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19 **Epithelio-mesenchymal Transition in the Embryo and Adult: General Remarks**

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Epithelio-mesenchymal transition is a process of major importance to the development of the vertebrate embryo, which early on is composed only of epithelia. Transformation of certain of these epithelia to elongated, inwandering cells (EMT) produces all of the mesenchyme of the embryo, including the precursors of fibroblasts and all of the myoblasts that migrate to the limb and face. Later on, EMT is used in tissue remodeling (e.g., to bring about palate fusion), and certain mesenchymal cells, as in the kidney, may also transform back into epithelium (MET). These transformations between tissue phenotype are under the control of the E-cadherin gene (MET) or oncogenes, such as *c-src* and *c-fos* (EMT). Other genes that promote MET include *Pax2*, *wnt-1*, and *wnt-4*. Growth factors such as HGF/SF may promote MET or EMT *in vitro*, whereas TGFß3 and SHH are definitely involved in important *in vivo* embryonic EMTs. Both embryonic and adult epithelia can be induced to downregulate E-cadherin and turn on the *src* EMT signaling pathway by suspending them in collagen gels *in vitro*. *In vivo*, adult epithelia may turn on the EMT pathway by mistake to give rise to malignant cells that express the invasive motility of normal mesenchymal cells in abnormal places. E-cadherin transfection can revert some of these mesenchymal cells back to epithelia, except possibly in the case of malignant cells that are overexpressing the nuclear transcription factor, LEF-1. It will be important to understanding such pathologies as these to know more about the mechanisms of normal epithelio-mesenchymal transition in the embryo.

20 **Epithelio-Mesenchymal Transition in Embryonic Neural Cells: Causes and Consequences**

Don Newgreen, Joe Minichiello, Richard Kerr, Tiffany Symes and Peter Farlie. The Murdoch Institute, Parkville 3052, Victoria, Australia.

The transition of sessile epithelial cells of the avian dorsal neural tube into migratory mesenchymal neural crest cells is one of the most studied examples of epithelio-mesenchymal transition (EMT) and involves a down-regulation of N-cadherin function, alteration in the actin cytoskeleton and changes in integrin-based matrix adhesion/spreading. Neural crest molecular markers are also synthesised, including the transcription factor *Slug* (normally expressed before and during EMT) and the cell surface label HNK-1 (normally expressed after EMT). These changes can be induced in vitro by BMP-4, a natural inducer in vivo. In vitro inhibition of PK-C ι and ζ immediately induces EMT of neural epithelial cells. The direct effect of this is cytoskeletal reorganization, with rapid secondary changes in cell-cell adhesion via N-cadherin redistribution/down-regulation, and increased integrin-dependent cell attachment, spreading, and migration on matrix. Eventually, after a lag of hours, synthesis of *Slug* and HNK-1 occur. EMT can also be induced by transient Ca^{2+} deprivation. This acts within minutes and primarily destabilizes cadherin-based cell-cell adhesion, rapidly leading to changes in the cytoskeleton, and to cell spreading and migration on matrix. If the stimulus is maintained for 2–4 hours, neural crest markers are also synthesized. Exposure of neural epithelial cells in vitro to embryonic large chondroitin sulphate proteoglycan, a component of their environment in vivo, also induces EMT. In an epithelial model cell, mouse F9 EC cells, transfection of avian *Slug* augments EMT-like changes gradually, including loss of cadherin junctions, cytoskeletal alterations and increased cell spreading on culture substrates.

These results suggest i) that the complex molecular decisions of EMT can be established very rapidly by manipulation of the function of one (or a few) existing cellular effector molecule (say A → A'; where A for example is a cadherin which is active or inactive, depending on phosphorylation); ii) that this effector molecule is linked to orchestrate other effector molecules (e.g., B, C, D etc.; for example catenins, integrins, cytoskeletal molecules) to change their functional state (→B', →C', →D' etc.); iii) that the logic circuit connecting the effector molecules is constructed to enable B → B' to drive A → A', C → C', D → D', and so on; iv) that the logic circuit also alters gene activity, v) that the new gene product spectrum acts to promote or cement the EMT responses. Thus, an integrated EMT can be made rapidly but provisionally by cytoplasmic logic circuits, and this choice can be ratified generically for long term maintainance. This two stage model allows the same complex response to be arrived at from different initiating molecular changes and satisfies a requirement for speed of EMT with permanence of the decision.

