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The expression of the imprinted gene *Ipl* is restricted to extra-embryonic tissues and embryonic lateral mesoderm during early mouse development

SALLY L. DUNWOODIE*,[1,2] and ROSA S.P. BEDDINGTON[1,†]

[1]*Department of Mammalian Development, National Institute for Medical Research, London, UK and*
[2]*The Victor Chang Cardiac Research Institute, Sydney, Australia*

ABSTRACT Genes with restricted expression within the developing embryo represent valuable tools as they allow distinct tissue types to be distinguished and studied. In order to identify genes that are expressed within a particular germ layer, a differential screen was performed using germ layer-specific cDNA libraries derived from gastrulation stage mouse embryos. The gene expression profiles of the germ layers were compared following the hybridisation of some 20,000 cDNA clones with probes derived from germ layer-specific *Ectoderm, Mesoderm* and *Endoderm* libraries. A cDNA clone *(50c15)* was identified that hybridised with the *Mesoderm*-derived probe but not *Ectoderm* or *Endoderm*. *50c15* derives from *Ipl/Tssc3/BWR1C,* an imprinted gene which in human maps to chromosome 11p15.5. This region has been associated with Beckwith-Weidemann Syndrome, Wilms' tumour and ovarian, breast and lung cancer. In the gastrulating mouse embryo, wholemount RNA *in situ* hybridisation revealed that *Ipl* expression is restricted not only to the mesodermal germ layer, but specifically to lateral mesoderm and the most posterior extent of the primitive streak from which lateral and extra-embryonic mesoderm is derived. Moreover, *Ipl* is expressed in extra-embryonic tissues prior to gastrulation and afterwards in extra-embryonic mesoderm, ectoderm and endoderm. This expression profile indicates that *Ipl* is a good molecular marker for embryonic mesoderm and extra-embryonic tissues. In addition heterotopic grafting studies indicate that nascent mesoderm, which expresses *Ipl,* is restricted in its potential and therefore may be committed to its fate.

KEY WORDS: *primitive streak, lateral mesoderm, extra-embryonic tissues, cell fate, Ipl*

Introduction

Genes expressed in a restricted manner can be effective tools for molecular embryologists. If expression is restricted to a particular tissue or a distinct cell type then, in the absence of morphological criteria, specific cells can be distinguished on the basis of molecular parameters. The requirement for molecular markers is considerable, as developmental biologists (amongst others) need to be able to distinguish between cells types in order to describe normal developmental events and indeed to interpret the phenotypic effects of mutation.

The complexity of mammalian development is evident in the number and array of cell types that are generated within the embryo, and in extra-embryonic tissues that are essential for the maintenance, nourishment and protection of the foetus within the uterus. These extra-embryonic tissues must be established early in gestation and indeed start developing at the blastocyst stage (3.5 dpc in mouse) with the formation of the inner cell mass (ICM) and trophectoderm cell lineages. (Fig. 1; Gardner, 1983; Rossant, 1986). However it is not a simple case of extra-embryonic tissues being derived from one cell lineage and the embryo from the other. While the trophectoderm only contributes to extra-embryonic derivatives such as trophoblastic giant cells, extra-embryonic ectoderm and the ectoplacental cone, the ICM gives rise to both embryonic and extra-

Abbreviations used in this paper: a, amnion; BWS, Beckwith-Wiedemann syndrome; D, distal; dpc, days post coitum; E, embryonic; eE, extra-embryonic; EB, early allantoic bud; ES, early streak stage; ES cell, embryonic stem cell; EST, expressed sequence tag; g, gut; ICM, inner cell mass; lm, lateral mesoderm; LS, late streak stage; ne, neuroectoderm; nt, neural tube; 0B, no allantoic bud; P, proximal/posterior; PBS, phosphate buffered saline; PFA, paraformaldehyde; plm, presumptive lateral mesoderm; PS, pre streak stage; so, somatic mesoderm; sp, splanchnic mesoderm; ye, endodermal yolksac; ym, mesodermal yolksac.

***Address correspondence to:** Dr. Sally Dunwoodie. Victor Chang Cardiac Research Institute, 384 Victoria Street, Darlinghurst, NSW 2010, Australia. Tel: +612-9295-8513 Fax: +612-9295-8501. e-mail: s.dunwoodie@victorchang.unsw.edu.au

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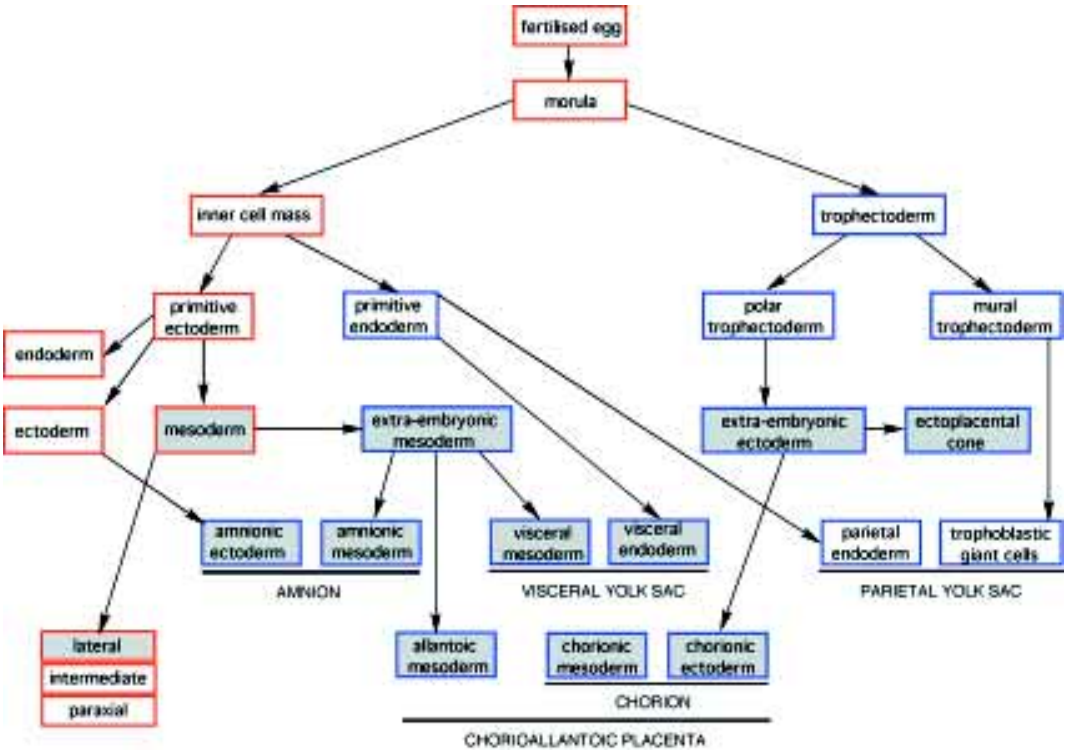

