Instituto Juan March
de Estudios e Investigaciones

120 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Common Molecules in Development and Carcinogenesis

Organized by

M. Takeichi and M. A. Nieto

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A. Cano
M. W. J. Ferguson
T. Gridley
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INTRODUCTION

M. Angela Nieto and Masatoshi Takeichi

Recent findings emerging from different research fields are reinforcing the idea that the same molecules and mechanisms operate during embryonic development and in the adult, under both physiological and pathological conditions.

In this workshop, held at the Juan March Foundation between February 26-28, 2001, a total of 50 scientists, including speakers, gathered to exchange ideas regarding the molecules and signalling pathways that are common to development and cancer. The link between development and tumorigenesis is stronger than ever, and may open new avenues in cancer research owing to the availability of experimentally amenable systems.

Different topics were covered in this workshop, and particular attention was paid to the molecules and signalling pathways triggering one of the key processes in morphogenesis and tumour progression: the Epithelial-Mesenchymal Transition (EMT). This process involves a dramatic change in cell phenotype by which a well-differentiated and polarised epithelial cell is converted into a mesenchymal cell with a leading edge that facilitates its migration through the extracellular matrix, and thus, the colonisation of different structures. The acquisition of this phenotype is fundamental for the formation of many tissues and organs during embryonic development and constitutes the first step in the metastatic process in tumours of epithelial origin.

Several signalling pathways have been implicated in triggering EMT. In particular, the TGF-β superfamily has been shown to induce EMT both during embryonic development, and in tumour invasion and metastasis. Members of this family participate in many different developmental processes including the control of neural induction, dorso-ventral patterning and organogenesis, and they promote the conversion of cells with an epidermoid carcinoma phenotype to a spindle morphology *in vitro*. The majority of these processes were revised at the meeting, where an additional very promising role in wound healing was presented.

Undoubtedly, adhesion molecules deserved to be paid special attention during this workshop as individual cells must adhere either to their neighbours or to the extracellular matrix around them. Amongst the main protein families that mediate these interactions are the cadherins and the integrins, and the correct functioning of these molecules is crucial both for tissue morphogenesis and tissue homeostasis in the adult. This has become even more evident

when discussing the number of genes involved in adhesion, since it seems that there has been a particular expansion of these molecules in humans. They are tightly regulated, not least because their malfunctioning produces serious consequences including embryonic lethality and the endowment of neo-plastic properties to tumour cells. Indeed, E-cadherin is thought to be an invasion-suppressor gene since its loss is considered a marker of poor clinical outcome. As the loss of E-cadherin is concomitant with the onset of EMT, it is extremely important to understand the mechanisms that regulate cadherin expression. Recently, members of the Snail family of transcription factors that repress cadherin expression have been implicated in EMT, both in different embryonic regions during development and during tumour progression. Indeed, results from several groups have shown that the direct repression of E-cadherin transcription is sufficient to trigger EMT. During the meeting we had the opportunity to discuss the role of these transcription factors in different systems and to learn about other transcription factors also involved in the regulation of cadherin expression.

Another molecule that occupies a central position in the regulation of cell adhesion, cell growth and tumorigenesis is β-catenin. Apart from its well-known role in cadherin-mediated adhesion processes, recent studies have established its role in a novel signal transduction pathway initiated by Wnt growth factors. This pathway is crucial for embryonic patterning and cell fate determination both in vertebrates and invertebrates through the control of gene transcription. Moreover, the association of β-catenin with the tumour suppressor gene APC reveals a role for this pathway in tumorigenesis. The study in different systems and the availability of several mouse models is helping to understand the important equilibrium between the diverse functions of β-catenin in a particular cell context.

Finally, a theme common to embryonic cell migration and neo-plastic progression is the production of extracellular matrix hydrolytic enzymes. Among them, the metalloproteinases attracted much attention, since a positive correlation between their expression and the invasive potential of tumours, both *in vitro* and *in vivo*, has been found.

The use of different experimental systems and approaches, including transgenic and mutant animals and sophisticated screening approaches, has uncovered a whole plethora of molecules involved in the cellular signalling pathways that lead to the acquisition of normal migratory behaviour or malignant properties. I hope and believe that this meeting has

contributed to enhance the communication between scientists working in these fields, and opens up new avenues of research and novel collaborations aimed at better understanding the mechanisms used by common molecules operating in both physiology and pathology processes. It should also served to emphasise the need for basic researchers and pathologists to work together in the hope of understanding the process of tumour progression, paving the way for the design of specific anti-invasive drugs.

M. A. Nieto and M. Takeichi

Session 1: Epithelial/mesenchymal transitions in development and tumorigenesis
Chair: M. Angela Nieto

Overview of epithelial-mesenchymal transformation

Elizabeth D. Hay

During epithelial-mesenchymal transformation (EMT) in the embryo, epithelial cells lose E-cadherin, tight junctions, desmosomes, keratin, and apical-basal polarity. They change to elongated, fibroblast-like cells that acquire front end-back end polarity, vimentin, and the motility mechanisms needed to invade extracellular matrix.[1] In early studies on mechanisms of EMT, Src , Ras, Mos, Fos, and antibodies to E-cadherin[2] were shown to transform MDCK cells to mesenchyme, and more recently downregulation of E-cadherin by snail transcription factor was also demonstrated to induce EMT in MDCK cells.[3] E-cadherin itself is a powerful inducer of epithelial genes in cell lines and also can transform primary embryonic fibroblasts to stratified, desmosome-rich epithelia with both adherens junctions and tight junctions. [1] Recently, it has been shown that LEF-1 competes with E-cadherin for β-catenin, a protein that stabilizes adherens junctions when bound to E-cadherin, but that enters the nucleus on binding to LEF-1 to form β-catenin/LEF-1 transcription complexes. In several *in vitro* systems, induction of EMT has been shown to be associated with activation of this transcription complex.[4] For direct proof of β-catenin/LEF-1 causative involvement in EMT, we induced colon carcinoma, MDCK cells, and corneal epithelium to transform to mesenchyme by infecting them with LEF-adenovirus (Kim and Hay, unpublished). This demonstration of LEF-1 potential to induce EMT *in vitro* strengthens the possibility that the β-catenin/LEF-1 transcription complex brings about EMT *in vivo.* Induction of EMT in embryonic hearts and palates by TGFβ might, in fact, be mediated by this complex, because it has now been shown that TGFβ alone or in collaboration with Wnt can activate LEF-1 using Smad 4 or Smad 3, 4 with or without β-catenin.[5] Interestingly, activation of the Ras-Raf-ERK/MAPKinase pathway in MDCK cells stimulates TGFβ secretion and synergizes with TGFβ to promote EMT. Other genes likely to be involved in EMT by effects on β-catenin and TGFβ signaling pathways include members of the snail/slug family. Moreover, the stimulatory effect of extracellular matrix and members of the β1 integrin pathway, such as Src, on EMT[1] may be mediated by cooperation between integrin and growth factor signaling pathways.[6]

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Control of cell migration by Eph receptors and ephrins

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Eph receptor tyrosine kinases and their ephrin ligands mediate cell contact dependent interactions and are expressed in complex patterns during vertebrate development. Evidence that ephrins transduce signals suggests that bi-directional signalling may occur upon their interaction with Eph receptor. Such signalling occurs at the interfaces of complementary Eph receptor ephrin expression domains, and within domains of overlapping expression. To obtain direct evidence for the role of Eph receptors and ephrins in early development we developed *in vivo* and *in vitro* assays of the effects on cell behaviour of uni-directional versus bi-directional signalling, and of complementary versus overlapping expression. These molecules appears to mediate repulsion in many developmental contexts, and our findings suggest that bi-directional repulsion restricts cell intermingling between adjacent domains. In addition, we found that Eph receptor or ephrin-B activation can restrict cell-cell communication via gap junctions. However, in other contexts, Eph receptors and ephrins can upregulate adhesion, and a number of possible mechanisms have been suggested to underlie repulsion versus adhesion responses. I will discuss our recent studies revealing novel developmental roles of Eph receptors and ephrins, and mechanisms by which they may control cell behaviour.

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Growth factor signaling in branching morphogenesis and carcinoma progression

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The plasticity of epithelial cells is of critical importance during embryogenesis. Various signaling systems have been implicated in the remodeling of epithelia and in the conversion of epithelia into mesenchyme. The analysis of epithelial cell plasticity in *Drosophila* has has led to the identification of key genes in some of these processes. We investigated the mechanism of branching morphogenesis in the lung, making use of recent work on *Drosophila* tracheal development. Branchless and breathless, which are orthologs of fibroblast growth factor (FGF) and FGF receptors, control several key stages of tracheal placode branching. The *sprouty* gene, which encodes a cysteine-rich protein, was found to regulate branchless signaling via breathless, its cognate tyrosine kinase surface receptor. We and others have shown that FGF 10, an ortholog of branchless, is a key positive regulator of lung branching morphogenesis. Mouse *Sprouty2,* an ortholog of *Drosophila sprouty,* is expressed in the peripheral endoderm in embryonic lung and downregulated in the clefts between new branches at E12.5. *mSprouty2* is expressed in a domain restricted in time and space, adjacent to that of *Fgf10* in the peripheral mesenchyme. FGF10 beads upregulated the expression of *mSprouty2* in adjacent epithelium in embryonic lung explant culture. Lung cultures treated with exogenous FGF10 showed greater branching and higher levels of *mSprouty2* mRNA. Conversely, *Fgf10* antisense oligonucleotides reduced branching and decreased *mSprouty2* mRNA levels. Studies of transgenic mouse embryos expressing *Sprouty 2* in the lung confirm the crucial importance of this gene in branching morphogenesis. Epithelial cell plasticity may also be involved in carcinoma progression. We have studied a malignant bladder epithelial cell line (NBT-II) that can undergo reversible conversion to motile fibroblastic-like cells (epithelium-mesenchyme transition, EMT) upon exposure to several growth factors. Scatter activity involves early and transient activation of c-Src. Conversion also requires activation of the Ras-MAP kinase pathway. Slug, a transcription factor related to snail in *Drosophila*, is also induced early, before the dissociation of epithelial cells. Snail is a zinc finger transcriptional repressor produced during early gastrulation in invaginating mesodermal cells. Cells producing slug lose their desmosomes and are converted into stationary fibroblast-like cells. NBT-II epithelial cells can also undergo EMT upon exposure to native collagens and laminin 5. Activation of the $\alpha 2\beta 1$ integrin by type 1 collagen induced specific tyrosine phosphorylation of FAK and paxillin. Transient expression of a paxillin gene with mutations affecting two critical tyrosines strongly inhibited NBT-II cell motility on a collagen-coated substrate. We will present a model summarizing our current view of the mechanisms governing EMT in NBT-II cells. We have investigated whether epithelial cell plasticity is involved in tumor progression by analyzing the behavior of NBT-II cells expressing growth/scatter factors constitutively or following inducion. FGF-1-expressing cells have a fibroblastic morphology *in vitro* and were much more tumorigenic in nude mice than mocked transfected NBT-II cells. The role of FGF-2 was also investigated in NBT-II cells rendered autocrine for this growth factor. Autocrine behavior was required for NBT-II cells to become invasive, angiogenic and tumorigenic. NBT-II cells expressing a high-

