

## Review

# Expression of MAL and MAL2, two elements of the protein machinery for raft-mediated transport, in normal and neoplastic human tissue

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**Summary.** Polarized transport of lipids and proteins to the apical and basolateral membrane subdomains is essential for the functioning of epithelial cells. Apical transport is mediated by a direct route from the Golgi and an indirect route, referred to as transcytosis, involving the transport of the protein to the basolateral membrane followed by its internalization and subsequent transcellular transport to the apical subdomain. MAL and MAL2 have been demonstrated to be essential components of the machinery for the direct and indirect routes, respectively. Herein, we review the range of expression of MAL and MAL2 in normal human tissue and compare it with that of neoplastic tissue. Our analysis provides insight into the potential use of MAL- and MAL2-mediated pathways in many types of epithelial cells as well as in nonepithelial cells. In addition, the specific alterations in MAL and/or MAL2 expression observed in specific types of carcinoma provides a basis to understand the loss of the polarized phenotype that frequently accompanies the neoplastic transformation process. This points out potential applications of MAL and MAL2 as markers for tumor characterization.

**Key words:** Lipid rafts, MAL family proteins, Epithelial cells, Polarized transport, Carcinomas

### Lipid rafts and polarized transport

Polarized epithelial cells display two highly specialized plasma membrane subdomains. The free surface, the apical membrane, faces the external milieu whereas the basolateral membrane faces the body's

interior. Epithelial cells take up ions, water, proteins, macromolecules and even entire pathogens through these subdomains and are able to target them to specific cell compartments or transport them across the cell from one side of the epithelial barrier to the other. These transport processes include absorption by enterocytes and renal tubular cells, secretion by hepatocytes, endocrine cells, and exocrine cells and gaseous exchange by alveolar and capillary endothelial cells. The performance of these functions depends on the correct organization of the cell surface into structurally and physiologically distinct apical and basolateral subdomains equipped with specific sets of proteins and lipids (Matter and Mellman, 1994; Mostov et al., 2000).

Selective transport of proteins to the apical membrane takes place in epithelial cells by two different routes (Fig. 1), referred to as the direct and the indirect pathways (Matter and Mellman, 1994; Mostov et al., 2000). Newly synthesized apical proteins relying on the direct route are packaged after their passage through the Golgi into vesicular carriers destined for the apical surface. In contrast, proteins transported by the indirect route, also referred to as transcytosis, are first targeted to the basolateral surface and then endocytosed and transported across the cell to the apical surface. All epithelia appear to use the indirect pathway, whereas the direct pathway is used to a greater or lesser extent depending on the particular tissue. Hepatocytes and hepatocyte-related cell lines (such as hepatoma HepG2 cells) mostly rely on the indirect pathway, whereas other epithelia such as kidney or intestinal epithelia and most epithelial cell lines (e.g., renal MDCK cells and intestinal Caco-2 cells) use both pathways to varying degrees (Matter and Mellman, 1994; Mostov et al., 2000).

Despite considerable advances, the mechanisms of sorting and intracellular transport of proteins are not completely understood. For a long time, lipids were thought to play a merely passive role as simple building