Fig. 1. Summary of tissue lineages that constitute the mouse embryo. *Tissues that give rise to the embryo proper and to extra-embryonic cells are bordered in red, extra-embryonic tissues are bordered in blue and those that express* Ipl *are coloured grey. Adapted from Hogan* et al. *(1984).*

embryonic tissues (Gardner, 1982; Gardner, 1983). At implantation the ICM comprises two tissue lineages: primitive ectoderm (epiblast) and primitive endoderm. During gastrulation the epiblast differentiates into the embryonic germ layers (ectoderm, mesoderm and endoderm), and also gives rise to all extra-embryonic mesoderm (yolk sac, amnion, chorion and allantois) as well as amniotic ectoderm (Gardner and Rossant, 1979). The primitive endoderm colonises the extra-embryonic parietal and visceral endoderm in the yolk sacs surrounding the embryo. The compound nature of extra-embryonic tissues is evident as amnion, chorion and visceral yolk sac are bilayered consisting of mesoderm with either ectoderm or endoderm. Moreover, the complex origin of the chorioallantoic placenta is apparent, as it is comprised of trophectoderm-derived extra-embryonic ectoderm (chorionic ectoderm) and the ICM-derived chorionic mesoderm and allantois (Fig. 1). These tissue lineages result from a hierarchy of decisions considered to be irreversible. Thus all the states that precede terminal differentiation embody a reduction in potency compared with the previous state (Slack, 1983). Mapping the fate of cells has enabled tissue lineages depicted in Figure 1 to be circumscribed, and it is clear that an extensive array of embryonic and extra-embryonic tissues is derived from nascent mesoderm. A fate map has been drawn for nascent mesoderm (Parameswaran and Tam, 1995) but maps such as this do not define states of determination therefore grafting studies are required to address whether cells are irreversibly determined to fulfil their fate. Moreover, in order to distinguish between the progressive states of commitment towards determination, molecular markers specific for each state are required.

To identify novel genes that are expressed in a germ layer-specific manner a series of cDNA libraries were generated from 7.5dpc mouse embryos (Harrison *et al.*, 1995). Libraries were derived from the whole embryonic portion of the conceptus *(Embryonic Region)*, the individual germ layers *(Ectoderm, Mesoderm, Endoderm)* and the avenue during gastrulation through which the mesoderm and endoderm arise *(Primitive Streak)*. Through subtractive hybridisation these libraries have led to the identification of novel genes that are expressed differentially within the developing embryo (Dunwoodie *et al.*, 1997; 1998; Harrison *et al.*, 1995; 2000). Here we report the identification of a cDNA clone *50c15* that was isolated from these libraries using a differential hybridisation approach to identify genes with enriched or specific expression in nascent mesoderm. *50c15* represents the mouse homologue of *IPL/TSSC3/BWR1C (Imprinted in placenta and liver / Tumor suppressing subtransferable candidate 3 / Beckwith-Wiedemann Region 1C)* a gene identified because it lies within a region that is associated with Beckwith-Weidemann Syndrome, Wilms' tumour and ovarian, breast and lung cancer (Hu *et al.*, 1997; Qian *et al.*, 1997; Schwienbacher *et al.*, 1998).

At 7.5 dpc, the developmental stage from which the libraries were derived, *Ipl* expression is restricted to the proximal nascent mesoderm and the posterior primitive streak. As gastrulation proceeds expression is restricted to splanchnic and somatic (lateral) mesoderm which like extra-embryonic mesoderm is derived from the posterior primitive streak. Accordingly, *Ipl* is expressed by nascent extra-embryonic mesoderm and later by the mesodermal component of the amnion, chorion and yolk sac, as well as by the allantois. Unlike in the embryo, the expression of *Ipl* in extra-embryonic tissues is not restricted to mesoderm as it is detected prior to gastrulation in extra-embryonic ectoderm and later in derivatives of extra-embryonic ectoderm and endoderm.

Results

Isolation and Characterisation of the 50c15 *cDNA*

A differential screen was designed to identify in mouse genes that are predominantly expressed in the mesodermal germ layer of the embryo. The *Ectoderm, Mesoderm* and *Endoderm* cDNA

libraries were radioactively labelled and each was hybridised to an identical grid consisting of 20,000 *Embryonic Region* cDNA clones. Two identical *Embryonic Region* cDNA clones, designated *50c15*, hybridised to the *Mesoderm* library but not the *Ectoderm* or *Endoderm*. The relative representation of *50c15* in the *Ectoderm, Mesoderm* and *Endoderm* libraries was determined by Southern blot analysis. Consistent with the screen, *50c15* cDNA was 25 times more abundant in the *Mesoderm* library than the *Endoderm* library and was not detectable in the *Ectoderm* library (Fig. 2A). In addition, *50c15* cDNA was detected in *Primitive Streak* and an undifferentiated *Embryonic Stem Cell* library.

Northern blot analysis using the *50c15* cDNA as a probe showed transcripts were readily detectable in undifferentiated Embryonic Stem (ES) cells, 11.5 dpc embryos (including yolk sac) and 11.5dpc placenta (Fig. 2B). Southern blot analysis showed that the *50c15* cDNA probe hybridised with genomic DNA fragments from human, mouse, dog, chicken, zebrafish, *Xenopus* and *Drosophila* (Fig. 3). Sequence analysis revealed that *50c15* (accession number Y15443) consisted of 721 nucleotides and represented the mouse homologue of *IPL /TSSC3/BWR1C (Imprinted in placenta and liver / Tumor suppressing subtransferable candidate 3 / Beckwith-Wiedemann Region 1C;* Hu *et al.*, 1997; Qian *et al.*, 1997; Schwienbacher *et al.*, 1998). A protein of approximately 16k Daltons was produced following *in vitro* translation using the *50c15* cDNA as template; a size consistent with the cDNA encoding 144 amino acids (data not shown). Ipl contains a pleckstrin homology (PH)-like domain. The PH domain consists of 120 amino acids and is found in many proteins involved in signal transduction or cytoskeletal reorganisation (Shaw, 1996). TDAG51 and Tih1 display the greatest homology to Ipl; TDAG51 contains two PH-like domains while Ipl and Tih1 consist almost entirely of a single PH-like domain (Frank *et al.*, 1999).