molecular mass isoform of FGF-2 became highly metastatic in the lung via a FGF receptor-independent pathway. Recent studies of human bladder carcinoma have demonstrated the multifunctional properties of some growth factors, which act positively via enhanced autocrine loops or negatively, as suppressors of tumor progression. This work clearly shows that similar mechanisms control epithelial morphogenesis in development and tumor progression.

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Molecular aspects of cell interactions during intestinal development and tumorigenesis

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Endoderm/epithelium–mesenchyme cell interactions are essential for both intestinal development and homeostasis. Heterotopic associations of fetal endoderm and mesenchyme have been used to delineated the respective role of these tissue anlagen in the morpho-functional development of the gut (1).

We have focused our attention towards two families of genes involved in this cellular cross-talk: the laminins and the Cdx genes.

Among other extracellular matrix molecules, laminins are major constituants of the basement membrane (BM), a tridimensional molecular structure located at the interface between the epithelial and stromal cells. The precise composition of the BM is specific to each developmental stage and to the crypt (proliferative compartment)-villus (differentiation compartment) location in the adult gut (2,3). We have analyzed the regulation and role of laminin-1 and -5, on the epithelial cell behavior, the former being expressed in the crypt, the latter in the villus BM. The combination of various experimental approaches (inhibition of the expression or overexpression in colonic cancer cell lines, injection of the cells in nude mice, promoter studies) leads to the conclusion that laminin-1 is crucial for the assembly of the BM components (in particular of laminin-10), for the basal segregation of the $\alpha 6\beta 4$ integrin and for cell proliferation and polarisation; laminin-5, whose promoter is up-regulated by HGF/SF is rather involved in cell spreading and migration (4,5).

Cdx1 and Cdx2, are the mammalian homologues of the Drosophila caudal homeobox gene; they are specifically expressed in the intestinal epithelium from fetal stages up to adult life (6); Cdx1 is expressed in the crypt cells, whereas Cdx2 is predominantly expressed in the villus cells. Using proximo-distal heterotopic associations of fetal rat endoderm and mesenchyme, we showed that the expression of Cdx genes was dependent upon the nature of the mesenchyme, and provided arguments of the role played by Cdx1 and Cdx2 in defining the positional identity along the length of gut in mammals (7). We showed that laminin-1 induces an increased expression of Cdx2 in cultured intestinal epithelial cells; besides, overexpression of Cdx2 leads to changes in cell-cell and cell-matrix adhesion molecules and to an increased cell differentiation, paralleled by an inhibition of the expression of Cdx1 (8). Together with observations made by other groups, it is suggested that these genes participate in the control of intestinal homeostasis.

It has been reported that Cdx1 and Cdx2 expression is altered in colorectal cancers; the possibility that these genes may be targets of oncogenic pathways has been tested for the Wnt/β-catenin and the PTEN/PI3K pathways which are both activated during development and cancerogenesis.

Firstly, in collaboration with Dr.Kemler (Freiburg) and with Dr.Clevers (Utrecht), arguments have been provided suggesting that Cdx1, but not Cdx2, is a target of the Wnt

pathway during intestinal development (9). Indeed, Cdx1 expression is upregulated in intestinal endoderms upon coculture on NIH3T3 cells transfected to produce Wnt1. In addition, Cdx1 expression is no more detectable in the fetal intestine of mice deficient for the Tcf4 factor (activated by Wnt signalling). Beside development, we have also demonstrated that the oncogenic activation of the β-catenin/Tcf4 pathway stimulates Cdx1 in human colon adenocarcinoma cell lines, whereas no effect was observed in an intestinal mesenchymal cell line.

Secondly, it appears that Cdx2, but not Cdx1, is a specific target of the pathway that involves the tumor suppressor gene PTEN, an antagonist of the PI3K pathway (10); collaboration with Dr.Evers (Galveston) and with Dr.Cristofano (NY)]. Indeed, (i) the CDX2 protein and the phosphatase encoded by PTEN share a similar pattern along the longitudinal and crypt-villus axes of the gut, (ii) Cdx2 expression is reduced in the colon epithelium and in the intestinal polyps of $PTEN^{+/-}$ mice, and (iii) PTEN transfection or any treatment to antagonize PI3K activity stimulate Cdx2 expression in the colon adenocarcinoma cell line HT29.

Taken together, these data demonstrate that intestinal development and homeostasis depend on epithelial-mesenchymal cell interactions that involve various laminin variants and the Cdx1 and Cdx2 homeobox genes. Interestingly, the latter are regulated by distinct developmental signalling pathways that are frequently activated during colon tumorigenesis.

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Session 2: TGF-β/BMP signalling in development and cancer
Chair: David Wilkinson

Regulation of BMP signaling in the extracellular space by Chordin, Xolloid and Twisted gastrulation

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Dorsal-ventral patterning in vertebrate and *Drosophila* embryos requires a conserved system of extracellular proteins to generate a gradient of positional information. The components involved include Bone Morphogenetic proteins (BMP/Dpp), a BMP antagonist (Chordin/Short gastrulation), and a secreted metalloproteinase (Xolloid/Tolloid) that cleaves Chd/Sog allowing reactivation of BMP signalling. Here we introduce an additional player, Xenopus Twisted gastrulation (xTsg). xTsg is expressed ventrally as part of the BMP-4 synexpression group and encodes a secreted protein that binds BMP and Chordin. Biochemical studies indicate that xTsg can modulate BMP signaling by three different mechanisms: 1) xTsg forms ternary complexes with Chordin and BMP and makes full-length Chordin a better BMP-antagonist, 2) xTsg removes BMP from the inactive complexes formed between the growth factor and the Chordin fragments generated by Xolloid cleavage, in this aspect xTsg will potentiate BMP signaling, and 3) at higher concentrations xTsg can compete the binding of BMP to its cognate receptor. Overexpression and loss-of-function studies on Xenopus embryos indicates that, in vivo, xTsg behaves as a ventralizing agent, probably by competing the residual anti-BMP activity of Chordin fragments. In this way xTsg provides a permissive signal that allows high levels of BMP signaling in the embryo. Drosophila Tsg also binds BMPs and this mechanism probably explains why Twisted gastrulation is necessary to acquire the highest levels of Dpp signaling in the fly embryo.

TGFß isoforms in palate development, wound healing and fibrosis

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During mammalian palate development bilateral shelves elevate from a vertical position along side the tongue to a horizontal position above the dorsum of the tongue, contact and fuse with each other. Fusion of the midline epithelial seam involves initial cell adherence, degeneration and migration of the medial edge epithelial sea cells and mesenchymal condensation across the palate. This event is frequently disrupted leading to the birth defect - Cleft Palate. TGF-ß3 localises specifically to the medial edge epithelia during palatal fusion. Suppression of TGF-ß3 activity using a ibodies or antisense oligonucleotides in cultures of paired palatal shelves results in a failure of palatal fusion. Transgenic deletion of the TGF-ß3 gene results in cleft palate due to a failure of palatal shelf fusion in the homozygous null embryos. Analysis of the palatal medial edge epithelia of the TGF-ß3 -/- embryos reveals they have fewer microvilli: a phenotype, which can be reversed by exogenous administration of TGF-ß3 in culture, which also induces palatal fusion. These effects are likely transduced by alterations in the cyto-skeleton.

Fetal wounds heal without scarring, whereas adult wounds scar. Scarring is a major medical problem. Amongst the many cellular and molecular differences between scar free fetal healing and scar forming adult healing are the ratio of TGF-ß isoforms at the wound site. Adult wounds have high levels of TGF-ß1 and 2 (largely der ed from inflammatory cells such as monocytes and macrophages) and low levels of TGF-ß3, whereas fetal wounds have high levels of TGF-ß3 (derived from keratinocytes and fibroblasts), but low levels of TGF-ß1 and 2 (as there is a much reduced inflammatory r ponse in the fetus). Experimental manipulation of the TGF-ß profile of healing adult rodent wounds by exogenous neutralisation of TGF-ß1 and 2 (using neutralising antibodies) or elevation of TGF-ß3 (by addition of recombinant protein) results in scar free adult healing. These manipulations are also associated with alterations in the organisation of the extracellular matrix and the inflammatory cell profile.

Experimental wounding of TGF-ß3 -/- embryos reveals a delay in wound healing characterised by a failure of the epithelial and mesenchymal cells to migrate. This results in a scar. This deficit in mesenchymal cell migration can be mimicked in vitro and specifically rescued by TGF-ß3, but not TGF-ß1 and 2 suggesting isoform specific receptor recognition and signalling.

These findings have been extended from experimental investigations in rodents through to comparable studies in pig and potential human scar preventing pharmaceutical agents are entering human clinical trial as a result. Interestingly TGF-ß3 mutations may render humans susceptible to cleft palate and also susceptible to adverse scarring.