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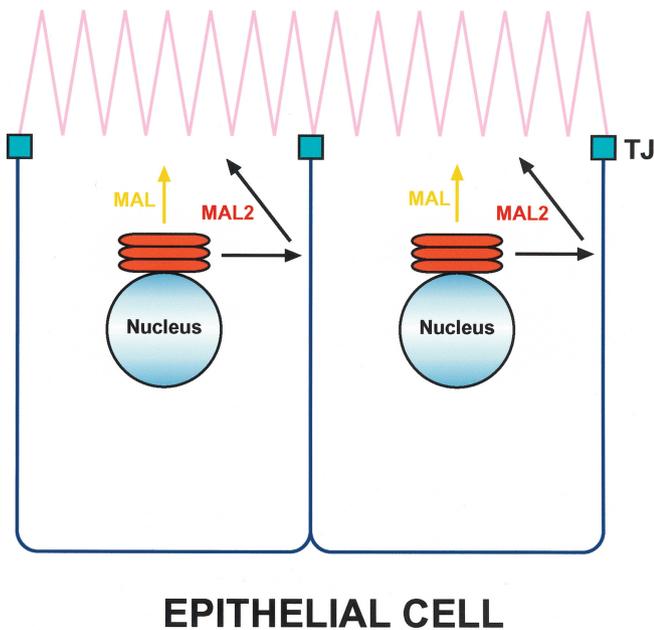
blocks for membranes delimiting intracellular compartments. However, during the past decade, a new model has emerged to account for lipid diversity (Simons and Ikonen, 1997). This model proposes the existence of biological membranes of lipid microdomains or rafts that have a high sphingolipid and cholesterol content and are organized in a tightly packed manner; unlike the loosely packed, disordered phospholipids present in the majority of membranes (Fig. 2A). The tight packing of lipids in rafts confers resistance to solubilization by non-ionic detergents at low temperatures, which allows their isolation as an insoluble membrane fraction (Brown and Rose, 1992). Several lines of evidence indicate the *in vivo* existence of rafts and argue against the possibility that they are simply artifacts of the detergent-solubilization procedure (Jacobson and Dietrich, 1999). Rafts appear to be mobile, dynamic entities that move laterally along the plane of the plasma membrane and traffic continuously between the plasma membrane and internal compartments (Nichols et al., 2001).

Rafts were originally proposed as membrane platforms in the Golgi used for the formation of transport carriers destined for the apical surface (Simons and Wandinger-Ness, 1990). According to this model,

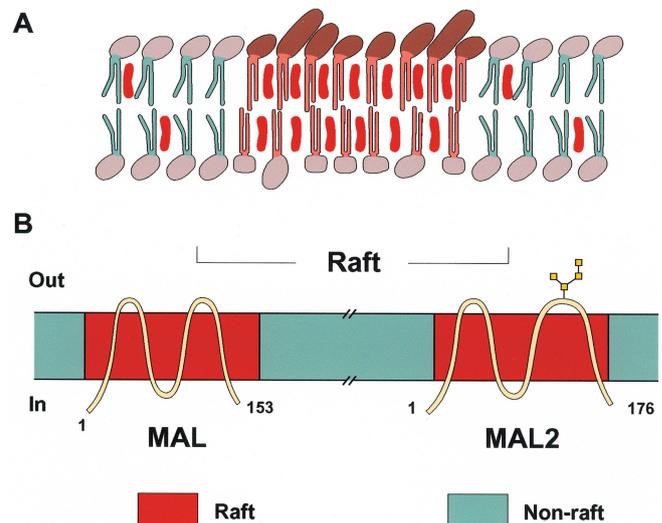
the direct apical transport pathway is mediated by the selective integration of apical cargo proteins into rafts that subsequently originate the transport vesicles. To be operative as a transport route, it was postulated that rafts used specialized protein sorting machinery (Simons and Wandinger-Ness, 1990). This machinery would consist of a minimal set of proteins to ensure the processes of vesicle formation, cargo recruiting, targeting and fusion to the apical surface.

### Machinery for the direct apical route: the MAL protein

The MAL gene was originally identified by Alonso and Weissman (1987) during a search for genes differentially expressed during T-cell development. Later on, the MAL gene was also found to be expressed in polarized epithelial cell lines (Zacchetti et al., 1995; Martín-Belmonte et al., 1998) and myelin-forming cells (Kim et al., 1995; Schaeren-Wiemers et al., 1995). The MAL gene encodes a 17-kD integral membrane protein (Fig. 2B), referred to as MAL, with selective residence in raft membranes in all the cell types in which it is expressed (Kim et al., 1995; Millán and Alonso, 1998; Martín-Belmonte et al., 1998). MAL is known to play an essential role as an element of the machinery for direct transport of apical proteins, as its depletion severely



**Fig. 1.** Pathways for apical transport in polarized epithelial cells. Apical transport in epithelial cells is achieved by two main routes. Newly synthesized proteins using the direct route are packed in transport carriers destined for the apical surface after their passage through the Golgi. A direct pathway to the apical surface involving rafts is mediated by the MAL protein. Membrane proteins using the indirect pathway are first transported to the basolateral domain and are then internalized and transported across the cell by transcytosis. Transcytotic transport is mediated by the MAL2 protein. TJ, tight junction.