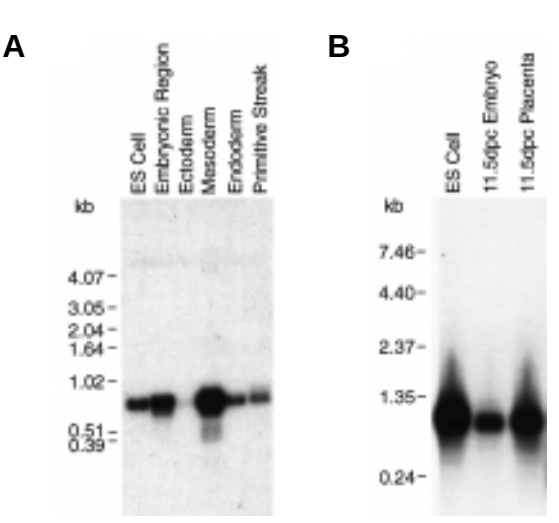

Fig. 2. Library Southern blot analysis and Northern analysis with the *50c15* cDNA as probe. (A) *Detection of* 50c15 *cDNA by Southern blot analysis. The* 50c15 *cDNA (approximately 800 bp) was detected in the* Embryonic Region, Mesoderm, Endoderm *and* Primitive Streak *libraries and in an undifferentiated* Embryonic Stem (ES) Cell *library. The intensity of the hybridisation signal was quantified using the Image Quant program and the following values (corrected for differences in DNA loading) were assigned:* Embryonic Stem Cell, *5;* Embryonic Region, *20;* Ectoderm, *0;* Mesoderm, *100;* Endoderm, *4;* Primitive Streak, *5.* **(B)** *The transcript (approximately 800 nucleotides) was detected in 2.5 μg of polyA(+) RNA isolated from undifferentiated Embryonic Stem (ES) cells, in 11.5 dpc embryos with their yolk sac and in 11.5 dpc placentas.*

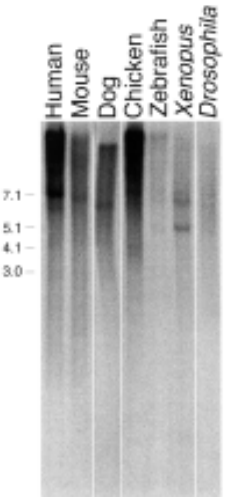

Fig. 3. Genomic Southern Zoo blot analysis. *Hybridisation of the* 50c15 *cDNA probe was detected in human, mouse, dog, chicken, zebrafish,* Xenopus *and* Drosophila.

Ipl *is expressed in Embryonic and Extra-Embryonic Tissues*

RNA *in situ* hybridisation was performed in wholemount using the 5*0c15* cDNA as a probe in order to localise *Ipl* transcripts. *Ipl* was expressed predominantly in extra-embryonic tissues derived from both the trophectoderm and the ICM while within the embryo expression was restricted to the posterior primitive streak and lateral mesoderm. Prior to gastrulation *Ipl* expression was restricted to extra-embryonic ectoderm and the ectoplacental cone (Fig. 4 A,E). During the initial stages of gastrulation mesoderm is induced at the embryonic/extra-embryonic junction where it delaminates from the epiblast through the primitive streak and spreads into both extra-embryonic and embryonic regions of the conceptus. *Ipl* was expressed at the early primitive streak stage in the first mesoderm induced during gastrulation (Fig. 4B) and by the late primitive streak stage was clearly expressed in both embryonic and extra-embryonic nascent mesoderm (Fig. 4 C,F,G). Although at this stage mesoderm in the embryonic region surrounds the entire epiblast, *Ipl* is expressed only in proximal/posterior mesoderm (Fig. 4C). Fate mapping studies demonstrate that this mesoderm will become lateral embryonic mesoderm and extra-embryonic mesoderm (Parameswaran and Tam, 1995). By the headfold stage (8 dpc), expression was restricted to the posterior primitive streak from which lateral and extra-embryonic mesoderm are derived (Beddington, 1982; Lawson *et al.*, 1991; Tam and Beddington, 1987). Expression of *Ipl* was also evident in most extra-embryonic tissues of the conceptus. The level of expression appeared highest in the ectoplacental cone, the chorion and the allantois but this may be due to the greater cell density of these tissues compared to the visceral yolk sac and the amnion (Fig. 4 D,H; data not shown).

This pattern of *Ipl* expression persisted at the 4 somite stage, expression being maintained in extra-embryonic tissues and in embryonic lateral mesoderm (Fig. 4 I,J). In wholemount it appeared that *Ipl* was expressed at elevated levels at the junction between the lateral mesoderm of the embryo and the extra-embryonic region. Transverse sections confirmed the expression of *Ipl* in both splanchnic and somatic lateral mesoderm, and showed a greater

