool in this field, which can be easily used to study the involvement of this pathway in any developmental processes at any stage of development. It can also be effectively used in cancer research to study the biology of this pathway in cancer and to test the effectiveness of anti-cancer therapeutic agents to fight cancer.

Appendix I: Sagital sectioning through E9.0-9.5 *Tcf/Lef-lacZ* embryo



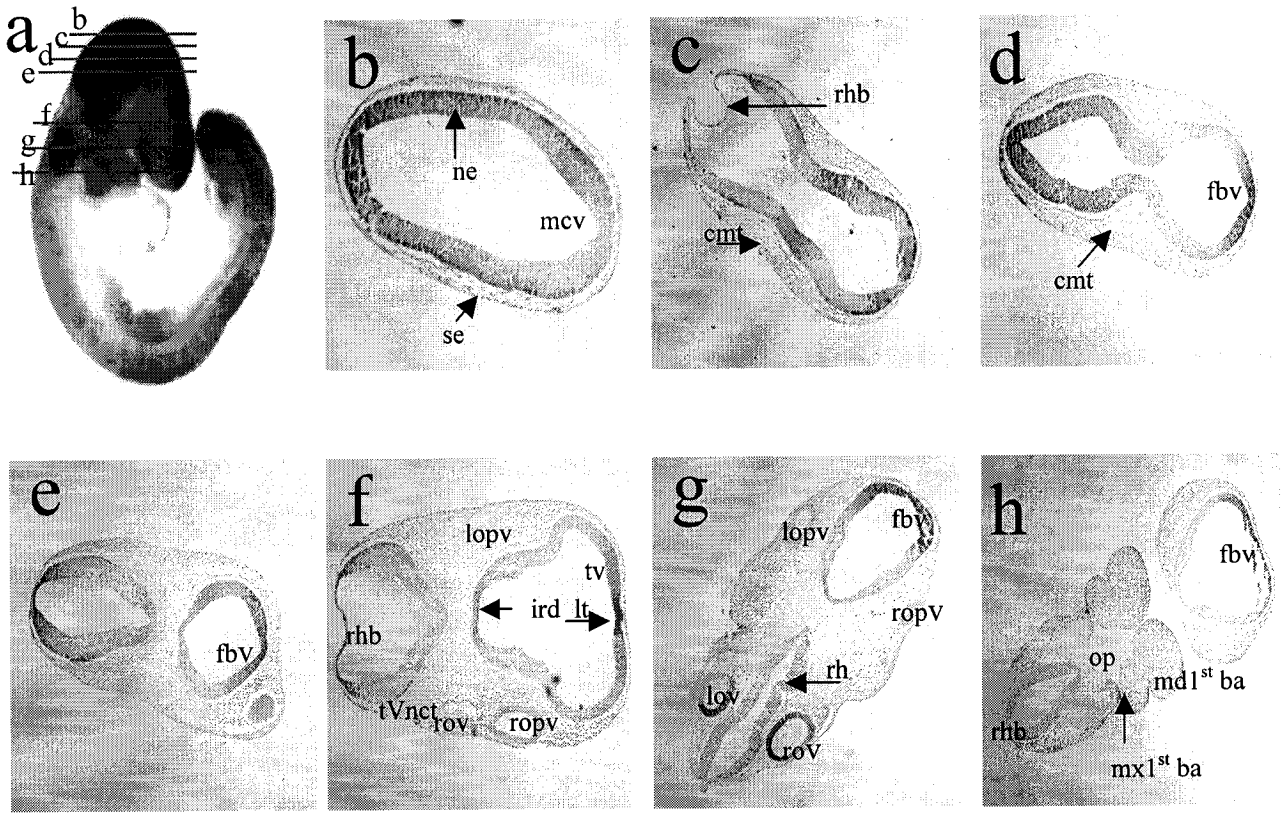
Appendix I Legend

(a)- Whole mount lacZ staining of E9.0-9.5 *Tcf/lef-lacZ* embryo.

(b-h)- Serial Sagital sections through the embryo starting from the right side (b) until halfway of the embryo (h).

Abbreviations: (s = somite, h = heart, 1^{st} ba = first branchial arch, rov = right otic vesicle, hb = hind brain, mb = midbrain, fb = forebrain, ropv = right optic vesicle).

