

INSIDE LAB INVEST

Laboratory Investigation (2006) 86, 101–105.
doi:10.1038/labinvest.3700382

Alternate candidate genes for bladder ‘pre-neoplasia’

Over 20 years of world-wide research has established the paradigm of examining the morphological lesions of human tissue dysplasia for the molecular signatures of neoplasia. The colonic mucosa was a master tissue of interest, but over time most other human tissues have yielded molecular secrets to investigators. However, the fundamental question remains of how the neoplastic sequence of somatic mutations begins. Like astrophysicists seeking the origin(s) of the universe, we must look ever earlier in the cancerous sequence. Such an opportunity has arisen through an elegant series of articles by Czerniak *et al* published previously in these pages,^{1–3} in which whole-organ mapping of bladder urothelial dysplasia is performed. This has included: establishing whole-organ mapping of the histology and molecular features of bladder pre-neoplasia as a valid experimental technique, comprehensive analysis of gene expression patterns along the papillary and nonpapillary pathways of bladder neoplasia, and identification of chromosomal regions 13q14 and 17p13 as containing the key tumor suppressor gene loci RB1 and p53, respectively. In the first instance, bladder cancer is a good human tumor model for studies of early events in carcinogenesis, due to its simple anatomy and the ability to perform complete histologic and molecular mapping of the organ mucosa. In the last instance, these authors identified deleted chromosomal regions in anatomic clusters surrounding the foci of clonal expansion of intraurothelial neoplasia. Importantly, the genetic mapping suggested that non-coding DNA sequences characterized by a high concentration of repetitive sequences might predispose to chromosomal deletions in histologically normal regions of the mucosa, and hence create risk for neoplasia.

In the current issue, this research team has focused further on chromosome 13, to identify alternative candidate genes involved in clonal expansion of *in situ* urothelial dysplasia (Kim *et al*⁴ (p. 175)). The allelic patterns of 40 microsatellite markers mapping to all regions of chromosome 13 were examined in bladders resected from eight patients with invasive urothelial carcinoma, using the whole-organ mapping approach. Particular attention was given to 79 single-nucleotide polymorphisms (SNPs) located in the 13q14 region containing the RB1 gene. Four clusters of allelic

losses mapping to distinct regions of chromosome 13 were identified, with SNP markers mapping to the 13q14 region showing allelic losses associated with early clonal expansion of intraurothelial neoplasia. These losses appear to represent an incipient event in histologically normal urothelial mucosa, as the losses were present in large areas of bladder mucosa encompassing not only invasive cancer and adjacent high-grade dysplasia but also areas of low-grade dysplasia extending to areas of histologically normal urothelium. Inactivation of RB1 gene *per se* occurred *subsequent* to the incipient and anatomically widespread allelic losses in the perichromosomal region, and was associated with onset of severe dysplasia/carcinoma within a limited anatomic region. RB1 sequencing was performed, along with examination of the methylation status of the RB1 promoter and RB1 protein expression patterns in the mucosa. RB1 gene inactivation was not necessarily present in regions of allelic loss in the adjacent 13q14 chromosomal regions, indicating that adjacent 13q14 allelic alterations are more frequent than RB1 itself in bladder neoplasia. The data suggest as well that genes near RB1 rather than RB1 itself are involved in the initial *in situ* expansion of a neoplastic epithelial cell clone. The authors also gave consideration to the clinical diagnostic value of these findings, by examining a large set of bladder tumors. Incipient allelic losses in the 13q14 region could be identified in approximately 32% of 25 bladder tumor tissue samples. These data were extended to examine voided urine samples of 21 patients with bladder cancer: the 13q14 allelic losses were identified in 38%.