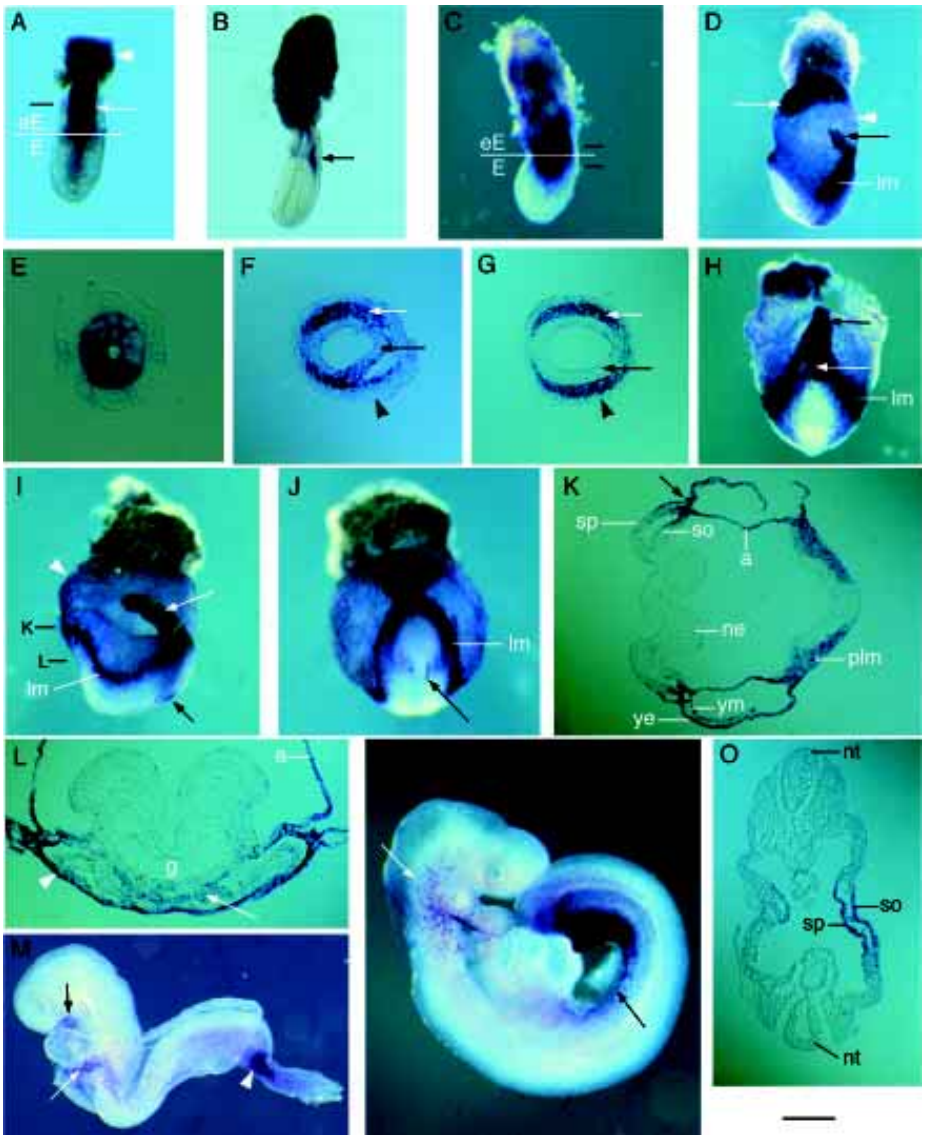

Fig. 4. *Ipl* transcript accumulation between 5.5 and 9.5dpc of mouse development. (A) *Lateral view of 5.5 dpc embryo shows* Ipl *transcripts localised to the ectoplacental cone (white arrow head) and the extra-embryonic ectoderm (white arrow). A line indicates the embryonic (E) and extra-embryonic (eE) boundary.* **(B)** *Lateral view of an early primitive streak stage embryo (posterior to right).* Ipl *transcripts localised to the ectoplacental cone and the first mesoderm to arise from the primitive streak (arrow).* **(C)** *Full length primitive streak stage embryo (posterior to right).* Ipl *transcripts are localised to nascent extra-embryonic mesoderm and proximoposterior nascent embryonic mesoderm. A line indicates the embryonic (E) and extra-embryonic (eE) boundary.* **(D)** *Lateral view of head fold stage embryo (posterior to right).* Ipl *transcripts were localised to all extra-embryonic tissues; ectoplacental cone, chorion (white arrow), yolk sac (white arrow head), allantois (black arrow) and amnion plus presumptive lateral mesoderm (lm).* **(E)** *Transverse section of embryo in (A); level of section indicated by solid black line.* Ipl *transcripts were localised to the extra-embryonic ectoderm but not the visceral endoderm.* **(F)** *Transverse section (posterior to right) in the extra-embryonic region of embryo in (C); the level of the section indicated by solid black line.* Ipl *transcripts were localised to the nascent extra-embryonic mesoderm (white arrow) just detected in the extra-embryonic ectoderm (black arrow) but not detected in the extra-embryonic visceral endoderm (black arrow head).* **(G)** *Transverse section (posterior to right) in the embryonic region of embryo in (C); level of section indicated by solid black line.* Ipl *transcripts were localised to the nascent embryonic mesoderm (white arrow), but not to the ectoderm (black arrow) or endoderm (black arrow head).* **(H)** *Posterior view of a head fold stage embryo, similar stage to the embryo in (D).* Ipl *transcripts were localised to the allantois (black arrow), the posterior primitive streak (white arrow) and the presumptive lateral mesoderm (lm).* **(I)** *and* **(J)** *The same 4 somite stage embryo lateral view (posterior to right) and posterior view, respectively.* Ipl *transcripts were localised to the ectoplacental cone, yolk sac (white arrow head), lateral mesoderm (lm), in bilateral strips extending from the posterior lateral mesoderm towards the node and the ventral node (black arrow). Expression around the node in (J) appears asymmetric (black arrow).* **(K)** *Transverse section of embryo in (I), the level of the section indicated by solid line.* Ipl *transcripts are localised to the presumptive lateral mesoderm (plm), splanchnic (sp) and somatic (so) lateral mesoderm, the amnion (a), both the endodermal (ye) and mesodermal (ym) components of the yolk sac. Note that transcript accumulation is greatest at the embryonic/extra-embryonic junction (black arrow). Neuroectoderm (ne).* **(L)** *Transverse section of embryo in (I), the level of the section indicated by solid line.* Ipl *transcripts were detected throughout the anterior lateral splanchnic mesoderm (white arrow) which is the presumptive cardiac mesoderm. Gut (g), amnion (a), endoderm (arrow head).* **(M)** *Lateral view of an 8 somite stage embryo (posterior to right).* Ipl *transcripts are localised to the base of the allantois (arrow head) and 3 stripes along the pericardium (black arrow). Expression to the posterior of the heart is due to attached yolk sac (white arrow).* **(N)** *Lateral view of a 9.5 dpc embryo.* Ipl *transcripts are localised to posterior lateral mesoderm (black arrow) and to surface ectoderm over the hindbrain and branchial arches (white arrow).* **(O)** *Transverse section (anterior to the top) of embryo in (N), the level of the section indicated by two lines.* Ipl *transcripts are localised to the splanchnic (sp) and somatic (so) mesoderm. Neural tube (nt). Scale bar: A, 150 μm; B, 225 μm; C, 200 μm; D,E, 220 μm; F,G, 100 μm; H, 170 μm; I,J, 280 μm; K, 170 μm; L, 700 μm; M, 300 μm; N, 385 μm; O, 290 μm.*

density of *Ipl* transcript accumulation at the junction of this lateral mesoderm with the visceral yolk sac and the amnion (Fig. 4K). More rostrally, *Ipl* transcripts were detected in the presumptive cardiac mesoderm, a derivative of splanchnic mesoderm (Fig. 4L). A new domain of expression was also apparent which extended from the nascent lateral mesoderm to the node. Transcripts were also present in ventrolateral cells of the node (Fig. 4 I,J). *Ipl* continued to be expressed in extra-embryonic tissues until at least 14.5 dpc (data not shown). In the embryo, *Ipl* expression continued in posterolateral mesoderm and was evident at the base of the allantois until at least 9.5 dpc (Fig. 4 M,N,O). Up until 8 dpc expression of *Ipl* within the embryo was largely restricted to lateral mesoderm, but from 8.5 dpc additional sites of expression in the pericardium and cranial surface ectoderm were detected. *Ipl* was not expressed throughout the pericardium but rather in finger-like streams of cells projecting from where the pericardium abutted the

surface ectoderm (Fig. 4M). *lpl* expression in cranial surface ectoderm appeared patchy and specifically elevated in the vicinity of the branchial arches (Fig. 4N).

Is Nascent Proximal/Posterior Mesoderm irreversibly Determined to Achieve its Fate?

