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MULTIPLE FACETS OF SIALOMUCIN COMPLEX/MUC4, A MEMBRANE MUCIN AND ERBB2 LIGAND, IN TUMORS AND TISSUES (Y2K UPDATE)

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1. ABSTRACT

Sialomucin complex (SMC, MUC4) is a high M_r glycoprotein heterodimer, composed of mucin (ASGP-1) and transmembrane (ASGP-2) subunits. ASGP-2 contains two EGF-like domains and acts as an intramembrane ligand for the receptor tyrosine kinase ErbB2. Transfection studies with SMC DNAs showed that SMC expression could markedly reduce both cell-cell and cell-matrix interactions *in vitro* and increase the growth of primary tumors and the formation of metastatic foci of human A375 melanoma cells as xenotransplants in nude mice, possibly through the ability to suppress apoptosis. SMC is expressed in most vulnerable epithelia as a protective agent, which is found in both membrane and soluble forms at luminal surfaces and secreted into fluids such as milk and tears. SMC appears to be constitutively expressed by most accessible epithelia, notable exceptions being the mammary gland and uterine luminal epithelium, in which it is tightly regulated during pregnancy. Down-regulation at the luminal uterine surface appears necessary for blastocyst implantation. TGF-b is a potent repressor of SMC expression in the mammary gland and uterus, though by different mechanisms. These combined results suggest that SMC has multiple functions in epithelia and is tightly regulated in those tissues where its special functions are required.

2. INTRODUCTION

2.1. Mucins

Mucins were originally described as the glycoprotein components of epithelial mucus secretions and defined by their high M_r values and content of O-linked oligosaccharides (1). These original mucins were primarily gel-forming species. With the advent of molecular cloning, two other classes have been defined, the membrane mucins and small, soluble mucins. Mucins from eight different human genes have now been reasonably well characterized (MUCs 1-4, 5AC, 5B, 6, 7) (Table 1), though not all have been completely sequenced. All eight mucins have one or two types of tandem repeat domains, which are highly O-glycosylated, but not highly conserved between species (2). Mucins also have unique domains, which are more highly conserved. An example is the von Willebrand factor domains found in gel-forming mucins MUCs 2, 5AC, 5B

Table 1. Human mucins

Mucin	Class/Family	Expressed in	Special domains	Chromosome
MUC1	membrane/1	most epithelia	cytoplasmic	1q21-24
MUC2	gel-forming/2	Intestine, airway	vWf	11p15.5
MUC3	membrane?/3	intestine, airway	EGF	7q22
SMC/MUC4	membrane/3	most epithelia	EGF	3q29
MUC5AC	gel-forming/2	airway, etc	vWf	11p15.5
MUC5B	gel-forming/2	airway, etc	vWf	11p15.5
MUC6	gel-forming?/2	GI tract, airway	vWf?	11p15.5
MUC7	small, soluble/4	oral cav., airway	none?	4q13-21

vWf, von Willebrand factor; EGF, epidermal growth factor; SMC, sialomucin complex.

and 6. Genetic analyses of human and rodent mucins suggest that the eight can be divided into four families (Table 1). The gel-forming mucins have similar N- and C-terminal domains, which are related to von Willebrand factor domains involved in multimer formation. All of these genes are found in a single chromosomal location. Membrane mucins comprise two families: MUC1 and MUC3/MUC4. The designation of MUC3 as a membrane mucin is based on the sequences of the rat (3) and mouse (4) homologs and the similarities of their C-terminal regions to rat sialomucin complex (SMC) (5) and human MUC4 (6). MUC3 and MUC4 are found on different chromosomes and are more highly diverged than the gel-forming family members. MUC1 (2) and the small, soluble mucin MUC7 (7) appear to be unrelated to any of the other mucins, based on available sequence data.

Functionally, mucins can be divided into three classes: membrane, gel-forming and small soluble. The gel-forming mucins form disulfide-crosslinked gels and are a major constituent of the classical mucus secretions protecting vulnerable epithelia (1). The small soluble MUC7 is found in less viscous secretions, such as saliva, from which it was originally isolated as MG2 (8). Mucins can generally be classified also by the cells in which they are synthesized, though the data on this aspect are as yet incomplete. Gel-forming mucins are made in and secreted from mucous cells of glandular tissue and goblet cells of luminal epithelia (1). The other classes, including membrane mucins, are made in serous cells (9) or luminal surface epithelial cells (10, 11). Mucins are secreted by both constitutive and regulated mechanisms (12), depending on the cell type and stimuli. Studies on mucin biosynthesis have concentrated mostly on the gel-forming mucins, for which the pathways can be quite complex (13). The individual steps include apoprotein synthesis with co-translational N-glycosylation, dimerization with disulfide formation, O-glycosylation, multimerization and secretion. The specifics of glycosylation are under continuing investigation, but it is clear that the processes are tissue- and cell type-dependent (2). Both mucin protein expression and glycosylation of mucins appear to be altered with the differentiation state of cells and with neoplastic transformation (2, 13, 14). It is this latter phenomenon which has contributed to much of the recent interest in mucins as markers for tumor progression and prognosis (15).