This study provides key evidence for the presence of critical alternative candidate genes mapping to the 13q14 region. These are involved in clonal expansion of urothelial neoplasia antecedent to inactivation of the RB1 gene. Allelic alterations in such alternative genes can be detected in the voided urine of a subset of patients with bladder cancer, pointing to the potential clinical utility of these findings. Most notably, this research demonstrates the power of whole-organ histologic and molecular mapping for identifying the genes of ‘pre-neoplasia’, before morphological changes are evident.

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Biobanking and RNA stability: protecting your investment

As detailed in a recent editorial by Crawford and Tykocinski,¹ academic pathology will have an ever-increasing role as the ‘enabler’ of human tissue research in the post-genomic era. Pathologists already play key roles in procuring and banking well-characterized, high-quality tissue, enabling research to link molecular information about human disease with relevant clinical information from well-maintained clinical databases. In the true sense of a bank, these stored tissues and databases have become the currency that drives both basic and translational research. The value of such collections depends most critically on the quality of the stored material. As ‘biobank tellers’, pathologists have become responsible not only for storage but also for optimal collection of these precious deposits. Here, a critically important challenge is the acquisition of human tissues for extraction of the RNA used in large-scale expression microarrays or in single gene expression studies: RNA fragility and degradation are omnipresent concerns.

Inside this issue, **Micke *et al***² (p. 202) present a practical approach to human tissue collection that maintains RNA integrity even after delayed freezing. These investigators carefully tested several different conditions for preserving or stabilizing RNA from surgically resected, fresh tonsil and colon tissue. RNA structural integrity was studied by microchip electrophoresis, and expression levels of *cfos*, *HIF1 α* , *Bcl2*, *PCNA*, *TGF β 1* and *SMAD7* were evaluated by RT-PCR. Surprisingly, RNA remained stable for up to 16 h under several conditions. However, measured gene expression levels were most stable when samples were kept on ice prior to freezing. In contrast, more variable gene expression was observed when samples were stored at room temperature, in normal saline, or in a commercial RNA-stabilizing buffer. Notably, the changes in measured gene expression appeared to be more due to *bona fide* alterations in gene expression under these ‘stressed’ conditions, as opposed to nonspecific degradation of RNA message. The results suggest that non-fixed human tissue specimens may be transported on ice over a period of hours without adversely affecting RNA quality or expression levels. This work is of critical value to pathologists and other investigators who are charged

with protecting the valuable investment of banked human tissues, thereby enhancing their ability to enable future research and discovery.

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Mesenchymal stem cell alchemy in the pancreas

In last month’s issue, the generation of pancreatic cells from stem cells was explored using hepatic stem cells.¹ As the embryology of the hepatopancreatic cell layer is an early ontologic event, switching on of a ‘master’ pancreas gene, *Pdx1*, through gene therapy provided the potential for generating pancreatic cells from adult tissue. In the current issue, **Seeberger *et al***² (p. 141) isolated mesenchymal stem cells from the pancreas itself, to explore their plasticity and potential for growth into useful cell types. This was based on the premise that pancreatic progenitor cells may reside within the pancreatic ductal epithelium, acinar tissue, and/or pancreatic small cells. Utilization of a non-cadaveric source of pancreatic cells, particularly if they could be expanded *in vitro* on the basis of stem cell-like properties, may be a highly valuable therapeutic strategy.

The authors were able to isolate a pancreatic cell fraction enriched in ductal epithelium, from which cell aggregates could be established in culture. Outgrowths of ‘fibroblast-like’ spindle cells were characterized as mesenchymal stem cells (MSCs), on the basis of their expression of defining cell surface antigens. In keeping with their putative MSC properties, these cells were successfully differentiated *in vitro* into a remarkable series of mesenchymal phenotypes: osteocytes, adipocytes, and chondrocytes. These MSCs also were capable of differentiating into cells of endodermal origin: hepatocytes (on the basis of albumin expression and other specific hepatocyte genes), and pancreatic endoderm (on the basis of expressing *Pdx1*—the ‘master gene’ for pancreatic differentiation). In the latter instance, additional genes expressed included *NeuroD*, *Neurogenin 3*, *Pax4*, *Insulin*, and *Glucagon*—all phenotypically relevant for pancreatic islet cell differentiation. The key variable in all of these experiments was the culture conditions. Unlike the experiments of Tang *et al*,¹ gene transduction was not required. These authors have thus demonstrated that multipotent mesenchymal stem cells residing within the adult exocrine pancreas could represent a progenitor cell capable of generating functional islet cells. The convergence of scientific investigations on stem cell generation of pancreatic islet cells,

especially their ability to generate insulin, has clear and profound implications on potential treatment for diabetes.