The posterior primitive streak expresses *lpl* (Fig. 4H). Fate mapping studies show that when posterior primitive streak cells are labelled, they give rise to lateral embryonic mesoderm and to extra-embryonic mesoderm (Beddington, 1982, 1994; Smith *et al.*, 1994; Tam and Beddington, 1987). The nascent mesoderm that will give rise to these two distinct lineages is located in the proximal/posterior position in primitive streak stage embryos (Parameswaran and Tam, 1995). Consistent with this *lpl* is expressed in proximal/posterior nascent mesoderm and later by lateral embryonic mesoderm and extra-embryonic mesoderm (Fig. 4). This suggests that lateral and extra-embryonic mesoderm express *lpl* upon induction and that expression continues through the nascent mesoderm stage to and beyond differentiation. Does this mean that nascent mesoderm in the proximal/posterior aspect of the embryo can only form lateral embryonic mesoderm and extra-embryonic mesoderm? That is, is it irreversibly determined to fulfil its fate? Or does it have greater potential, which would allow it to differentiate into other mesodermal tissue types? Cell fate can be determined by grafting cells from a specific site in 'marked' donor embryos to various sites in host embryos and determining which tissues are colonised by the grafted cells. Grafting cells to the same site will reveal their natural fate; grafting these cells to another (heterotopic) site addresses if they are committed to this fate. In this case if grafted cells become fully integrated into differentiated tissues of the host and behave like host cells, then they are not irreversibly determined to achieve their fate. If, however, they are unable to integrate into host tissue, this indicates that they are committed to their fate. Nascent mesoderm was taken from the proximal/posterior position (from late primitive streak stage embryos) and grafted to a heterotopic (distal) site in host embryos. After 24 hours in culture, only rarely did donor cells integrate into their new environment. They were never seen to integrate into paraxial mesoderm of the head or trunk while integration did occur in some instances into intermediate and lateral mesoderm (Fig. 5 F,H,J,L and Table 1). As a control, orthotopic grafting of proximal/posterior nascent mesoderm resulted in donor cell integration into lateral mesoderm and extra-embryonic tissues confirming the fate of these cells (Fig. 5 A,C,D; Table 1; Parameswaran and Tam, 1995). These data suggest that proximal/posterior nascent mesoderm has reduced potential (in this context) and so may be committed to its fate as it cannot integrate into paraxial mesoderm. We next determined if nascent mesoderm from another (distal) site would also display this restricted potential when grafted to a heterotopic location. When distal nascent mesoderm was introduced to the proximal/posterior position they were able to integrate, without exception, into lateral mesoderm and into the differentiated components of the extra-embryonic mesodermal, yolk sac, amnion and allantois (Fig. 5 B,E and Table 1). Orthotopic grafting of distal nascent mesoderm was also performed and showed that donor cells integrated into paraxial mesoderm (the predominant site of integration) as well as into intermediate and lateral sites confirming the fate of these cells (Fig. 5 G,I,K,L, Table 1 and) (Parameswaran and Tam, 1995). These data indicate that distally located nascent mesoderm is not restricted to its fate and therefore does not represent tissue that is irreversibly committed.

Discussion

Does lpl *show Lineage-Restricted Expression in Mesoderm?*

Derivatives of embryonic mesoderm are many and diverse and include tissues such as striated muscle, bone, genital ridges and kidney. Nascent embryonic mesoderm, from which these tissues

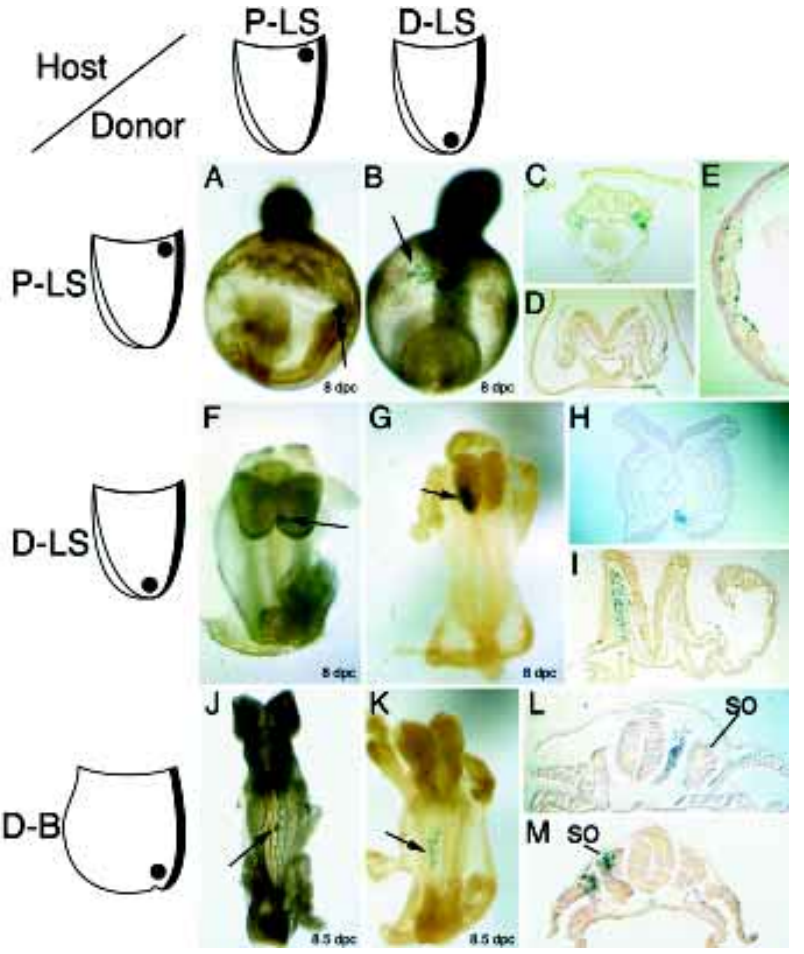

Fig. 5. Distribution of donor-derived cells following grafts to orthotopic and heterotopic sites. *The location of donor cells, and in hosts the graft position are indicated in diagrams of late primitive streak stage (LS) and no allantoic bud/early allantoic bud (B) embryos and designated proximal/posterior (P) or distal (D).* **(A,C,D)** *Proximal/posterior mesoderm grafted to the proximal/posterior site integrates into the base of the allantois (C) as well as somatic and splanchnic mesoderm of the embryo (D).* **(B,E)** *Distal mesoderm grafted to the proximal/posterior location integrates into the endothelial cells (mesoderm) of the visceral yolksac (E).* **(F,H,J,L)** *Proximal/posterior mesoderm grafted to the distal site does not integrate into paraxial mesoderm of the head (F,H) or trunk (J,L).* **(J,I,K,M)** *Distal mesoderm grafted to the distal site integrates into paraxial mesoderm of the head (G,I) or trunk (K,M). Scale bar: A,B, 350 μm; C,100 μm; D 375 μm; E, 60 μm; F, 210 μm; G,J,K, 222 μm; I, 395 μm; M,L, 885 μm.*

are derived, is morphologically homogeneous and questions concerning how diversity is achieved and when it is instigated remain. It is clear that nascent mesoderm is regionalised with respect to fate since discrete domains give rise to distinct mesodermal tissue types (Parameswaran and Tam, 1995). The first mesoderm to be induced (proximal mesoderm) gives rise to extra-embryonic mesoderm and as gastrulation proceeds nascent mesoderm distal to this is fated to become embryonic lateral, and then paraxial. By the late primitive streak stage the extra-embryonic mesoderm has been displaced into the extra-embryonic cavity while the embryonic wings of nascent mesoderm can be divided into regions that will give rise to lateral (proximal/posterior), cardiac (proximal/anterior) and paraxial (distal). It is likely that before morphological distinctions are apparent, molecular heterogeneity arises within this nascent tissue. Here we report that *Ipl* is expressed in the posterior primitive streak, proximal/posterior nascent mesoderm that will give rise to extra-embryonic and embryonic lateral mesoderm, and then in differentiated extra-embryonic and lateral embryonic mesodermal tissue. It is tempting to speculate that *Ipl* expression acts as a lineage tracer for this subset of mesoderm. This issue could be addressed with the use of a dual transgene system that irreversibly marks cells that have expressed *Ipl*, such a system has been described by Chai *et al.* (2000).