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Growth factor and transcriptional regulation of cell transformation in the embryonic heart

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During heart development, the precursor cells of the mitral and tricuspid valves are formed by an epithelial-mesenchymal cell transformation of endothelial cells lining the heart lumen. This process of cell transformation has been extensively studied by utilization of collagen gel tissue culture assays and chick tissue. It was shown that the endothelial response was due to a localized stimulus from the adjacent myocardium. Early studies exploring growth factors in the transformation process pointed to a role for TGFß. Though any exogenous TGFß isoform could mediate cell transformation, antisense oligonucleotide studies indicated a major role for TGFß3. However, when TGFß2 KO mice proved to have heart defects, the role of TGFß2 in chick hearts was re-examined. Our recent studies show that TGFß2 and TGFß3 have separate and sequential activities in mediating transformation. Antibodies against TGFß2 block cell-cell separation and blocking antibodies against TGFß3 inhibit subsequent cell invasion. Studies with TGFß Type II and III receptors showed a similar division of roles, Type III receptor antibody blocked cell-cell separation while Type II receptor antibody blocked cell invasion. Independent signal transduction via these receptors was confirmed by differences in the regulation of several markers expressed by mesenchymal cells. To explore the regulation of epithelial-mesenchymal cell transformation in this system, antisense oligonucleotides were prepared for a variety of transcription factors associated with embryonic cell transformations. Antisense oligonucleotides for Slug and Mox-1 were particularly effective in blocking transformation *in vitro*. Antisense Slug oligos blocked cell-cell separation while antisense Mox-1 oligos blocked cell invasion. Direct analysis showed that Slug mRNA and protein were regulated by TGFß2 and that Mox-1 protein expression was regulated by TGFß3. Analysis of the proximal Mox-1 promoter has identified a region with a novel isoform-specific regulation of a reporter. Thus Slug is regulated by TGFß2 and the Type II receptor while Mox-1 is regulated by TGFß3 and Type II receptor. These data argue that epithelial-mesenchymal cell transformation is regulated, in part, by largely independent regulation of different transcription factors needed for distinct elements of the process. Additional signal transduction pathways including Wnt and HGF are known to be involved in this transformation in the heart. Ongoing studies are aimed at integrating the TGFß-mediated pathways with these other components of the transformation stimulus.

Cell adhesion and cytoskeleton dynamics during epithelial fusion in *Drosophila*

Enrique Martín-Blanco

Morphogenesis depends on the coordination of cellular activities during development. One of the best-studied morphogenetic processes is *Drosophila* embryonic dorsal closure. During closure epidermal sheets stretch and meet at the dorsal midline. Late in the *Drosophila* life, adult structures merge in a process, parallel to dorsal closure, that implements the encounter of imaginal cells. Both processes are driven by cell shape changes, share regulatory controls and result from similar cellular activities Dorsal closure and imaginal fusion are mediated by the activity of the JNK cascade. Mutants for members of this pathway show a dorsal hole and no elongation of lateral cells. Loss of function conditions for these genes during metamorphosis lead to disc fusion failure. We have found that, for both processes, JNK signaling is essential to promote emission of filopodia out of leading edge cells. Furthermore, interference with JNK signaling provokes detachment of intervening cells from the active spreading epithelia. Interestingly, we have found that integrins participate in epithelial spreading. Mutant alleles for myospheroid (mys), a *Drosophila* homologue of b-integrin, show epithelial detachment and dorsal phenotypes indistinguishable of those of JNK mutants. Mys accumulates in leading cells and it is transcriptionally regulated by JNK signaling. Moreover, Mys accumulates along the edge of imaginal discs and interference with Mys levels leads to disc detachments. Control of cytoskeleton dynamics and cell adhesion by JNK signaling might be a general mechanism leading processes such as ventral enclosure in *C. elegans*, palate fusion in mice, wound healing in vertebrates or tumor metastasis.

Session 3: Wnt and β catenin in development and cancer
Chair: Masatoshi Takeichi

Multiple Wnt signaling pathways in vertebrates

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Based on gain-of-function assays in Xenopus and zebrafish embryos, we have previously reported that different Wnts elicit one of two classes of responses. One group of Wnts stimulates elevation of dorsal regulatory genes leading to duplication of the embryonic axes, while another group of Wnts leads to changes in morphogenetic movements. Subsequent study has revealed that the former group of Wnts activates the canonical-catenin signaling pathway, while the latter group of Wnts appears to work in a-catenin-independent manner. These observations prompted us to investigate whether multiple Wnt signaling pathways exist in vertebrates.

Assays of mouse and rat Frizzled homologs in explants of Xenopus embryos revealed that these receptors preferentially activate one of two apparently distinct signaling pathways. One group of receptors leads to activation of known -catenin target genes, while other receptors are much less efficient in eliciting gene activation. Instead, these receptors preferentially stimulate CamKII, PKC, and intracellular calcium release. Based on these results we have proposed the existence of a-catenin-independent Frizzled signaling pathway, the Wnt/calcium pathway. While many important questions remain to be resolved, it is increasingly apparent that Wnt signaling in vertebrates is more complex than had been presumed based on genetic analyses in Drosophila.

Given the evidence supporting the presence of distinct Wnt/Frizzled signaling pathways in vertebrates, we have initiated screens with DNA microarrays to determine whether these apparently distinct signalling pathways have distinct effects on gene expression, and whether some genes may be regulated by both pathways. Preliminary results of this project will be presented.

Cadherins and catenins in development and cancer

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E-cadherin is a tumor suppressor protein with a well-established role in cell-cell adhesion. Adhesion could contribute to tumor suppression by physically joining cells or by facilitating other juxtacrine signaling events. Alternatively, E-cadherin tumor suppressor activity could result by influencing the nuclear signaling function of β-catenin, a known proto-oncogene. Indeed, in developing *Xenopus* embryos, cadherin overexpression antagonizes the signaling activity of β-catenin during embryonic axis induction by binding and sequestering β-catenin at the membrane and depleting it from the cytosolic and nuclear pools. By analogy, E-cadherin could regulate cell growth by binding β-catenin and antagonizing its nuclear signaling activity.

We have chosen to study the tumor suppressor activity of E-cadherin using the SW480 colorectal tumor cell line, because β-catenin is implicate in colorectal tumorigenesis and these cells are known to accumulate high levels of β-catenin due to a mutation in the APC gene. Expression of E-cadherin significantly inhibits the growth of this cell line. To distinguish between an adhesion- versus a β-catenin signaling-dependent mechanism, the growth inhibitory properties of chimeric cadherin constructs that specifically lack either adhesive function or β-catenin-binding activity were determined. Growth inhibitory activity is retained by all constructs that have the β-catenin binding region of the cytoplasmic domain but not by E-cadherin constructs that exhibit adhesive activity, but lack the β-catenin binding region. This growth suppression correlates with a reduction in β-catenin/TCF reporter gene activity. Importantly, direct inhibition of β-catenin/TCF signaling inhibits the growth of SW480 cells, and the growth inhibitory activity of E-cadherin is rescued by constitutively activated forms of TCF. Thus, the growth suppressor activity of E-cadherin is adhesion-independent and results from an inhibition of the β-catenin/TCF signaling pathway, suggesting that loss of E-cadherin expression can contribute to up-regulation of this pathway in human cancers.

In contrast to the mechanism of β-catenin signaling inhibition in the early *Xenopus* embryo, E-cadherin-mediated growth suppression in SW480 cells was not accompanied by depletion of β-catenin from the cytosolic and nuclear pools. One possible explanation is that a minor fraction of the β-catenin in this cell line may is active in nuclear signaling and that this minor active pool of β-catenin is selectively sequestered by E-cadherin. Indeed, we find that a sizable fraction of the β-catenin in SW480 cells is not competent to interact with either the cadherin cytoplasmic domain or with the TCF transcription factor. Therefore, there is a large pool of β-catenin that appears to be inactive with regards both to cell adhesion function and TCF-mediated transcriptional signaling, and E-cadherin may inhibit β-catenin signaling by depleting the subfraction of active β-catenin from the cytosol and nucleus. Thus, the physiological relationship between the two functions of β-catenin is more complex and subject to an additional layer of regulation. The molecular basis for the formation of these distinct cytosolic pools of β-catenin and its role in regulating β-catenin-mediated signaling is being investigated. It will be interesting to determine whether the interactions between cadherins and β-catenin signaling are similarly regulated in developing tissues.

Role of Beta-Catenin/armadillo in cell adhesion and signal transduction

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Beta-Catenin/armadillo is a component of both the cadherin cell adhesion system and the wnt signaling pathway. Wnt signaling increases the levels of cytosolic beta-catenin by preventing its ubiquination and degradation via proteasomes. This allows direct interaction of beta-catenin with transcription factors of the LEF/TCF family and modulation of gene expression. We have shown previously that the protein conductin forms a complex with beta-catenin, the tumor suppressor gene product APC, and GSK3 beta. Conductin induces beta-catenin degradation, whereas mutants of conductin that are deficient in complex formation stabilize beta-catenin. Fragments of APC that contain a conductin-binding domain also block beta-catenin degradation. Thus, conductin and the related protein axin are components of the multiprotein complex that direct beta-catenin to degradation.

In beta-catenin, we have now identified amino acids that distinctly affect APC, conduction or LEF-1/TCF binding. These residues form separate clusters in the superhelix built by armadillo repeats. Point mutations in one, APC or conductin binding sites, do not stabilize beta-catenin. Mutants unable to interact with both APC and conductin are fully stabilized. For degradation, it is thus sufficient if conductin or APC are recruited indirectly to beta-catenin and thus to the degradation complex.

We also generated beta-catenin-deficient mouse embryos and observed a defect in anterior-posterior axis formation at E5.5, as visualized by the absence of Hex and Hesx and the mislocation of Cerberus-like and Lim-1 expression in the visceral endoderm. Subsequently, no mesoderm and head structures are generated, but beta-catenin-deficient cells can contribute to these tissues in chimeric embryos. Intercellular adhesion is maintained since plakoglobin substitutes for beta-catenin. Our data suggest that beta-catenin-mediated signalling is essential in anterior-posterior axis formation of the pre-gastrula mouse embryo.