Appendix II: Transverse sectioning through E9.0-9.5 *Tcf/Lef-lacZ* embryo



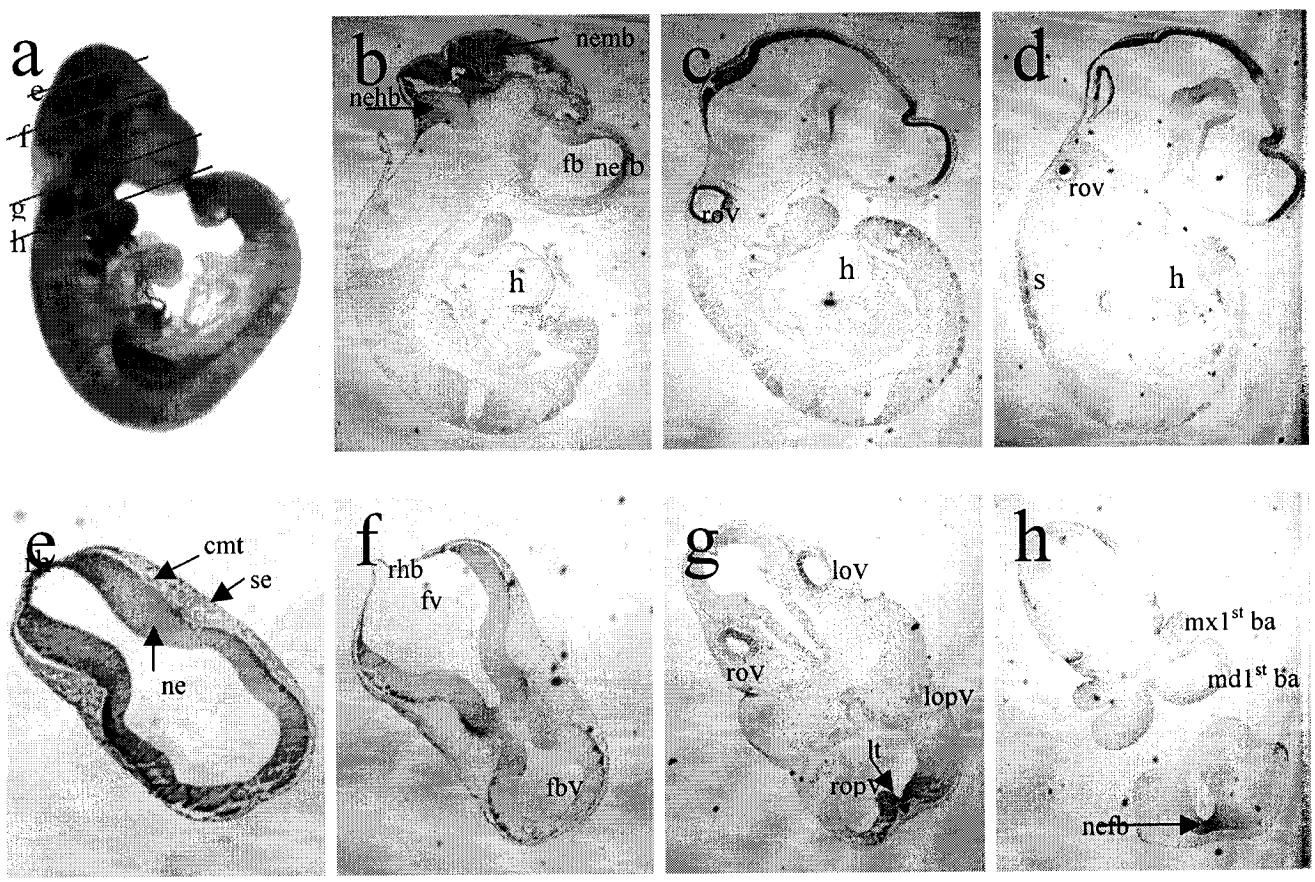
Appendix II legend

(a)- Whole mount lacZ staining of E9.0-9.5 *Tcf/lef-lacZ* embryo.

(b-h)- Serial transverse sections through the head region starting from top (b) until bottom of head structure (h) as indicated in (a).

Abbreviations: (se = surface ectoderm, ne = neuroepithelium, mcv = mesencephalic vesicle, cmt = cephalic mesenchyme tissue, rhb = roof of hindbrain, fbv = forebrain vesicle, tVnct = trigerminal (V) neural crest tissue, rov = right otic vesicle, lov = left otic vesicle, ropv = right optic vesicle, lopv = left optic vesicle, ird = infundibular recess of diencephalons, lt = lamina terminalis, rh = rhombomere, op = oropharynx, mx1stba = maxillary component of first branchial arch, md1stba = mandibular component of first branchial arch).

Appendix III: Transverse and sagital sectioning through E10.0 *Tcf/Lef-lacZ* embryo