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IBD: just another break in the wall

Inflammatory bowel disease (IBD), namely Crohn's disease (CD) and ulcerative colitis (UC), is characterized by chronic relapsing intestinal injury. The mucosal inflammation that is present in these diseases has captured the attention of many investigators as being the primary causal event. However, others have suggested that it is disruption of epithelial function that is critical to disease pathogenesis, engendering the characteristic immune response. For example, it is well recognized that integrity of the wall, or barrier, formed by the epithelium is compromised in patients with IBD, as well as a subset of their healthy first-degree relatives.¹ In CD patients whose disease is in remission, compromised barrier function may even predict disease relapse. Thus, compromised intestinal barrier function may have an important primary role in disease pathogenesis.

Despite the apparent clinical importance of the epithelial barrier, the mechanisms of barrier dysfunction in IBD are poorly defined. It is likely, however, that partial disruption of the epithelial tight junction, which seals the paracellular space, is a major component of the observed barrier dysfunction. Recently, *in vitro* studies have suggested that TNF, which is elevated in both CD and UC, disrupts the intestinal epithelial tight junction by upregulating myosin light chain kinase (MLCK) expression and activity.^{2,3} This is consistent with a wealth of data, primarily from *in vitro* studies, suggesting that MLCK is a critical physiological and pathophysiological regulator of the tight junction. Despite this, MLCK expression and activity have never been assessed in IBD patients. The hypothesis that MLCK is involved in IBD pathogenesis, which has been developed using cell culture and animal models, has, therefore, not been tested.

In this issue of *Lab Invest*, Blair *et al*⁴ (p. 191) ask if the reported *in vitro* observations of increased MLCK expression and activity in TNF-treated cultured cells are relevant to disease mechanisms in human patients. The authors carefully studied the relationships between intestinal epithelial MLCK

expression, MLCK activity, and disease activity in CD and UC patients. Using quantitative immunofluorescent staining methods, MLCK expression and activity was assessed in patient tissues. The data show that MLCK expression is increased in patients with IBD. This was true for patients with active as well as inactive disease. Moreover, the degree to which expression was increased correlated with disease activity. MLCK activity was also increased in IBD patients and this too correlated with the degree of disease activity.

This report is thus the first study to probe the role of MLCK in human IBD. Although the data presented are correlative, an acknowledged ethical and technical limitation of human studies, the data are consistent with a model that has been proposed based on work using cell culture and animal models.⁵ Together with previous mechanistic studies, the present data strongly suggest that epithelial MLCK is involved in human IBD. In the context of previous work in IBD patients, the data also suggest that the loss of intestinal epithelial barrier function in these patients is due to increased MLCK expression and enzymatic activity. These observations advance our understanding of IBD pathogenesis and may help to identify novel therapies for IBD patients. Therefore, though a single step, all in all this is yet another opportunity to repair breaks in the wall.

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Totally tubular?

Acute tubular necrosis (ATN), caused by ischemia or other nephrotoxic insults, is the most common cause of acute renal failure in hospitalized patients.

The renal failure that accompanies ATN is characterized histologically by tubular epithelial cell necrosis and apoptosis. Subsequent tubule repopulation restores normal tubular architecture and function. Treatments that augment this repair process could shorten hospital stays and reduce the need for dialysis. The identity of the cell population responsible for the repair of the tubules is, however, unknown. Do renal tubules, like many epithelial structures, have resident, pluripotent, lineage restricted stem cells that can be mobilized in response to damage? Or are bone marrow-derived cells, which have been shown not to be restricted to a hematopoietic lineage, involved?