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The E-cadherin cell adhesion complex in cell-cell adhesion and signaling during mouse development

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The cell adhesion molecule E-cadherin mediates the compaction process of mouse preimplantation embryos and is important for the maintenance and function of epithelial cell layers. β-catenin is a central component of the E-cadherin cell adhesion complex and plays in addition an essential role in the Wingless/Wnt signaling pathway.

During mouse preimplantation development the components of the E-cadherin-catenin complex are derived from both maternal and zygotic gene activity. The adhesion complex is increasingly accumulated and stored in a nonfunctional form, ready to be used for compaction and the formation of the trophectoderm cell layer. β-Catenin is the major tyrosine-phosphorylated protein in oocytes and early cleavage-stage embryos and the relative amount of phosphorylated β-catenin is greatly reduced during the morula-blastocyst transition. From these and other experiments we propose that tyrosine phosphorylation negatively regulates the adhesive strength of E-cadherin. In contrast, phosphorylation of the E-cadherin cytoplasmic domain by CKII and GSK3β increases binding of E-cadherin and β-catenin. Thus post-translational modifications of the components of the E-cadherincatenin complex represent one way to modulate the strength of cell-cell adhesion.

In Wnt-stimulated cells, β-catenin is stabilized in the cytoplasm and, together with LEF-1/TCF transcription factors, controls the transcription of specific target genes. To identify target genes of the Wnt/β-catenin signaling pathway in early mouse embryonic development we have established a co-culture system consisting of NIH3T3 fibroblasts expressing different Wnt's as feeder layer cells and embryonic stem (ES) cells expressing a green fluorescent protein (GFP) reporter gene transcriptionally regulated by the TCF/β-catenin complex. ES cells specifically respond to Wnt signal as monitored by GFP expression. In GFP-positive ES cells we observe expression of Brachyury and we found the Brachyury ist transcriptionally regulated by TCF/ß-catenin. We also provide evidence *in vitro* and *in vivo* that the Cdx1 homeobox gene is a direct transcriptional target of the Wnt/β-catenin signaling pathway. Upon Wnt stimulation, expression of Cdx1 can be induced in mouse embryonic stem (ES) cells as well as in undifferentiated rat embryonic endoderm. Tcf4-deficient mouse embryos show abrogation of Cdx1 protein in the small intestinal epithelium, making Tcf4 a likely candidate to transduce Wnt signal in this part of gut. The promoter region of the *Cdx1* gene contains several Tcf-binding motifs, and these bind Tcf/Lef1/β-catenin complexes and mediate β-catenin-dependent transactivation. This transcriptional regulation of the homeobox gene Cdx1 by Wnt/β-catenin signaling underlines the importance of this signaling pathway in mammalian endoderm development.

To study the role of β-catenin during brain morphogenesis, we specifically inactivated the β-catenin gene in the region of Wnt1 expression using the Cre/loxP system. β-catenin gene deletion resulted in dramatic brain malformation and failure of craniofacial development.

Absence of part of the midbrain and all of the cerebellum is reminiscent of the conventional Wnt1 knockout, suggesting that Wnt1 acts through β-catenin in controlling midbrain-hindbrain development. The additonal craniofacial phenotype, not observed in embryos lacking Wnt1, indicates a role of β-catenin in the fate of neural crest cells. Analysis of neural tube explants shows that β-catenin is efficiently eliminated in migrating neural crest cell precursors. This together with an increased apoptosis in cells migrating to the cranial ganglia and in areas of prechondrogenic condensations suggests that removal of β-catenin affects neural crest cell survival and/or differentiation. Our results demonstrate the pivotal role of β-catenin in morphogenetic processes during brain and craniofacial development.

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Role of the TCF family of transcription factors in intestinal polyp formation

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Colorectal cancer is one the most prevalent types of neoplasias in the developed countries. About 50 % of the western population will develop colorectal polyps during a normal life span. Eventually, some of these individuals will develop a colorectal tumor. Colorectal tumor development is a multistep process and these initially benign polyps represent the gate used by the epithelial intestinal cells to shuttle into the carcinogenic sequence.

Genetic and molecular studies in the last decade have demonstrated that the key event for polyp development in the intestine is the abnormal activation of the Wnt pathway. Around 85 % of the colorectal tumors show mutations in the APC gene while most of the remaining 15 % have mutations in b-catenin. Either genetic alteration leads to the nuclear accumulation of b-catenin, a protein present normally in the adherens junctions. The ultimate consequence of this process is the interaction of b-catenin with the Tcf family of transcription factors. This interaction leads to the expression of a still poorly characterized set of genes in the epithelial intestinal cells that most likely are responsible for tumor initiation (1,2).

Although considerable effort has been done in the identification of the genes activated by the b-catenin-Tcf complex in colorectal cancer cells, the contribution of these target genes to polyp formation remains unclear. To address this question, we have established colorectal cancer cell lines expressing a dominant negative form of the Tcf-4 or Tcf-1 transcription factors under the control of a doxycyclin inducible promoter. Upon addition of doxycyclin, these cell lines stop proliferating and are rapidly arrested in the G1 phase of the cell cycle. We have analyzed the changes in the gene expression profile caused by the expression of the DN-Tcf factors using a 25000 EST array chip. Around 25 genes are downregulated in these cell lines upon addition of doxycyclin while more than 200 are upregulated. Analysis of the expression of these b-catenin/Tcf target genes in normal and tumor tissue indicates that most of the downregulated genes are expressed in tumors but also in normal proliferating crypt cells of the intestine while several upregulated genes are well-known markers of intestinal differentiation. This observation supports the previous model in which the b-catenin/Tcf mediated transcription is determining or maintaining the undifferentiated phenotype of the epithelial intestinal crypt cell (2,3). Moreover, these results also suggest that polyp formation represents a disregulated expansion of the crypt compartment. This process might be caused by either an increase in the levels of the b-catenin/Tcf target genes in polyp cells or by an impaired ability of the crypt cells to properly switch-off the WNT pathway during their migration towards the differentiated compartment.

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Session 4: The Snail gene family in development and tumour progression
Chair: Jean P. Thiery

Snail family of proteins in Drosophila gastrulation and neurogenesis

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The Snail family comprises of proteins that contain four to six zinc fingers and function as transcriptional regulators during embryonic development, cancer formation, and apoptosis (Hemavathy et al., 2000). Drosophila Snail was the first member isolated and serves as the prototype (Boulay et al., 1987). Snail is essential for mesoderm invagination and differentiation in the early embryo. It has been demonstrated that Snail functions as a transcriptional repressor, and such function is essential for mesoderm formation (Kosman et al., 1991; Leptin, 1991; Ip et al., 1992; Hemavathy et al., 1997). Snail also acts redundantly with Escargot (French for snail), another member of this zinc-finger protein family, to control the embryonic wing disc development (Fuse et al., 1996). Expression pattern analyses revealed that Snail is expressed not only in early mesoderm and embryonic wing disc primodia, but also in neuroblasts (Alberga et al., 1991; Kosman et al., 1991; Leptin, 1991; Ip et al., 1994). Total loss of function of Snail, however, does not lead to significant phenotype in the developing CNS.

The absence of CNS phenotype in loss-of-function mutants of *snail* is due to the redundant function provided by another member of the family called Worniu (Chinese for snail), as well as by Escargot to a lesser extend. In deletion mutants which uncover these three genes, the CNS is highly abnormal (Ashraf et al., 1999). The ventral nerve cord is largely underdeveloped as revealed by multiple neuronal markers. One of the early CNS markers that is severely affected is *fushi tarazu* (*ftz*) (Goodman and Doe, 1993). *ftz* is initially expressed in many ganglion mother cells (GMC) and later on in many neurons. In the deletion mutants, *ftz* expression in GMC is almost abolished, and such defect can be rescued efficiently by transgenes of the *snail* family (Ashraf et al., 1999).

The CNS development of Drosophila has been well characterized. Clusters of cells in the neuroectoderm receive instruction from proneural genes to become competent to form neuroblasts (Goodman and Doe, 1993). Through the process of lateral inhibition, one of the cells in each cluster is selected to become neuroblast and delaminates from the ectoderm (Rooke and Xu, 1998). Delaminated neuroblasts have stem cell property, whereby each goes through repeated asymmetric cell division to generate a number of GMC (Lu et al., 2000). During or soon after delamination, genes such as *inscuteable*, *miranda*, *staufen*, and *prospero* are expressed. One of the functions of these genes is to control the subcellular localization within the neuroblasts of *prospero* mRNA and Prospero protein, which are segregated preferentially into GMC after cell division. Prospero is a key regulator in determining GMC fate, by controlling the expression of neural genes such as *ftz* and the precise one round of cell division to produce post-mitotic neurons (Lu et al., 2000; Li and Vaessin, 2000).

Since *ftz* expression in GMC is highly defective in the mutants, we surmised that Snail family of proteins may function at a regulatory step in neuroblasts or GMC (Ashraf et al., 1999). We now find that Snail family of proteins have functions within neuroblasts. In the absence of these proteins, the delaminated neuroblasts have significantly lower level of *inscuteable* RNA expression. While the expression of other genes that are involved in asymmetric neuroblast division including *miranda*, *staufen*, and *prospero* appear to be normal, the asymmetric localization of *prospero* RNA does not take place, and the strong Prospero protein staining in GMC normally resulted from asymmetric division is lost. These defects can be rescued by transgenic expression of Snail, Worniu, or Escargot. Thus, some intrinsic properties of asymmetric division is dependent on the presence of Snail family of proteins. The almost complete absence of Prospero protein staining in GMC in the deletion mutants is somewhat stronger than the phenotype of zygotic *inscuteable* null mutations. This points to the notion that the loss of *snail* family of genes causes more defects than just the misregulation of *inscuteable*. Accordingly we found that expression of *string* promoter-lacZ reporters (Lehman et al., 1999) was highly abnormal in the deletion mutants. Together, these results support the idea that both asymmetric localization and cell division of neuroblasts are regulated by the Snail family of proteins. However, *string* and *inscuteable* may not be the direct transcription targets. This postulation is based on the experiments demonstrating that mutations of the two Drosophila C-terminal binding protein (dCtBP) co-repressor interaction motifs (Nibu et al. 1998) in Snail hamper the ability of Snail to rescue the CNS defects. Perhaps Snail family of proteins interact with dCtBP to repress a yet to be identified target gene which in turn is required to repress *inscuteable* and *string* transcription in neuroblasts.