Appendix III legend

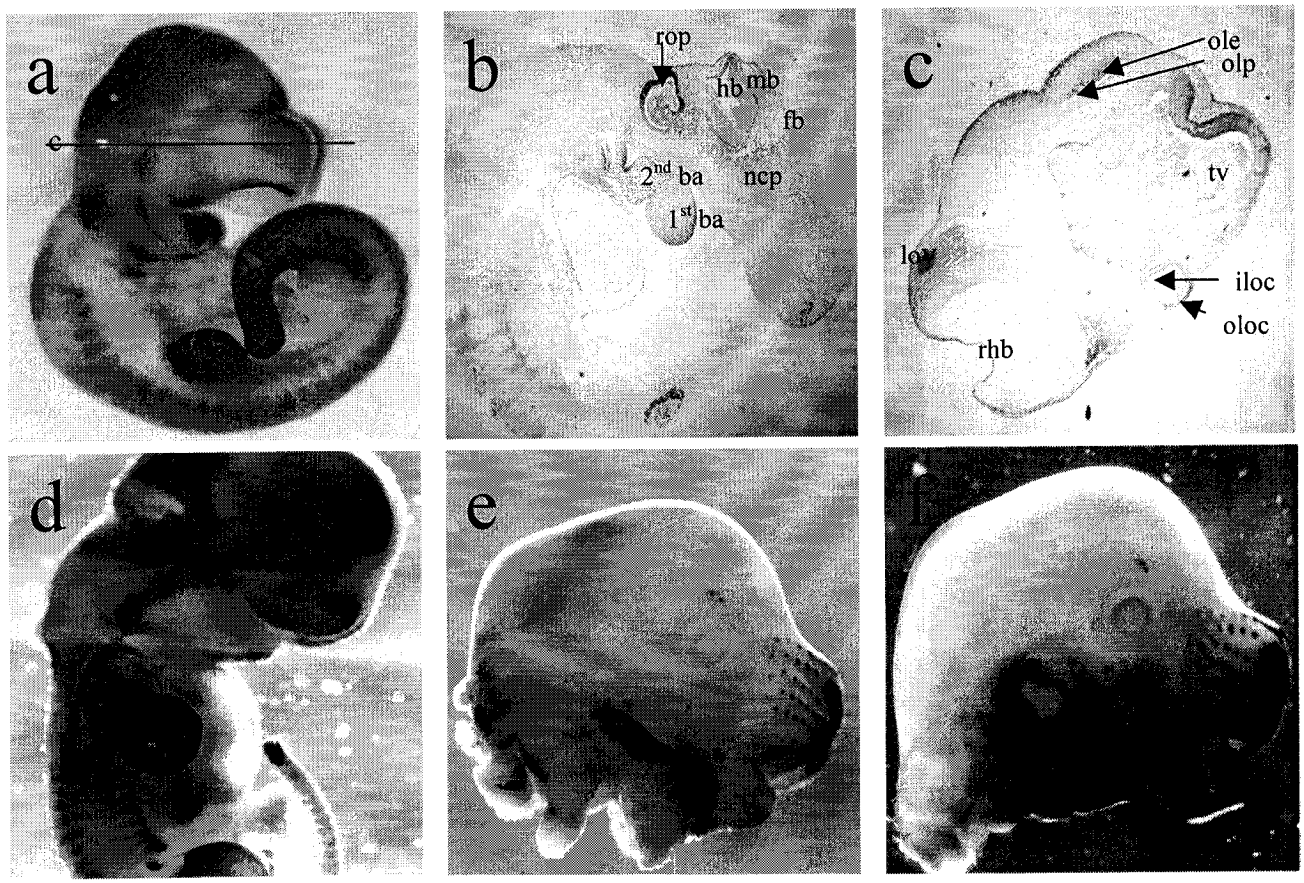
(a)- Whole mount lacZ staining of E10.0 *Tcf/lef-lacZ* embryo.

(b-d)- Serial Sagital sections through the embryo starting from the right side (b) until halfway of the embryo (d).

(e-h) Serial Transverse sections through the head region starting from top (e) until bottom of head structure (h) as indicated in (a).

Abbreviations: (s = somite, h = heart, nehb= neuroepithelial lining of the hindbrain, nemb= neuroepithelial lining of the midbrain, nefb= neuroepithelial lining of the forebrain, fb = forebrain, se = surface ectoderm, ne = neuroepithelium, cmt = cephalic mesenchyme tissue, lt = lamina terminalis, rhb = roof of hindbrain, fv = fourth ventricle, fbv = forebrain vesicle, rov = right otic vesicle, lov = left otic vesicle, ropv = right optic vesicle, lopv = left optic vesicle, mx1stba = maxillary component of first branchial arch, md1stba = mandibular component of first branchial arch).

Appendix IV: Reporter gene activation in the head region of E10.5-13.5 *Tcf/Lef-lacZ* embryo



Appendix IV legend

(a) Whole mount lacZ staining of E10.5 *Tcf/lef-lacZ* embryo.

(b) Sagital section through the embryo.

(c) Transverse section through the head region.

