Instituto Juan March de Estudios e Investigaciones

46 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Molecular Mechanisms Involved in Epithelial Cell Differentiation

Organized by

H. Beug, A. Zweibaum and F. X. Real

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C. Birchmeier
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G. F. Vande Woude
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INTRODUCTION

Francisco X. Real

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Epithelia cover the body surfaces that are in direct contact with the environment. In addition to providing mechanical protection, epithelial cells secrete a large number of molecules that participate in resistance to infection and the digestive process, among others.

Because of the many different functions they perform, epithelial tissues are affected in a large number of pathological processes (i.e. inflammation, atrophy, metaplasia) and epithelial-derived tumors constitute 85 % of human cancers.

In spite of their importance, understanding the mechanisms that control epithelial cell renewal and differentiation is well behind our knowledge of these processes in other cell types. First, epithelial tissues are not as readily accessible as, for example, hematopoietic cells. Second, there are only few instances in which normal epithelial cells can be cultured in vitro and conditions permissive for cell differentiation have only been worked out for a small number of models. Because of these problems, tumor cell lines are often used and, in some cases, they have proven extremely useful in unraveling both the steps and the mechanisms controlling cell maturation. In addition, tissue-specific differences in extracellular matrix and mesenchyme, as well as other factors, may also play a significant role.

The main aim of the Workshop was to update the mechanisms that are involved in the control of cell differentiation in epithelia.

The identification of stem cells in epithelia remains an important but difficult task. Two different approaches were reported that have provided insight into this issue in the intestine and the skin, respectively. In the intestine, cell replication has been identified in situ using a variety of techniques to identify DNA synthesis whereas cell culture methods, and the selective isolation of cells on the basis of their phenotype, has been applied to the study of the epidermis.

In the last few years, major advances have been made in the identification of molecules that control morphogenesis, such as hepatocyte growth factor. In vitro studies are beginning to allow the dissection of morphogenesis and cytodifferentiation in epithelia, particularly in the breast.

An issue of continued debate is the use of cultured cell models vs. transgenic models. When considering the former, a great emphasis was placed on the role of the extracellular matrix, the mesenchyme, and other connective tissue components in epithelial cell differentiation. Regarding the latter, emphasis was placed on the need to complement "animal phenotype" studies with biochemical and molecular analysis using cultured cells derived from transgenic animals. Finally, the possibility of introducing genes in tissue explants and analyzing their effect both in vitro and upon injection in experimental animals was proposed as an alternative strategy to analyze gene function without the need to generate transgenic animals.

Several studies addressed the plasticity of the epithelial cell phenotype, in particular epithelial-mesenchymal transitions. The peptides (i.e. hepatocyte growth factor, transforming growth factor beta, fibroblast growth factor) and receptors that are able to induce such changes are being identified and their role during development, tissue regeneration, and neoplasia is being examined. In these processes, dramatic changes in the expression of tissue-specific genes and

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adhesion molecules take place. Furthermore, the induction of protease production upon loss of the differentiated epithelial phenotype was reported as a factor contributing to the acquisition of migrating properties and - in the case of tumor cells - to the metastatic process.

Regarding gene regulation by transcription factors, current evidence points to the existence of few really tissue-specific factors and the current challenge is to understand how multimolecular complexes are formed that interact with nucleic acid sequences not only in the vicinity of transcribed sequences but also at distance.

The Workshop brought together scientists from diverse fields and with different interests, emphasizing the advantages and disadvantages of the various approaches used. The recent advances should improve our understanding of both basic questions and pathological processes.

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FIRST SESSION: POLARITY AND MORPHOGENESIS

Chairperson: Francisco X. Real

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Anne Müsch*, Huaxi Xu‡†, Charles Yeaman, Dennis Shields† & Enrique Rodriguez-Boulan.* **Mechanisms involved in the assembly of post-Golgi vesicles in polarized and non polarized cells.**
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The Trans Golgi Network (TGN) is a major sorting center in the secretory pathway, where basolateral membranes of epithelial cells, to regulated and constitutive secretory pathways, and to lysosomes, are packaged into distinct post-TGN vesicles. Sorting of basolateral membrane proteins is mediated by discrete signals in the cytoplasmic domain, whereas sorting of apical membrane proteins, lysosomal hydrolases and presumably secretory proteins is directed by luminal signals. To characterize specific "basolateral", "apical" and "regulated" vesicle assembly mechanisms, we performed competition experiments using basolateral signal peptides corresponding to the cytoplasmic tails of Vesicular Stomatitis Virus G protein (VSVG), a mutant basolateral form of influenza hemagglutinin (HAY), and the TGN marker TGN 38. The VSVG peptide strongly inhibited the vesicular release of VSVG from the Trans Golgi Network (TGN) of semi-intact epithelial (MDCK), fibroblastic (3T3) and regulated secretory (GH3 pituitary) cells. The peptide did not inhibit the vesicular release of HA in MDCK cells and two polypeptide hormones in GH3 cells and only affected partially the release of HA in 3T3 fibroblasts. Our results indicate that a basolateral-like, signal-mediated constitutive pathway from TGN to plasma membrane exists in all three cell types and that an apical-like pathway is present in fibroblasts. The data support cargo protein involvement, not bulk flow, in the formation of post-TGN vesicles. Data on the sorting of apical and basolateral proteins in the TGN obtained with an adenovirus mediated gene transfer system will also be discussed.

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INDUCTION OF EPITHELIAL TUBULOGENESIS IN VITRO

Roberto Montesano

Dept. of Morphology, University Medical Center, Geneva (CH)

A crucial event in the ontogeny of most parenchymal organs is the formation of branching epithelial tubules. Although it has long been recognized that epithelial tubulogenesis is controlled by the adjacent mesenchyme, the molecular mechanisms involved in these inductive interactions are still poorly understood. Our approach to this problem has been to study the effect of mesenchyme-derived diffusible factors on the morphogenetic properties of well differentiated epithelial cells grown in a three-dimensional collagen matrix.

Madin-Darby canine kidney (MDCK) epithelial cells retain several properties of kidney tubule epithelium and generate tubule-like structures *in vivo,* but form spherical cysts when grown within three-dimensional collagen gels (1). To investigate whether the morphogenetic properties of MDCK cells might be influenced by diffusible factors released by neighbouring mesenchymal or stromal cells, we designed an *in vitro* system in which MDCK cells are cocultured in collagen gels with embryonic or adult fibroblasts under conditions precluding heterocellular contact. We found that MDCK cells suspended within a collagen gel contiguous to a fibroblast-populated gel layer formed branching tubules, instead of the spherical cysts that developed under control conditions, i.e. in the absence of fibroblasts. In addition, MDCK cells grown as a monolayer on a cell-free collagen gel cast on top of a fibroblast-containing gel layer invaded the underlying collagen matrix, within which they formed a network of branching tubules. Finally, fibroblast-conditioned medium (CM) stimulated tubule formation by MDCK cells grown in collagen gels (2). The effect of CM was not mimicked by any of a number of well characterized growth and differentiation factors, including EGF, bFGF, aFGF, TGF-ß1, IGF-I, IGF-II, PDGF, or KGF (2).

While we were attempting to identify the fibroblast-derived factor which induced tubule formation by MDCK cells, it was reported that hepatocyte growth factor (HGF), a polypeptide considered to be a humoral mediator of liver regeneration (3), is produced by cultured fibroblasts and stimulates the proliferation of a broad spectrum of epithelial cell types (4). These new data prompted us to explore the effect of HGF in our system. We found that MDCK cells grown in collagen gels in the presence of HGF formed branching tubular structures. In addition, MDCK cells grown in the presence of fibroblast CM that had been preincubated with anti-HGF antibodies exclusively formed spherical cysts, which demonstrated that the fibroblast-derived tubulogenic factor is HGF (5). These studies have revealed a previously unsuspected property of HGF, i.e. the ability to transmit molecular information that determines the spatial arrangement of epithelial cells. It is noteworthy that no other molecule has heretofore been reported to have similar properties. Interestingly, HGF has been shown to

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be identical to scatter factor (SF), a protein which induces dispersion of MDCK cells in monolayer culture (6). This raises the intriguing question of how the same cytokine induces formation of well organized tubules in collagen gels, while causing cell dissociation in conventional cultures. We found that HGF induces MDCK cells grown *on the surface* of a collagen gel to scatter, as they do on a plastic substratum. This finding suggests that the critical factor which determines whether MDCK cells will dissociate or organize into tubules in response to HGF/SF is the geometry of cell-matrix interactions (i.e. two-dimensional versus three-dimensional interactions), rather than the chemical nature of the substratum (5).

Is the paracrine type of epithelial-mesenchymal interaction describe above more widely applicable to the morphogenesis of other epithelial tissues? To address this issue, we investigated whether diffusible factors released by fibroblasts would promote formation of duct-like structures by mammary gland epithelial cells embedded in collagen gels. Using a clone (TAC-2) derived in our laboratory from the normal murine mammary gland (NMuMG) epithelial cell line, we found that addition of fibroblast CM to cultures of TAC-2 cells stimulated the development of an extensive system of highly arborized duct-like structures, which in appropriate sections were seen to contain a central lumen. The effect of fibroblast CM was abrogated by antibodies to HGF, whereas addition of exogenous HGF to the cultures mimicked the tubulogenic activity of fibroblast CM. The effect of fibroblast CM or HGF was markedly potentiated by the simultaneous addition of hydrocortisone, which also enhanced lumen formation (7). These results indicate that the tubulogenic activity of HGF is not restricted to a single epithelial type, and suggest that HGF is an important stromal mediator of mammary gland ductal morphogenesis.

Taken together, the results obtained with kidney-derived MDCK cells and TAC-2 mammary gland epithelial cells suggest that HGF may be one of the long-sought paracrine mediators of morphogenetic epithelial-mesenchymal interactions.

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SECOND SESSION: STEM CELLS

Chairperson: Alain Zweibaum

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STEM CELLS IN THE SMALL INTESTINAL EPITHELIUM, CS Potten, CRC Epithelial Biology, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester M20 9BX, UK

Proliferation occurs in discrete closed units - the crypts, in the small intestine where proliferation is as fast as in any tissues in the body. The cells ultimately responsible for this cell replacement, the stem cells, can be shown by cell migration studies, a variety of cell kinetic experiments and mathematical modelling to be located at the base of the crypts immediately above the differentiated Paneth cells (1,2). The stem cell location in the colon is likely to be at the absolute base of the crypts. This spatial organisation, in the absence of stem cell specific markers, offers the ability to study the behaviour and characteristics of the cells at the specific stem cell location. Clonal regeneration assays, a test of the functional capacity of stem cells, have been extensively used. These show that each crypt contains up to 30 clonogenic cells, far less than the total number of 150 proliferating cells. The size of the clonogenic compartment appears to be dependent upon the level of damage induced in order to study these cells. After medium sized doses there are about 6 clonogenic cells and after high doses about 30 cells that can perform clonogenic function (3). A certain level of spontaneous apoptosis occurs in the stem cell region which is believed to be a reflection of stem cell homeostatic mechanisms in operation. Up to about 6 cells in the stem cell region of the crypt exhibit an exquisite radiosensitivity consistent with a lack of repair capacity and an intolerance of minor levels of DNA damage. Once damaged, these cells switch on the apoptosis program. This represents a protective mechanism operating on the functional stem cell compartment which may account for the surprisingly rare cancer incidence in this rapidly proliferating tissue. This process does not appear to be functioning in the large bowel (4). Taken as a whole, the observations suggest a 3-tier stem cell compartment with about 6 exquisitely sensitive steady state functional stem cells, a further 6 cells with clonogenic capacity, higher radio resistance and good repair capacity. Finally, there are about 24 even more resistant reserve clonogenic cells and about 120-130 rapidly proliferating cells with no apparent stem cell attributes. In the colon, differences exist in the effectiveness of these processes (possibly related to bcl-2 expression), which may account in part for the higher cancer risk.