21 **Structural and Signaling Functions of β-catenin During Epithelio-Fibroblastoid Cell Transitions**

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Members of the Transforming Growth Factor-β family (TGF-β family) induce Ras-transformed mammary epithelial cells to undergo a transition from an epithelial (sessile) to a fibroblastoid (invasive) phenotype. This cell transition occurs most efficiently in vivo or in reconstituted collagen matrices. By contrast, the non-tumorigenic parental cells under identical conditions are arrested in the cell cycle. Based on these findings, we postulate a dual effect of TGFβ. On the one hand, TGFβ restricts the proliferative capacity of normal or benign cells; on the other hand, epithelial cells that have progressed beyond a certain stage of tumorigenicity, are converted to a fibroblastoid phenotype. Recent experiments show that epithelial-fibroblastoid conversion is initiated by disruption of adherens junctions and dissociation of E-cadherin/β-catenin complexes. During this process, E-cadherin remains located at the plasma membrane and β-catenin is dispersed throughout cytoplasm and nucleus. While TGF-β reduces the steady state levels of both E-cadherin and β-catenin, the uncomplexed pool of β-catenin increases substantially. Experiments employing reporter constructs show that Tcf/Lef-specific transcription activity correlates with the presence of uncomplexed β-catenin. In Ras-transformed cells, the transactivating potential of β-catenin/Tcf complexes is significantly more pronounced than in the parental EpH4 cells. Interestingly, the potential of a given cell to respond to TGF-β and to undergo epithelial-fibroblastoid transition is restricted to a limited time period.

22 **Proteoglycan-Dependent Interaction in the Implantation Process**

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Heparan sulfate proteoglycans (HSPGs) mediate growth factor binding and cell adhesion in various systems. Studies in mouse embryos as well as human fetal-maternal interface tissue indicate that the HSPG, perlecan, as well as various HSPG binding proteins are expressed in complementary patterns at sites of embryo-uterine interactions. HSPG binding proteins found at these sites include HB-EGF, amphiregulin and heparin/heparan sulfate interacting protein (HIP/L29). Furthermore, HS polysaccharides at these sites have been shown to bind FGF-2. Expression of HSPGs and many HSPG-binding proteins is highly regulated with regard to the development of the embryo and generation of a receptive uterine state, requiring complex interactions between ovarian steroids and embryo-derived factors.

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23 **Integrin Signaling and Trophoblast Phenotype Conversion During Mouse Blastocyst Implantation**

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Trophoblast cells, which comprise the outer layer of the blastocyst, form the first transporting epithelium during preimplantation embryonic development. These cells establish an interface with the uterine endometrium during blastocyst implantation and subsequently form the placenta and fetal membranes. At the outset of implantation, the trophoblast cells convert from a polarized epithelium to invasive cells that provide the embryo with access to the maternal blood supply. Phenotype conversion occurs during blastocyst differentiation and is marked by the acquisition of adhesion-competence within the apical domain of the trophoblast plasma membrane. Apical adhesion is fully realized only after trophoblast cells contact an extracellular matrix, which leads rapidly to the up regulation of integrin-mediated adhesion, dissociation of the trophoblast and cell migration into the endometrium. To investigate the ontogeny of adhesion at the apical surface of trophoblast cells, we have assayed integrin-mediated fibronectin binding activity (FBA) on the surface of intact mouse blastocysts. Maximal FBA required contact with immobilized or soluble fibronectin and was up regulated through a process dependent upon protein trafficking, but not *de novo* protein synthesis. The up regulation of FBA was specifically inhibited by antibodies against fibronectin-binding integrins, including $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$. Integrin-mediated outside-in signaling required the activities of protein tyrosine kinase, Rho-like GTPase and phospholipase C, which elevated intracellular Ca^{2+} levels. Ca^{2+} mobilization produced an inside-out signal that ultimately increased FBA through a calmodulin-independent mechanism. Intracellular vesicles that accumulated within the cytoplasm of trophoblast cells during blastocyst differentiation appeared to undergo Ca^{2+}-mediated exocytosis at this time. We speculate that proteins sequestered within the vesicles that are either secreted or inserted into the apical plasma membrane serve to strengthen integrin binding. Ligand-induced integrin signaling may also regulate gene expression and other cytological processes that control trophoblast phenotype conversion. Supported by NIH grant HD36764

24 **Adhesiveness of the apical membrane of uterine epithelial cells: a cascade of molecular alterations that leads to stable cell-cell binding**