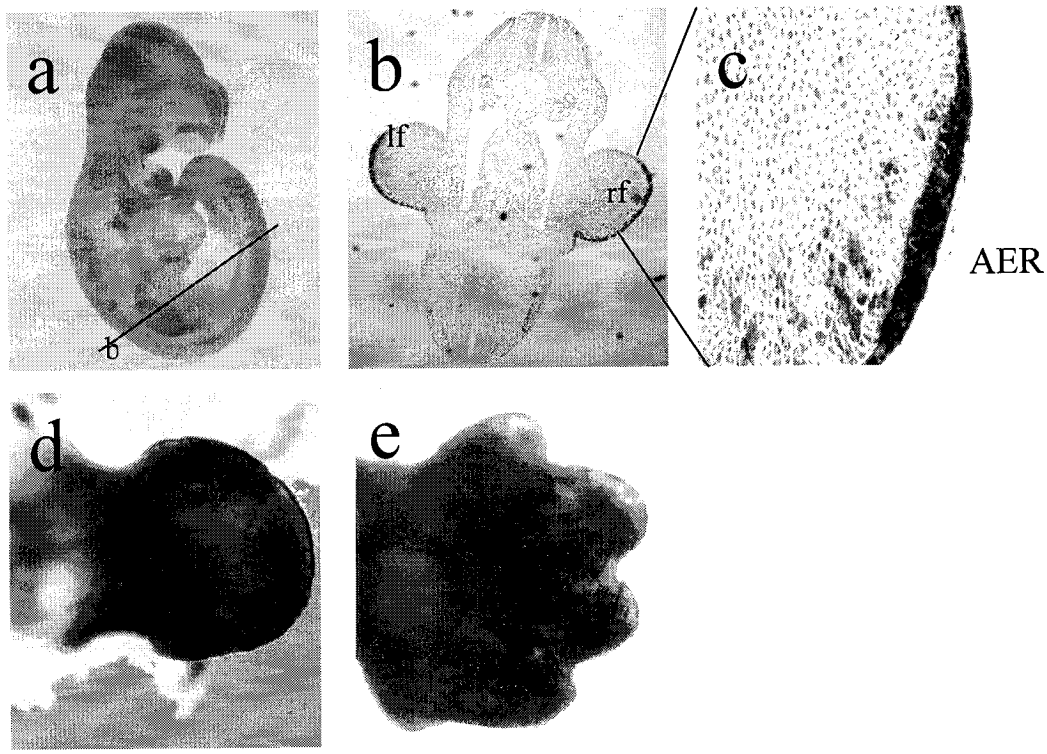
(d) Whole mount lacZ staining of E11.5 *Tcf/lef-lacZ* embryo.

(e) Whole mount lacZ staining of E12.5 head *Tcf/lef-lacZ* embryo.

(f) Whole mount lacZ staining of E13.5 head *Tcf/lef-lacZ* embryo.

Abbreviations: (rop = right otic pit, lop = left otic pit, hb = hindbrain, mb = midbrain, fb = forebrain, 1^{st} ba = first branchial arch, 2^{nd} ba = second branchial arch, ncp = neural component of the pituitary, oloc = outer layer of optic cup, iloc = inner layer of optic cup, olp = olfactory pit, ole = olfactory epithelium, tv = telencephalic vesicle, rhb = roof of hindbrain).

Appendix V: *Tcf/Lef-lacZ* reporter gene activation during limb development



Appendix V legend

(a) Whole mount lacZ staining of E10.5 *Tcf/lef-lacZ* embryo.

(b) Transverse section through the forelimb of the embryo.

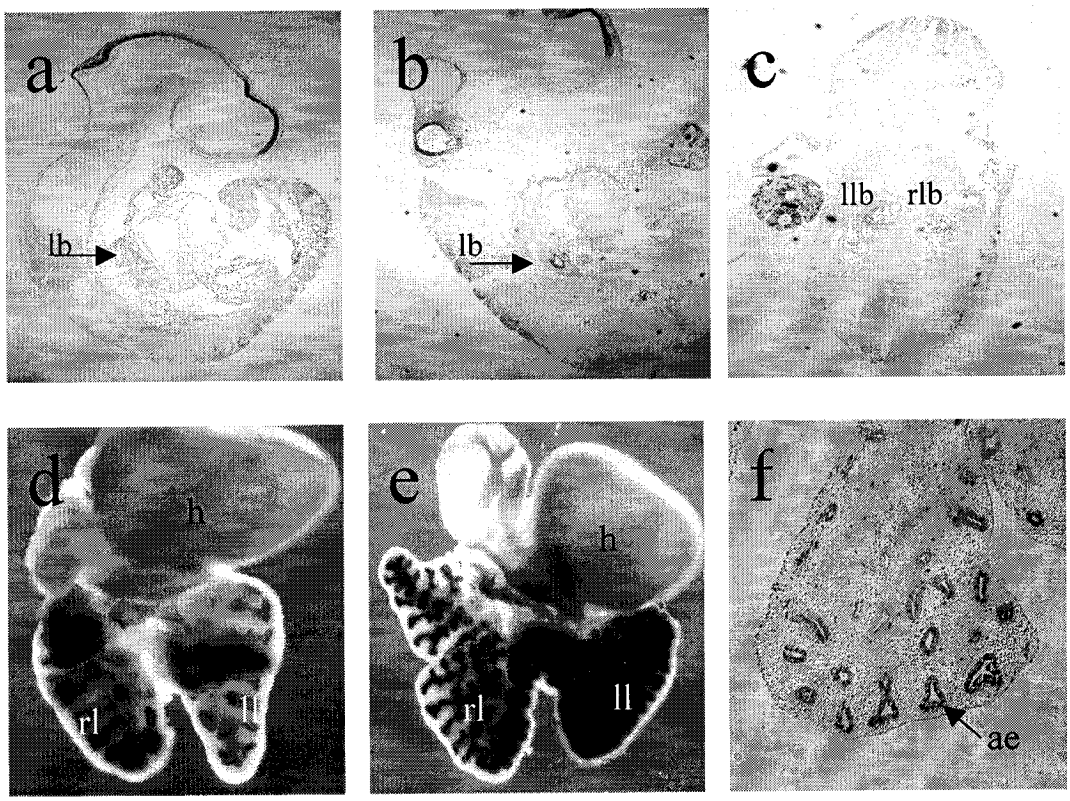
(c) 40X magnification of section in b.