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ISOLATION AND LOCALISATION OF HUMAN EPIDERMAL STEM CELLS

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The epidermis is made up of multiple layers of keratinocytes. Proliferation takes place in the basal layer of cells that adhere, via receptors of the integrin family, to the underlying basement membrane. Cells that initiate terminal differentiation detach from the basement membrane as a result of integrin inactivation and move into the first suprabasal layer. Terminal differentiation occurs as cells move through the suprabasal layers to the surface of the tissue. Terminally differentiated cells are continually shed from the epidermis and epidermal self-renewal depends on proliferation of stem cells in the basal layer. In addition to stem cells there are other proliferative keratinocytes, called transit amplifying cells, which are destined to initiate terminal differentiation after a small number of rounds of division. My laboratory has recently shown that stem cells express higher levels of integrins than transit amplifying cells and that this can be used to determine the location of stem cells in epidermis from different body-sites. Furthermore, stem cells can be isolated directly from skin and their behaviour in culture can be analysed. We are currently looking for additional stem cell markers and I shall present our recent data.

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THIRD SESSION:

DIFFERENTIATION - IN VITRO MODELS

Chairpersons:
Hartmut Beug and Enrique Rodríguez-Boulan

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Induction of the hepatic phenotype by over-expression of HNF-4. Gerald Spath and Mary C. Weiss. Unité de Génétique de la Differenciation, URA CNRS 1149, Department of Molcular Biology, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris, France.

The techniques of reverse genetics have been used in a number of laboratories to identify and clone a series of transcription factors that are enriched in the hepatocyte. In cultured cells of hepatic origin, only two of them, HNF1 and HNF4, show expression patterns that are strictly correleated with the differentiation profile of the cells: in dedifferentiated hepatoma cell variants as well as in hepatoma-fibroblast hybrids showing extinction of the hepatic cell phenotype, neither of these factors is produced. In addition, it has been shown that HNF4 is a positive regulator of the HNF1 gene. Consequently, HNF4 can be considered the most likely candidate for a regulator of hepatic cell differentiation.

In order to examine the effects of forced expression of exogenous HNF1 and HNF4 on 1) the expression state of the endogenous HNF1 gene and 2) the differentiation state of the recipient cell, expression vectors have been prepared that contain cDNA for the factors as well as for a VSV tag: the exogenous and endogenous mRNAs can be distinguished by size, and the exogenous proteins are detectable by immunofluorescence.

Clones of dedifferentiated variant C2 and H5 rat hepatoma cells have been isolated which express exogenous HNF1 and HNF4. In all cases, the presence of exogenous HNF1 leads to the activation of expression of the endogenous HNF1 gene, confirming the existence of a suspected positive regulatory loop. However, only exogenous HNF4 expression leads to a modification of the cell's phenotype, and only in the case of transfection into H5 cells. The steps in the re-acquisition of hepatic morphology and polarization as well as in the re-expression of hepatic functions will be presented.

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Apical delivery in human intestinal epithelial cells: a potential role for oligosaccharide side chains.

Alain Zweibaum, INSERM U178, Villejuif, France.

Differentiated human colon carcinoma cell lines such as Caco-2 and HT-29 have been extensively used as *in vitro* models to study the apical delivery of proteins, as they express, after confluency, the differentiation characteristics of either intestinal enterocytes (Caco-2[1] and some HT-29 subpopulations like those selected by adaptation to glucose deprivation, i.e. HT29-Glc^{-}[2]) or goblet cells (HT-29 subpopulations and more particularly those selected for their resitance to methotrexate, quoted as HT29-MTX cells[3]). Unlike in the human intestine, where apically secreted glycoproteins, like mucins, or apically addressed glycoproteins, like brush border membrane-associated hydrolases, constitutively express ABH blood group terminal sugars[4], the corresponding proteins in Caco-2 or HT-29 cells show modifications of glycosylation with ABH terminal sugars being replaced by sialylated sugars. A potential role of these oligosaccharide side-chains in the intracellular traffic of glycoproteins relies on results obtained with HT29-Glc^{-} and HT29-MTX cells. These cells are characterized, after confluency, by the presence in both populations of an apical brush border whose membrane is endowed with dipeptidylpeptidase-IV (DPP-IV), MUC1 and CEA and, in MTX cells, of mucins which accumulate in the supranuclear compartment of the cells and are secreted in the culture medium. The major carbohydrate component of these mucins has been shown to be NeuAcα2,3Galβ1-3GalNAc-*O*.[5] In a recent work the groups of G. Huet (INSERM U377, Lille, France), P. Delannoy (UST, Lille, France) and F.X. Real (IMIM, Barcelona, Spain) have shown that treatment of HT29-MTX cells with the sugar analog GalNAc-α-*O*-benzyl resulted in a decreased secretion of mucins which show a lower sialic acid content and an increased content of T antigen, i.e. Galβ1-3GalNAc-*O*. In this work it was shown that GalNAc-α-*O*-benzyl was metabolized to Galβ1-3GalNAc-α-*O*-benzyl which occurs to be a potent inhibitor of the *O*-glycan α-2,3-sialyltransferase[6]. In collaboration with these three groups we have recently observed that this effect was not restricted to MTX cells and mucins, but also involved apically addressed proteins such as DPP-IV, MUC1 and CEA. Permanent treatment of both mucus-secreting and enterocytic HT-29 cells by GalNAc-α-*O*-benzyl results in (1) a considerable swelling of the cells which show a 6-fold increase of their volume without modification of their viability or morphological differentiation, i.e. cell polarity and presence of an apical brush border, (2) a generalized intracytoplasmic accumulation of DPP-IV, MUC1 and CEA which are absent from the brush border, in contrast to villin (which is not known to be glycosylated) and (3) a lower sialic acid, and a an increased content of T antigen in these proteins as substantiated by their reactivity to the lectins MAA (specific for NeuAcα2,3Gal) and PNA (specific for Galβ1,3GalNAc). The arrest of the intracellular traffic of these glycoproteins occurs without there being any effect on their mRNA level or total protein content. It is totally and rapidly reversible within less than 48h after removal of the drug. Altogether these results show that drug-induced modifications of oligosaccharide side-chains result in an arrest of the intracellular traffic of apically delivered glycoproteins in HT-29 cells. These results raise the question of whether oligosaccharide side-chains, like eg ABH determinants in the normal, are involved in the biosynthetic traffic of apically delivered glycoproteins.

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Epithelial-mesenchymal transdifferentiation and malignant progression of mouse skin carcinogenesis

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In mouse epidermal carcinogenesis progression to malignant carcinomas involves de progressive loss of the epithelial phenotype. Thus, carcinomas can be classified by histological criteria ranging from well differentiated squamous cell carcinomas (SCC) to highly invasive undifferentiated spindle cell carcinomas (SpCC). Such changes are also observed *in vitro* , in cell lines derived from tumors or transformed in culture by different approaches, and are associated with down-regulation of cell adhesion receptors, such as E-cadherin (1), alterations in the expression/organization of cytoskeletal proteins (2), and the acquisition of a fibroblastoid or spindle cell morphology.

Whereas the events responsible for loss of differentiation in squamous carciomas are presently unknown, we have recently shown that transformed keratinocytes that escape to the block of proliferation exerted by transforming growth factor-β_1 (TGF-β_1) in normal epithelial cells, when cultured in the presence of this factor, elicits a reversible and sequential conversion from an epithelial to a fibroblast-like phenotype (3). This conversion correlates with down-regulation of E-cadherin and cytokeratins and up-regulation of vimentin.

The tumorigenic properties of TGF-β_1-treated cell cultures were studied by transplantation in nude mice. TGF-β_1-treated cell cultures with a fibroblast-like morphology produced highly anaplastic SpCC, whereas untreated epithelial cells induced well differentiated SCC; and cultures in an intermediate step of reversion to epithelial, showing an epithelioid phenotype, gave rise to tumors containing both differentiated and undifferentiated regions, a type of tumor described in the literature as carcinosarcoma. Thus, the sequential tumoral stages that take place during progression from SCC to SpCC in *in vivo* chemical carcinogenesis could be reproduced *in vitro* by treatment of transformed keratinocytes with TGF-β_1. These results suggest a role for TGF-β_1 in late stages of malignant progression in mouse epidermal carcinogenesis.

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CELL DIFFERENTIATION IN THE HUMAN EXOCRINE PANCREAS. Francisco X. Real, Maya R. Vilá, and Teresa Adell. Institut Municipal d'Investigació Mèdica, 08003-Barcelona, Spain.

The exocrine and endocrine pancreas derive from poorly characterized protodifferentiated cells located in fetal ducts. To better understand the mechanisms involved in cell differentiation, we have: 1) established better differentiation markers, 2) developed methods for the culture of normal human exocrine pancreas, and 3) examined the role of growth factors in pancreas cell proliferation and differentiation.

Acinar cells are well characterized at the molecular levels and genes encoding the digestive enzymes have been isolated and characterized. In contrast, less is known regarding molecular markers restricted to ductal cells. We have identified differentiation markers which are present in ductal cells but not in acinar cells: CK7, CK19, MUC3, DPP-IV, and CFTR (reviewed in 1). However, none of these markers is exclusively expressed in the pancreas.

To examine cell differentiation, we have developed methods for the culture of normal pancreas epithelium. Ninety percent of cells in the exocrine pancreas have an acinar phenotype and in vitro culture is associated with the loss of acinar differentiation, indicated by a marked decrease in the expression of amylase, lipase, and chymotrypsinogen. Concomitantly, a ductal phenotype is activated, as determined by the expression of CK7, CK19, and CFTR and the capacity to respond to secretin (2). This phenotypical change cannot be explained on the basis of ductal cell outgrowth. Expression of acinar genes has been shown to correlate strongly with the presence of a transcriptional complex designated PTF1 which includes p48, a DNA-binding protein specific of the exocrine pancreas (3). The regulation of p48 and PTF1 levels in this system is currently being examined.

Because normal exocrine pancreas cultures have a limited lifespan, we have examined which growth factors stimulate their in vitro proliferation: HGF is the most potent mitogen and TGF alpha and KGF are also stimulatory (4).

Finally, we have examined the effect HGF on the phenotype of pancreas cancer cells. IMIM-PC-2 cells were selected because their phenotype is similar to that of normal ducts (growth as a polarized monolayer, formation of domes, and expression of all cytokeratins typical of normal ductal cells) (5). IMIM-PC-2 cells overexpress c-met and its levels are down-regulated in postconfluence, when cells acquire a differentiated phenotype (6). HGF does not stimulate the proliferation of cells seeded at low density but it induces scattering. When HGF is added after confluence, it induces foci of unpolarized cells which tend to detach and grow in suspension. Upon subculture in the presence of HGF, these cells attach, show a fibroblastic aspect ,and an up-regulated expression of vimentin. Similarly, chronic treatment (>4 weeks) of IMIM-PC-2 cells with HGF is accompanied by the acquisition of a fibroblastic aspect and vimentin up-regulation.