2.2. Membrane mucins

The first membrane mucin, epiglycanin, was discovered as a consequence of its expression on allotransplantable, but not nonallotransplantable, mouse mammary tumors (16). Unfortunately, this mucin has never been cloned and sequenced, so its characterization has lagged and its relationship to human mucins has never been defined. MUC1 and MUC4/SMC are by far the best characterized of the class. Membrane MUC1 is found on the surfaces of most simple epithelia and believed to play a role in the protection of these surfaces (2). The ability of membrane mucins to protect cells was originally proposed as the mechanism by which epiglycanin promoted allotransplantability of tumor cells, by masking histocompatibility antigens recognized by immune killer cells (17). Subsequent transfection studies have verified the potent anti-adhesive effects of these membrane mucins when expressed on the surfaces of tumor cells (18, 19, 20, 21). Membrane mucins may have a second function, cell signaling. MUC1 has a highly conserved cytoplasmic domain which can be tyrosine-phosphorylated (22) and may couple to cytoplasmic signaling pathways. How this event is linked to extracellular signaling agents or intracellular downstream effectors remains to be clarified. Evidence has been presented for an interaction of phosphorylated MUC1 with Grb2, an adaptor protein linking Ras of the Ras/MAPK mitogenic pathway to its activators on membranes (23). MUC1 also is proposed to interact with b-catenin (24), an important component of the Wnt signaling pathway (25) and of cadherin-type cell-cell junctions (26), though the nature of the interaction and its role in cell behavior are unclear. MUCs 3 and 4 have extracellular EGF-like domains (27). In the case of Muc4, one of these domains can participate in complex formation with the receptor tyrosine kinase ErbB2 (28), as described below. Most importantly, these studies suggest that membrane mucins have at least two functions, anti-recognition (anti-adhesion) and cell signaling, both of which may be critical contributors to tumor progression. Membrane mucins also may play important roles in normal cell functions, such as participation in epithelial development.

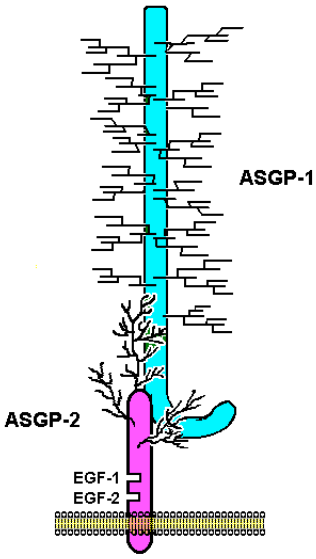

Figure 1: Model of sialomucin complex. Reprinted from ref. 27.

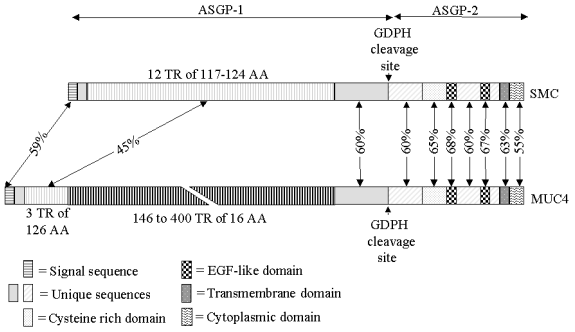

Figure 2: Comparisons of rat SMC and human MUC4. Figure courtesy of Dr. Jean-Pierre Aubert, redrawn by Shari Price-Schiavi.

The term "membrane mucin" is somewhat a misnomer, because all four known members of this class (MUC1, MUC3, MUC4/SMC, epiglycanin) can also be found in soluble forms, produced by alternative splicing (29) and/or proteolysis (30). In the case of MUC3 cloning and sequencing of human cDNAs did not reveal a transmembrane sequence (31), in contrast to the rat and mouse cDNAs (3, 4). The likely explanation is that a nonmembrane alternative splice form was sequenced in the human, though this question needs to be examined by both biochemical and molecular biological studies. Interestingly, both MUC1 and SMC/MUC4 are produced as heterodimeric complexes from a single gene product via a proteolytic cleavage during the early stages of their transit to the cell surface (32, 33, 34). Their cleavage sites appear to be unrelated (14). However, the proposed cleavage site in SMC/MUC4 is found in MUC2 (14). MUC2 cleavage produces the "link" protein (35) proposed to function in multimer formation. Both MUC1 and SMC/MUC4 have been shown to undergo recycling from the cell surface during a maturation process that adds additional carbohydrate to the mucin subunits (36, 37).