(d) Right limb of E11.5 *Tcf/lef-lacZ* embryo.

(e) Right limb of E12.5 *Tcf/lef-lacZ* embryo.

Abbreviations: (lfl = left forelimb, rfl = right forelimb, AER = Apical Ectodermal Ridge).

Appendix VI: *Tcf/Lef-lacZ* reporter gene activation during lung development



Appendix VI legend

(a) Sagital section through E9.5 *Tcf/lef-lacZ* embryo. Arrow points to the developing lung bud

(b) Sagital section through E10.5 *Tcf/lef-lacZ* embryo. Arrow points to the developing lung bud.

(c) Transverse section through E10.0 *Tcf/lef-lacZ* embryo, showing the two developing lung buds.

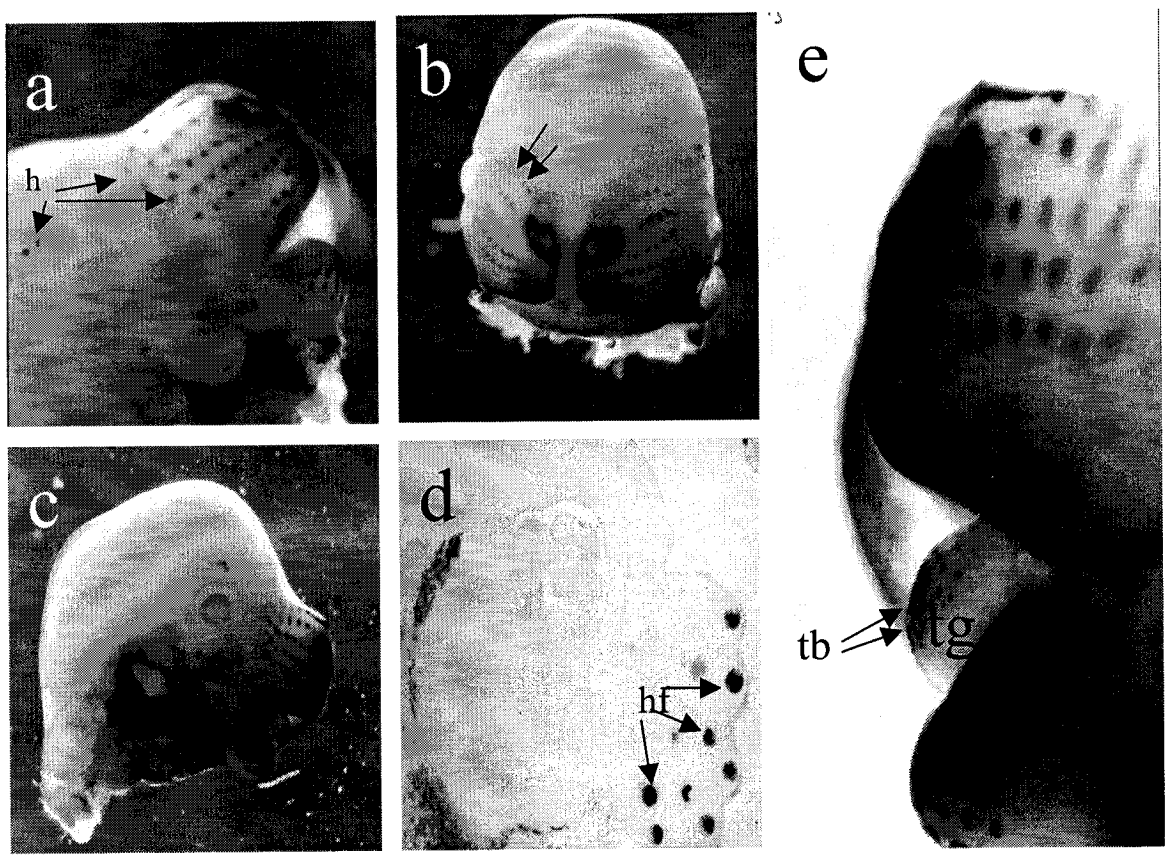
(d) Whole mount lacZ staining of E12.5 *Tcf/lef-lacZ* lung.

(e) Whole mount lacZ staining of E13.5 *Tcf/lef-lacZ* lung.

(f) Transverse section of E14.5 *Tcf/lef-lacZ* lung stained for β-galactosidase activity.

Abbreviations: (lb = lung bud, llb = left lung bud, rlb = right lung bud, rl = righ lung, ll = left lung, h = heart, ae = airway epithelium).