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These results suggest that pancreatic acinar and ductal cells show a high degree of plasticity *in vitro*. Normal pancreatic cultures and tumor cell lines display phenotypes that are similar to those of chronic pancreatitis and pancreas tumor tissues, respectively, suggesting that they are adequate cellular models for the study of pancreatic pathology.

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EFFECTS OF THYROID HORMONE AND ITS RECEPTOR THE erbA ONCOGENE ON THE PHENOTYPE AND GENE EXPRESSION OF MAMMARY EPITHELIAL CELLS

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Thyroid hormone (triiodothyronine, T3) is a main regulator of growth, development and metabolism in vertebrates modulating gene expression and physiology in multiple cell types. T3 receptors are encoded by two genes (TR/c-erbA α and β) which are the cellular counterparts of the retroviral v-erbA oncogene involved in avian erythroleukemia. The clinical and experimental studies suggesting the involvement of T3 and its receptors in carcinogenesis (1), together with the expression of thyroid receptors in normal mammary glands and breast cancer cells (2), the homology between thyroid and estrogen/progesterone receptors (3), the alterations in the erbA loci (4) and the contradictory reports on the beneficial or adverse effects of T3 on human breast cancer (5) prompted us to analyze T3/erbA and v-erbA actions on mammary epithelial cells. To this, we have used an spontaneously immortalized mouse mammary epithelial cell line (EpH4) which shows a normal differentiated phenotype.

EpH4 cells express low but significant levels of thyroid receptors. However, the RNA concentration for the non-hormone binding TR/c-erbAα2 form was higher than that corresponding to the active TR/c-erbAα1, and TR/c-erbAβ is not expressed. To clearly reveal T3 effects in EpH4 cells, the low level of endogenous T3 receptors was increased by introduction and expression of an exogenous c-erbA (chicken TR/c-erbAα) gene using retroviral vectors. Parallel cultures were infected with retroviruses encoding the v-erbA gene. Expression of the erbA genes was verified in Northern blots and hormone binding assays. Since the ability to degrade extracellular matrix is a prerequisite for epithelial cell plasticity, morphogenesis and metastasis, we studied the expression of protease genes. T3 treatment increases the levels of the 1.9 kb stromelysin 1 (ST-1) and the 1.7/1.9 kb stromelysin 2 (ST-2) mRNAs in a way that correlates with the amount of active thyroid receptor expressed. This increase is quite slow, reaching the maximum after 40 h of T3 treatment. In line with this, run-on experiments showed that elevated ST-1/-2 mRNA levels do not result from increases in the transcription of these genes. These effects seem to be quite specific since no alterations were found in the expression of genes coding for other

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proteases or protease inhibitors. Hormonal effects on stromelysin and also collagenolytic activities were analyzed by zymography. In both cases, T3 enhanced the secretion in cells expressing elevated TR/c-erbA levels (6).

We have also studied the effects of T3/c-erbA and v-erbA on the differentiated epithelial phenotype of EpH4 cells. Activated TR/c-erbA has profound effects on the polarized phenotype preventing the formation of normal compact monolayers as shown by the inhibition of the transepithelial electrical resistance of cells grown on porous filters, and causing a partial redistribution of apical and basolateral markers. Thus, T3 treatment of EpH4+TR/c-erbA cells leads to the expression of the apical marker DPP IV also on the lateral plasma membrane and to the diffuse distribution over the whole lateral domain of several components of the adherent junctions (E-cadherin, β-catenin, fodrin). Furthermore, addition of T3 to c-erbA-expressing cells causes the disruption of the tridimensional tubular structures formed in collagen gels, similar alterations in the distribution of polarized proteins and in the ordered deposition of components of the basement membrane (laminin). These results show that activated c-erbA affect the normal epithelial phenotype and morphogenetic potential of EpH4 cells (6).

On the other hand, v-erbA abolished completely the ability of the cells to form domes when cultured on plastic. This action reflects also an inhibitory effect on the polarized phenotype of EpH4 cells. Besides this, v-erbA-expressing cells are absolutely unable to develop organized structures in collagen gels. instead, they fail to attach to collagen gel growing to confluence on the surface of the dish. V-erbA also inhibits partially the expression of some epithelial markers (Z0-1, E-cadherin) and concomitantly enhances that of mesenchymal markers such as fibronectin. Together, these results show that the mutated thyroid receptor v-erbA alters the normal differentiated phenotype of mammary epithelial EpH4 cells.

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MODULATION OF THE POLARIZED EPITHELIAL PHENOTYPE BY A CONDITIONAL c-JunER PROTEIN

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Epithelial cells exhibit very specialized features: first, cellular polarity, characterized by structurally and functionally distinct domains (apical and basolateral); and second, the tight interactions via different types of junctions between neighbouring cells within a cellular monolayer. Both of these properties can be affected in carcinoma cells.

Members of the EGF (epidermal growth factor) receptor family are known to be specifically involved in mammary carcinogenesis. We sought to examine c-Jun, as one of the nuclear targets of activated receptors, in normal mammary epithelial cells. For this. we used a c-JunER fusion protein which was tightly controlled by estradiol. Activation of the JunER by hormone resulted in the transcriptional regulation of a variety of AP-1 target genes. More importantly, hormone-activated JunER induced a reversible loss of epithelial polarity, a disruption of intercellular junctions and normal barrier function and the formation of irregular mulilayers. Loss of epithelial polarity involved complete redistribution of both apical and basolateral proteins to the entire plasma membrane. The redistribution of uvomorulin and β-catenin was accompanied by a destabilization of complexes formed between these two proteins, leading to an enrichment of β-catenin in the detergent-soluble fraction.

Uninduced cells were able to form three-dimensional tubular structures in collagen I gels which were disrupted upon JunER activation, leading to irregular cell aggregates. Interestingly, the JunER-induced disruption of tubular structures was dependent on active signaling by growth factors. Moreover, the effects of JunER could be mimicked in normal cells by the addition of acidic FGF (fibroblast growth factor). These data suggest that a possible function of c-Jun in epithelial cells is to modulate epithelial polarity and regulate tissue organization, processes which may be equally important for both normal breast development and as initiating steps in carcinogenesis.

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FOURTH SESSION: ADHESION MOLECULES

Chairperson: Mina Bissell

Instituto Juan March (Madrid)

The Role of Mesenchymal Ligands for Epithelial Tyrosine Kinase Receptors in the Morphogenesis and Differentiation of Epithelia

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Growth and morphogenesis of epithelia represent essential steps in the development of many eukaryotic organs. A series of development studies have demonstrated that growth and morphogenesis of epithelia are closely regulated by mesenchymal epithelial interactions. Recent evidence seggests, that epithelial tyrosine kinase receptors and their mesenchymal ligands are good candidates for this signal transfer.

We have examined the role of two mesenchymal ligands of epithelial tyrosine kinases in mouse mammary gland morphogenesis. In organ cultures, scatter factor/hepatocyte growth factor (SF/HGF) promoted branching of the ductal trees but inhibited differentiation. Neu differentiation factor/heregulin (NDF/HRG) stimulated lobulo-alveolar budding and the production of milk proteins. These functional effects parallel the expression of the factors in vivo: SF/HGF is produced in mesenchymal cells during ductal branching in the virgin animal, NDF/HRG during lobulo-alveolar development at pregnancy. Branching morphogenesis and lobulo-alveolar differentiation could be abolished by blocking expression of the endogenous ligands by the respective antisense oligonucleotides. We thus show that the major postnatal morphogenic periods of mammary gland development are dependent on sequential mesenchymal-epithelial interactions mediated by SF/HGF and NDF/HRG.

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Role of E-cadherin in the epithelial phenotype and invasion properties of transformed epidermal keratinocytes.

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The cell-cell adhesion molecule E-cadherin (ECD) plays a determinant role in the acquisition and maintenance of the architecture of most epithelial tissues. Loss of ECD is frequently observed in a wide variety of epithelial carcinomas and an anti-invasive role has been proposed for this molecule (1). Our previous studies in the mouse skin carcinogenesis model showed that ECD expression is downregulated during progression of squamous cell carcinomas, whereas the development of fully undifferentiated spindle cell carcinomas is accompanied by the complete loss of ECD and P-cadherin (PCD)(2).

Using the mouse epidermal system, we are investigating the molecular mechanisms responsible for the downregulation of ECD and PCD as well as the influence of ECD in the epithelial phenotype and in the invasion/metastatic properties of malignant murine keratinocytes. A cell system constituted by a squamous carcinoma cell line, HaCa4 (ECD-/PCD+) and derived cell clones obtained by reintroduction of ECD cDNA has shown that the expression of ECD suppresses the in vitro invasion/migratory properties of parental HaCa4 cells, but is not sufficient to block the in vivo metastatic behavior. Thus, some of the ECD+ cell clones (E62) remain as metastatic as the parental cells, whereas other ECD+ clones (E24) are non-metastatic. Interestingly, E24 cells exhibit a more differentiated phenotype with a strong tendency to stratify in culture and express lower levels of oncogenic v-H-ras (3). To further understand the role of of ECD in the invasion/migratory process, the expression/activity of metalloproteinases in the HaCa4 and ECD-derived cell clones is being presently analysed.

On the other hand, the influence of ECD expression in the epithelial-fibroblast phenotype transition which occurs during development of spindle cell carcinomas is being studied, using a cell system constituted by the spindle carcinoma cell line, CarB, and derived cell clones obtained after stable transfection of ECD and/or plakoglobin. These studies indicate that the presence of stable ECD-catenin complexes is not sufficient to revert the spindle phenotype and suggest that other epidermal components lost in spindle cells (i.e. desmosomes, intermediate filament keratins) are needed for the maintenance of the epithelial phenotype in highly transformed keratinocytes.

The molecular mechanisms responsible for the downregulation of ECD and PCD during mouse skin tumor progression are being investigated through the analysis of the regulation of ECD and PCD promoters (4,5). These studies have identified 5' proximal sequences (GC-rich and CAAT-box) responsible for the expression of both cadherin genes, with a differential involvement of Sp1-like (PCD) and AP2-like (ECD) transcription factors. In addition, the palindromic E-pal element specific of ECD promoter appears to play a regulatory role, acting as a repressor in non-expressing keratinocytes. The nuclear binding assays carried out suggest that some of the transcription factors interacting with the 5' proximal sequences of the ECD promoter may be lost or inactivated in non-expressing keratinocytes.

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ROLE OF PROTEIN KINASE C IN DIFFERENTIATION OF HUMAN INTESTINAL EPITHELIAL CELLS

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Protein kinase C (PK-C) plays a crucial role in the signal transduction mechanism activated by growth factors, hormones and neurotransmitters. A role for PK-C in the development and progression of colon cancer has been suggested (1). However, the study of the effects of PK-C activation in these processes has been hampered by the lack of reproducible methods for the culture of normal intestinal epithelium and the undifferentiated phenotype of most colorectal cancer cell lines. recent progress in this area has evolved from the characterization of intestinal epithelial cell that differentiate in culture into goblet or absorptive cells. we have used the mucus-secreting HT-29 M6 cells (2) to analyze the role of PK-C in goblet cell differentiation.