**Fig. 2.** Model of lipid raft structure and function in biological membranes. **A.** Schematic model of lipid raft structure. Rafts are membrane microdomains formed by high concentrations of sphingolipids (dark-brown-headed structures) and cholesterol (red bean-shaped structures) immersed in a phospholipid-rich (light-brown-headed structures) environment. Glycolipids and sphingomyelin are restricted to the outer leaflet of the bilayer whereas cholesterol and phospholipids are in both leaflets. Note that lipids in the rafts usually have long and saturated fatty acyl chains, whereas those in lipids excluded from these microdomains are shorter and unsaturated. **B.** Schematic model of the predicted structure of MAL2 and MAL. The glycosylated loop of MAL2 is indicated.

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reduces transport of specific proteins to the apical surface in the polarized epithelial MDCK cell line (Cheong et al., 1999; Puertollano et al., 1999; Martín-Belmonte et al., 2000, 2001).

The observations that MAL: 1) is present in surface clathrin-coated pits (Puertollano et al., 2001), 2) internalizes for recycling (Puertollano and Alonso, 1999), 3) uses clathrin for internalization, and 4) is included in the same vesicles with membrane receptors that use clathrin for apical internalization during translocation from the plasma membrane to the cell's interior (Martín-Belmonte et al., 2003), have recently led to the demonstration that, in addition to being an element of the apparatus for the direct apical route of exocytosis, MAL plays a role as machinery for clathrin-mediated endocytosis from the apical surface (Martín-Belmonte et al., 2003). MAL, therefore, could regulate the apical levels of membrane receptors by acting at both the secretory and endocytic pathways.

### **Machinery for the indirect apical route: the MAL2 protein**

Although the involvement of lipid rafts in the direct route of apical transport has gained substantial experimental support in recent years, their participation in other transport processes has been controversial. For instance, whereas the transcytosing polymeric immunoglobulin receptor appears to incorporate into rafts during movement to the apical membrane in intestinal cell explants (Hansen et al., 1999), it remains excluded from that fraction in epithelial MDCK cells (Sarnataro et al., 2000). However, recent evidence obtained from polarized hepatic cells clearly implies a role for rafts in the transcytotic pathway (de Marco et al., 2002; Nyasae et al., 2003; Slimane et al., 2003).

The fact that other transport pathways may use rafts raises the question of as to which protein machinery is acting in those processes. MAL is the founder member

**Table 1.** Summary of the distribution of MAL and MAL2 in different human tissues.

TISSUE/ORGAN	POSITIVE FOR MAL	POSITIVE FOR MAL2	NEGATIVE FOR BOTH MAL AND MAL2
Common structures	Axons, Mast cells	Neurons, Mast cells	Endothelial cells, Fibroblasts, Muscle cells
Skin	Ductal eccrin cells	Apical keratinized epithelium Sebaceous glands	Hair follicles, Melanocytes
Esophagus	Stratified squamous epithelium	Stratified squamous epithelium	Muscle cells, Submucose
Stomach	Parietal cells, Chief cells, Surface mucus cells	Parietal cells, Chief cells, Surface mucus cells	Muscle cells, Submucose
Small intestine	Crypt cells, Paneth cells , Enterocytes with microvilli, Lymphocytes (Peyer's patches)	Crypt cells, Paneth cells, Enterocytes with microvilli	Muscle cells, Submucose
Large intestine	Mucus cells	Mucus cells	Muscle cells, Submucose
Liver	Centrilobular hepatocytes, Intrahepatic ductal epithelium	Hepatocytes, Intrahepatic ductal epithelium	Kupffer cells, Endothelium
Pancreas	Acinar cells, Ductal cells Endocrine cells	Acinar cells, Ductal cells Endocrine cells	
Kidney	Distal convoluted tubules Collecting tubules Loop of Henle Collecting tubules	Distal convoluted tubules Glomerulus Loop of Henle Collecting tubules	Proximal convoluted tubules
Prostate	Ductal and acinar cells	Ductal and acinar cells	
Lymph node and tonsil	T cells, HEV endothelium	Dendritic cells, HEV endothelium	Sinusoidal cells, Macrophages, Other endothelia
Thymus	Cortical thymocytes Medullary thymocytes Hassall's corpuscles	Epithelial cells Hassall's corpuscles	
Bronchi and trachea	Respiratory epithelium Goblet cells	Respiratory epithelium Goblet cells	
Lung	Type 2 pneumocytes Mucus cells	Alveolar lining cells Type 2 pneumocytes, Mucus cells	
Thyroid	Thyrocytes	Thyrocytes	
Testis	Leydig cells, Sertoli cells	Leydig cells, Sertoli cells	Germinal cells
Adrenal gland	Medullary cells Zona reticularis, glomerulosa and fasciculata	Medullary cells Zona reticularis, glomerulosa and fasciculata	