2.3. Sialomucin complex/MUC4

Sialomucin complex was originally isolated from metastatic 13762 rat mammary adenocarcinoma ascites cells. It is a heterodimeric tumor cell surface glycoprotein complex composed of a high Mr sialomucin (ascites sialoglycoprotein-1, ASGP-1) (38) and a 120 kDa transmembrane N-glycosylated component ASGP-2 (39) (Figure 1). The complex is present at very high levels in the ascites cells (>10^6 copies/cell), and the sialomucin has been implicated in the metastatic potential of 13762 cells (40) and their resistance to killing by natural killer cells (41). Biosynthesis studies showed that the complex is synthesized as an approx. 300 kDa N-glycosylated precursor pSMC, which is cleaved to the two subunits early in its transit to the cell surface, before addition of most of the O-glycosyl groups to the mucin subunit (32). Molecular cloning and sequencing and Northern blots indicated that the precursor is made from a 9 kb transcript containing 0.1 and 0.9 kb 5' and 3' noncoding regions, respectively. The coding sequence for ASGP-1 contains a signal peptide, a 50 amino acid Ser/Thr-rich N-terminal domain, a large Ser/Thr-rich tandem repeat (TR) domain with repeats averaging 124 amino acids (1513 aa) and a third Ser/Thr-rich domain (609 aa) (Figure 2). ASGP-2 contains 7 domains: two hydrophilic N-glycosylated domains, two epidermal growth factor(EGF)-like domains, a non-EGF cysteine-rich domain, a transmembrane domain and a short cytoplasmic domain (42) (Figure 2). The EGF-like domains contain all of the consensus amino acid residues required for growth factor activity (5).

Recent studies have shown that SMC is the rat homolog of the human mucin MUC4 (6, 43, 44) (Figure 2). The relationship between rat SMC and human MUC4 had not been recognized previously because rat Muc4 does not have the 16 amino acid tandem repeat domain which was the identifying characteristic of human MUC4 (6, 44). Otherwise, they are highly similar, as shown in Figure 2, including a high degree of conservation of cysteine residues and of ASGP-2 N-glycosylation sites (6).

3. SMC/MUC4 FUNCTIONS

3.1. Anti-adhesion

To study the mechanism of anti-recognition effects of SMC, constructs containing 3, 5 and 8 copies of the 125-amino acid repeats (Figure 2) of rat SMC were prepared and subcloned into an inducible expression vector with a tetracycline-regulatable promoter (18). This is a tet-off system, in which removal of tetracycline induces expression of the SMC analogs. Transfection into A375 human melanoma cells provided the system for analyzing anti-adhesion effects. Overexpression of SMC in these cells reduced both cell-cell and cell-matrix interactions. The anti-adhesive effect was fully reversible with changes in tetracycline, and dependent on both the size of the SMC and the level of expression. Kinetics of binding of cells to fibronectin, laminin and collagens using transfected cells containing SMC with different numbers of mucin repeats in the presence and absence of tetracycline indicated that

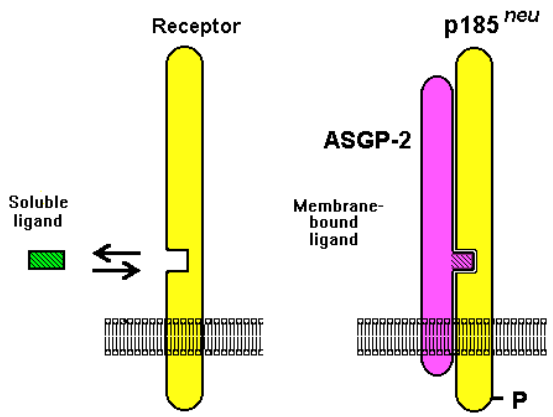

Figure 3: Model for intramembrane vs soluble ligand. $p185^{neu}$ = ErbB2. P, phosphotyrosine.

SMC blocks integrin-dependent cell-matrix adhesion by a nonspecific steric effect (18).

As noted above, SMC has been implicated in tumor metastasis. One of the mechanisms by which membrane mucins might promote metastasis is by allowing mucin-expressing tumor cells to escape destruction by the immune system. Overexpression of SMC in A375 cells inhibits tumor cell killing by lymphokine-activated killer cells (45). Experiments with constructs with different numbers of repeats demonstrated that resistance to immune cell killing depends on the size of the mucin molecule. Loss of killing correlated with loss of recognition, measured by a bead binding assay using MHC-conjugated beads. Tumor cell binding to the beads was inhibited by SMC overexpression and dependent on the number of SMC mucin repeats. Interestingly, SMC-overexpression also reduced antibody binding to cell adhesion receptors, such as I-CAM, on the A375 cells, an effect which could be prevented by antibody capping of the SMC. These results have potential implications for the use of antibody therapies for cells expressing SMC. For example, since SMC binds ErbB2 directly (28), antibodies such as Herceptin, which target ErbB2, may be less effective on cells overexpressing SMC.

3.2. ErbB2 Ligand

One of the intriguing features of SMC is the presence in ASGP-2 of two EGF-like domains, both of which have the conserved amino acid residues of active growth factors of this family. An intramembrane complex of ASGP-2 and ErbB2 has been demonstrated by co-immunoprecipitation experiments from detergent lysates in a number of systems, including 13762 ascites cell membranes, Sf9 insect cells expressing ASGP-2 and ErbB2, SMC-transfected A375, MCF-7 and Cos-7 cells, lactating rat mammary tissue and primary rat mammary epithelial cells. In the case of the insect cells, EGF receptor, ErbB2, ErbB3 and ErbB4 were expressed separately or in combination with ASGP-2. ASGP-2 was observed to be co-immunoprecipitated with ErbB2, but not the other receptors (28). No immunoprecipitation of ASGP-2 by anti-ErbB2 was observed in the cells expressing ASGP-2 without ErbB2. ASGP-2 could also be immunoprecipitated using anti-phosphotyrosine from cells expressing both ASGP-2 and ErbB2, but not from cells expressing only ASGP-2 (28). These results clearly show that membrane ASGP-2 can associate directly with membrane ErbB2. This novel *intramembrane complex* is illustrated and compared to the classical ligand-receptor complex in Figure 3.