TPA-induced PK-C activation prevented the accumulation of the differentiation markers dipeptidylpetidase IV (DPP-IV), villin and mucins in HT-29 M6 cells. This phorbol ester also downregulated the expression of mucins and DPP-IV in postconfluent differentiated cultures of these cells; both increased secretion and diminished mRNA levels of these markers were observed in cells treated with TPA. Sensitivity to this phorbol ester also defined to different steps in the process of differentiation; at early days HT-29 M6 cultures respond to TPA with loss of cell contacts and proliferation whereas in long-term cultures these paramenters were not affected. In both cases cellular levels of markers were decreased.

TPA also induced alterations in cell-matrix and cell-cell interactions in preconfluent HT-29 M6 cells. After incubation with this phorbol ester cells showed lower cell-cell aggregation, that correlated with alterations in the localization of the adhesion protein E-cadherin as analyzed by immunofluorescence. Detergent extraction evidenced that activation of PK-C caused the translocation of E-cadherin from a Triton-resistant cytoskeleton-associated fraction to a Triton-soluble, cytosolic fraction.

E-cadherin inactivation was accompanied by a rapid and

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transient increase in the total cellular content of tyrosine phosphorylation: several major substrates of 125-120 and 75-70 KDa were specially affected by TPA. Concomitantly with this increase in phosphorylation, we have detected the activation of c-src and FAK tyrosine kinase, two proteins that are found associated in HT-29 M6 cells. One of the proteins phosphorylated in tyrosine after activation of PK-C has been identified as p120-catenin, a c-src substrate that recently has been described to interact with E-cadherin (3). No changes in tyrosine phosphorylation of other E-cadherin-associated proteins as β-catenin were detected. These results suggest that TPA effects on E-cadherin inactivation might be mediated by c-src stimulation and p120-catenin phosphorylation.

We have also investigated the PK-C isoform responsible for triggering such effects. Immunoblotting with isoform-specific antibodies we have detected in these cells cPK-Cα, nPK-Cϵ, and aPK-Cζ. Using a cPK-C-selective TPA-analogue we have determined that activation of cPK-Cα is sufficient to produce E-cadherin solubilization. This conclusion has been confirmed transfecting a constitutively-activated mutant of this isoform regulated by an inducible promoter.

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CHARACTERIZATION AND POTENTIAL USE OF NEW IMMORTALIZED MOUSE INTESTINAL CELL LINES TO STUDY THE INDUCTIVE ROLE OF LAMINA PROPRIA AND LOCAL IMMUNE SYSTEM ON INTESTINAL TERMINAL DIFFERENTIATION.

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As already demonstrated for the villus-associated epithelium, the renewal of follicle-associated epithelium (FAE) found in Peyer's patches is dependant on the proliferation of stem cells located in the surrounding crypts. Two distinct spatio-temporal axes of migration/differentiation originate from the same ring of crypt stem cells. On one side of the crypt, villus-type differentiation leads to the differentiation into absortive enterocytes, enteroendocrine or goblet cells. On the other side, dome-type differentiation leads to the formation of absorptive enterocytes displaying several distinct features (loss of functions related to antibacterial defense and nutrition), and M cells, specialized in antigen and micro-organism sampling. Immune follicle cells seems to play a key role in the specific pattern of differentiation of FAE, as cross-talks between lamina propria and epithelial cells have been shown to be necessary to achieve villus enterocyte differentiation. To test this hypothesis of a two-way differentiation pattern, driven by underlying non-epithelial cells, we have developed new cultured models. A mouse cell line was derived by lipofection of FAE with the SV40 large T antigen controlled by the villin promoter. A clone of immortalized cells, referred as FAE-2 cells, exhibited a stable expression of SV40 nuclear large T antigen, and had a prolonged life span. FAE-2 cells formed confluent monolayers composed of dome-forming, polarized cuboid cells separated by tight junctions. Evidences of the epithelial and intestinal origin of FAE-2 cells were provided by the expression of an epithelial-specific actin network, ZO-1 protein, cytokeratins 46,51,55 Kd, cytokeratins 8-18, and villin. The alternative model used is the mouse intestinal crypt cell line m-IC$_{cl2}$ cells (Bens et al., 1995, Eur. J. Cell Biol., ECBO Supp. Abstract 656). In addition to classical epithelial and intestinal markers, this clonal cell line maintained in culture differentiated functions characteristic of intestinal crypt cells (intracellular localization of sucrase isomaltase, high level of expression of polymeric immunoglobulin receptors, intracellular Paneth-specific glycoconjugates and CFTR chloride channel activity). These two murine intestinal cell lines are used to study the lamina propria- or lymphoid follicle-mediated control of terminal differentiation, by co-culturing them with fibroblasts or lymphoid cells isolated from mouse Peyer's patches.

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EXPRESSION OF LAMININ-5 IN THE HUMAN INTESTINE AND IN VARIOUS COLON CARCINOMA CELL LINES

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Cell interactions between embryonic epithelial cell (endoderm) and stromal cells (mesenchyme) are determinant and prerequisite for intestinal morphogenesis and differentiation. Recent works argue in favour of a role of laminin-1, located in the subepithelial basement membrane, in these processes. Among the newly discovered variants of laminin, laminin-5 (LN5) is of potent interest. This isoform is a cross-shaped heterotrimeric molecule consisting of one heavy chain $\alpha3$ (165 Kd) and 2 light chains, $\beta3$ (140 Kd) and $\gamma2$ (100 Kd). In the skin, former experiments have demonstrated that LN5 is located in the hemidesmosomal complex, which also include the integrin $\alpha6\beta4$, a potential receptor of LN5. Expression of LN-5 is specifically impaired in lethal junctional epidermolysis bullosa, disease characterized by a dermal-epidermal disadhesion.

In the human intestine, immunocytochemical studies using the purified polyclonal antibodies SE85p (against $\alpha3$), SE144p (against $\gamma2$) and the monoclonal antibodies GB3 (against the mature molecule) show: 1) an early expression of LN-5 during intestinal morphogenesis; 2) an increasing gradient of intensity along the crypt-villus axis which parallels the differentiation of epithelial cells.

In order to correlate the expression of LN-5 and differentiation, we used colonic cancer cell lines displaying various differentiation properties. Western blot and immunoprecipitation assays have revealed that the undifferentiated HT29 cells as well as the two differentiated HT29-MTX and HT29-Fu subpopulations produced the 3 chains described in the skin, whereas in the Caco2 cells only the $\gamma2$ chain could be detected.

Although the repertoire of integrins expressed by HT29 and Caco2 cells differ, the adhesion ability on LN-5 is identical for both cell types. Tests of inhibition of adhesion, with various anti-integrin subunits antibodies, show that receptors of LN-5 are specific to the cell type. In the case of the Caco2 cells, the receptors of the ß1 family ($\alpha2\beta1$ and $\alpha6\beta1$) are involved in the binding of cells to LN-5 and concerning the HT29 cell line a cooperation between the ß4 ($\alpha6\beta4$) and the ß1 ($\alpha3\beta1$ and $\alpha2\beta1$) family is needed.

Finally the use of chimeric hybrid intestines between mouse and chick tissue components analyzed immunocytochemically with species-specific antibodies enabled us to study the cellular origin of LN-5. The $\gamma2$ chain is produced very early by the endoderm compartment, later relayed by the mesenchyme which gives the adult pattern of expression.

These preliminary results suggest a potential role of LN-5 in migration/differentiation of the intestinal epithelial cells rather than in adhesion as it has been described in the skin. They point to a complex mechanism of basement membrane formation as well as to the diversity of receptors implicated in the epithelial/mesenchymal interactions.

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patched requirements in the developing tergites of _Drosophila melanogaster_

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The segment polarity gene *patched* have been studied in morphogenetic clones of mutant cells carrying the loss-of-function allele *ptc* ^{6P} and in allelic series of viable combinations. Three different regions can be clonally determined according to tergite phenotypes of mutant cells. Clones are rare in an anterior region, in which non-autonomous polarity reversal phenotypes can be observed. Clones in medial region are devoided of pigment and differentiate trichomes ressembling those characteristic of the acrotergite suggesting a posterior transformation. These clones are also accompanied by non-autonomous polarity reversions inducing mirror-image phenotypes. A posterior region can also be limited in which phenotypes of non-autonomous loss of pigmentation and autonomous differentiation of ectopic bristles are observed.

patched alleles show an unique allelic series of pattern phenotypes. Pattern perturbations are enlargement of both an anterior and a posterior region. In posterior regions pattern elements are coordinately translocated when compared with wild type phenotypes, suggesting that *patched* is involved in a pattern controlling mechanism acting in tergites.

The patterns of expression of *wingless*, *engrailed*, and *patched* in *lac Z* enhancer trap lines are restricted to partially overlapping domains at the anterior-posterior compartment border. Those patterns provide an explanation to the allelic series of phenotypes suggesting a dependence on larval and histoblasts *wingless* function at the lineage restriction border modulated by *patched*. As reported in imaginal discs, expression is extremely reduced in the non-border histoblasts where clonal phenotypes have been detected. Clones with differential anterior-posterior non-autonomous phenotypes can be observed at any position in *patched* low-expression domain finally suggesting that positional cell fate in tergites is dependent on local less-expressed signals.

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FIFTH SESSION: ANIMAL MODELS

Chairperson: Walter Birchmeier

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CONTROL OF THE SQUAMOUS-SPINDLE TRANSITION AND E-CADHERIN EXPRESSION IN MOUSE SKIN TUMOURS.

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A change in cell phenotype is frequently observed in the advanced stage of mouse skin carcinogenesis, when a well differentiated squamous cell carcinoma progresses to an undifferentiated sarcoma like spindle cell carcinoma. The mechanism of this progression is still unknown and is probably due to the loss of factors controlling the epidermal differentiation program. In vivo, the squamous-spindle progression probably involves some intermediate stages which are neither squamous or spindle, but are characterized by a more plastic phenotype. The Sn161 is a squamous cell line in vitro, able to give rise to spindle carcinomas when injected s.c. in nude mouse. Squamous and spindle clones of this cell line have been obtained and all of them induce spindle cell carcinoma when injected s.c in nude mouse. Treatment of the squamous clones with TGFβ induced a squamous- spindle conversion. After 6h of treatment the cells showed delocalization of the adhesion molecule E-cadherin and further treatment caused a reduction in E-cadherin expression levels. No changes in the expression of other epidermal markers were observed. The capability of exogenous factors to modify the cell phenotype was confirmed by injecting the Sn161 cells into several tissues of the mouse. When injected s.c., the cells are in a more fibroblastic environment and give rise to tumours with a uniform spindle morphology.However injection intraperitoneally gave rise to nodules with poorly differentiated squamous areas mixed with spindle areas .Intravenous injection of Sn161 induced lung metastases with a similar morphology. The development of more squamous tumours is probably due to the influence exerted by the epithelial microenviroment.One feature of spindle tumours induced by s.c. injection of Sn161 cells is the absence of E-cadherin protein.Analysis of the transcriptional activity of the E-cadherin gene in spindle cells confirmed that the squamous-spindle switch is not due to the loss of a transcription factor controlling E-cadherin expression.The spindle cell lines A5 and H11 express no detectable E-cadherin protein. However RT-PCR showed that a low transcriptional activity is present in both spindle cell lines. This was supported by transient transfection experiment with a plasmid containing 6 kb of the E-cadherin promoter controlling a luciferase reporter gene. This showed positive expression of luciferase in both spindle cell lines, at a level corresponding to about 40% of the control.On the other hand the more aggressive Car B cell line shows complete absence of promoter activity. We conclude that development of invasive spindle cell tumours may take place in several steps. An early event may be delocalization and reduced expression of E-cadherin induced by TGFβ. Furher reduction in E-cadherin expression can occour by post-transcriptional alterations of mRNA levels, and finally by complete loss of promoter activity

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TYROSINE KINASE RECEPTOR IN THE CONTROL OF CELLULAR GROWTH, MOTILITY AND DIFFERENTIATION. Carmen Birchmeier, Eva Riethmacher-Sonnenberg, Friedhelm Bladt, Dieter Riethmacher and Dirk Meyer, Max-Delbrück-Centrum für Molekulare Medizin, Robert-Rössle-Straße 10, 13122 Berlin.