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Roles of snail-family transcriptional repressors during mouse embryogenesis

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In *Drosophila* two zygotic genes, *Snail* and *Twist*, are essential for mesoderm differentiation and morphogenesis during *Drosophila* embryogenesis. Molecular analysis of the *Twist* and *Snail* genes indicates that they encode proteins of the basic helix-loop-helix and zinc-finger families, respectively. *Twist* is a transcriptional activator of mesoderm-specific genes, while *Snail* acts to maintain proper germ layer boundaries by repressing the expression within the mesoderm of regulatory genes involved in ectodermal development.

We and others have cloned mouse genes encoding Snail-family proteins, termed *Snail* and *Slug* (Nieto et al., 1992; Smith et al., 1992; Jiang et al. 1998; Sefton et al., 1998), and we have constructed targeted mutations in both the *Snail* and *Slug* genes. Contrary to expectations from analysis of other vertebrates, the *Slug* gene is not required for formation and differentiation of mesoderm and neural crest in mice (Jiang et al. 1998). This is most likely due to the fact that the mouse *Slug* gene, unlike the *Slug* genes of other vertebrates, is not expressed in either early embryonic mesoderm or premigratory neural crest precursors in the dorsal neural tube (Jiang et al. 1998; Sefton et al., 1998). However, *Slug* mutant homozygotes exhibit a number of other defects, including postnatal growth retardation, eye defects, and defects in formation of the nails.

Embryos homozygous for a mutation of the mouse *Snail* gene die early during embryogenesis. The mutant embryos exhibit a defect in the epithelial-mesenchymal transition in the primitive streak of the embryo, which is required for proper formation of the mesoderm germ layer. In *Snail* homozygous mutant embryos, a mesoderm germ layer forms but the cells do not become truly mesenchymal. In the majority of *Snail* mutant embryos, cavities or lacunae form in the mesoderm cell layer, and the mesoderm cells abutting these lacunae exhibit an epithelial morphology. Cells lining these lacunae are more columnar and exhibit apical/basal polarity, with microvilli along the apical surface and the presence of electron-dense adhesive junctions that resemble adherens junctions. Preliminary expression studies indicate that E-cadherin expression is not downregulated in the primitive streak and mesoderm of the *Snail* mutant embryos. These data suggest that formation of the mesoderm cell layer can occur despite the retention of E-cadherin expression, but also show that downregulation of E-cadherin expression is required for mesoderm cells to acquire a mesenchymal morphology.

We are also testing for genetic interactions between a targeted mutation of the mouse *Twist* gene (Chen and Behringer, 1995) and the *Snail* and *Slug* mutations. Haploinsufficiency for the human *TWIST* gene is one of the major causes of Saethre-Chotzen Syndrome, one of the most common disorders of craniosynostosis (premature fusion of the calvarial bones of the skull). Studies have shown that mice heterozygous for the *Twist* null mutation exhibit mutant skeletal phenotypes, including hind leg digit duplications and subtle skull defects (Bourgeois et al., 1998). We are testing for genetic interactions by creating double mutant *Snail/ Twist*

and *Slug/ Twist* mice, and are analyzing the double mutant embryos and mice for synergistic effects. The current status of these studies will be described.

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The Snail gene family in evolution, development and tumour progression

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The Snail family of zinc-finger transcription factors plays a crucial role in embryonic development of vertebrates and invertebrates, with an evolutionarily conserved function in mesoderm development, and further roles in neural differentiation and vertebrate neural crest formation. Its function in mesoderm and neural crest delamination is mediated by the triggering of epithelial-mesenchymal transitions (EMT). Very recently, we have shown that Snail-mediated EMT is, at least in part, due to the direct repression of *E-cadherin* expression. Furthermore, we have shown that *Snail* has been co-opted to trigger the EMT associated with the acquisition of the malignant phenotype in tumours. Thus, these transcriptions factors are utilised for the same cellular process both during normal embryonic development and in pathological situations of the adult, such as tumour progression.

Several expected and unexpected observations make the study of this gene family from an evolutionary point of view extremely interesting. Firstly, the neural crest is believed to have been crucial in the formation of the "new head" of vertebrates. In addition, we have reported a unique swapping in the expression patterns and the roles of the two vertebrate family members (*Snail* and *Slug*) between avian and mammals. Thus, we have undertaken the study of the distribution and the evolutionary history of the Snail family.

The founding member of the family was the *snail* gene from *Drosophila melanogaster*, and until very recently, only three members had been characterized in *Drosophila* (*snail*, *escargot* and *scratch*), one in urochordates and cephalochordates (*snail*) and two in vertebrates (*snail* and *slug*). Vertebrate *snail* genes are more related between themselves than to any of the fly genes, so a simple picture of a unique gene present before the protostome/deuterostome radiation and independent duplication events in the arthropod and vertebrate lineages that leads to the present situation is assumed.

However, in the last two years, three new members of the family have been described: *ces1* from *Caenorhabditis elegans*, *worniu* from *Drosophila*, and *Smuc* from mouse. In our attempt to analyse the phylogenetic relationships of the *Snail* family genes, we have identified several previously undescribed *Snail*-type genes from a variety of model organisms. In the light of this new data, and in the search for ancestral and derived functions, I will present a new classification of the gene family and discuss the roles of these genes in neural crest formation and in general, in epithelial to mesenchymal transitions during early development and tumour progression.

Regulation of E-cadherin expression by snail transcriptional repressor

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It has been recently shown that ectopic expression of snail protein, a mesenchymal transcriptional repressor, induces downregulation of E-cadherin mRNA (1, 2). Snail blocks E-cadherin expression through its binding to three 5'-CACCTG-3' boxes present in E-cadherin promoter (1). Moreover, snail expression in epithelial cells induces the acquisition of a fibroblastic phenotype, characterised by the inhibition of epithelium specific genes (CK-18, MUC-1) and the induction of mesenchymal specific genes (fibronectin, LEF-1). Moreover, snail expression is also required for gastrulation and for the induction of mesenchymal genes in the early phases of development (3). We show here that induction of snail in epithelial cells stimulates the expression of one of these mesenchymal genes ZEB1. Like snail, ZEB1 is also a transcriptional repressor and can inhibit E-cadherin promoter activity although with lower efficiency than snail. This result suggest a possible role of ZEB1 in snail-induced transcriptional repression of E-cadherin.

Although expression of snail and E-cadherin is generally contrary, we have detected several cell lines that express both mRNAs. In these cell lines (for instance, EpH4) Snail subcellular localisation was determined by immunofluorescence and was found to be restricted to the cytosol, whereas it was detected in the nucleus in cell lines not expressing E-cadherin. A study of snail deletion mutants indicate that a PS-rich sequence present in the N-terminal domain of this molecule is involved in retention in the cytosol. This result evidences a new level of regulation of snail function in epithelial cells.

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Induction of snail expression by activation of the PTH(rP)receptor is necessary and sufficient for proper epithelio-mesenchyme transition in the formation of parietal endoderm in the early mouse embryo

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The formation of parietal endoderm (PE) in the periimplantation mouse embryo is the earliest example of an epithelio-mesenchyme transition (EMT) during murine embryogenesis. This process can be mimicked *in vitro* using F9 EC cells. We and others have established that parathyroid related peptide (PTHrP) and its receptor play an important role in this process both *in vivo* and *in vitro*. *In vitro*, using isolated Inner Cell Masses (ICM) from blastocysts, it was shown by others that endogenously produced PTHrP enhances EMT and differentiation to PE, especially on suboptimal FN concentrations. The PTHrP receptor signals predominantly through activation of protein kinase A (PKA), and addition of cell permeable cAMP analogs mimicks the PTHrP effect. Recently we identified the transcription factor snail as an immediate early target gene to the action of PTHrP in F9 cells.

Embryos lacking the PTHrP receptor do form PE *in vivo*, which however seems not to function properly, based on the small size of the embryos already at day 8.5. The embryos die around embryonic day 12.5, which might be a consequence of this growth retardation as well. In PE isolated from d 6.5 embryos, we found that in -/- embryos, in contrast to wt and heterozygous embryos, a large proportion of cells have not undergone EMT and instead remain attached to one another. On low amounts of FN, the cells do migrate, but as sheets of cells, rather than as solitary cells. This is reflected in the continued presence of E-cadherin and β-catenin at sites of cell-cell contact, which is absent in normal PE. Importantly, incubation of the receptor -/- PE with dbcAMP or culturing it on high amounts of FN induces EMT, showing that the cells are still responsive to elevated cAMP levels and the inductive effect of FN. This treatment results in elevated snail levels, indicating snail is pivotal in the process. Indeed, overexpressing snail in F9EC aggregates results in enhanced formation of PE which cannot be further optimised by PTHrP addition. These results indicate that i) There is no or little redundancy *in vivo* for PTH(rP) receptor functioning in inducing complete EMT in PE, ii) Elevation of snail levels is sufficient to induce EMT, suggesting this is a crucial event for proper PE differentiation and iii) EMT is necessary for PE to function properly.