of a family of proteins, known as the MAL protein family, with structural and biochemical similarities (Pérez et al., 1997). Earlier on, we proposed that members of the MAL family could play a role as machinery for raft-mediated transport processes (Pérez et al., 1997). Supporting this proposal, MAL2 (Wilson et al., 2001) (Fig. 2B), a member of the MAL family, has recently been shown to be an essential component of the machinery for basolateral-to-apical transcytosis in hepatoma HepG2 cells (de Marco et al., 2002).

### **The expression of machinery for the direct and indirect routes of apical transport in human epithelial cells**

The demonstrated role (Fig. 1) of MAL and MAL2 as machinery for the direct and indirect routes of apical transport, respectively, allows the prediction of the potential use of the direct and indirect transport pathways by specific types of epithelial cells, if we assume that the expression of MAL and MAL2 is indicative of the functioning of the respective route. Therefore, we have recently used specific mAb to either MAL or MAL2 in a survey of human tissues to obtain information on the potential use of different apical transport pathways by specific types of epithelial cells (Marazuela et al., 2003, 2004a). We found that MAL and MAL2 expression are tissue- and cell-type-specific. Some cell types express both molecules while other types express only one or neither of them (Table 1). The use of the MAL- and/or MAL2-mediated pathways in a few selected examples for all the scenarios found is discussed below.

#### *Epithelial cells expressing MAL and MAL2*

The simultaneous expression of MAL and MAL2 by parietal (oxyntic) cells, goblet cells and type-2 pneumocytes (Table 1) suggests that these cell types use both the direct and indirect routes for apical transport. These routes are probably required to enable the specialized roles performed by these cell types, such as ion transport by parietal cells, mucus transport by goblet cells and surfactant release by type 2 pneumocytes.

#### *Differential use of MAL-mediated transport pathways*

An example of a differential use of the direct transport pathway mediated by MAL in related epithelial cell types is that of pneumocytes (Fig. 3a,b). The flat, type-1, pneumocytes involved in gaseous exchange do not express detectable levels of MAL although they are positive for MAL2 expression. In contrast, round, type-2 pneumocytes, which secrete surfactant, were found to express both MAL and MAL2. An interesting scenario arises in the liver, where all the hepatocytes are positive for MAL2 expression but only those localized in the centrilobular area express MAL (Fig. 3c,d). This probably reflects heterogeneity in the use of the direct