We are analyzing the biological and physiological role of receptor tyrosine kinases, c-ros, c-met and c-erbB2 and c-erbB3. These receptors were identified because of their oncogenic potential when mutated. Therefore, the signal transmitted by these receptors are commonly expected to be mitogenic. Experiments in cell culture have shown that these receptors can not only regulate cellular growth, but also motility, morphogenesis and differentiation.

The c-ros receptor encodes an receptor tyrosine kinase with an as yet unknown ligand. The receptor is exclusively expressed in epithelial cells. Transcripts for c-ros are found during development in the Wolffian duct, in a Wolffian duct derivative, the ureter, and in intestine and lung. In the adult, a single site of expression is detected, epithelia of the caput epididymis, which develop from the Wolffian duct. We have introduced a targeted mutations into the c-ros gene by homologous recombination. Embryonal stem cell technology was used to create mice that carry the mutation. Homozygous mutant females appear healthy and normal, in contrast to homozygous males which are infertile. Chimeric animals that consist of c-ros -/- and c-ros +/+ cells transmit mutant and wild-type alleles with the same frequency, demonstrating that the c-ros receptor does not act in a cell autonomous manner during in male germ cell development or function. Indeed, the primary defect was found in epithelia of the epididymis. Large columnar epithelial cells typical of the initial epididymal segment are absent in mutant males and replaced by low columnar epithelial cells typical of more distal parts of the epididymis in control animals. This primary defect in epididymal epithelia affects sperm maturation and function in vivo.

The c-met receptor encodes a tyrosine kinase receptor. The specific ligand is SF/HGF. We have determined the expression patterns of SF/HGF and c-met during development and found c-met expression predominantly in epithelia, whereas the ligand is expressed in the surrounding mesenchyme. Additional sites of c-met expression are neuronal cells and, in the early embryo, muscle precursor cells. we have introduced a targeted mutation into the SF/HGF and c-met genes by homologous recombination and analyzed animals which carry such mutations in a homozygous state. As expected, we find identical defects in SF/HGF and c-met mutant animals, demonstrating the absence of a second c-met ligand (or the absence of a second SF/HGF receptor). Homozygous mutant animals die during development in a relatively broad window of time between E12.5 and E16.5. Various defects can be observed at the time of death. 1) A defect in liver development, resulting in decreased liver size and damage to the parenchyma that varies in serverity. In extreme cases, this leads to a loss of the entire parenchymal compartment. Also, c-met -/- cells cannot contribute to the adult liver in chimera that consist of c-met -/- and c-met +/+ cells, demonstration a cell autonomous role of c-met in development and/or growth of liver parenchyma. SF/HGF has thus a growth and cytoprotective function in liver development. 2) A defect in development of the placenta, and particularly the labyrinth layer. In this layer, exchange of oxygen and nutrients between the maternal and the embryonal compartments take place, obviously an essential function for survival and growth of the embryo. The absence of this layer might be responsible for the observed embryonic lethality. This defect also might cause the developmental retardation of homozygous mutant embryos that is first observed around E14.5 and gets more pronounced at later developmental stages. 3) Absence of developing muscles in the limbs, diaphragm and tip of the tongue, a result of the absence of migration of myogenic precursor cells. These muscle precursor cells derive from the ventrolateral edge of the somite, and upon external stimulation, desegregate

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from the somite, undergo a epithelial mesenchymal conversion and migrate as single cells into the anlage of the limb, diaphragm and tongue. The dermomyotomal cells in the ventrolateral edge of the somite express the c-met receptor, whereas SF/HGF is produced by the target sites the cells migrate to. In conclusion, we observe multiple, independent functions of SF/HGF and c-met in vivo, which resemble the activities described in vitro: the factor and its receptor can regulate growth, survival and migration of cells.

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Extracellular Matrix As a Master Switch in Differentiation, Apoptosis and Cancer

In the last decade, we have established "designer microenvironments" in culture and in transgenic mice for studying how tissue-specific gene expression is maintained in the mammary gland of both mice and men. We have shown that the extracellular matrix(ECM) regulates milk protein gene expression at multiple levels. It also regulates growth factor expression and growth signals. The ratio of ECM-degrading proteinases and their inhibitors are crucial, and a disturbance in this ratio leads to formation and dissemination of breast cancer. The mechanism by which the ECM directs these functions will be discussed.

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2. Boudreau, N., Myers, C., Bissell, MJ. (1995) *Trends in Cell Biology*

3. Ronnov -Jessen, L., Petersen, OW., Koteliansky, VE., Bissell, MJ. (1995) *Journal of Clinical Investigation* 95:859-873

4. Streuli, CH., Schmidhauser, C., Bailey, N., Yurchenco, P., Skubitz, APN., Roskelley, C., Bissell, MJ., (1995) *Journal of Cell Biology*

5. Lin, QL., Dempsey, P., Coffey, RJ., Bissell, MJ. (1995)*Journal of Cell Biology* 129:1115-1126

6. Petersen OW., Ronnov-Jessen, L., Bissell, MJ., (1995)*The Breast Journal* 1:22-35

7. Desperes, PY., Hara, E., Bissell, MJ., Campisi, J.(1995) *Molecular Cell Biology.* 3398-3404

8. Werb, Z. and Bissell, MJ. (eds) (1995) *Seminars In Cancer Biology* "Basic Science Aspects of Breast Cancer"

9. Jones, PL., Boudreau, N., Myers, CA., Erickson, HP., Bissell, MJ. (1995) *Journal of Cell Science* 108:519-527

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11. Roskelley, C., Bissell, MJ. (In Press) *Biochemistry & Cell Biology*

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ROLE OF EGF-R IN EPITHELIAL PROLIFERATION AND DIFFERENTIATION

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The epidermal growth factor receptor (EGFR) belongs to a family of tyrosine kinase receptors and is activated by several ligands including EGF and TGFα. In the mouse blastocyst EGFR is detected on the trophectoderm which gives rise to the trophoblasts, the specialized epithelial cells of the placenta (1). Later in gestation EGF binding activity can be detected in various organs, but only a few studies have localized the EGFR in specific tissues or cell types (2). The broad expression pattern of EGFR indicates that it might influence many cellular processes like proliferation, differentiation and morphogenesis.

In order to examine how these distinct functions might be regulated *in vivo* the EGFR gene was inactivated in embryonic stem (ES) cells by replacing the first exon with an E.coli *lac*Z gene and independently targeted ES clones were used to generate mice lacking functional EGFR. Homozygote mutant mice are retarded in growth and die at different stages of embryonic development depending on their genetic background (3). In an inbred 129/Sv background mutant mice die at midgestation whereas in a mixed 129/Sv x C57BL/6 they can survive until birth and even to postnatal day 20 in a 129/Sv x C57BL/6 x MF1 background. Death in utero probably results from a defect in the placentas, which are smaller in size and have an underdeveloped spongiotrophoblast layer. Newborn mutant mice have open eyes, underdeveloped whiskers, immature lungs and structural defects in their epidermis correlating well with the expression pattern of EGFR as monitored by β-galactosidase activity. These results indicate that EGF-R primarily affects epithelial proliferation and/or differentiation in a strain-dependent manner. Evidence that the genetic background most likely acts on the development of the extraembryonic tissues will be presented.

(1) J.C. Cross et al., Science 266, 1508-1518, 1994

(2) R. Derynck, Adv. Cancer Res. 58, 27-52, 1992

(3) M. Sibilia and E.F. Wagner, Science 269, 234-238, 1995

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The Role of the E-Cadherin/Catenin Complex in Tumor Progression and Embryonic Development

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E-Cadherin is a transmembrane molecule mediating Ca-dependent homotypic cell-cell interactions between epithelial cells. The loss of E-cadherin expression or function correlates with enhanced aggressiveness and de-differentiation of many human carcinomas and correlates with a non-favourable clinical prognosis. Within the cell, E-cadherin is associated with the catenins in so-called adherens junctions. This intracellular interaction appears to be regulated by the phosphorylation of the catenins and is necessary for the homotypic extracellular interaction of two E-cadherin molecules in cell-cell contacts. Yet another molecule with which the catenins can interact is the adenomatous polyposis coli (APC) tumor suppressor gene product, probably working co-operatively to regulate cell adhesion and growth. To study the involvement of junctional components in differentiation of epithelial cells during tumor progression and embryonic development we established transgenic mouse lines carrying a defined mutation in the g-catenin/plakoglobin gene. By crossing these animals with other transgenic mice carrying defined mutations in other junctional components (E-cadherin, APC, b-catenin), the reconstitution of multistep processes in carcinogenesis might become feasible.

Our working hypothesis is that in E-cadherin or g-catenin +/- mice, the wild-type alleles may be lost during tumor progression and thus render tumor cells more invasive and metastatic. The inactivation of the second allele, a loss-of-heterozygosity event, has been described in human as well as in animal cancer. We therefore crossed our heterozygous mutant animals with *Min* +/- mice (animals with a mutation in the *Apc* gene, which develop intestinal adenomas) and studied the consequences of an induced loss-of-heterozygosity event by the genetic induction of tumors. We expected that tumor bearing double mutant animals would develop metastatic lesions more rapidly and would die earlier than mice with an intact E-cadherin or catenin gene. These metastasis might be composed of cells with an E-cadherin or catenin -/- genotype. The analysis of the animals resulting from such crossings will be presented.

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Expression of a dominant negative mutant of the epidermal growth factor receptor in the epidermis of transgenic mice. Murillas, R.; Larcher, F.; Santos, M.; Conti, C.; Ullrich, A. and Jorcano, J.L. Dpto. Biología Molecular, CIEMAT 28040 Madrid, España

Signaling through epidermal growth factor receptor (EGFR) seems to be a fundamental mechanism in the phisiology of epidermis. We have generated transgenic mice overexpressing a truncated form of the EGFR gene (lacking the citoplasmic domain) under the control of the keratin 5 promoter. As expected, the expression of the transgene occurrs in the basal layer of estratified epithelia. Mice with a lower level of transgene expression show a phenotype of wavy hair and curly whiskers resembling the waved-1 and waved-2 mutants. However, mice showing a higher level of transgene expression display a much more severe phenotype characterized by striking alterations in hair follicle and skin architecture.Hair follicles show different degrees of degeneration ranging from mild vacuolization to severe necrosis and infiltration by inflamatory elements. Finally the hair follicle dissappears leaving only the hair shaft. Dermis and fat layer appear completely disorganized and interfollicular epidermis displays hiperplasia. We have carried out Western Blot experiments showing complete inhibition of EGFR autophosphorilation in response to EGF injection in the epidermis of our transgenic mice. Thus, signal transduction through the EGFR seemed to be either abolished or greatly reduced in our transgenic mice. We are currently performing skin carcinogenesis experiments on our loss of function EGFR transgenic mice to determine whether tumor development is inhibited .