Session 5: Cell adhesion and extracellular matrix in development and cancer
Chair: Barry M. Gumbiner

Regulation of morphogenetic cell movement by cadherins and catenins

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Regulation of cell adhesion is thought to be essential for promoting a variety of morphogenetic processes, including cell migration and rearrangement. The cadherin adhesion system plays a central role in cell-cell adhesion, and therefore elucidating its regulatory mechanism is important. We previously found that a human colon carcinoma cell line, Colo205, express cadherins and associated catenins at normal levels. Nevertheless, they cannot undergo normal cell-cell aggregation, suggesting that the cadherin system is physiologically suppressed. Interestingly, if these cells are transfected with a mutant cadherin, designated as JM(-), in which the juxtamembrane (JM) region of the cytoplasmic domain was deleted, cadherin-dependent cell association was restored. This finding suggested that the JM domain has some regulatory role in cadherin function. Since p120-catenin is known to interact with the JM domain, this molecule can be one of regulators to interact with this domain. Indeed, when Colo205 cells were transfected with a N-terminus-truncated p120-catenin, it induced their cadherin-dependent aggregation, implying that the endogenous p120-catenin negatively regulated cadherin activity in Colo205 cells, and this activity was abolished by the removal of the N-terminus (1).

We hypothesized that the original role of the JM domain is to regulate cell adhesion in normal morphogenetic processes. To test this idea, we focused on myotome morphogenesis as a model, in which myocytes undergo coordinated cell migration and rearrangement during somite differentiation. We injected various cadherin constructs into developing somites, and examined the effect of their expressions on myotome development. Ectopic expression of the JM(-) cadherin in developing somites blocked the normal alignment of myocytes; their medio-lateral spreading was inhibited, leaving them as unorganized cell clumps, suggesting that this cadherin construct inhibited the migration and/or rearrangement of myocytes. This finding supports the idea that regulation of cadherin activity is required for morphogenetic cell movement, and the JM domain is involved in this regulation. A point-mutant cadherin to which p120-catenin is unable to bind had no effect on myocyte migration, indicating that this catenin is not involved in the above phenomenon. Other unknown factors may modulate cadherin activity through the JM domain, and, in turn, regulate cell rearrangement. From these observations, we conclude that the regulation of cadherin activity via the JM domain is important for morphogenetic cell movement or rearrangement.

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Genomic approaches to cell adhesion

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Invasion and metastasis (collectively malignancy) are the processes that make cancer a dangerous disease. We understand much less about them than we do about the initiation and development of primary tumors, yet malignancy is what kills. So, there is a pressing need to understand the molecular and cellular changes that contribute to invasion and metastasis. These processes involve loss of positional controls, which are intrinsically more complex than the loss of growth controls involved in primary tumor growth. Invasion requires both loss of adhesion for "home base" and acquisition of invasive and migratory properties, which themselves require acquisition of novel adhesive interactions. Good examples exist in human tumors both of loss of adhesion (e.g., cadherins in colon and stomach carcinomas) and gain of adhesion (e.g., integrins in malignant melanomas and many carcinomas). Changes in cell adhesion probably also contribute to the extravasation of tumor cells from the vasculature. Despite these anecdotal cases, we do not have a good picture of the changes in cell adhesion that contribute to the many steps required for a successful metastasis. In part this is because the processes are complex; in part it is because we have until very recently lacked a sufficiently complete picture of the molecules involved in cell adhesion. The situation has changed radically in recent years and we now have the possibility to attempt a detailed inventory of the changes in cell adhesion associated with the multiple steps of invasion and metastasis.

This prospect comes from several advances. First, during the past couple of decades, we have developed an understanding of many of the proteins that mediate ceil-cell and cell-matrix adhesion (1). Second, the completion, or near completion, of the human genome sequence offers the imminent prospect of preparing a largely complete list of adhesion receptors and their ligands. We can already do that for flies and worms and comparison of those sequences with what we already know of cell adhesion molecules in vertebrates provides some informative insights into the evolution of cell adhesion (2). Third, the availability of DNA arrays and other post-genomic approaches allows rapid screening for changes in the expression of genes and proteins in primary tumors and cells in various stages of malignancy. Initial applications of this approach have yielded new insights into changes in molecules involved in cell adhesion and migration that occur during malignant progression (3). Further application of this strategy promises to reveal many further such changes. Compilation of inventories of such changes offer improved diagnosis and open up routes to new therapies. This is particularly the case for cell surface adhesion receptors, such receptors are already being successfully targeted using antithrombotic and other drugs and seem likely targets for drugs designed to block invasion, metastasis and angiogenesis (4).

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Transcriptional repression of *E-cadherin* expression by Snail and E2A factors

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The invasion of tumour cells into adjacent connective tissues involves the loss of cell-cell interactions together with the gain of proteolytic and migratory properties, and is frequently associated with epithelial-mesenchymal transitions (EMTs), a crucial process for the generation of different tissues during embryonic development. Strong cell-cell adhesion in both embryonic and adult epithelial tissues is mainly dependent on the E-cadherin/catenin adhesion system. Indeed, the loss of E-cadherin mediated intercellular interactions is determinant for the acquisition of the invasive phenotype in epithelial tumours and for EMT processes that take place during early embryonic development.

We are investigating the molecular mechanisms involved in *E-cadherin* downregulation during tumour progression. Previous work on the mouse *E-cadherin* promoter showed that the E-pal element (containing two adjacent E-boxes) plays a repressor role in E-cadherin deficient carcinoma cells and fibroblasts, and supported the hypothesis that repression through the E-pal element should be dominant over the activation of constitutive positive acting factors interacting with the GC-rich and CCAAT box proximal elements[1,2]. To identify transcriptional repressors of E-cadherin expression we recently developed a one-hybrid yeast system based in the use of the E-pal element as bait and of a cDNA library from NIH3T3 fibroblasts as prey. This system has allowed the identification of the zinc-finger factor Snail, previously implicated in EMTs in embryos, as a potent repressor of E-cadherin expression[3]. Epithelial cells that ectopically express Snail adopt a fibroblastoid phenotype and acquire tumorigenic and invasive properties. Endogenous *Snail* is expressed in invasive mouse and human carcinoma cell lines where *E-cadherin* expression has been lost. Furthermore, the presence of *Snail* expression at the invasive front of squamous cell carcinomas and in dedifferentiated carcinomas also supports its implication in E-cadherin downregulation and in EMTs during the invasion process[3].

A second factor isolated in the one-hybrid screening with high abundance (32% of the clones) corresponded to the mouse E47 bHLH factor, one of the two major products of *E2A* gene. Ectopic expression of E47 in epithelial MDCK cells leads to a dramatic EMT, concomitant with the loss of E-cadherin and the acquisition of invasive and tumorigenic properties. Repression of *E-cadherin* expression by E47 occurs at transcriptional level through the binding to the E-boxes of the E-pal element. Interestingly, endogenous *E2A* is expressed in invasive E-cadherin deficient carcinoma cell lines but it is absent from epithelial cell lines. This pattern correlates with the expression of *E2A* observed in early mouse embryos, where it is absent from the different epithelia. Thus, E12/E47 may be considered as an additional repressor of *E-cadherin* expression both during development and in tumour progression. The relationship between Snail family factors and E2A factors in the repression of *E-cadherin* expression is presently being investigated.

Taken together, our results support the hypothesis that the same molecules and regulatory mechanisms are utilised for the same cellular processes during normal embryonic development and in pathological events in the adult such as cancer progression.

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How matrix metalloproteinases regulate development and neoplasia

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Matrix metalloproteinases (MMPs) are enzymes degrade most components of the extracellular matrix(ECM), including collagens, laminin, fibrin and proteoglycans. They are regulated by a family of physiological inhibitors, the tissue inhibitor of metalloproteinases (TIMPs). The 20 members of the family have been identified to date are all secreted or transmembrane molecules. MMPs participate in: (1) degradation of ECM to allow cell migration through the extracellular matrix; (2) alteration of the ECM micro-environment resulting in alteration in cellular behavior; (3) modulation of biologically active molecules by direct cleavage or release from ECM stores; and (4) regulation of the activity of other proteases. Cell attachment, migration, proliferation, differentiation and apoptosis are all regulated by MMPs.

We have focused on the matrix metalloproteinase (MMP) familyand have sought to understand its role(s) during development and neoplastic progression in transgenic mice. Endochondral bone formation presents a particularly interesting developmental challenge. During this process an avascular tissue (cartilage) is converted into one of the most highly vascularized tissues (bone) in the vertebrate body. Ossification begins with invasion of the calcified hypertrophic cartilage by capillaries. This neovascularization of the growth plates is accompanied by apoptosis of the terminal hypertrophic chondrocytes, degradation of the cartilage matrix and deposition of bone matrix by osteoblasts. Remodeling of the bone matrix by chondroclasts results in a cavity filled with vascular channels containing hematopoietic cells. We have shown that gelatinase B/MMP-9 makes VEGF, the angiogenic factor made by hypertrophic chondrocytes available. The functional VEGF then co-ordinates matrix degradation and the recruitment of endothelial cells, chondroclasts and osteoblasts to allow the osteogenesis to proceed.

During carcinogenesis of pancreatic islets in transgenic mice, an angiogenic switch activates the quiescent vasculature. Paradoxically, vascular endothelial growth factor (VEGF) and its receptors are expressed constitutively. Nevertheless, a synthetic inhibitor of VEGF signaling impairs angiogenic switching and tumor growth. MMP-2/gelatinase-A and MMP-9/gelatinase-B, are upregulated in angiogenic lesions. MMP-9 can render normal islets angiogenic, releasing VEGF. MMP inhibitors reduce angiogenic switching, tumor number and growth, as does genetic ablation of MMP-9. Absence of MMP-2 does not impair induction of angiogenesis, but retards tumor growth. Thus, MMP-9 is a component of the angiogenic switch.

In a mouse model of multistage tumorigenesis elicited by the HPV16 oncogenes, MMP-9 is upregulated in angiogenic dysplasias and invasive cancers of the epidermis. Mice lacking MMP-9 reveal this MMP normally amplifies keratinocyte hyperproliferation , and accelerates angiogenesis; furthermore it enhances the formation of invasive tumors, and yet restricts their malignant de-differentiation. Notably, MMP-9 is expressed in reactive stromal cells, including fibroblasts, mast cells and neutrophils. Bone marrow transfer of MMP-9 sufficient cells is sufficient to restore wild type carcinogenesis, unequivocally demonstrating

that inflammatory cells are co-conspirators not innocent bystanders to carcinoma development.