Complex formation was further examined by expressing the extracellular domains of ASGP-2 (ASGP-2-ECD) and ErbB2 (ErbB2-ECD) in High 5 (secretory) insect cells individually and together. Conditioned medium from each of the cell cultures was then immunoprecipitated with anti-ErbB2. Alternatively, conditioned media from cells individually infected with ASGP-2-ECD and ErbB2-ECD were mixed and incubated before immunoprecipitation. Anti-ASGP-2 immuno-blotting of the immunoprecipitates showed that ASGP-2-ECD was co-immunoprecipitated with ErbB2-ECD only when they were expressed in the same cell. Surprisingly, when soluble ASGP-2-ECD and soluble ErbB2-ECD from the conditioned media of insect cells individually expressing the proteins were mixed, no complex was observed by immunoprecipitation. These results indicate that, at least in this system, the ligand and receptor must be expressed in the same cell (an intracrine response) to form a complex, likely interacting in an intracellular compartment before they reach the cell surface. This conclusion is consistent with our observations from numerous experiments that soluble, recombinant ASGP-2 added to insect cells or mammalian cells expressing ErbB2 on their cell surfaces does not form an ASGP-2-receptor complex or stimulate receptor phosphorylation. To characterize the soluble ASGP-2-ECD/ErbB2-ECD secreted complex, the insect cells were metabolically labeled with amino acid. The fluorogram indicated that no components other than ASGP-2 and ErbB2 could be detected in the complex. Studies on C-terminal deletion mutants of ASGP-2 implicate EGF1 (more N-terminal EGF domain, Figs. 1 and 2) as the domain responsible for association of ASGP-2-ECD with ErbB2-ECD (28).

Two separate studies indicate that complex formation with ASGP-2 activates ErbB2 phosphorylation. First, anti-phosphotyrosine immunoblot analyses indicate that receptor phosphorylation is increased in insect cells expressing ErbB2 plus ASGP-2, but not those expressing ErbB2 alone or the other three receptors plus ASGP-2. Second, SMC-transfected A375 cells treated with or without tetracycline to regulate SMC expression exhibited greater ErbB2 phosphorylation in the SMC-overexpressing cells than in the SMC negative cells, indicating that SMC (ASGP-2) can activate phosphorylation of ErbB2. Furthermore, when these cells were treated with the ErbB3 ligand neuregulin, which activates ErbB2/ErbB3 complex, ErbB2 phosphorylation was further increased, showing that SMC can potentiate the effects of the neuregulin. ErbB3 phosphorylation was also potentiated in this experiment. We have proposed that SMC is acting as an intramembrane, autocrine modulator of ErbB2 (Figure 4). By

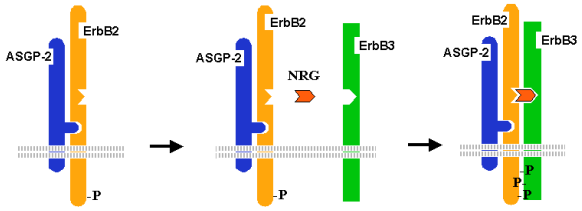

Figure 4: Model for potentiation of ErbB2 and ErbB3 phosphorylation by ASGP-2. NRG, neuregulin.

this mechanism an intracellular complex is formed between SMC ASGP-2 EGF1 domain and ErbB2. This complex migrates to the cell surface, where it can form a receptor heterodimer with neuregulin-activated ErbB3, thus potentiating the phosphorylation of both receptors (Figure 4).

4. SMC/MUC4 IN TUMOR PROGRESSION

4.1. Effect on Primary Tumor Growth

The ability of SMC to potentiate phosphorylation of ErbB2 and ErbB3 could promote tumor progression. To address this issue, A375 cells were used for tumor growth and metastasis studies *in vivo*. The key in these experiments was the ability of tetracycline to regulate the expression of SMC in the A375 cells when given to the animals in their drinking water *in vivo* (tetracycline therapy). The effect of SMC overexpression on primary tumor growth was tested by subcutaneous injection. Tumors in animals without tetracycline therapy (SMC-expressing) grew at a faster rate than those in animals receiving tetracycline, particularly at later stages, indicating that SMC can promote tumor growth of primary tumors. These studies raise the question of the mechanism by which SMC promotes tumor progression. *In vitro* studies of the A375 cells indicated that SMC expression does not greatly enhance proliferation, consistent with a failure to activate MAP kinase (28). However, SMC-expressing tumors show a marked decrease in apoptotic cells. Moreover, SMC expression in the A375 cells substantially represses apoptosis induced by serum deprivation (S. Jepson, unpublished observations). The reduced apoptotic rate could provide an explanation for the SMC effect on the growth of the primary tumors.