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At embryo implantation, two epithelia, trophoblast and uterine epithelium, initiate an adhesive interaction via their apical plasma membrane. With respect to the uterine epithelium, this interaction seems to be possible only in a specific state called receptivity which is hormonally controlled. The adhesive properties of the apical surface of uterine cells might be faciliated by changes in the epithelial phenotype. In the study reported here, we developed an in vitro model for implantation in the human using endometrial RL95-2 and HEC-1-A cell lines. Data obtained suggest that apical adhesiveness of these cells is due to a loss of apico-basal polarity and a re-organization of adhesion molecules enabling an integrin-mediated signaling pathway. Using a novel approach of force measurements with the atomic force microscope we have characterized parameters of these specific adhesive interactions. Data presented here are consistent with the concept that uterine epithelial cells in the receptive state possess a reorganized epithelial phenotype, i.e. a non-polarized architecture and, thus, a luminal plasma membrane equipped with appropriate adhesion molecules; if trophoblast cells are positioned onto the surface for sufficient periods of time, a cascade of events can be initiated that leads to the formation of strong adhesion at the apical cell pole.

123 **Electrolyte sensitive mechanism in embryonic renal collecting duct epithelia under serum free culture conditions**

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During kidney development the embryonic collecting duct develops into the functional Principal (P) and Intercalated (IC) cells, which controll the sodium, water and pH balance in the body. Up to now it is unknown by which mechanism the different cell types are developing. We assume that an electrolyte sensitive mechanism is involved.
Embryonic renal collecting duct tissue was isolated by microsurgical methods and placed on a tissue carrier and in a perfusion culture container. For 14 days the tissue was cultured in six media with increasing sodium concentrations (IMDM / 117; M 199 / 138; MCM / 142; WME / 144; BME / 146; DMEM / 157 mmol/l) under serum free conditions.
After culture the generated tissue was investigated by immunohistochemical markers. We found that independent of the used culture media in all of the experimental series an estabilished renal collecting duct epithelium in excellent morphological condition was harvested. The immunohistochemical testing revealed that constitutive proteins such as Na/K ATPase, Laminin, P_{CD} 9 and Cytokeratin 19 were found in all of the experimental series. In contrast, facultative protein binding demonstrated by mab 703, mab 503 or PNA were only found in individual series.
Thus, each culture medium produces a very specific differentiation pattern. Further experiments are necessary to investigate if the differentiation profile is evocated by increasing sodium concentrations or if additional factors are involved.

124 **Different Chondroitin and Dermatan Sulphate Proteoglycans can Inhibit Cell Spreading and Migration.**

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Neural crest cell adhesion, spreading and movement is guided by extracellular matrix molecules in a positive and negative fashion. The positive molecules contain specific sequences that bind to cell surface integrin receptors. Chondroitin sulfate proteoglycans (CSPGs) inhibit these behaviours in a way which is poorly understood. Yamagata *et al.* [J. Biol. Chem. **264**, 8012-8018 (1989)] suggested that substrate-bound CSPGs must have many CS chains to influence cell behaviour, as this multivalency influences the conformation or clustering, and hence the function, of cell surface receptors.
This study aims to determine which parts of CSPGs and the related dermatan sulfate (DS) PGs are functionally important. We compared the abilities of aggrecan, biglycan, decorin and neurocan to inhibit neural crest cell attachment, spreading and migration on fibronectin in vitro, since these four PGs have different core proteins with varying numbers of CS and DS chains of different sizes. The PGs were present either bound to fibronectin substrates or in the culture medium. Cell spreading was the most sensitive measure of the inhibition, then cell movement and lastly, cell outgrowth. All four PGs inhibited neural crest cell spreading and movement when bound to the substrate. Digestion of aggrecan, biglycan or decorin with chondroitinases abolished the inhibition, suggesting that this depends on the sugar chains, not the core protein. Digestion of neurocan with chondroitinases reduced but did not abolish the inhibitory effect, suggesting that the core protein has some inhibitory activity. Thus, inhibition of neural crest cell behaviour by substrate-bound PGs is relatively non-specific, since the PGs need not have any particular core protein or large numbers of CS or DS chains. When presented in the medium, only aggrecan inhibited neural crest cell spreading, and this inhibition was abolished by chondroitinase. This suggests that PG specificity isw revealed in some circumstances. These findings suggest that the multivalency hypothesis requires re-examination, and that the mode of presentation of the PGs must be considered in experiments of this type.