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The two-handed zinc finger protein SIP1 downregulates E-cadherin and induces invasion

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The epithelial cell-cell adhesion protein E-cadherin is a genuine tumor suppressor as well as an established invasion suppressor. Lobular breast cancers and diffuse gastric cancers frequently harbor E-cadherin-inactivating mutations. However, in various other epithelial tumors transcriptional downregulation of E-cadherin appears to be a major event during tumor progression. Increased levels of the zinc finger transcription factor Snail were reported to mediate such reversible, though malignant E-cadherin suppression. Here, we report that the DNA-binding protein SIP1 (Smad-interacting protein 1; ZEB2) downregulates the E-cadherin promoter activity of man and mouse. This two-handed multi-zinc finger protein binds to both of two E2-boxes in the E-cadherin promoter sequences, and mutation of either the zinc fingers or the E2-boxes abrogates the transcriptional suppression. Moreover, conditional expression of SIP1 in E-cadherin-positive MDCK cells likewise affected E-cadherin transcription, resulting in a striking loss of E-cadherin-mediated intercellular adhesion. Concomitantly, invasion into collagen type 1 was induced. This dramatic SIP1 effect could not be ascribed to Snail induction, and it was accompanied neither by loss of b-catenin at the cell surface, nor by activation of LEF/TCF activity. Further, we showed that SIP1 is highly expressed in several E-cadherin-negative human carcinoma cell lines including cell lines lacking Snail expression, but also in normal nonepithelial cell types. How SIP1 is induced in such cells needs further investigation. How SIP1 is induced in such cells needs further investigation. SIP1 contains several motifs predicted to interact with the transcriptional repressor CtBP, but these domains could be mutationally inactivated without loss of the E-cadherin suppression effect. In conclusion, SIP1 appears to be a novel and potent invasion promoter protein in malignant epithelial tumors, acting negatively on E-cadherin expression via promoter binding. Presently, we generate suitable SIP

POSTERS

Mechanisms of head induction in vertebrates

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The *Xenopus* cerberus gene encodes a secreted factor expressed in the Spemann organizer that induces ectopic head formation when its mRNA is injected into *Xenopus* embryos. In the mouse, the related gene cerberus-like, Cer-l, is expressed in the Anterior Visceral Endoderm (AVE) and mesendoderm that underlies the presumptive anterior neural plate. Both factors function in the extracellular space as multivalent growth-factor antagonists inhibiting BMP, Nodal and Wnt proteins. Recent embryological and genetic evidence indicates that in vertebrates, the head and the trunk are induced by different sets of signals, originated by distinctly separated cell populations: in mice, the Head Organizer is located in the AVE and the Trunk Organizer in the Anterior Primitive Streak and node; in amphibians, the Spemann's Organizer (the homologous structure of the node in the mouse) partially overlaps with the anterior endoderm cells expressing cerberus. This raises the question whether in the mouse, these two signalling centers are independent or functionally interdependent with each other. Evidence for the above mentioned data will be shown as well as that the BMP antagonists chordin and noggin expressed in the node and its derivatives interact for correct forebrain development. A case of genetic interaction is not verified in cer-l; noggin double mutants, leading to a view of a complex genetic network at the level of the several BMP antagonists in the patterning of the early mouse embryo.

p85α, a regulatory subunit of class IA PI3K, stimulates the transcriptional activity of ß-catenin/LEF-1

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The canonical Wnt signalling pathway is critical during development and carcinogenesis. Activation of this pathway results in the cytoplasmic accumulation and nuclear translocation of ß-catenin where it is thought to interact with members of the LEF/Tcf family of transcription factors, thereby activating gene transcription. Recently, a Ras-dependent interaction between ß-catenin and the regulatory subunit of the class IA PI3K, p85α, was demonstrated in mouse keratinocytes (Espada et al. 1999. J. Cell Biol. 146: 967) suggesting a potential conecction between the Ras and Wnt signalling pathways. Gene reporter assays indicate that the transcriptional activity of the ß-catenin/LEF-1 co1plex is stimulated when p85α is coexpressed. This stimulation is further enhanced by the chemical inhibition of PI3K catalytic activity. Moreover, microinjection of recombinant p85α is sufficient to promote the nuclear translocation of ß-catenin, and microinjection of recombinant ß-catenin induces the cytoplasmatic and nuclear accumulation of p85α. This is the first evidence to suggest a role for p85α as a transcriptional coactivator in the canonical Wnt signaling pathway.

CSFRP1 controls proliferation and axonogenesis in the developing chick neural retina

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Secreted frizzled related (SFRPs) are a new class of molecules that share extensive homologies with the N-terminal, extracellular, cystein-rich domain (CRD) of the frizzled family, putative receptors of the Wnt family of signalling molecules. SFRPs lacks however of the transmembrane and intracellular domains of the frizzled receptors, instead they show in their C-terminal portion similarities of the with the so-called "basic netrin module" (NTR). Recently, we have isolated in chick a new member of this family. The nucleotide sequence of this gene shares 62% homology with that of the Xenopus FrzA and 81-82% with those of the mouse and human SFRP1, respectively. On the basis of these homologies we named this gene cSfrp1. In situ hybridisation analysis indicates that cSfrp1 is strongly expressed since gastrula stages in the most anterior portion of the embryo, with a pattern which overlaps with that of Otx2 and is complementary to that of cWnt8c. Later cSfrp1 localisation extends to mesodermal and neural plate tissue with a highly dynamic expression pattern that suggests that this molecule might be involved in different processes during embryonic development. In the eye, cSfrp1 is expressed with a pattern consistent with its involvement in cell differentiation. To address this issue we have generated a source of recombinant protein and analysed its effect in culture of E5 dissociated retinal cells. cSFRP1 induced a dramatic decrease of retinal cell proliferation as determined by BrdU incorporation. In parallel, the number of cells that exit the cells cycle and begin to express the neuronal markers, such as Tuj1 and islet1/2, increased. Further, soluble cSFRP1 induced profuse neurite outgrowth from retina explants grown over polylysine coated dishes. Whether cSFRP1 exerts these activities through the Wnt signalling pathway or through and independent signalling mechanisms remains to be established

De-differentiation induced by TGF-beta in fetal hepatocytes confers them resistance to its apoptotic effect

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Transforming growth factor-beta (TGF-β1) induces death of fetal hepatocytes by an apoptotic mechanism. However, always 40-50% cells survive. The process of cell survival is coincident with changes in morphology and phenotype. TGF-β treated cells progressively acquire fibroblast-like appearance; they are flat, are spindle-like and migrate to the empty spaces. Furthermore, cells elicit an epithelial-mesenchymal transition. Co-expression of vimentin and cytokeratin filaments and F-actin stress fibers can be observed in most of the fibroblast-like cells. Expression of liver specific genes is very low in these cells; this effect is due to the decrease of their rate of transcription analyzed in "run on" experiments. Surviving cells also present a decreased DNA-binding activity for liver-enriched transcription factors (HNF-1, HNF-3 and HNF-4). In contrast, a high expression of protooncogenes, such as c-fos, c-jun, c-myc and H-ras and an increase in AP-1 DNA-binding activity can be observed. Taking together all these results, we propose that TGF-β could be promoting some phenotypic, morphological and molecular changes in fetal hepatocytes that allow them to escape from its apoptotic effect. According with this, TGF-β, secreted at high concentrations during hepatocarcinogenesis might induce death of normal cells while providing a selective advantage for the survival of cells that are partially transformed and/or less differentiated and unresponsive to the factor.

rSNA, a retro-SNAIL gene expressed in human cell lines and tissues

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Snail is a zinc-finger transcription factor initially identified in *Drosophila* where mutant embryos show defects in mesoderm formation1. Two members of this family (Snail and Slug) have been described in vertebrates where they have been implicated in mesoderm formation and neural crest migration from the neural tube (see 2 and references therein). This property led us to suggest that Snail genes are required to release cells from an epithelium, permitting them to migrate, in a process known as epithelial-to-mesenchymal transition (EMT)3. It has also been shown that mouse and human Snail genes are involved in the EMT that takes place during tumour progression4,5. This process, mediated by the direct transcriptional repression of E-cadherin expression by Snail, leads to the acquisition of invasive properties and is associated with the degree of dedifferentiation and aggressiveness in tumours4. RT-PCR analysis of human SNAIL expression revealed that, in addition to the expected transcript, an extremely similar but different cDNA could be amplified from different tumour cell lines. Database analyses indicated that it corresponded to a sequence previously described as a non-functional pseudogene located in a different chromosome6. However, our data show that it constitutes a real transcription unit originated from a LINE1-mediated retrotranscription of a bona fide SNAIL transcript followed by genome insertion. Sequence analysis revealed a complete and non-interrupted open reading frame, where mutations have accumulated without disrupting the coding region. This has led us to name it retro-SNAIL (rSNA), to distinguish it from non-functional pseudogenes. In order to investigate the functional role of rSNA we have analysed its distribution in a panel of human tissues and tumour cell lines and the phenotypical changes that occur upon its stable expression in epithelial cells. The possible functional relevance of this transcription unit will be discussed in view of the results presented.