Appendix VII: *Tcf/Lef-lacZ* reporter gene activation during hair follicle and taste bud development



Appendix VII legend

(a) Whole mount LacZ staining of E12.5 *Tcf/lef-lacZ* head (lateral view). Arrows point to developing hair follicles.

(b) Whole mount LacZ staining of E12.5 *Tcf/lef-lacZ* head (front view). Arrows point to developing hair follicles.

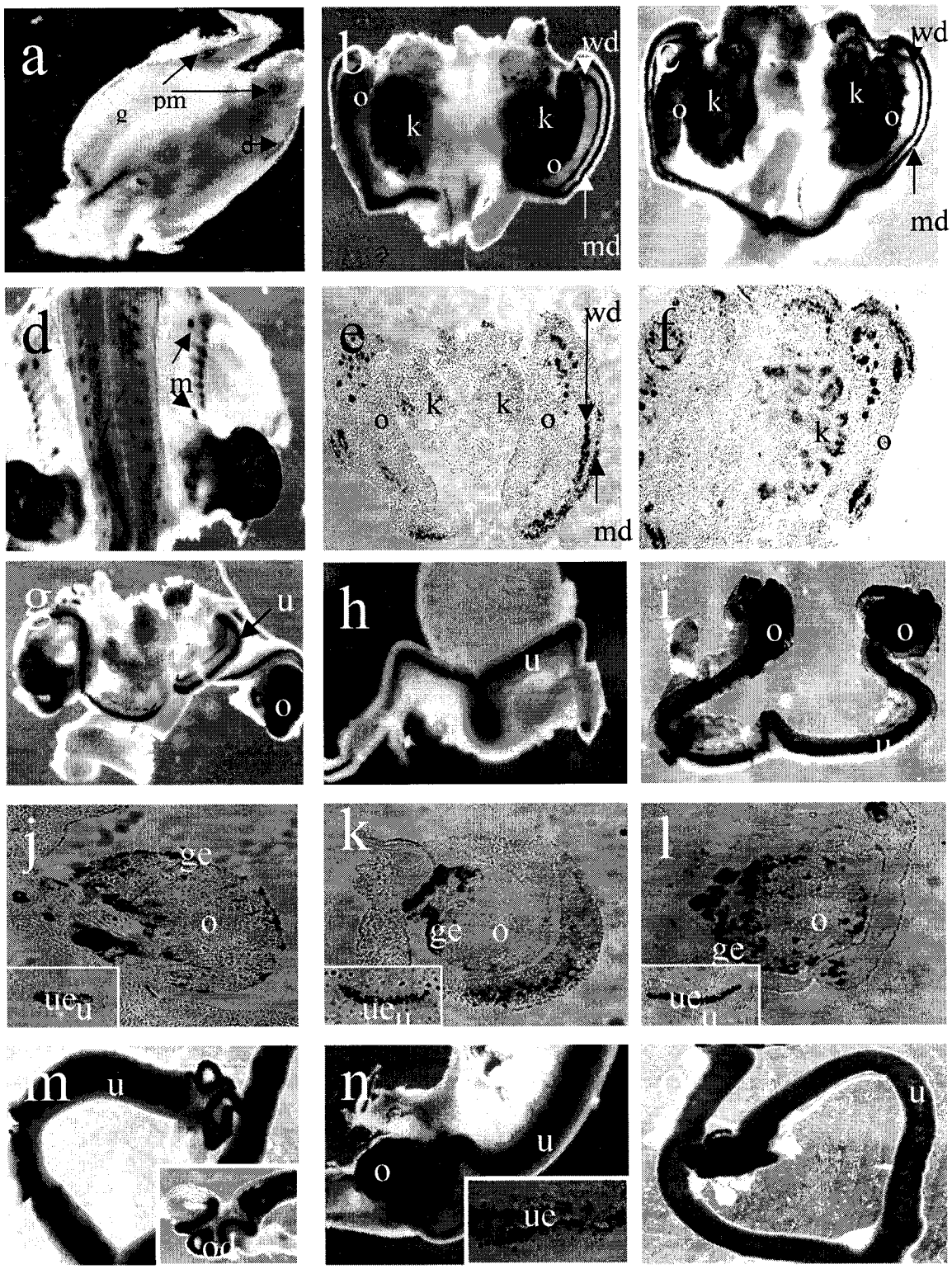
(c) Whole mount LacZ staining of E13.5 *Tcf/lef-lacZ* head Lateral view.

(d) Sagital section through the head of E12.5 *Tcf/lef-lacZ*. Arrows point to developing hair follicles.

(e) Whole mount LacZ staining of E13.5 *Tcf/lef-lacZ* head. Arrows point to developing taste buds.

Abbreviatione: (hf = hair follicle, tg = Tongue, tb = taste bud).

Appendix VIII: *Tcf/Lef-lacZ* reporter gene activation during female reproductive tract development



Appendix VIII legend

(a) Whole mount LacZ staining of the genital ridge region of E11.5 *Tcf/lef-lacZ* embryo. The sex of the embryo is not distinguished at this stage.