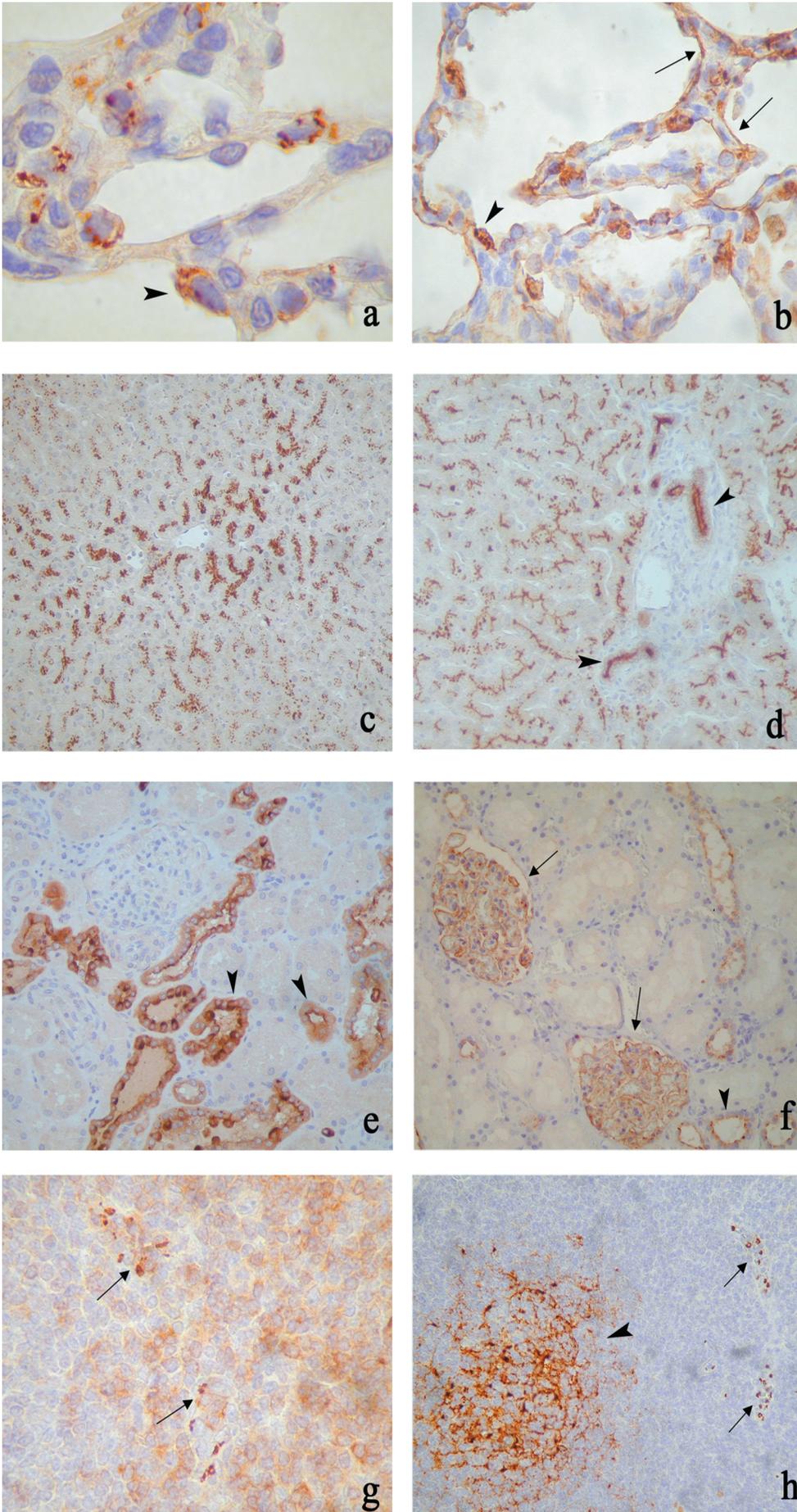
pathway by hepatocytes related to the distance from the terminal hepatic venule.

#### *Differential use of both MAL- and MAL2-mediated transport pathways*

There are two good examples of the differential use of both the direct and indirect routes mediated by MAL and MAL2, respectively, in related epithelial cell types. The first is the epithelium lining the renal proximal convoluted tubules, which was negative for MAL2 and MAL expression, whereas that of the distal convoluted tubules was strongly positive (Fig. 3e,f). A second pair of related cell types worth comparing is that of the flattened endothelial cells of normal blood vessels and the cuboidal endothelium of the high endothelial venules (HEVs) of lymphoid organs (Fig. 3g,h). These were found to be negative and positive, respectively, for both MAL and MAL2 expression, yet while they have similar roles in lining blood vessels and regulating blood coagulation, additionally, HEVs are the main site for constitutive extravasation during lymphocyte recirculation.