Is Proximal/Posterior Nascent Mesoderm irreversibly Committed to its Fate?

Orthotopic grafting experiments show that nascent mesoderm in the proximal/posterior region is capable of being manipulated, and of integrating fully into mesoderm of host embryos (Fig. 5 B,E and Table 1). However when transplanted to a more distal site, proximal/posterior mesoderm which expresses *Ipl,* is unable to contribute to paraxial mesoderm of either the head or trunk (Fig 5. F,H,J,L and Table 1). This indicates that inherent differences exist between proximal/posterior and distal nascent mesoderm. It is unclear whether this difference represents partial commitment to fate or complete commitment such that the cells are irreversible determination. A true test of irreversible determination requires that the grafted cells display characteristic morphology indicative of a fully determined state that is, the formation of differentiated tissue. This was not observed and the grafted cells remained clumped. It is more likely that these cells are not irreversibly determined but rather partially committed to their fate. One measure of this would be the persistent expression of *Ipl*. However, due to technical difficulties we were unable to assay for gene expression in the *lacZ*-positive grafted tissue following embryo culture. Nevertheless, it is clear that nascent mesoderm, that expresses *Ipl,* is in part committed to its fate since a difference exists between nascent mesoderm in proximal/posterior and distal sites.

TABLE 1

THE CONTRIBUTION OF GRAFTED CELLS TO EMBRYONIC TISSUES IN HOST EMBRYOS

	donor cell integration								
	extra-embryonic mesoderm				embryonic mesoderm				
Host /Donor	yolk sac	amnion	allantois	no	lateral	intermediate	paraxial	no	embryos
P to P	6	2	5	0	4[1]	0	0	0	9
D to P	7	1	1	1[2]	3[3]	0	0	0	9
D to D	0	0	0	0	8[4]	3[5]	16[6]	1[7]	17
P to D	0	0	0	0	4[8]	2[9]	0	12[10]	21

(1) 2x somatic mesoderm, 2x splanchnic mesoderm; (2) base of allantois; (3) splanchnic mesoderm; (4) 6x head, 2x trunk; (5) trunk; (6) 12x head, 4x trunk; (7) 1x trunk adjacent to intermediate mesoderm; (8) 2x head, 2x trunk; (9) trunk; (10) head: 1x lateral mesoderm, 3x paraxial mesoderm. trunk: 1x lateral mesoderm, 5x paraxial mesoderm, 2x dorsal to neural tube. (P) proximal/posterior and (D) distal nascent mesoderm. "no" indicates that grafted cells remained clumped and did not integrate into host embryos.

Ipl *Expression may be Required for the Development and Function of Extra-Embryonic Tissues*

Our expression analysis of *Ipl* begins at 5.5 dpc and this appears to be the earliest stage at which expression can be detected, as no *Ipl*-derived EST's are present in the database prior to this time. Although *Ipl* is almost exclusively restricted to lateral mesoderm in the embryo, expression occurs in all extra-embryonic tissues from the headfold stage (Fig. 4D). Extra-embryonic tissues are bilayered (except for the allantois) and consist of mesoderm and either ectoderm or endoderm Activation of *Ipl* in extra-embryonic tissues occurs at different developmental stages such that expression is first detected in extra-embryonic ectoderm at 5.5 dpc prior to the onset of gastrulation (Fig. 4 A,E). Following mesoderm induction *Ipl* is next expressed in presumptive extra-embryonic mesoderm (Fig. 4 B,C,F,G) and then later in endoderm of the visceral yolk sac by the neural plate stage (Fig. 4D). The different developmental stages at which *Ipl* expression is induced in extra-embryonic tissue suggests that the timing of this event is not co-ordinated. Since *Ipl* is expressed in all extra-embryonic tissue it does suggest that it may be intrinsic to the development and/or function of these tissues.

Ipl, *Imprinting and Foetal Growth*

IPL maps to human chromosome 11p15.5 and in mouse this gene maps to a syntenic region on chromosome 7. These chromosomal regions contain several known imprinted genes and *IPL* is imprinted in both human and mouse, the maternal allele showing higher levels of expression (Qian *et al.*, 1997). In human, the imprinting syndrome Beckwith-Wiedemann syndrome (BWS; OMIM 130650) maps to chromosome 11p15.5 and deregulation of imprinting in this region is implicated in this disease (Mannens and Wilde, 1997). BWS is characterised by neonatal gigantism, overgrowth of organs such as liver and kidney, and an increased risk of developing childhood tumours. It is possible that *IPL* plays a role in the development of this disorder since in humans it is expressed in kidney and liver in both the foetus and adult as well as in the placenta (Frank *et al.*, 1999; Qian *et al.*, 1997).

Many of the imprinted genes that map to human chromosome 11p15.5 affect growth. *IGF2* is an important foetal growth factor which is paternally expressed (DeChiara *et al.*, 1990; 1991; Ferguson-Smith *et al.*, 1991). *CDKNIC* is maternally expressed and exerts a negative effect on cell proliferation, while *H19* is a maternally expressed gene which can act as a tumour suppressor (Bartolomei *et al.*, 1991; Hao *et al.*, 1993; Lee *et al.*, 1995; Matsuoka *et al.*, 1995). It is possible that *IPL* may also be involved in growth regulation in addition to being implicated in BWS. *IPL* maps within the tumour-suppressing subchromosomal fragment from 11p15.5 (Hu *et al.*, 1997). It also shares considerable homology with TDAG51, which can induce Fas expression and Fas-mediated apoptosis of T cell hybridoma cells (Park *et al.*, 1996). Furthermore, approximately 50% of advanced breast cancer in humans shows loss of heterozygosity of 11p15.5 and defective Fas-mediated apoptosis has been reported in some breast cancer cell lines (Park *et al.*, 1996; Winqvist *et al.*, 1995). Thus *IPL* may be required for normal Fas expression and so act as a negative regulator of growth in some circumstances.