125 **Epithelio-Mesenchymal Transformation of Embryonic Neural Cells is induced by Inhibition of an Atypical Protein Kinase C.**

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Epithelio-mesenchymal transition (EMT) involves re-organization of cell-cell and cell-substrate adhesion molecules and the actin cytoskeleton. In embryonic neural epithelium *in vitro,* EMT is induced by several broad and narrow specificity kinase inhibitors, all of which share PK-C inhibitory ability. A non-inhibitory analogue and potent inhibitors of PK-A, PK-G, MLCK and PTK were not active. This suggests the involvement of PK-C, however the most specific PK-C inhibitors showed lower than expected bioactivity. Treatment with several kinases in combination indicated that EMT induction did not require simultaneous inhibition of several different kinases. The relative ineffectiveness of modulators of conventional calcium-regulated PK-C; calcium levels, and DAG functions suggest that an atypical PK-C was involved. Immunoblots of neural tissue revealed the presence of the atypical isoforms ι (or λ) and ζ as well as other isoforms. However, only the atypical isoforms were enriched at the apical margins of the neural epithelia, overlapping with N-cadherin, and F-actin, the molecules which undergo major structural and functional alterations in EMT. These results demonstrate, i) EMT in embryonic neural epithelium *in vitro* can be induced by inhibiting kinases, probably an atypical PK-C, and ii) atypical PK-C isoforms are in the tissue at the appropriate stage and subcellular site of cells undergoing EMT.

126 Smooth muscle foam cell formation: Influence of macrophages and cytokines

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One of the early changes in the arterial intima during the process of atherosclerosis is the accumulation of both lipid-laden macrophages (MΦ) and foamy smooth muscle cells (SMC). The aim of our studies is to elucidate the mechanisms that lead to foam cell formation in SMC and to determine whether scavenger receptors also participate in the uptake of modified lipoproteins in this cell type. Since MΦ are supposed to play a crucial role in foam cell formation in SMC, we established direct and indirect co-culture systems comprised of SMC and varying numbers of MΦ to test the influence of MΦ on the uptake of DiI-acetyl-LDL (DiI-Ac-LDL) in SMC. Furthermore, we studied whether several factors, that are known to influence scavenger receptor activity and the uptake of DiI-Ac-LDL in MΦ, likewise affect these activities in SMC. Our experiments showed that the uptake of DiI-Ac-LDL by SMC is elevated when the cells were directly co-cultured with increasing numbers of lipid-laden MΦ. We found a moderate basal expression of scavenger receptors in porcine SMC that was slightly enhanced when the cells were stimulated with PMA. The uptake of DiI-Ac-LDL in SMC was increased very efficiently after treatment of the cells with mixtures of calcimycin and phorbol esters. The influence of MΦ on the uptake of DiI-Ac-LDL in SMC may be a consequence of the cellular release of cytokines into the culture medium (paracrine or autocrine mechanisms). If so, enhancement of DiI-Ac-LDL uptake in the indirect co-culture system should be found. This has yet to be examined. The synergistic effects of calcimycin and phorbol ester in SMC are explicable, since phorbol ester mimics diacylglycerol and enhances the affinity of protein kinase C for calcium. Competition experiments showed that the uptake of DiI-Ac-LDL was considerably decreased in the presence of a twentyfold surplus of Ac-LDL, which means that uptake of DiI-Ac-LDL was very specific. Presently we are investigating whether the uptake of DiI-Ac-LDL in SMC can be blocked by an antibody against type I and type II scavenger receptors in order to show that transformation of SMC into foam cells is due to the uptake of modified lipoproteins via scavenger receptors.

127 **Correlation of invasion with differential expression of (a5ß1) and (a6ß1) integrins in Ras-transformed mammary epithelial cells.**

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Integrin-mediated signaling and integrin modulation of mitogen signals probably play an important role in tumor growth and behaviour.We investigated the expression and localization of ß1-integrins which include cell surface receptors for collagens (Col),laminin(Lam) and fibronectin (Fn). In the mouse mammary epithelial cell line EpH4 transfected with v-Ha-Ras (Ep-Ras), we found that this oncogene did not effect polarization but (1) enhanced adhesion to Col,Lam and Fn, (2) increased the level of a2ß1,a3ß1,a5ß1 and a6ß1 and caused an aberrant overglycosilated ß1 chain.
Interestingly,Ep-Ras cells undergo TGF-ß induced epithelial-mesenchymal transition (EMT) in the nude mouse via a TGF-ß autocrine loop. This spindle-shaped,invasive phenotype (Ep5-Tu) expressed relatively low levels of a2ß1,a3ß1 and a6ß1 protein and mRNA compared to Ep-Ras cells. The Fn-receptor a5ß1 was the only one of the tested integrins which remained strongly expressed at the protein level. Confocal microscopy indicated that both the a5 and a6 subunit was now detected on the apical surface . In addition, surface expression of the a5ß1 and a6ß1 was upregulated as assayed by surface biotinylation and an apparent assembly of Fn occured at the cell surface of this phenotype.
EMT by exogenous TGF-ß1 could be also demonstrated in vitro. The mesenchymal cells displayed increased levels of the Fn-receptor, Fn assembly and apical localization of the a5 and a6 subunits.
In conclusion, data suggest a role for a5ß1,a6ß1 and Fn assembly for migration/invasion of mammary carcinoma cells after TGF-ß dependent EMT which seems to be rather frequent in human carcinomas.