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Analysis of the molecular clock that controls somitogenesis

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Among the theoretical models attempting to explain somitogenesis, some suggested the existence of an oscillator or clock in the presomitic cells [1, 2]. The purpose of such a clock was to generate a temporal periodicity wich could then be translated into the basic spatial metameric pattern of the somites. Analysis in our laboratory of the expression of an avian hairy-homologue called c-hairy1 in the presomitic mesoderm (PSM), provided the first molecular evidence for the existence of such a clock in these cells [3]. c-hairy1 is expressed in a very dynamic anterior-posterior expression sequence in the PSM. This dynamic expression is independent of cell movements and does not result from the propagation of a signal in the plane of the PSM, but rather corresponds to an intrinsic property of the cells. In addition, our laboratory and others have shown that lunatic fringe is expressed rhythmically in chick and mouse PSM [4,5]. Lunatic fringe is a vertebrate homolog of a *Drosophila* gene that plays a role in modulating Notch-Delta signalling. The lunatic fringe null mutant mice retain the metameric pattern, but somitogenesis and Notch signalling are abnormal [6,7]. This suggests that the segmentation clock lies upstream of the Notch-Delta pathway in vertebrate somitogenesis, and that the clock might act by modulating the Notch signalling pathway to generate a periodic arrangement of boundaries. Current work in the laboratory is primarely focussed on the molecular analysis of the clock mechanisms. We are currently analyzing the status of the protein products of the cycling genes c-hairy1 and lunatic fringe to define the correlation between mRNA and protein level of expression along the rotro-caudal PSM as well as the possible contribution of post-translational modifications of different proteins in the molecular clock machinery. To evaluate the relative contributions to the clock mechanism made by cell-cell communication systems such as Notch signalling, we are analyzing the expression of cycling genes at the single cell level.

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Role of β1-integrins in mammary gland development

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Cell-matrix interactions mediated by integrins are important regulators of cell growth, programmed cell death and cytodifferentiation. To study the role of β1-integrin in mammary gland organization, we have used an approach of "dominant-negative mutation". We have created transgenic mice that express in the mammary gland a chimeric molecule consisting of a cytoplasmic and transmembrane domains of the β1-integrin subunit and an extracellular domain of the T-cell differentiation antigen CD4. Such a chimera neither binds to α-integrin subunits, nor interacts with the matrix integrin ligands, however, it competes with endogenous β1-integrin in binding to signalling molecules, and therefore exhibits an effect of a dominant-negative mutation. We have observed a retarded development of the transgenic glands in pregnancy and in lactation due to decreased proliferation and increased apoptosis rates. Differentiation of secretory epithelial cells was retarded as well. At the onset of lactation, the amount of the whey acidic protein (WAP) and β-casein gene transcripts was significantly diminished in transgenic glands and reached the level similar to that observed in wild-type glands by day 5 of lactation. In involution, the apoptosis rates were similar in the transgenic and wild-type glands, whereas the levels of WAP and β-casein transcripts dropped much quicker in the transgenic mice, indicating a precocious de-differentiation of secretory epithelium.

We have analyzed the different intracellular signalling pathways that can be responsible for the impaired growth and differentiation control in the transgenic glands during pregnancy and lactation. Our results show a defect in the activation of the MAPK (ERK and JNK) pathways via the Shc and PI3K pathways, while FAK activation is not altered. On the other hand, preliminary results suggest that the levels of STAT5 (the transcription factor involved in the activation of the expression of milk protein genes) accesible to signalling are diminished in the transgenic glands, accounting for precocious de-differentiation in involution. Our observations prove that *in vivo*, β1-integrins are involved in control of proliferation, apoptosis, differentiation, and maintenance of baso-apical polarity of mammary epithelial cells, and therefore they are essential for normal mammary gland development and function.

The Ras/Raf pathway and the regulation of integrin activity during *Drosophila* development

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Interactions between integrins cell adhesion receptors and their extracellular ligands are central to cell migration. One important aspect of the integrin function during the process of cell migration is the regulation of its activity. It is vital that integrins only form attachments at the appropriate time and can be inactivated to allow morphogenetic changes such as epithelium to mesenchyme transitions and the subsequent migration of mesenchymal cells. Experiments in vitro have shown that the small GTPases of the Ras family are among the intracellular molecules required to modulate integrin function. We have used the Drosophila wing as a model system to study the role of the Ras/Raf pathway in regulating integrin activation in a developing organism. The Position Specific (PS) integrins are required during morphogenesis to hold the dorsal and ventral wing epithelia together. We have seen that expression of constitutively activated integrins leads to a failure in this adhesion which produces a wing blister. We have looked for suppressors of this lack of adhesion phenotype and found that expression of dominant negative forms of Ras or Raf suppresses the blister phenotype. Furthermore, expression of activated forms of these molecules in the wing results in an integrin-like phenotype where the dorsal and ventral wing surfaces are separated. Taken together, our preliminary data suggest that in Drosophila the Ras/Raf pathway might be involved in a negative feed back loop that regulates PS integrin-mediated adhesion. Further details of this regulation and its developmental consequences will be presented.

TGF-β3 and palatal fusion

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The isolated cleft palate is a frequent congenital malformation caused, amongst others, by an altered expression of Transforming Growth Factor beta 3 (TGF-b3): TGF-b3 null mutant mice show cleft palate as the only craniofacial malformation (Proetzel et al., 1995; Kaartinen et al., 1995), whilst mutations of the TGF-b3 gene have been found in isolated palatal clefts in humans (Lidral et al., 1998). Up to date, TGF-b3 has been reported to cause medial edge epithelial (MEE) cells differentiation (Taya et al.1998; Martínez-Álvarez et al., 1996, 2000a, b), transformation into mesenchyme (Kaartinen et al., 1997; Sun et al., 1998) and death (Martínez-Álvarez et al., 2000a). The aim of this work was to determine whether TGF-b3 has a role on MEE cells adhesion and intercalation. Immunohistochemistry and labelling of MEE cells in culture by lipophilic cell markers were performed on TGF-b3 null and wild type palates, 8.5 embryonic day chick embryos or both mouse and chick palate cultures. First, our results show differences in the pattern of expression of the adhesion or adhesion related molecules E-cadherin, vinculin and b-catenin between the multilayered TGF-b3 wild type and the monolayered null palatal MEE. The extracellular matrix molecule chondroitin sulphate proteoglycan (CSPG), which seems to be relevant during palatal shelf adhesion and is expressed on the TGF-b3 wild type MEE surface, is absent in the TGF-b3 null palates. Furthermore, both CSPG synthesis and palatal shelf adhesion are clearly induced in chicken palate cultures when TGF-b3 is added to the culture medium. Second, marking MEE cells from each palatal shelf with DiI or DiO in culture has allowed the observation of their intercalation by the time of the elongation and disappearance of the midline epithelial seam. TGF-b3 seems to be inducing this mechanism, as it is altered in TGF-b3 null palate cultures. The incapacity of TGF-b3 null MEE cells all to form a multilayered epithelium, to intermingle when opposing epithelia are placed in contact in culture and to become mesenchyme (Kaartinen et al., 1997; Sun et al., 1998) suggests that TGF-b3 could be inducing MEE cell differentiation towards a "mobile" phenotype. This would also explain why both TGF-b3 null superficial MEE cells do not show the actin caused cellular bulges that are observed in TGF-b3 wild type palates (Martínez-Álvarez et al., 2000b) and are unable to synthesise CSPG prior to palatal fusion. Either an alteration of the actin cytoskeleton organisation/composition or a failure on the linkage/distribution of adhesion related molecules (as can be inferred by the distorted patterns of expression of b-catenin and vinculin in TGF-b3 null mice) could be the primary effect of TGF-b3 on MEE cells. As a consequence, TGF-b3 null MEE cells would be unable to establish proper contacts both amongst themselves and with the opposing epithelium, resulting in disturbed palatal fusion and cleft palate. Further investigations are being carried out to demonstrate this hypothesis.

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Vitronectin regulates Sonic hedgehog activity during cerebellum development through CREB phosphorylation

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During development of the cerebellum, Sonic hedgehog (SHH) is expressed in migrating and settled Purkinje neurons and is directly responsible for proliferation of granule cell precursors in the external germinal layer. We have previously demonstrated that SHH interacts with vitronectin in the differentiation of spinal motor neurons. Here, we analysed whether similar interactions between SHH and extracellular matrix glycoproteins regulate subsequent steps of granule cell development. Laminins are expressed in the outer most external germinal layer where proliferation of granule cell precursors is maximal. Consistent with this expression pattern, laminin significantly increases SHH induced proliferation in primary cultures of cerebellar granule cells. Vitronectin and its integrin receptor subunits alpha-v are expressed in the inner part of the external germinal layer where granule cell precursors exit the cell cycle and commence to differentiate. In cultures, vitronectin is able to overcome SHH-induced proliferation, thus allowing granule cell differentiation. Our studies indicate that the pathway in granule cell precursors responsible for the conversion of a proliferative SHH-mediated response to a differentiation signal depends on CREB. Vitronectin stimulates phosphorylation of cyclic-AMP responsive element-binding protein (CREB), and over-expression of CREB is sufficient to induce granule cell differentiation in the presence of SHH. Taken together, these data suggest that granule neuron differentiation is regulated by the vitronectin-induced phosphorylation of CREB, a critical event that terminates SHH-mediated proliferation and permits the differentiation program to proceed in these cells.

Transcriptional repression in response to Wingless signalling: the role of Teashirt

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During development, extracellular signals often act at multiple thresholds to specify distinct transcriptional and cellular responses. For example, in the embryonic midgut of *Drosophila*, low levels of Wingless stimulate the transcription of homeotic genes whereas high Wingless levels repress these genes(1, 2). Wingless-mediated transcriptional activation is conferred by *Drosophila* T cell factor (dTCF) and its co-activator Armadillo (3-7), but the nuclear factors mediating transcriptional repression are unknown. Here we show that teashirt is required for Wingless-mediated repression of Ultrabithorax in the midgut. Teashirt is also a repressor of the homeotic gene labial in this tissue. Furthermore, the target sequence for Tsh within the Ultrabithorax midgut enhancer coincides with the response sequence mediating repression of this enhancer by high Wingless levels. Finally, we demonstrate that the zinc finger protein Teashirt behaves as a transcriptional repressor in transfected mammalian cells and that it harbours a repression domain in its N-terminus which binds the CtBP co-repressor. It thus appears that the response to high Wingless levels in the *Drosophila* midgut is indirect and based on transcriptional activation of the Teashirt repressor.

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