(b) Whole mount LacZ staining of the genital ridge region of E12.5 female *Tcf/lef-lacZ* embryo.

(c) Whole mount LacZ staining of the genital ridge region of E13.5 female *Tcf/lef-lacZ* embryo.

(d) Whole mount LacZ staining of the trunk region of E11.5 Tcf-lacZ embryo. Arrows point to the forming mammary glands.

(e) Cross-section through the genital region of E12.5 female *Tcf/lef-lacZ* embryo.

(f) Cross-section through the genital region of E13.5 female *Tcf/lef-lacZ* embryo.

(g) Whole mount LacZ staining of the of E15.5 *Tcf/lef-lacZ* embryo reproductive tract.

(h) Whole mount LacZ staining of the of E16.5 *Tcf/lef-lacZ* embryo reproductive tract.

(i) Whole mount LacZ staining of the of E18.5 *Tcf/lef-lacZ* embryo reproductive tract.

(j) Cross-section through the ovary of E15.5 female *Tcf/lef-lacZ* embryo. Inset is sagital-section through the uterus.

(k) Cross-section through the ovary of E16.5 female *Tcf/lef-lacZ* embryo. Inset is sagital-section through the uterus.

(l) Cross-section through the ovary of E18.5 female *Tcf/lef-lacZ* embryo. Inset is sagital-section through the uterus.

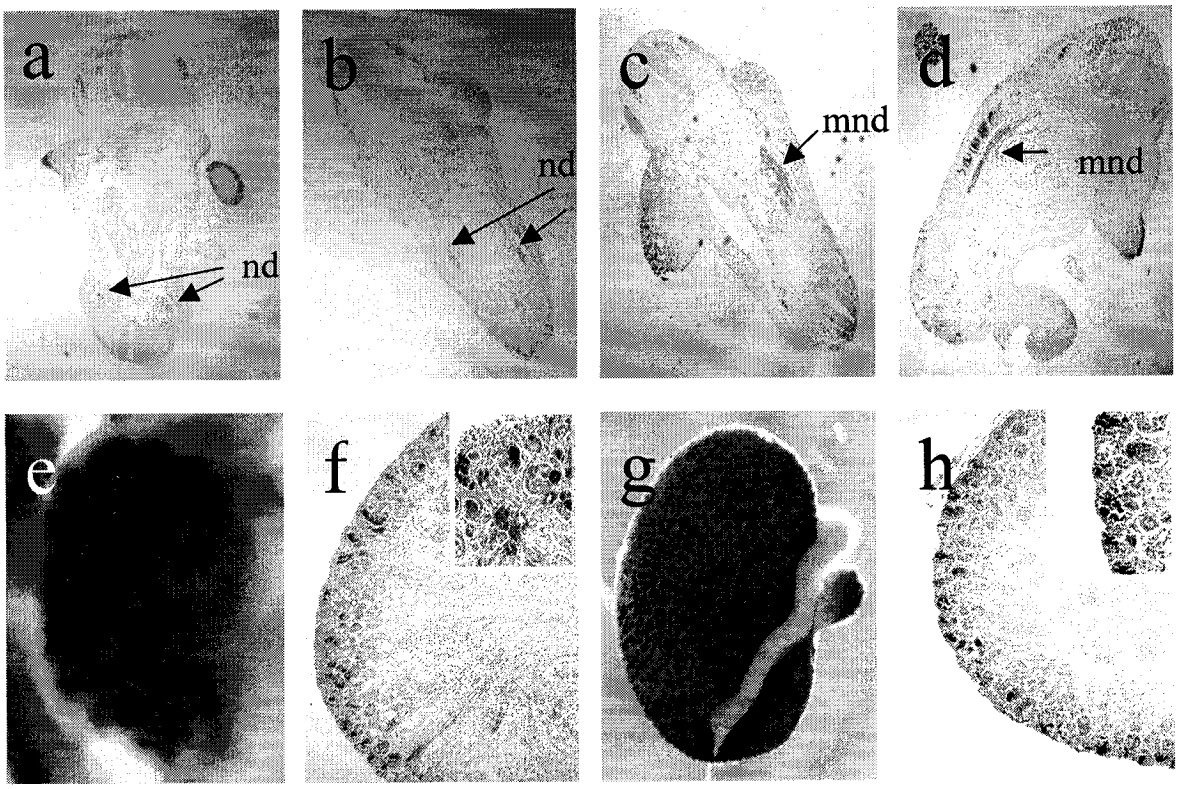
(m) Whole mount LacZ staining of day 7 *Tcf/lef-lacZ* female uterus. Inset is oviduct region. Note the restricted staining in the oviduct region at this time.

(n) Whole mount LacZ staining of day 10 *Tcf/lef-lacZ* female uterus. Inset is sagital-section through the uterus.

(o) Whole mount LacZ staining of day 21 *Tcf/lef-lacZ* female uerus.