#### **MAL- and MAL2-mediated transport pathways in nonepithelial cells**

In the case of nonepithelial cells, it is worth emphasizing that T lymphocytes express MAL but not MAL2, the opposite being true in peripheral neurons. Although none of these cell types polarizes segregating typical apical and basolateral surfaces, they are nevertheless polarized cells. T cells acquire polarized morphology during migration, displaying a leading edge at the front, which contains chemokine receptors, and a protrusion at the rear, referred to as the uropod, which concentrates adhesion molecules. The plasma membrane of the neurons is subdivided into an axon and several dendrites, which have a different protein and lipid composition and have clearly distinct functions. The expression of MAL (T cells) and MAL2 (neurons) suggests the existence of transport pathways in these cells reminiscent of the direct and indirect routes, respectively, of polarized epithelia (Alonso and Millán, 2001; Millán et al., 2002). Follicular dendritic cells, which contain numerous membranous projections, are involved in trapping antigens to display them to activate lymphocytes. The specific expression of MAL2 in follicular dendritic cells (Fig. 3h) might be related to the intense membrane trafficking that takes place in these antigen-presenting cells. Another interesting case is that of mast cells, which express both MAL and MAL2. MAL and MAL2 were localized to cytoplasmic granules, suggesting the involvement of these two proteins in the intensive secretory activity of mast cells, which includes the secretion of biogenic amines (histamine), serine proteases (tryptase, chymase), proteoglycans (heparin and chondroitin sulfate), lipid mediators (platelet-activating factor, prostaglandin D<sub>2</sub>,



**Fig. 3.** Expression of MAL and MAL2 in normal tissues. **a.** MAL staining in the lung. While type-2 pneumocytes are positive (arrowhead), no staining is found in alveolar lining cells. x 100. **b.** MAL2 staining in the lung. Alveolar lining cells (arrows) and type-2 pneumocytes (arrowhead) are positive. x 100. **c.** MAL staining in the liver. Granular staining is found in the centrilobular area. x 40. **d.** MAL2 staining in the liver. Granular staining is found in hepatocytes. Strong reactivity is found in bile canaliculi (arrowheads). x 40. **e.** MAL staining in the kidney cortex. The glomeruli and proximal convoluted tubules are negative. Distal convoluted tubules are highly stained (arrowheads) (x 20). Distal convoluted tubules give a differential pattern between cells, with more pronounced staining in some cells and at the apical border. **f.** MAL2 staining in the kidney cortex. The glomeruli (arrows) and distal convoluted tubules are highly stained (arrowhead). x 20. **g.** MAL staining in the lymph node. Staining is confined to paracortical lymphocytes (T-cell zone) and sporadic lymphocytes in the follicle. While no staining is found in other endothelial cells, specific staining is detected in high endothelial venules (arrows). x 40. **h.** MAL2 staining in the lymph node. Staining is confined to follicular dendritic cells (arrowhead). While no staining is found in other endothelial cells, specific staining is detected in high endothelial venules (arrows). x 40

leukotrienes, etc.) and cytokines (IL-3, TNF- $\alpha$ , MIP-1 $\alpha$ , IL-4, etc.).

### Expression of MAL and MAL2 in tumor cells

The essential role of MAL and MAL2 in raft-mediated traffic and their specific expression in polarized epithelial cells, secretory cells, and other cell types such as neurons, T lymphocytes, dendritic cells and mast cells imply that alterations in the expression and/or distribution would probably be reflected in the abnormal functioning of the cells. It is plausible that this alteration is accompanied, at least in some cases, by changes in the protein sorting machinery with the result that the analysis of the MAL proteolipid family expression could aid detection of incipient neoplasms and improve our understanding of tumoral transformation.

A compilation of reported data regarding MAL and MAL2 expression in human tumors is presented in Table 2. A close correlation of MAL protein expression with carcinogenesis and/or progression of human cancer has recently been found in esophageal cancer (Mimori et al., 2003), a neoplasm with great malignant potential. The MAL gene is strongly expressed in normal esophageal epithelial cells but becomes extinguished in esophageal carcinoma. Tumor suppressor activity of MAL was demonstrated in esophageal tumors expressing exogenous MAL, as ectopic expression of MAL prevented tumor progression and led to increased death of the tumor cells by apoptosis (Mimori et al., 2003).