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The Role of Erbb2, FGF4 and Wnt-4 in Development of Mammary Epithelium studied by expressing the genes in transplanted mammary gland.

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To investigate the possible role of genes such as erbB2, FGF4 and Wnt4 in development of the three-dimensional structure of mouse mammary epithelium we have expressed them in the epithelium in vivo. For this we have used our unique 'tissue reconstitution' method for expressing oncogenes in mouse mammary epithelium. Normal epithelial cells are isolated from one mouse, oncogenes are inserted in brief primary culture using helper-free retrovirus, and the cells are implanted into the 'cleared' mammary fat pad of another mouse. A 'cleared' mammary fat pad is one from which the endogenous epithelium has been removed early in development. The transplanted cells reconstitute an epithelial 'tree' in the fat pad, with some of the cells expressing the introduced gene. This approach has advantages over expressing genes in transgenic mice, in particular it gives clones of cells expressing the inserted gene against a background of normal cells; and hormone-insensitive promoters can be used [Edwards PAW (1993) Cancer Surveys 16, 79-96; Edwards PAW, Abram CL and Bradbury JM (1995) Journal of Mammary Gland Biology and Neoplasia, Plenum Press, vol. 1, in press].

We have previously reported that expressing the oncogene *neu*, the mutant rat form of erbB2, caused a spectrum of lesions that resembled lesions found in human breasts, including atypical hyperplasias, carcinomas-in-situ and frank carcinoma [Bradbury, Arno & Edwards, Oncogene 8, 1551-1558 (1993)]. The mildest/earliest changes that might represent the effect of overactive erbB2 without progression events seem to be foci of premature alveolus development on ducts in the virgin, suggesting that inappropriate expression of erbB2 can cause alveoli to form. One explanation of this would be that erbB2 is involved in the development or maintenance of alveoli in the normal mammary gland. However, this interpretation may be over simple, as we also find that expressing FGF4 in virgin gland causes alveoli to appear, and as this gene is also expressed in lactation it too may be involved in development or maintenance of alveoli.

When we expressed Wnt-4 in virgin epithelium, the growth pattern showed additional branching as in early pregnancy. As expression of Wnt-4 normally rises at the beginning of pregnancy it may be a key signal in the development of new branches in early pregnancy [Bradbury, Edwards, Niemeyer and Dale Dev Biol, *in press.*].

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SIXTH SESSION:
EPITHELIAL CELLS, EXTRACELLULAR MATRIX AND THE MESSENCHYME

Chairpersons: Fiona Watt and George F. Vande Woude

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Cell interactions and Basement Membrane molecules in intestinal epithelial differentiation

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Intestinal epithelial cell differentiation occurs during development as well as along the crypt to villus axis from undifferentiated embryonic endodermal cells and from crypt stem cells respectively. Differentiation of gut epithelial cells requires the presence of undifferentiated mesenchymal cells in the fetal intestine and of fibroblasts from the lamina propria in the adult organ (1,2). The current knowledge indicates that the molecular mechanisms of these mesenchymal-epithelial cell interactions involve on one hand local paracrine soluble factors (FGFs, TGFβs, HGF) via specific receptors and on the other hand basement membrane (BM) molecules via integrins. Basement membranes are composed mainly of laminins (LN), type IV collagen, nidogen and perlecan. In the last years 7 different isoforms of laminins and at least 3 types of collagen IV molecules have been discovered.

The intestinal subepithelial BM molecular composition varies as a function of developmental stages and of crypt to villus localisation in parallel to morphogenesis and epithelial cell differentiation (3). As an example, in the human adult intestinal mucosa laminin-1 is found to be located in the BM lining the villus epithelial cells, while laminin-2 is restricted to the crypt BM; the first expression of LN-2 is delayed as compared to that of LN-1 (4).

In an *in vitro* coculture system allowing to mimick subnormal mesenchymal-epithelial cell interactions, we could demonstrate that BM formation - requires heterologous cell contacts, - is progressive, and precedes epithelial differentiation. This cellular cooperation has been further documented by the development as grafts of interspecies chick-rodent endodermal-mesenchymal associations. The immunocytochemical survey of these hybrid intestines showed that the BM is composed of molecules produced by both cell compartments : some of them being exclusively of epithelial origin, others of mesenchymal origin and others are produced by both cell types (3).

The role of individual BM components on intestinal epithelial cell differentiation has been approached by the use of antibodies blocking a specific molecule, and of antisense messenger RNA : 1) In endodermal-fibroblastic cocultures, anti-laminin antibodies inhibit the expression of epithelial differentiation markers (lactase); 2) Stable transfection of Caco2 cells with an eucaryotic vector carrying an anti-sense cDNA of LN-1 α1 chain alters the

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polarization/differentiation of the cell, and the assembly of BM molecules at the Caco2/fibroblastic interface in cocultures. These data emphasize the major role of $\alpha1$ constituent chain of laminin-1 on the BM formation and on epithelial cell differentiation. On the other hand, the absence LN-2 $\alpha2$ chain expression in a mutant mouse strain (*dy/dy*), does not lead to detectable changes in intestinal morphology or differentiation (4).

Finally, in preliminary experiments, we found that two clonal cell lines exhibit : - differential proliferative and differentiation response to TGFß and IL_2, - opposite effects on epithelial proliferation and differentiation. Attempts are now directed to better characterize mesenchymal cell populations involved in normal epithelial-mesenchymal cell interactions and to analyze their deregulation in pathological situations.

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CD44 isosoforms in tumor progression and embryonic development

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Despite tremendous efforts in the last decades, the identification of proteins that are exclusively expressed on tumor cells has not been successful. Interestingly, in several instances, tumors make use of proteins that are otherwise important in embryonic development, suggesting that such "fetooncoproteins" confer functions to tumors that are predominantly required during embryogenesis (eg. migration, homing to specific tissues). Examples of such proteins are N-CAM, CEA and a recently identified, metastasis-specific marker, CD44v. CD44v describes a subgroup of the CD44 protein family. The members of this protein family are encoded by one gene, but differ in primary amino acid sequence due to alternative splicing. The subgroup CD44v includes all those members of the CD44 family that carry one or several of 10 "variant" exon sequences in the extracellular part of the transmembrane protein. In the smallest isoform, CD44s (standard), all these variant exon sequences are excised.
Variants of CD44 have recently gained interest for several reasons: i) they are causally involved in metastasis formation in rat systems(1); ii) they are expressed on human mammary and colon carcinomas, and they predict poor prognosis(2); and iii) they are transiently expressed on immune cells upon antigenic stimulation in vivo and in vitro and functional interference by CD44v-specific antibodies abolishes the immune response(3).
Whereas CD44s is expressed on many different cells in the adult and during embryo development, the expression of CD44v is highly restricted to a few tissues. In embryos, the apical ectodermal ridge (AER), a structure composed of epithelial cells that induces limb formation in the neighbouring mesodermal layers, represents an exquisite example. The function of CD44v in AER is absolutely required for this induction process.

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CROSSING THE DIGESTIVE BARRIER : IN VITRO RECONSTITUTION OF PEYER'S PATCH FOLLICLE-ASSOCIATED EPITHELIUM PROPERTIES AND M CELL FORMATION.

Pringault, E.

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By sampling antigens and micro-organisms through the digestive barrier, M cells, found scattered in the follicle-associated epithelium (FAE) of intestinal Peyer's Patches (PP), have key role in the acquisition of a local, mucosal immunity. Since M cells are a minority cell population, most studies have been restricted to morphological analyses, precluding biochemical studies. Furthermore, attemps to identify specific markers have failed. Whether M cells are of epithelial intestinal origin has been controversial, and so far, has not been yet demonstrated.

Our in vivo studies have shown that 1/mouse M cells express cytokeratins and villin but not vimentin, supporting their intestinal epithelial origin, 2/ that villin has a cytoplasmic distribution in M cells in contrast to adjacent absorptive enterocytes, indicating that the F-actin network differs greatly in these two cells types, and 3/ that the cytoplasmic distribution of villin provides an additional criteria to identify M cells by optical microscopy (Kerneis et al, 1995).

M cells establish intimate contact with underlying B and T lymphocytes, as well as antigen presenting cells. To explore the role of this lympho-epithelial contact on M cell differentiation, we decided to develop experimental models, in vivo and in vitro.

We first tried to induce in vivo the formation of M cells from intestinal cells, by ectopic transfer of Peyer's patch lymphoid cells.

1/ In vivo induction of Peyer's patch-like structures: freshly isolated lymphoid follicle cells were injected under the duodenal mucosa of a recipient mouse, in a location devoided of endogenous follicle or PP. After several days, we have observed the formation of structures similar to PP. The epithelium covering these induced patches displayed the characteristics of FAE and contained typical M cells. In contrast, the injection of thymocytes of three month old mice did not induce these structures. FAE and M cell differentiation could therefore be specifically induced from any intestinal crypt from which normally originate a villus-type differentiation.

Thus, we tried to establish co-culture conditions with immnune and epithelial cells which mimic the in situ situation.

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2/ In vitro induction of FAE properties: a co-culture system composed of intestinal cells and follicle cells has been developed. Caco-2 clone1 cells, selected for a well developed homogenous brush-border and a widespread sucrase-isomaltase expression (Costa de Beauregard et al, 1995), were grown up-side-down on 3 µm porous filters. After confluency, lymphoid follicle cells were introduced in the basolateral chamber and co-cultured with intestinal cells for 1 to 7 days. Follicle cells passed through the filter, digested the extracellular matrix and homed into the epithelial monolayer. Epithelial polarity and tightness were maintained. We observed that decrease of apical expression of sucrase isomaltase, disorganization of the brush border, and occasional apparition of apical membrane ruffles were correlated with the homing of lymphoid follicle cells. These changes in apical surface properties were followed by the acquisition of a vectorial (apical to basolateral) translocation of inert particles (fluorescent latex beads). Thus, lympho-epithelial contacts have converted well differentiated villus-type absorptive enterocytes into epithelial cells sharing structural and functional properties with FAE and M cells.
Such experimental models for the induction of M cell properties will be used to study lympho-epithelial contact and translocation of micro-organisms at the molecular level.