4.2. Effect on Metastasis

The effect of SMC overexpression on tumor progression was further examined using a tail vein injection metastasis assay (M. Komatsu, unpublished observations). Three groups of animals were used: A) injected cells with SMC ON with no tetracycline therapy; B) injected cells with SMC ON, with tetracycline therapy; and C) injected cells with SMC OFF, with tetracycline therapy. Microscopic metastatic foci were counted in histological sections of paraffin-embedded lung tissue. Both groups A and B exhibited similar high numbers of metastatic foci compared to group C or to animals injected with parental (non-transfected) A375 cells. These results indicate that SMC expression can strongly promote metastasis (group A vs C). Moreover, formation of metastatic foci appears to be a rapid event, occurring more quickly than transcriptional downregulation and turnover of the SMC (group B). Interestingly, measurements of the sizes of the metastatic foci indicated that they were not significantly different for the three groups. Thus, SMC overexpression does not appear to play a role in growth of the metastatic foci, in contrast to the results observed with the primary tumors. A possible explanation is that the larger primary tumors have reached the limits of their local growth support and require additional growth mechanisms than the smaller metastatic foci. These results clearly show that SMC can promote formation of metastatic foci.

An important question is whether SMC can promote metastases from a primary tumor. SMC-expressing A375 cells are highly aggressive. Tumors injected into the flank of the nude mice grew into the body cavity and invaded the internal organs, including the lungs. Thus, a remote site was required for the studies on metastasis to the lungs from a primary tumor. For these experiments the tumors were injected subcutaneously into the foot of tetracycline-treated or untreated rats. Primary tumors were removed by amputation at the knee after a short period of tumor growth. The amputees were maintained for an additional four weeks to allow growth of the metastatic foci to a detectable size. Half (5/10) of the untreated animals, but none of those receiving tetracycline therapy, had metastases (M. Komatsu, unpublished observations). These studies support our hypothesis that SMC can promote tumor progression and metastasis.

An important part of the metastasis process is extravasation of the tumor cells from the circulation. This extravasation is believed to mimic selectin-dependent migration of leukocytes from the blood into tissues (46). However, the anti-adhesive effects of SMC should block this mechanism unless those effects are specificallly overridden. Interestingly, overexpressed SMC in the metastatic A375 cells carries the selectin ligand Sle^x. Thus, SMC could be directly involved in the tumor extravasation process as the carrier for Sle^x in the cascade of events required for metastasis. The presence of the ligand on the extended mucin structure would also make it more accessible for interaction with selectins on other cells, such as platelets and leukocytes for formation of tumor emboli, and endothelial cells for initiation of the extravasation process.

Our combined results described have allowed us to propose a working model for metastasis in which SMC can be invoked in as many as four steps (Figure 5): dissociation from primary tumor via anti-adhesive effects, escape from immune cell killing via anti-recognition effects, formation of emboli via selectin binding mechanisms (not shown) and extravasation via selectin binding mechanisms.

5. SMC/MUC4 IN THE MAMMARY GLAND AND MAMMARY TUMORS

5.1. SMC/MUC4 Expression Levels

SMC is abundantly expressed on highly metastatic rat mammary ascites tumor cells. Thus, it was of interest to know the levels of SMC expressed by normal mammary

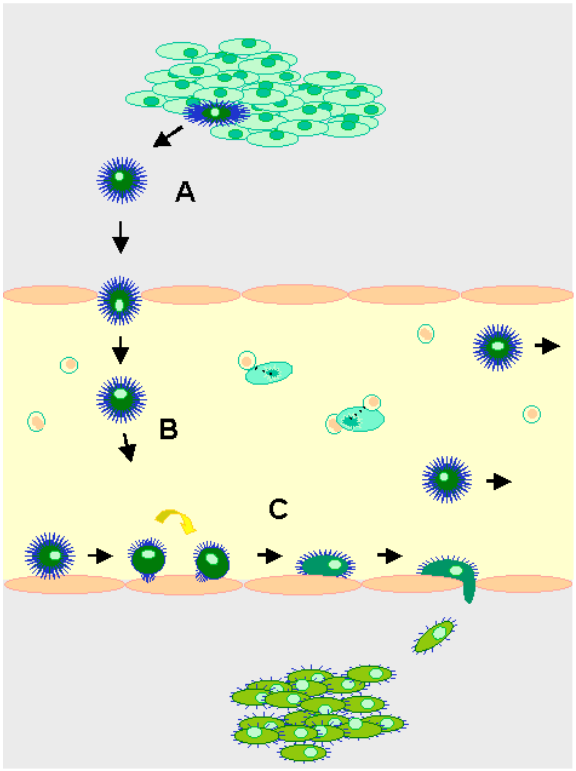

Figure 5: Model for SMC involvement in three major steps of metastasis: (A) dissociation from primary tumor, (B) escape from immune cell killing and (C) extravasation.

tissue. Immunoblot analyses, using monoclonal antibodies prepared against the complex, demonstrated SMC levels about 100-fold less in rat lactating mammary gland than those in the ascites tumor (47). Expression in the virgin gland is about 100-fold lower still. Mammary gland contains a soluble, secretable form of SMC which is not found in the ascites cells; milk and mammary gland also have the membrane form. Immunolocalization studies with anti-ASGP-2 indicate that SMC is present in virgin and lactating mammary tissue in the apical regions of ductal and secretory epithelial cells. SMC expression is up-regulated in mammary gland during pregnancy for secretion into milk, in which the concentration is about 0.1 mg/ml. Thus, there are three important differences in SMC between the normal virgin gland and ascites tumors: expression level, production of soluble form and localization to the apical cell surface domain.