If *IPL* were a negative regulator of growth its expression pattern and imprint would be consistent with the maternal/paternal conflict model of Moore and Haig (1991). The conflict model postulates that the more resources an embryo acquires from its mother, the larger it is at birth and the more likely it is to survive and reproduce, a situation, which benefits the father. However, the greater the nutrient demands of the pregnancy, the more detrimental this will be to the potential future reproduction to the mother. This model is based on a previous proposal that imprinting has evolved in mammals to restrain the proliferative growth of the placenta and it is well documented that there is a correlation between size of the placenta and size of the embryo (Hall, 1990). Therefore, the prediction from the model is that preferential paternal expression will function to increase the nutrient demands on the mother, while preferential maternal expression reduces those demands. Therefore, maternal expression of *IPL*, if it were a negative regulator of growth, would tend to reduce placenta size and thus alleviate nutritional demands on the mother. The expression of *Ipl* in extra-embryonic tissues in the mouse may contribute to regulating the size of the placenta from extremely early developmental stages.

Materials and Methods

Differential cDNA Library Screen

cDNA inserts from the *Ectoderm, Mesoderm* and *Endoderm* libraries were isolated from the pSPORT1 plasmid, α^{32}P dCTP-labelled and hybridised to identical arrays of 20,000 cDNA clones from the *Embryonic Region* library according to Harrison *et al.* (1995).

Southern and Northern Blot Analysis

cDNA library Southern blot analysis: cDNA inserts were released from pSPORT1 using *Not*I/*Sal*I restriction enzymes, 1µg of DNA was size fractionated in 1% agarose, transferred to nylon membrane (Hybond-N+; Amersham) and cross-linked to the membrane by exposure to UV light. The membrane was hybridised at 65ºC in a sodium phosphate buffer (Church and Gilbert, 1984) with a probe concentration of 1x10^6 cpm/ml hybridisation buffer and the filter was washed at 65ºC in 0.2xSSC, 0.1% SDS.

Genomic DNA Southern blot: Genomic DNA was isolated using standard methods and digested with *Eco*RI, size fractionated in 0.8% agarose and transferred and fixed to hybridisation membrane (Hybond N+; Amersham). The membrane was hybridised at 37ºC in 20% formamide, 5xSSC, 5X Denhardt's and washed at 50ºC in 2xSSC, 0.1 %SDS. The entire *Ipl* cDNA Fragment was released from pSPORT1 with *Not*I/*Sal*I restriction enzymes, gel purified by QIAEX gel extraction (QIAGEN) and α^{32}P dCTP-labelled using a Random-Primed DNA Labelling Kit (Roche).

Northern blot analysis: Total RNA was isolated from 11.5 dpc embryos and yolk sac, 11.5 dpc placenta and from CGR8 ES cells (Wilson *et al.*, 1993) according to Chomczynski and Sacchi (1987). Poly(A)+ RNA was isolated using the polyA Tract system (Promega). The RNA was denatured and size fractionated (1% agarose, 2.2M formaldehyde) and transferred to nylon membrane (Hybond N+; Amersham). The membrane was hybridised and washed as described for the cDNA library Southern.

Embryo Recovery

Embryos were collected from timed C57BL6 x DBA matings. Noon on the day of appearance of the vaginal plug was designated 0.5 dpc. Embryos were dissected from the uterus and Reichert's membrane removed as described by Beddington (1987) in M2 medium (Hogan *et al.*, 1984) containing 10% foetal calf serum (Advanced Protein Products) instead of bovine serum albumin. Embryos for wholemount RNA *in situ* hybridisation were rinsed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS. Embryos for RNA isolation were immediately dissolved in denaturing solution and RNA isolated according to Chomczynski and Sacchi (1987).

RNA In Situ *Hybridisation to Mouse Embryos*

Antisense riboprobes were derived from the full length *Ipl* cDNA. Wholemount RNA *in situ* hybridisation was carried out as previously described (Dunwoodie *et al.*, 1997). The length of proteinase K treatment varied; 5.5 - 6.5 dpc (5 minutes), 7.0 - 8.5 dpc (10 minutes), 9.0 dpc and older (15 minutes.) Embryos were post-fixed in 4% PFA, 0.1% glutaraldehyde in PBS.

Cell Grafting, Embryo Culture and Analysis of Transgene Expression

Gastrulating mouse embryos were collected at 7.5 dpc and staged according to Downs and Davies (1993). Donor cells were dissected from proximal/posterior nascent mesoderm at the late streak stage (LS) from a gene trap mouse line *(Pty)* that expresses the *lacZ* transgene in all cells of the embryo (Skarnes *et al.*, 1995). Host embryos at neural plate stages (0B/EB) were derived from C57BL6 x DBA matings. Nascent mesoderm was dissected from donor embryos as described in Harrison *et al.* (1995). Mesoderm to be grafted was then isolated from the rest of the nascent mesoderm using fine glass needles, dissected into clumps of approximately 15-30 cells, grafted by hand into the proximal/posterior or distal nascent mesoderm and cultured for 24 hours as described previously (Beddington, 1982). The colonisation pattern of donor cells was visualised after staining for β-galactosidase activity as described by Beddington (1994).

Embryo Sections

After wholemount RNA *in situ* hybridisation, embryos were processed for paraffin sectioning by dehydration through an ethanol series, cleared in Histoclear (National Diagnostics) and embedded in paraffin wax (Histoplast, m.p. 56ºC). Sections (7 µm) were dewaxed in Histoclear (5 minutes) and mounted under coverslips in DPX mountant (BDH).

Photography

Low power photographs were taken using a dissecting microscope (Nikon), higher power photographs were taken in a compound microscope (Zeiss Axiophot) using tungsten film (Kodak 64T).

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References

BARTOLOMEI, M.S., ZEMEL, S. and TILGHMAN, S.M. (1991). Parental imprinting of the mouse H19 gene. *Nature* 351: 153-155.

BEDDINGTON, R.S.P. (1982). An autoradiographic analysis of tissue potency in different regions of the embryonic ectoderm during gastrulation in the mouse. *J Embryol Exp Morphol* 69: 265-285.

BEDDINGTON, R.S.P. (1994). Induction of a second neural axis by the mouse node. *Development* 120: 613-620.

BEDDINGTON, R.S.P. (1987). Isolation, culture and manipulation of post-implantation mouse embryos. In *Mammalian Development*: A practical approach. (Eds. Monk, M.) Oxford IRL Press. Oxford, pp. 43-70.

CHAI, Y., JIANG, X., ITO, Y., BRINGAS, P., JR., HAN, J., ROWITCH, D.H., SORIANO, P., MCMAHON, A.P. and SUCOV, H.M. (2000). Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* 127: 1671-1679.

CHOMCZYNSKI, P. and SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal Biochem* 162: 156-159.

CHURCH, G.M. and GILBERT, W. (1984). Genomic sequencing. *Proc Natl Acad Sci USA* 81: 1991-1995.

DECHIARA, T.M., EFSTRATIADIS, A. and ROBERTSON, E.J. (1990). A growth-deficiency phenotype in heterozygous mice carrying an insulin- like growth factor II gene disrupted by targeting. *Nature* 345: 78-80.