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MET:HGF/SF, TUMORIGENESIS AND METASTASIS

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The product of *met* protooncogene is the tyrosine kinase growth factor receptor for hepatocyte growth factor/scatter factor (HGF/SF). The *met* protooncogene is expressed in most adult and embryonic tissues, especially in epithelial cells. HGF/SF is expressed predominantly by mesenchymal cells, stroma and fibroblasts, and is a paracrine mediator of mitogenic, motogenic, and morphogenic responses in epithelial cells expressing Met. The *met* protooncogene is amplified in spontaneous transformants of NIH/3T3 cells and overexpression of Met efficiently transforms NIH/3T3 cells. We have shown that this transformation is due to an autocrine mechanism (1). We have recently demonstrated that Met is overexpressed in human sarcoma cell lines as well as in primary sarcomas, suggesting that similar autocrine signaling may contribute to the development of these human tumors (2).

p53 is a potent regulator of cell growth and division and loss of its wild-type function is a major step in the development and progression of many human cancers. Mice heterozygous and homozygous for loss-of-function mutations in p53 (p53 +/- and p53 -/-) appear normal at birth, but are highly predisposed to the development of a variety of tumors. Sarcomas occur at high frequency in p53-deficient mice as well as in individuals with Li-Fraumeni syndrome. Both human sarcoma tumors and sarcomas from the p53-deficient animals express high levels of Met (3). We have shown that late passage fibroblast cell lines established from p53-deficient animals overexpress Met and can be tumorigenic in athymic nude mice, suggesting that progression occurs *in vitro*. The tumor explants display increased HGF/SF expression and Met turnover, indicating that autocrine Met activation is selected during the growth of the tumor. Thus, inappropriate Met expression contributes to sarcomagenesis, and this event appears to be greatly enhanced by the loss of wild-type p53. Loss of p53 function does not by itself cause transformation, but inappropriate Met expression may be a leading factor in sarcomagenesis.

The expression of mitogenic, motogenic, and angiogenic responses attributed to Met:HGF/SF signaling in a variety of tissues led us to ask whether sarcoma cells transformed through an autocrine mechanism are invasive and metastatic *in vitro* and *in vivo* (4). Met:HGF/SF transformed NIH/3T3 cells are invasive *in vitro* and display enhanced protease activity necessary for the invasive phenotype. In experimental and spontaneous metastasis assays, these cells metastasize to the lung, but are also

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invasive in other tissues such as the heart, diaphragm, salivary gland, and retroperitoneum (4). We have also shown that C127 mouse cells and SKLMS-1, a human leiomyosarcoma cell line, becomes invasive *in vitro* and metastatic *in vivo* with Met:HGF/SF paracrine or autocrine signaling, respectively. Moreover, we have shown that Met:HGF/SF activation in SKLMS-1 cells induces the expression of the urokinase plasminogen activator (uPA) proteolysis network and Met:HGF/SF signaling is, therefore, likely to contribute to the generation of the invasive metastatic phenotype in these human sarcoma cells.

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REGULATION OF EPITHELIAL SPECIFIC TRANSCRIPTION IN PAPILLOMAVIRUSES.

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We are using papillomaviruses as a model system to understand epithelial specific transcription and differentiation. Papillomaviruses are causing a variety of epithelial neoplasias, and the association of some types, like HPV-16, with cervical cancer, makes them the most important viruses involved in human carcinogenesis. The epitheliotropism of papillomaviruses does not reside in the infection process, but in the specificity of the viral gene expression for epithelial cells. Strangely, the cis-reponsive elements that activate viral transcription are recognized by factors that are ubiquitous rather than unique for epithelial cells. This is reminiscent of similar observations made in transcriptional studies of keratin and other cellular genes expressed in epithelia. At least 6 different transcription factors bind the enhancers of different HPV types, namely AP1, NFI, OCT1, SP1, TEF1, and YY1. As these viruses have no sequences in common except these motifs, they are likely to represent the minimal complement to induce epithelial specific transcription, and the question is, why they fail to exert functions although they bind in non-epithelial cells. An important clue to understand this phenomenon comes from the NFI transcription factor family, which is derived from 4 different genes. The NFI-C gene expresses the ubiquitous transcription factor CTF, while the NFI-X gene expresses a repressor, which antagonized the CTF function. This repressor is absent from epithelial cells, which results in a net-epithelial specific activation. Our research aims to understand why this repressor is not made in epithelial cells, additional epithelial specific mechanisms governing the synergism between CTF and heterologous transcription factors, and the role of these processes for regulation of cellular genes during epithelial differentiation.

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CHARACTERIZATION OF NUCLEAR PROTEIN BINDING SITES IN THE PROMOTER OF HUMAN KERATIN K17 GENE

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Keratin K17, while not present in healthy skin, is expressed under various pathological conditions, including psoriasis and cutaneous allergic reactions. The regulatory circuits involved in transcription of the human keratin K17 gene are poorly understood. To begin an analysis of the molecular mechanisms that regulate K17 gene transcription, we have studied the interactions between the nuclear proteins and the promoter region of the human K17 gene. That promoter region contained 450 bp upstream from the translation initiation site. For the studies, we have used electrophoretic mobility shift assays, computer analysis, site-directed mutagenesis and DNA-mediated cell transfection. In addition to the previously characterized interferon γ responsive elements, we have identified eight protein binding sites in the promoter. Five of them bind known transcription factors NF1, AP2 and Sp1, and three others bind still unidentified proteins. Using site-directed mutagenesis, we have demonstrated the importance of the protein binding sites for the promoter function involved in both constitutive and interferon-induced expression of the K17 keratin gene.

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POSTERS

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THE MSP RECEPTOR GENE (*RON*) IS INVOLVED IN DEVELOPMENT OF EPITHELIAL, BONE AND NEURO-ENDOCRINE TISSUES

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Macrophage Stimulating Protein (MSP) was originally isolated as a chemotactic factor for peritoneal macrophages. We previously showed that the MSP receptor is the tyrosine kinase encoded by the proto-oncogene *RON*. To elucidate the biological role of MSP we studied the expression of the *Ron* receptor *in vivo*, and the response to the factor *in vitro*. *RON* specific transcripts were detectable in the mouse liver from early embryonal life (day 12.5 p.c.) through adult life. Adrenal gland, spinal ganglia, skin, lung and - unexpectedly - ossification centers of developing mandible, clavicle and ribs were also positive at later stages (day 13.5-16.5 p.c.). From day 17.5 *RON* was expressed in the gut epithelium and in a specific area of the central nervous system, corresponding to the nucleus of the hypoglossus. In adult mouse tissues *RON* transcripts were observed in brain, adrenal glands, gastro-intestinal tract, testis and kidney. Epithelial, osteoclast-like and neuroendocrine cells express the *Ron* receptor and respond to MSP *in vitro*. In PC12 cells, while NGF induced growth arrest and morphological differentiation, MSP behaved as a strong mitogen.These findings show that the *Ron* receptor and its ligand are involved in the development of epithelial tissues, bones, and neuro-endocrine derivatives driving cells towards the proliferation program.

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ACTIVATION OF PK-Cα PROMOTES TRANSLOCATION OF E-CADHERIN AND CELL SCATTERING IN HT-29 M6 CELLS.

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The intestinal cell line HT-29 M6 is able to differentiate after confluence to a mucosecretor phenotype (1). Incubation of this cell line with the phorbol ester TPA, an activator of the PK-C, induces the scattering of the cell colonies, effect accompanied by the decrease of the cell-cell contacts and the loss of the differentiation capacity (2, 3).

Here we show that TPA affects the localisation and levels of E-cadherin, a protein that plays a crucial role in stablishing of the homotypical contacts (4). TPA promotes the rapid dissociation of the cadherin from the citoskeleton and its posterior desappearance. Other proteins associated to E-cadherin such as α and β catenins do not modify nor their asssociation with the citoskeleton neither their levels.

The implication of the distinct PK-C isoforms in this effect was investigated using specific activators. TMTA, a phorbol ester that only stimulates the subfamily of the calcium-sensitive PK-C isoforms (cPK-Cs) (5), induces cellular scattering and the dissociation of the cadherin from the cytoskeleton with identical kinetics to TPA. Among the cPK-Cs activated by TMTA, only cPK-Cα was detected in HT-29 M6 cells. The specificity of TMTA to cPK-Cα was verified using in vitro protein kinase assays and by its capacity to translocate and deplete only this isoform.

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ABSTRACT

MOLECULAR CLONING OF A SET OF cDNAS OVEREXPRESSED DURING EPITHELIAL COMMITTMENT AND DIFFERENTIATION

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We have taken advantage of the distinct differentiation potentials of the HT-29 and HT-29/M6 cell lines to isolate, by subtractive hybridization a group of cDNAs encoding transcripts overexpressed in the committment and differentiation of HT29/M6 cells to the mucus-secreting phenotype (1). We isolated 54 cDNA clones that were sequenced by their 5' end to generate a set of epithelial ESTs. These cDNA clones were classified in four groups: 1-transcripts encoded by the mitochondrial genome (9 clones encoding the 12S mt-rRNA, 16S mt-rRNA, and cytochrome c oxidase); 2-transcripts that included an Alu repeat (10 clones); 3-transcripts encoding components of the protein syntheses machinery (23 clones encoding a number of ribosomal proteins and elongation factors); 4-transcripts not included in any of the other groups (11 clones encoding ferritin, cytokeratin 18, TCHTP, the EMK kinase and several previously unknown proteins). Northern blot analysis showed the presence of HT29/M6-specific mt-RNA precursors. Several of the clones encoded products greatly overexpressed in HT-29/M6 cells. In this sense, steady-state levels of the transcripts that included Alu repeats in their putative 3'UTR were higher in the HT-29/M6 cells than in HT-29 cells, suggesting a possible involvement of the Alu elements in the process of HT-29/M6 differentiation.

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HEPATOCYTE GROWTH FACTOR-INDUCED SCATTERING IS ASSOCIATED WITH DOWN-REGULATION OF E-CADHERIN AND APC. Rosanna Paciucci, Montse Torà, Victor Díaz, and Francisco X. Real. Institut Municipal d'Investigació Mèdica, 08003-Barcelona (Spain).

Hepatocyte growth factor (HGF) is a potent mitogen for normal exocrine pancreas cultures (1). In addition, c-met and HGF levels are up-regulated in pancreas cancer tissues (2), suggesting their participation in pancreas cancer progression.

We have examined the motogenic effect of HGF on pancreas cancer cell lines displaying differentiated or undifferentiated features (3). Differentiation was defined on the basis of morphological aspect and growth pattern, ultrastructure, development of transmonolayer resistance, and expression of differentiation markers.

Only 1/5 cell lines examined (IMIM-PC-2) responded to HGF with scattering; however, this effect was only observed when cells were cultured on plastic in the presence of <1% FBS. Although HGF-induced scattering results from the loss of intercellular contacts, no clear effects on E-cadherin expression have been reported. We have used immunofluorescence to examine the expression of E-cadherin, and other cell surface proteins involved in cell adhesion (APC, CEA, and MUC1), in cells undergoing scattering. The levels of cytoskeleton-associated E-cadherin were much lower in IMIM-PC-2 cells cultured in FBS-free medium than in cells cultured in medium supplemented with 10 % FBS. Treatment of serum-starved IMIM-PC-2 cells with HGF for 1 h resulted in the down-regulation of E-cadherin, detected using western blotting; after 24 h of treatment, cell surface E-cadherin and APC were undetectable by immunofluorescence. EACA inhibited the motogenic effect of HGF and this effect was accompanied by an increase in the levels of E-cadherin and APC in areas of cell-cell contacts; in contrast, the levels or distribution of CEA or MUC1 were not altered. Similar effects were observed when IMIM-PC-2 cells were exposed to EACA in the absence of HGF.

IMIM-PC-2 cells constitutively express uPA and treatment with HGF led to an increase in the levels of uPA, but not of tPA, transcripts.