128 **Effects of serum replacement on steroid metabolism and morphology of human endometrial epithelial cells**

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One of the most important steps in steroid metabolism of endometrial epithelial cells is the inactivation of estradiol by converting it to estrone. In human endometrium this reaction is catalysed either by the microsomal enzyme 17ß-hydroxysteroid dehydrogenase type 2 (17ß-HSD2) or by the peroxisomal enzyme 17ßHSD4. In order to elucidate the particular function of each of these enzymes the human endometrial epithelial cell lines HEC-1-A and RL95-2 were examined with respect to their estradiol metabolic capabilities, in comparison to human endometrium in vivo. Digoxigenin-labelled riboprobes were prepared which hybridized to the mRNA-transcripts of 17ßHSD1-4. 17ß-HSD2 and 17ß-HSD4 were detected in total RNA-preparations from HEC-1-A- and RL95-2-cells as well as from human endometrial tissue. The known localization in glandular epithelium could be demonstrated by means of in-situ-hybridization on frozen sections of human endometrium. For metabolic studies fetal calf serum in the cell culture meduium was reduced stepwise by a defined supplement (TCM™, ICN Biomedicals, Eschwege). Maximal serum concentration was 10%, minimal serum concentration was 0.25%. This treatment caused a decrease in 17ß-HSD2-transcription, but an increase in mRNA expression of 17ß-HSD4. Moreover changes in cell morphology were observed by immunofluorescence staining of cytoskeletal proteins (actin, tubulin). This finding may be interesting as we have shown earlier that there are kovalent as well as non-kovalent interactions between 17ß -HSD4 and actin in vivo. The particular factor causing these changes still has to be identified. It was concluded that estradiol degradation is differentially regulated and that this is accompanied by distinct morphological changes.

129 **Tumor Cells Induce α-Smooth Muscle Actin Expression in Quiescent Fibroblasts in 3-D Coculture**

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Myofibroblasts identified by the expression of α-smooth muscle actin (α-SMA) have been considered as the most significant stromal cell type not only in numerous reactive lesions but also in the desmoplastic reaction of scirrhous carcinomas. These phenotypically altered fibroblasts have been shown to compromise the majority of the interstitial stroma in human breast carcinomas and may fundamentally modulate the peritumoral extracellular matrix (ECM).

The aim of our project is to mimic the development of scirrhous breast carcinomas in a well-defined 3-D culture system in order to investigate fibroblast-tumor cell interactions involved in desmoplasia and myofibroblast differentiation. Experiments have been carried out using four different breast carcinoma cell types, two human adult skin fibroblast cell lines and fibroblasts grown out from breast tumor biopsy specimen. The heterologous 3-D coculture system applied is based upon a previously described model for human bladder cancer (Schuster et al., J. Urol. 151, 1707-1711, 1994) utilizing a liquid overlay technique for 3-D aggregation and growth. Cocultivation of breast cancer spheroids and fibroblast aggregates was initiated at sizes of 300 µm - 400 µm.

As shown by immunohistochemistry 10-20% of the normal human skin fibroblasts expressed α-SMA in exponential and confluent monolayer culture while the proportion of α-SMA positive cells accounted for 40-60% in fibroblasts outgrown from tumor biopsies. Cultivation of fibroblasts in 3-D aggregates resulted in a loss of α-SMA positivity accompanying cell quiescence also verified by Western blotting. Cocultivation with breast tumor spheroids (T47D and MCF7 but not BT474) was associated with an induction of αSMA expression in fibroblasts outgrown from breast tumor material (3 out of 4 different fibroblast cultures) but not in normal human skin fibroblast. α-SMA immunoreactivity in 3-D coculture was shown in particular for fibroblasts located in direct neighborhood to tumor cells.

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