The 13762 ascites tumors exhibit a 3-fold SMC gene amplification and a 5-fold increase in SMC transcript level compared to virgin mammary gland, far short of the 10,000-fold difference in SMC protein level. As noted previously, SMC expression in the rat mammary gland is sharply increased during pregnancy. Moreover, SMC regulation during pregnancy appears to be predominantly post-transcriptional. Thus, transcriptional regulation of SMC must happen at an earlier stage of mammary development.

5.2. Transcript Regulation

Rat mammary epithelial cells (MEC) in primary culture were used to address the question of how SMC transcript levels are regulated. Transcript upregulation in these cells is induced by serum, an effect which can be substantially mimicked by insulin or insulin-like growth factor (IGF), but not EGF, heregulin or PDGF (X. Zhu, unpublished observations). The induced expression of SMC and its transcript by serum, insulin or IGF correlated with activation of Erk1/Erk2 MAP kinases, and could be be blocked by either PD98058 or UO126, specific inhibitors of MEK activity, the kinase which activates Erk of the Ras-MAPK transcriptional activation pathway. The SMC upregulation could be reproduced in the absence of growth factor by transfection of MEC with a constitutively active MEK-2 (S218/222D). The growth factor responses indicated that prolonged, not transient, stimulation of MAPK was necessary for SMC transcript expression. Thus, Erk activation appears to be necessary for SMC gene expression in the mammary gland, suggesting the presence of transcriptional upregulation as well as post-transcriptional repression (see below) mechanisms. Importantly, the effects of the MAPK pathway inhibitors and TGF-b in suppressing SMC expression are additive, indicating that they involve different pathways.

Prolactin may also play a role in regulating SMC transcript levels during mammary development, based on our studies with the Rama37 putative stem cell line (48). Rama37 cells can be induced by appropriate stimuli to differentiate into alveolar-like or myoepithelial phenotypes (49). For example, when treated with prolactin, they behave like mammary secretory epithelial cells, forming domes or blisters as a consequence of fluid secretion at their apical surfaces. Most importantly, the prolactin treatment induces a robust expression of SMC (P. Li, unpublished observations). Interestingly, tyrosine phosphorylation of ErbB2 appears concomitantly with SMC expression. One of the markers of mammary alveolar differentiation is peanut lectin-binding carbohydrate (50). Immunoprecipitation experiments indicate that this marker is carried by SMC, as well as other glycoproteins. Surprisingly, the marker is on ASGP-2, which is predominantly N-glycosylated, rather than the mucin subunit ASGP-1, which binds peanut lectin in the 13762 ascites tumor cells (51). This result indicates that there are interesting glycosylation differences between the differentiated rat mammary epithelial cells and their tumors.

Putative SMC promoters from both the 13762 ascites tumor and normal tissue have been cloned using PCR-based DNA walking (S. Price-Schiavi, unpublished observations), 600 bp from ascites tumor DNA and 2.4 kb from normal rat liver DNA. The normal and tumor sequences were identical for the 600 bp they have in common. Examination of the 2.4 kb region shows a number of sequences that resemble known canonical promoter/transcription factor sequence elements, including a STAT5 regulatory element located just 5' of the transcription start site and likely to play a role in prolactin regulation. Preliminary reporter studies have established that the 600 bp and 2.4 kb flanking regions of the SMC

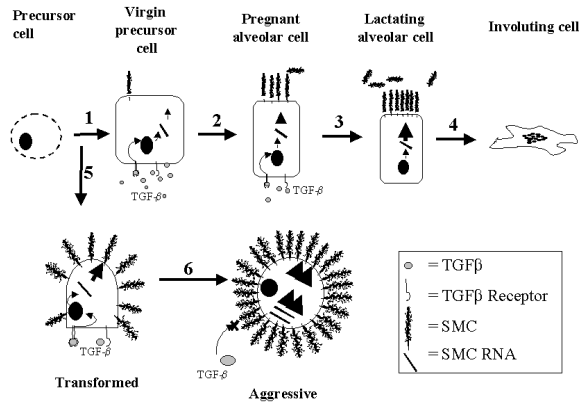

Figure 6: Hypothetical model for regulation of SMC expression during mammary development and mammary tumor progression. 1) Prolactin/IGF upregulation of SMC transcripts; 2) Loss of translational repression by extracellular matrix; 3) Loss of alveolar active TGF-b; 4) Loss of SMC during involution; 5) Neoplastic transformation with loss of repressive mechanism 2; 6) loss of TGF-b responsiveness.

gene exhibit promoter activity which is cell type specific. Interestingly, the shorter construct (0.6 kb) expresses higher reporter activity than the larger one (2.4 kb). A likely explanation for this observation is the presence of negative regulatory elements within the promoter sequence.