MAL overexpression in cutaneous T-cell lymphoma was associated with resistance to  $\alpha$ -interferon therapy (Tracey et al., 2002). While the majority of patients who achieved complete remission with  $\alpha$ -interferon therapy did not express MAL, most patients with a slow response did express MAL. The up-regulation of MAL in the resistant cells suggests that MAL-containing rafts might be important for maintaining normal membrane trafficking and signaling in these tumors (Tracey et al.,

2002). Although MAL expression is normally absent from normal B lymphocytes, MAL expression has been found in primary mediastinal B-cell lymphoma (Copie-Bergman et al., 1999, 2002; Pileri et al., 2003), a rare tumor that arises from a specific subset of resident thymic medullary B cells.

We have documented changes in MAL proteolipid family expression in specific types of renal carcinoma (Marazuela et al., 2003, 2004a). In this regard, the expression and/or distribution of MAL and/or MAL2 were altered in specific types of renal carcinoma (Table 2 and Fig. 4). Renal clear cell carcinoma, a histological variant of renal carcinoma related to cytoplasmic accumulation of lipids, whose pathogenesis is still a source of controversy, did not stain with MAL (Fig. 4a). In addition, MAL2 was also absent from the majority of renal clear cell carcinomas, although in some specimens MAL2 expression was detected (Fig. 4b). Conversely, renal oncocytomas, which are benign renal tumors, showed intense MAL staining (Fig. 4c) but did not express MAL2 (Fig. 4d). Other types of renal carcinoma, including chromophobe, papillary (Fig. 4 e,f) and granular carcinomas (not shown), were positive for both MAL and MAL2 expression. In thyroid follicular cell-derived carcinomas, while papillary and follicular carcinoma presented diffuse staining with both MAL and MAL2, anaplastic carcinoma showed MAL but not MAL2 expression (Marazuela et al., 2003, 2004b).