These results suggest that: 1) serum starvation affects the motogenic response to HGF, possibly by down-regulating cytoskeleton-associated E-cadherin, 2) scattering is accompanied by changes in the cell surface expression of E-cadherin and APC, and 3) induction of uPA may contribute to the decreased expression of cell adhesion molecules.

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EPITHELIAL-MESENCHYMAL TRANSITION: influence of the cell cycle position in FGF1-scattering effect by: C. Rebut-Bonneton, J.P. Thiery and B. Boyer. UMR 144, CNRS, Institut Curie. 12 rue Lhomond 75005 Paris

The rat bladder carcinoma cell line NBT-II exhibits two mutually exclusive responses to acidic FGF (FGF1): at high cell density, FGF1 is a potent mitogen that drives cells into the S phase whereas, at low density, FGF1 acts as a scattering agent that converts the epithelial NBT-II cells into fibroblastic-like motile cells. Because of these FGF1 properties, we investigated whether the scattering effect of FGF1 could depend on the position of cells in the cell cycle.

Cell synchronization was realized by two different ways: serum deprivation or, addition into the culture medium of agents like lovastatin and aphidicolin which arrest cell progression into cell cycle at different positions. Lovastatin is known to arrest cells at early G1, whereas aphidicolin ,which prevents mitotic cell division by interfering with the activity of DNA polymerase, arrests cells at late G1.

If cells were synchronized at G0 by serum deprivation, at early G1 or at late G1, they scattered more readily in response to FGF1 than unsynchronized cells. We can therefore hypothesize that FGF1 drives cells towards a critical point in G1 phase after which cells are directed to either cell dispersion (for low density culture) or mitogenesis (for high density culture). This hypothesis is currently examined by measuring some parameters which are known to be modulated throughout the cell cycle: MAPK activity, cyclin D and A levels or activities and phosphorylation of retinoblastoma protein.

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Identification of a 210 kD protein that is specific to differentiating human keratinocytes and is a new member of the desmoplakin/ BPA/plectin gene family

Christiana Ruhrberg and Fiona M. Watt

We have identified a new protein, p210, that is highly homologous to the intermediate filament associated proteins desmoplakin, plectin and bullous pemphigoid antigen (BPA) and is a new member of this protein family. Desmoplakin and BPA appear to be involved in the anchoring of keratin filaments to cell adhesion structures. Whereas BPA can only be found in the hemidesmosmes of basal keratinocytes, desmoplakin has a more widespread tissue distribution and is present in all living layers of the epidermis. In contrast, the localistion of the p210 protein is restricted to the upper layers of the skin, and the expression of the p210 mRNA is upregulated during the differentiation of keratinocytes. We have evidence that the p210 protein is membrane associated, and we furthermore know that it becomes crosslinked into the cornified envelope which is assembled under the cell membrane. Interestingly, the p210 protein also contains a region homologous to the desmoplakin domain that has recently been shown to mediate the binding of specific keratins. The role of the p210 protein may therefore be to anchor differentiation-specific keratins and the cornified envelope to the cell membrane, a function that would appear crucially important for the architecture of terminally differentiated keratinocytes. We are currently investigating whether the p210 protein is able to bind differentiation-specific keratins.

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CADHERINS REGULATE CELL-CELL INTERACTIONS IMPORTANT FOR MORPHOGENESIS OF THE ENDOCRINE PANCREAS.

In this study we have investigated the importance of cadherins for cell-cell interactions mediating the organization of endocrine cells into pancreatic islets of Langerhans in transgenic mice. Truncated mouse E-cadherin, with nearly all its extracellular amino acids deleted, was overexpressed in pancreatic β-cells in transgenic mice. The E-cadherin mutant competed with endogenous E-cadherin (and possibly other cadherins) for interactions with cytoplasmic components, which resulted in removal of cadherin(s) from the cell surface of most β-cells during early stages of pancreas ontogeny. At E17.5-18.0 in mice, when islets normally organize their cell types into a characteristic cell-architecture, transgenic β-cells were found dispersed in the tissue as interstitial clusters and individual cells. α-Cells selectively aggregated into islet-like clusters devoid of β-cells. Thus, we have for the first time showed, *in vivo*, that E-cadherin and potentially other cadherins regulate adhesive properties of β-cells which are essential for the aggregation of endocrine cells into islets, and possibly also for the organization of islets. β-cells which expressed high levels of truncated E-cadherin increased their expression of β-catenin. Surprisingly, between E17.5-18.0 and birth the number of β-cells which expressed enough of the mutant to compete out cadherin-activity rapidly decrease. We have indirect evidence that downregulation of the mutant occured at a transcriptional level. Endocrine cells are then organized into normal islets. The adult transgenic show no signs of pancreas deficiencies.

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Somatic Gene Therapy for Cystic Fibrosis by Epithelial Stem Cell Mutation Correction

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Cystic fibrosis (CF) is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which encodes a cAMP regulated chloride ion channel. Abnormal ion transport across epithelial cell membranes results in abnormal water and ion transport giving rise to highly viscous mucoid secretions in exocrine tissues. The resulting complications lead to premature death primarily as a result of chronic pulmonary infection. Gastrointestinal problems also contribute to the morbidity with 10-20% of patients suffering from meconium ileus and/or recurrent intestinal obstruction in later life. Current gene therapy strategies for CF are directed primarily at the respiratory epithelium and are by no means perfect. They face problems of targeting gene delivery to the correct cell type, efficiency and frequency of delivery, possible immune response to gene delivery vectors and expression profiles very different to that of wild type *CFTR*. In an attempt to overcome the various problems encountered by current CF gene therapy strategies we aim to correct the *Cftr* mutation in intestinal epithelial stem cells of the CF mouse model using homologous recombination before ultimately grafting the cells back into the mouse intestine. The goal of this approach is to obtain functional wild type Cftr in intestinal tissue under the control of its endogenous promoter sequences. This will allow corrected *Cftr* to be expressed there with the same precise temporal and spatial control as heterozygote mice which show no phenotype. At the same time, by targeting the crypt stem cells the treatment should involve a single delivery.

To this end intestinal epithelial stem cells from the mouse will be grown in primary cultures where they will be targeted. Cells which have been targeted will be selected for before being grafted into the mouse, either subcutaneously or onto denuded intestinal muscle. This technique of intestinal stem cell grafting has already been shown to generate neomucosa ie. a circumferential layer of epithelium which contains crypt/villus structures. The neomucosa has been characterized by others and shown to display progressive cytodifferentiation, brush border enzyme expression and the presence of all cell types.

Targeting vectors are currently under construction for correction of the *Cftr* mutation. The primary culture technique for small intestinal epithelium is now established using mouse tissue and characterisation of cell types is under way. Subcutaneous grafting of stem cells into syngeneic mice has recently been attempted.

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EPITHELIAL MEMBRANE PROTEIN-1 IS EXPRESSED IN GASTRIC PIT EPITHELIA

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Peripheral myelin protein 22 (PMP22) is expressed in many tissues of epithelial origin but mainly by Schwann cells as a component of compact myelin of the peripheral nervous system (PNS). Mutations affecting PMP22 including gene duplications, deletions and point mutations are associated with hereditary motor and sensory neuropathies. Although these phenotypes are restricted to the PNS, PMP22 is thought to play a dual role in myelin formation and in cell proliferation. One hypothesis for the lack of phenotype outside of the peripheral nervous system is compensatory mechanisms via related molecules.

We have cloned and characterized epithelial membrane protein-1 (EMP-1), a putative four-transmembrane protein of 160 amino acids with 40% amino acid identity to PMP22. EMP-1 and PMP22 are co-expressed in most tissues with the exception of the liver where neither can be detected. EMP-1 is prominently expressed in the gastrointestinal tract, skin, lung and brain. In the corpus gastricum, EMP-1 protein can be localised to epithelial cells of the gastric pit and isthmus of the gastric gland with a plasmamembrane associated sub-cellular distribution.

PMP22 was also identified as the growth arrest specific sequence-3, (gas3). We show that EMP-1 and PMP22 mRNA levels are inversely regulated in the degenerating rat sciatic nerve after injury and by growth arrest in NIH3T3 fibroblasts, with EMP-1 becomming more prominant in the proliferative cell state. These data support a finding of upregulation of an untranslated region of the EMP-1 mRNA upon serum induced entry into G1 phase of the cell cycle. These findings and the expression of EMP-1 in the proliferation and differentiation zones of the gastric mucosa proposes an intriguing link with epithelial cell differentiation state.

Our discovery of EMP-1 as the second member of a novel gene family led to the identification of the lens-specific membrane protein 20 (MP20) as a third but distant relative. The proteins of this family are likely to serve similar functions related to cell proliferation and differentiation in a variety of cell types and may be able to compensate functionally for mutations in other family members.

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Growth factors and differentiation promoters modulate the cytoskeletal organization during the epithelial-mesenchymal transition of cultured neonatal rat hepatocytes.

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Neonatal rat hepatocytes when cultured for different times in the absence of added growth factors lose some of their typical differentiation markers (albumin, α-fetoprotein, glutathione-S transferase subunit pi) and acquire a migrating morphology. Previous studies from our laboratory have demonstrated that they dedifferentiate by epithelial-mesenchymal transition. This event involve a change in the expression of the intermediate filament (IF) proteins and a exchange between pre-existing cytokeratin network by vimentin filaments.

Confocal scanning laser microscopy was used to determine the spatial distribution of cytokeratin and vimentin IF networks. Vimentin expressing hepatocytes were mainly located on the periphery of epithelial clusters and presented a migratory morphology, suggesting that vimentin expression was related to the loss of cell-cell contact. Horizontal and vertical dual imaging of double immunofluorescence with anti-vimentin and anti-cytokeratin antibodies indicated that both filament colocalize strongly. Three-dimensional reconstruction of optical sections revealed that newly synthesized vimentin assembled following the pre-existing cytokeratin network and, when present, both filaments scaffolds codistributed inside cultured hepatocytes.

To determine the possible role of dedifferentiating and differentiating factors in the epithelial-mesenchymal transition of cultured neonatal rat hepatocytes we grow liver cells in presence of EGF (10ng/ml), HGF(5 ng/ml), TGF-β (0.5ng/ml), dimethylsulfoxide (DMSO) (2%) and nicotinamide (10mM). Both growth factors (HGF and EGF) induced higher vimentin expression in cultured neonatal rat hepatocytes compared to non-treated cultures. However, while vimentin-induced EGF-treated cells had an epithelial morphology, vimentin-induced HGF-treated cells presented a fibroblastic morphology. In addition, EGF-treatment retain albumin expression for long time in cultured hepatocytes. TGF-β induce coexpression of vimentin and cytokeratin up to 24h of culture and later the number of hepatocytes in the culture were considerably reduced, suggesting that TGF-β could triggers cell death. In contrast, DMSO and nicotinamide (two differentiating factors) inhibited vimentin expression in cultured neonatal hepatocytes and retain their epithelial morphology even after 1 week in culture. Thus, differentiating factors inhibit the epithelial-mesenchymal transition of cultured neonatal rat hepatocytes.

These data provide evidence that the EMT could be regulated by growth and differentiating factors and suggest that the EMT capacity of neonatal liver cells could have a direct consequences on the differentiation program that the neonatal hepatocytes follow during postnatal development.

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List of Invited Speakers

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