5.3. Post-transcriptional Regulation

SMC transcript levels are high in virgin, pregnant and lactating rats, but protein expression is repressed until mid-pregnancy (52). SMC protein, but not transcript, levels are significantly reduced when mammary cells are cultured in Matrigel, a reconstituted basement membrane which stimulates casein expression. SMC precursor is synthesized in Matrigel at a 10-fold lower rate. Pulse labeling studies on the synthesis of SMC precursor in MEC implicate a translational regulatory mechanism, an unusual control mechanism for a milk protein. Matrigel has no effect on either the level of SMC or its transcript in cultured 13762 mammary tumor cells. None of the extracellular matrix components tested (laminins, collagens, fibronectin) reproduced the effects of Matrigel. Although most hormones, cytokines and growth factors, including estrogen, EGF, TGF-b, heregulin and hepatocyte growth factor, have no effect on SMC expression in MEC, TGF-b strongly down-regulates it, suggesting that TGF-b may play a significant role in SMC regulation (52). In contrast, TGF-b is ineffective in blocking SMC expression in 13762 mammary tumor cells. These results indicate that SMC is post-transcriptionally regulated by both TGF-b and the extracellular environment in normal mammary gland, but by neither in 13762 mammary adenocarcinoma cells, a regulatory dysregulation imposing an overexpression of SMC in the tumor cells.

The mechanism by which TGF-b downregulates SMC has been investigated by pulse-chase analyses in MEC (S. Price-Schiavi, unpublished observations). Synthesis of precursor was not affected by TGF-b, ruling out a translational mechanism. The primary effect was a reduction in the amount of ASGP-2 produced from precursor, suggesting that cleavage of the precursor is repressed in the presence of TGF-b. This mechanism is different from that observed with Matrigel, indicating that TGF-b is not responsible for the effects of Matrigel on the MEC. The Matrigel effect may be due to changes in cell shape or polarization of the MEC in Matrigel vs on plastic. Regardless, our results demonstrate a new type of regulatory effect by TGF-b. Two types of experiments suggest that the TGF-b effect on SMC expression does not involve transcriptional changes. 1) It is not inhibited by cycloheximide, suggesting that new protein synthesis is not required. 2) It is rapid, requiring less than 6 hr.

SMC expression can also be reduced substantially when either MEC or ascites cells are treated with tunicamycin, which blocks N-glycosylation. This treatment essentially eliminates SMC expression within 24 hr, probably by triggering the degradation of newly synthesized, incompletely folded SMC. The tunicamycin block was used to examine the turnover of mature SMC in MEC in the presence and absence of TGF-b. No differences were observed, indicating that turnover of the mature molecule does not contribute to its lowered expression in the presence of TGF-b. The half-life of SMC under these conditions was 8 hr. The half-life for 13762 tumor cells was 12 hr. Thus, turnover differences do not appear to make a substantial contribution to the approx. 10^4 difference in expression levels between the tumor cells and epithelial cells.

From these combined studies (Table 2) we can propose a model for the regulation of SMC in the mammary gland and its tumors (Figure 6). SMC transcript expression is induced by prolactin-dependent differentiation and requires the presence of serum factors, including insulin and/or IGF. However, synthesis of the SMC precursor protein is repressed by an effect of the basement membrane, mimicked in culture by Matrigel. SMC production is further repressed by an effect of TGF-b on SMC processing. The repression mechanisms are at least partially relieved during mid-pregnancy and alveolar differentiation in preparation for lactation. The key events, possibly related, are likely a reduction in active TGF-b and a change in the basement membrane interactions of the alveolar cells, which are surrounded by myoepithelial cells. The repression is also relieved upon removal of MEC from the animal, presumably by removing TGF-b from the epithelial cell environment and by releasing interactions with the basement membrane, allowing biosynthesis of SMC under the culture conditions we have described. In the 13762 tumor cells these repression mechanisms appear to have been disrupted. This disruption, in combination with the 5-fold amplification of the gene and overexpression of the transcript, contribute to SMC gross overexpression in this tumor cell line (42). Loss of TGF-b responsiveness is a significant factor in tumor progression, and may be involved in the overexpression of SMC in the 13762 ascites tumor cells. Studies of TGF-beta receptor

Table 2. Regulation of SMC expression in mammary gland

Cell type	Stage	Agent	Mechanism	Effect
Undifferentiated	Prepubertal	Prolactin	Transcriptional?	Increase
Precursor?	Virgin	Serum/IGF/insulin	Transcriptional	Increase
Luminal/alveolar?	Pregnant	Basement membrane	Translational?	Repress
Luminal/alveolar?	Pregnant	TGF-b	Post-translational	Repress

in primary human breast carcinomas and their associated lymph node metastases indicate that loss of TGF-b responsiveness can occur due to mutations in its Type I receptor kinase (53). This mutation is found predominantly in metastatic lesions rather than primary tumors. Thus, the results are consistent with our model for breast cancer progression involving enhanced expression of SMC (52).