A variety of evidence in both humans and mice support the ability of bone marrow-derived cells to become renal tubular cells. These studies are based on transplantation of bone marrow-derived cells (BMCs) that differ phenotypically or genetically from the host. The most commonly used genetic markers are the Y-chromosome or transgenes, such as GFP or β -galactosidase. For example, tubule cells containing a Y-chromosome can be detected in kidneys of male humans, and mice, who have received kidneys from a female donor. More interestingly, infusion of bone marrow-derived mesenchymal stem cells into mice limited loss of renal function and tubular damage induced by cisplatin, suggesting that these cells are involved in repair and represent a potential treatment for ATN.¹ However, the frequency with which BMCs are reported to contribute to tissue repair in the kidney and other epithelial organs varies widely.

In the previous issue of *Lab Invest*, **Toyokawa et al**² (p. 72) use sophisticated imaging techniques to assess the contribution of BMCs to renal tubular repair. They demonstrate that two-dimensional imaging is simply insufficient to properly evaluate the contribution of hemopoietic cells to tissue repair. Toyokawa *et al* show that two-dimensional imaging overestimates the frequency of BMCs within renal tubules during repair. This careful comparison of the two-dimensional methods used previously with an elegant three-dimensional approach may explain the contradiction between early studies which concluded that BMCs contributed significantly to tubular repair and more recent studies concluding that resident kidney cells are the principal source of new epithelial cells, with BMCs making, at most, a minor contribution.³ Toyokawa *et al* used transgenic rats expressing enhanced green fluorescent protein (EGFP) as BMC donors for transplantation into wild-type EGFP-rats and, conversely, transplanted wild type EGFP-BMCs into EGFP-transgenic rats. At 6 weeks after BMC infusion rats were subjected to renal ischemia reperfusion injury using standard models. The presence, or absence, of EGFP, depending on the BMC source, was used to mark donor-derived cells and a simple F-actin stain helped to identify tubular epithelial cells. Thus, identification of BMCs within

tubules depended upon the presence of EGFP within a cell possessing an epithelial F-actin staining pattern. Standard two-dimensional imaging immunofluorescent imaging of 6 μ m sections suggested that between 0.18 and 0.68% of cells in the reconstituted tubules were of BMC origin. The rate was similar, regardless of whether EGFP+ or EGFP- cells were transferred, suggesting that EGFP transgene silencing is not responsible for the low frequency of tubular BMCs observed. Of course, the interstitium of the kidney is full of cells of hematopoietic origin that have developed along a hematopoietic lineage and would also express the GFP transgene. Could what looks like colocalization of GFP and F-actin in reality be two closely opposed cells, each staining with a single marker? In a parallel set of experiments the authors used the same experimental conditions and then took 50 μ m sections. Confocal optical sections were collected at 0.3 μ m intervals and a three-dimensional image was constructed. These three-dimensional data demonstrate that as few as 0.1% of the cells in the reconstituted tubules are of BMC origin. The authors therefore conclude that up to three-fourths of the cells identified as of BMC origin in two-dimensional imaging represent false positives. Thus, the major contribution to renal tubular repair comes from cells other than the BMCs. This work clearly demonstrates the difficulty in assessing three-dimensional structure from two-dimensional sections and confirms the importance of using three-dimensional techniques in assessing the involvement of BMCs in epithelial repair. Indeed, a similar three-dimensional analysis of colonic epithelium has recently been used to demonstrate the role of BMCs in colonic epithelial repair.⁴ Thus, it appears that the ability of BMCs to limit renal injury may reflect a role independent of transdifferentiation,⁵ leading us to conclude that the contribution of BMCs to renal repair is not totally tubular.

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