DECHIARA, T.M., ROBERTSON, E.J. and EFSTRATIADIS, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 64: 849-859.

DOWNS, K.M. and DAVIES, T. (1993). Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* 118: 1255-1266.

DUNWOODIE, S.L., HENRIQUE, D., HARRISON, S.M. and BEDDINGTON, R.S.P. (1997). Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. *Development* 124: 3065-3076.

DUNWOODIE, S.L., RODRIGUEZ, T.A. and BEDDINGTON, R.S.P. (1998). Msg1 and Mrg1, founding members of a gene family, show distinct patterns of gene expression during mouse embryogenesis. *Mech Dev* 72: 27-40.

FERGUSON-SMITH, A.C., CATTANACH, B.M., BARTON, S.C., BEECHEY, C.V. and SURANI, M.A. (1991). Embryological and molecular investigations of parental imprinting on mouse chromosome 7. *Nature* 351: 667-670.

FRANK, D., MENDELSOHN, C.L., CICCONE, E., SVENSSON, K., OHLSSON, R. and TYCKO, B. (1999). A novel pleckstrin homology-related gene family defined by Ipl/Tssc3, TDAG51, and Tih1: tissue-specific expression, chromosomal location, and parental imprinting. *Mamm Genome* 10: 1150-1159.

GARDNER, R.L. (1982). Investigation of cell lineage and differentiation in the extraembryonic endoderm of the mouse embryo. *J Embryol Exp Morphol* 68: 175-198.

GARDNER, R.L. (1983). Origin and differentiation of extraembryonic tissues in the mouse. *Int Rev Exp Pathol* 24: 63-133.

GARDNER, R.L. and ROSSANT, J. (1979). Investigation of the fate of 4-5 day post-coitum mouse inner cell mass cells by blastocyst injection. *J Embryol Exp Morphol* 52: 141-152.

HALL, J.G. (1990). Genomic imprinting: review and relevance to human diseases. *Am J Hum Genet* 46: 857-873.

HAO, Y., CRENSHAW, T., MOULTON, T., NEWCOMB, E. and TYCKO, B. (1993). Tumour-suppressor activity of H19 RNA. *Nature* 365: 764-767.

HARRISON, S.M., DUNWOODIE, S.L., ARKELL, R.M., LEHRACH, H. and BEDDINGTON, R.S.P. (1995). Isolation of novel tissue-specific genes from cDNA libraries representing the individual tissue constituents of the gastrulating mouse embryo. *Development* 121: 2479-2489.

HARRISON, S.M., HOUZELSTEIN, D., DUNWOODIE, S.L. and BEDDINGTON, R.S.P. (2000). Sp5, a new member of the Sp1 family, is dynamically expressed during development and genetically interacts with Brachyury. *Dev Biol* 227: 358-372.

HOGAN, B., BEDDINGTON, R., CONSTANTININ, F. and LACY, E. (Ed.) (1984). *Manipulating the mouse embryo: a laboratory manual.* Cold Spring Harbor Laboratory Press.

HU, R.J., LEE, M.P., CONNORS, T.D., JOHNSON, L.A., BURN, T.C., SU, K., LANDES, G.M. and FEINBERG, A.P. (1997). A 2.5-Mb transcript map of a tumor-suppressing subchromosomal transferable fragment from 11p15.5, and isolation and sequence analysis of three novel genes. *Genomics* 46: 9-17.

LAWSON, K.A., MENESES, J.J. and PEDERSEN, R.A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* 113: 891-911.

LEE, M.H., REYNISDOTTIR, I. and MASSAGUE, J. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev* 9: 639-649.

MANNENS, M. and WILDE, A. (1997). KVLQT1, the rhythm of imprinting. *Nat Genet* 15: 113-115.

MATSUOKA, S., EDWARDS, M.C., BAI, C., PARKER, S., ZHANG, P., BALDINI, A., HARPER, J.W. and ELLEDGE, S.J. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev* 9: 650-662.

MOORE, T. and HAIG, D. (1991). Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet* 7: 45-49.

PARAMESWARAN, M. and TAM, P.P. (1995). Regionalisation of cell fate and morphogenetic movement of the mesoderm during mouse gastrulation. *Dev Genet* 17: 16-28.

PARK, C.G., LEE, S.Y., KANDALA, G. and CHOI, Y. (1996). A novel gene product that couples TCR signaling to Fas(CD95) expression in activation-induced cell death. *Immunity* 4: 583-591.

QIAN, N., FRANK, D., O'KEEFE, D., DAO, D., ZHAO, L., YUAN, L., WANG, Q., KEATING, M., WALSH, C. and TYCKO, B. (1997). The IPL gene on chromosome 11p15.5 is imprinted in humans and mice and is similar to TDAG51, implicated in Fas expression and apoptosis. *Hum Mol Genet* 6: 2021-2029.

ROSSANT, J. (1986). Development of extraembryonic cell lineages in the mouse embryo. In *Experimental approaches to mammalian embryonic development*, (Eds. Rossant, J. and Pedersen, R.A.). Cambridge University Press, Cambridge, pp.97-120.

SCHWIENBACHER, C., SABBIONI, S., CAMPI, M., VERONESE, A., BERNARDI, G., MENEGATTI, A., HATADA, I., MUKAI, T., OHASHI, H., BARBANTI-BRODANO, G., CROCE, C.M. and NEGRINI, M. (1998). Transcriptional map of 170-kb region at chromosome 11p15.5: identification and mutational analysis of the BWR1A gene reveals the presence of mutations in tumor samples. *Proc Natl Acad Sci USA* 95: 3873-3878.

SHAW, G. (1996). The pleckstrin homology domain: an intriguing multifunctional protein module. *Bioessays* 18: 35-46.

SKARNES, W.C., MOSS, J.E., HURTLEY, S.M. and BEDDINGTON, R.S. (1995). Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc Natl Acad Sci USA* 92: 6592-6596.

SLACK, J.M.W. (1983). The concepts of experimental embryology. In *From egg to embryo. Determinative events in early embryology*, (Eds. Slack, J.M.W.). Cambridge University press, Cambridge, pp. 11-30.

SMITH, J.L., GESTELAND, K.M. and SCHOENWOLF, G.C. (1994). Prospective fate map of the mouse primitive streak at 7.5 days of gestation. *Dev Dyn* 201: 279-289.

TAM, P.P. and BEDDINGTON, R.S.P. (1987). The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development* 99: 109-126.

WILSON, V., RASHBASS, P. and BEDDINGTON, R.S.P. (1993). Chimeric analysis of T (Brachyury) gene function. *Development* 117: 1321-1331.

WINQVIST, R., HAMPTON, G.M., MANNERMAA, A., BLANCO, G., ALAVAIKKO, M., KIVINIEMI, H., TASKINEN, P.J., EVANS, G.A., WRIGHT, F.A. and NEWSHAM, I. (1995). Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. *Cancer Res* 55: 2660-2664.