Abbreviations: (g = gonad, pmd = paramesonephric duct "region of future oviduct" d = duct "future Wolffian and Mullerian duct", o = ovary, k = kidney, wd = Wolffian duct, md = Mullerian duct, mg = mammary gland, u= uterus, o = ovary, ge = germinal epithelium, ue = uterine epithelium, od = oviduct).

Appendix IX: *Tcf/Lef-lacZ* reporter gene activation during kidney development



Appendix IX legend

(a-d) Transverse sections through E9.5 (a & b), E10.0 (c) and E11.0day (d) Tcf-lacZ embryos.

(e) Whole mount staining of E12.5 *Tcf/lef-lacZ* kidney.

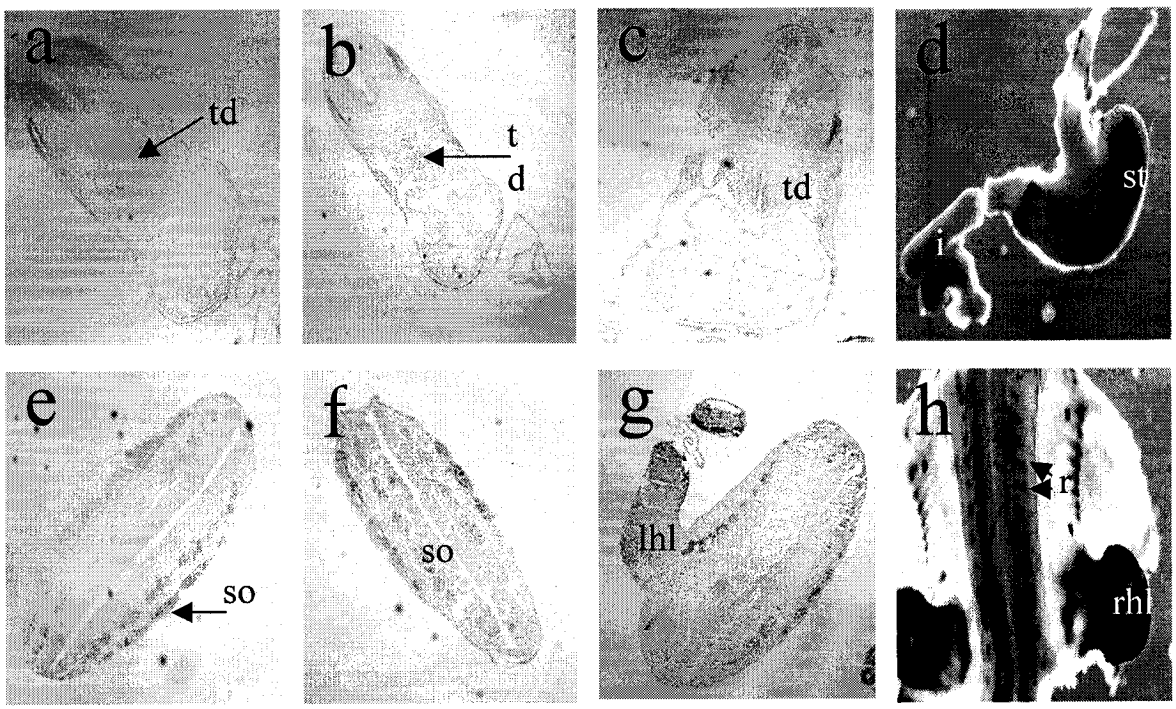
(f) Cross-section of E18.5 *Tcf/lef-lacZ* kidney (10X). Inset is 40X.

(g) Whole mount staining of Day 1 old *Tcf/lef-lacZ* kidney.

(h) Cross-section of Day 1 old *Tcf/lef-lacZ* kidney (10X). Inset is 40X.

Abbreviations: (nd = nephric duct, mnd = mesonephric duct).

Appendix X: *Tcf/Lef-lacZ* reporter gene activation during somitogenesis and gut development



Appendix X legend

(a) Cross-section of E9.5 *Tcf/lef-lacZ* embryo. Arrow points to the ventral bifurcation of the foregut where tracheal diverticulum (laryngo-tracheal groove) is forming.

(b) Cross-section of E10.0 *Tcf/lef-lacZ* embryo.

(c) Cross-section of E11.0 *Tcf/lef-lacZ* embryo.

(d) Whole mount LacZ staining of E12.5 stomach from *Tcf/lef-lacZ* embryo.

(e) Cross-section of E10.0 posterior region of *Tcf/lef-lacZ* embryo. Arrow points to the newly forming somites.

(f) Cross-section of E20.5 posterior region of *Tcf/lef-lacZ* embryo.

(g) Cross-section of E11.0 posterior region of *Tcf/lef-lacZ* embryo.

(h) Whole mount LacZ staining of E11.5 trunk region of *Tcf/lef-lacZ* embryo.

Abbreviations: (td = tracheal diverticulum, i= intestine, st = stomach, so = somite, lhl = left hind limb, rhl = right hind limb, r = rib).

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