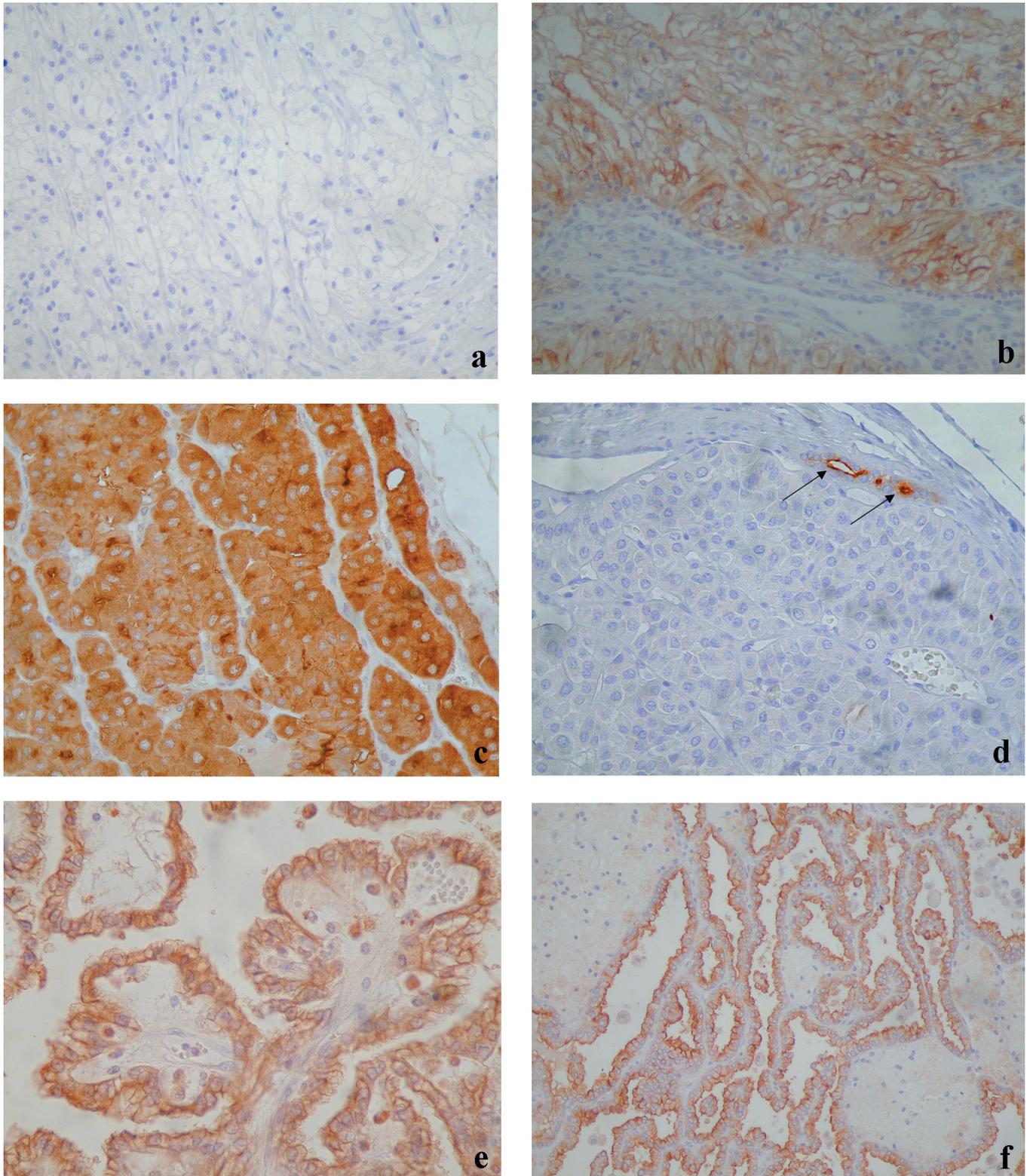
Taken together, these findings imply a relationship between the type of tumor and the expression of MAL and/or MAL2. It is therefore plausible that the study of alterations in the expression/distribution of the MAL family of molecules could aid detection of incipient neoplasms. The availability of the anti-human MAL and MAL2 mAb and their demonstrated use in both paraffin-embedded sections and cryosections may well allow the expression of MAL/MAL2 to be used as a novel tool for tumor characterization. In addition, as their mechanisms of action become better known, MAL and MAL2 may become excellent targets for cancer gene therapy.

**Table 2.** MAL and MAL2 expression in tumors.

	MAL EXPRESSION	MAL2 EXPRESSION	REFERENCES
<i>Renal carcinoma</i>			
Clear cell carcinoma	-	-/+ <sup>1</sup>	Marazuela et al., 2003, 2004a
Chromophobe carcinoma	+++	+++	
Papillary renal cell carcinoma	+++	+++	
Granular cell carcinoma	+++	+++	
Oncocytoma	+++	-	
<i>Lymphoma</i>			
Primary mediastinal large B-cell lymphoma	+++	N.D.	Copie-Bergman et al., 1999, 2002; Pileri et al., 2003
Cutaneous T-cell lymphoma	+++ <sup>2</sup>	N.D.	Tracey et al., 2002
<i>Esophageal carcinoma</i>	-	N.D.	Mimori et al., 2003

<sup>1</sup>: Results were variable. Although the majority of specimens were negative, some tumors were found to be positive for MAL2 expression.<sup>2</sup>: Overexpression in  $\alpha$ -interferon-resistant cells.

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**Fig. 4.** MAL and MAL2 expression in epithelial renal tumors. **a.** Clear cell carcinoma. No MAL staining is found in tumor cells. x 40. **b.** Clear cell carcinoma. Intense MAL2 staining is found in tumor cells in some specimens. x 40. **c.** Renal oncocytoma. The epithelium shows intense cytoplasmic granular staining for MAL. x 40. **d.** Renal oncocytoma. Tumor cells show no staining for MAL2. Normal staining is found in adjacent normal tubules (arrows). x 40. **e.** Papillary renal carcinoma. MAL staining is more intense on the apical side, with more pronounced staining in some cells. x 20. **f.** Papillary renal carcinoma. MAL2 staining is more intense on the apical side, with more pronounced staining in some cells. x 10

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