6. SMC/MUC4 IN THE FEMALE REPRODUCTIVE TRACT

6.1. SMC/MUC4 Expression in Different Tissues of the Female Reproductive Tract

Both membrane and nonmembrane forms of SMC are found in rat uterus as a complex of ASGP-1 and ASGP-2 (54). Immunocytochemical analyses indicate that the primary site of expression is at the luminal surface of the endometrium, though expression in endometrial glands was also observed. About 40% of the SMC, corresponding to the nonmembrane fraction, is removed by rinsing uterine preparations with saline, indicating that the soluble form is adsorbed loosely to the apical cell surfaces. In contrast to mammary gland, SMC is most highly expressed in the virgin animal, and its expression varies during the estrous cycle with the steady state level of transcript. The complex is present in a location consistent with steric inhibition of blastocyst implantation and is lost at the beginning of the period of receptivity for implantation. Moreover, the complex reappears immediately after the receptivity window on day 6. SMC expression appears to be regulated differently in the uterus, where it must be lost from the endometrial surface before blastocyst implantation can occur (54, 55), than it is in the mammary gland. Uterine luminal SMC is absent from ovariectomized rats, but is up-regulated when they are treated with estrogen and down-regulated by progesterone. Similar regulation is observed during the estrous cycle, when the amount of SMC parallels transcript levels in the luminal epithelial cells. Regulation of SMC in the rat closely parallels observations made on Muc1 in the mouse. Both implantation and loss of SMC expression can be blocked with RU486 (54). We propose that the down-regulation of SMC and its loss from the apical surface of the rat uterine lining contribute to the generation of the receptive state for implantation (Figure 7).

SMC is expressed in other regions of the female reproductive tract throughout the estrous cycle (56). In contrast to the uterus, no major quantitative changes are seen in the expression of SMC in the cervix, vagina, oviduct or uterine glandular cells during the phases of the estrous cycle (Table 3). However, significant changes are noted in the cellular localization of SMC in the different regions of the cervix and in the vagina as they undergo differentiative changes during the estrous cycle (56). Most importantly for its protective function, SMC is always found at the apical surface of the most superficial layers of the stratified epithelia. It is rarely found in the basal layers and is absent from the keratinized layer when it is present. SMC expression in the medial layers varies with the stage of the cycle. In the oviduct SMC is concentrated at the apical surface of the layer of columnar epithelial cells, where the membrane form of SMC appears particularly abundant. In contrast to the uterine luminal epithelia, SMC is present in the uterine glandular epithelia, cervix and vagina in ovariectomized rats (56) and does not change with hormone treatments. Thus, SMC expression is differentially regulated by steroid hormones in different regions of the female reproductive tract.

6.2. Regulation of SMC/MUC4 Expression

The question of the regulation of SMC expression in the uterus has been further investigated using primary cultures of rat uterine epithelial cells, which we isolated by methods described previously (57). Luminal epithelial cells were isolated from uteri of immature rats and cultured on Matrigel-coated permeable filters. Somewhat surprisingly, considering our results with mammary epithelial cells, the rat uterine luminal epithelial cells (RULECs) showed a robust expression of SMC in Matrigel. When these cells were cultured in the presence of estradiol, no increase in SMC expression in the cells was observed. Similarly, progesterone did not decrease SMC expression. When RULECs were continuously cultured in the presence of TGF-b, no expression of SMC was observed. Moreover, when the RULECs are co-cultured with uterine stromal fibroblasts, SMC expression is repressed. These results clearly show that RULECs express SMC, and that the expression responds to TGF-b and factors produced by co-cultured fibroblasts, but not to steroid hormones.

These studies, in combination with the observations on the mammary gland and other tissues of the female reproductive tract, suggest a model for SMC regulation. The most important postulate of that model is that the SMC gene is constitutively expressed in most epithelia, including those of the mammary gland and female reproductive tract. In mammary gland that expression appears to require specific activation of the Erk pathway. Insulin and IGF can act as activators, but EGF, heregulin and others cannot. Moreover, expression of the protein is blocked by TGF-b by a posttranscriptional effect. A similar model may be applied to the female reproductive tract. Constitutive expression is observed in all epithelial tissues, except the uterine luminal epithelium, which is a

Table 3. SMC/Muc4 expression in the female rat reproductive tract during the estrous cycle

Tissue	Stage I, Proestrous	Stage II/III, Estrous	Stage IV, Metestrous	Diestrous
OVIDUCT				
Isthmus,				
LE	+++	+++	+++	+++
GE	+++	+++	+++	+++
Ampulla and Infundibulum	+	+	+	+
UTERUS,				
LE	+	+++	+++	+
GE	+++	+++	+++	+++
CERVIX	++	+++	+++	++
VAGINA	+++	+++	+++	+++

LE = Luminal epithelium, GE = Glandular epithelium.

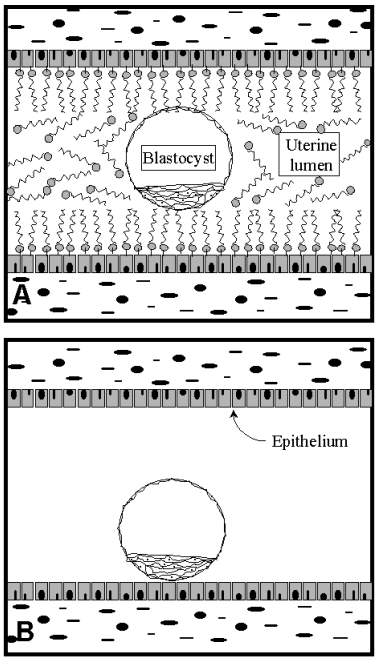

Figure 7: Generic model for role of SMC in protecting uterine epithelium and blocking implantation. Reprinted from ref. 54.

special case and has apparently evolved a downregulation mechanism to permit implantation. Since the expression of SMC in cultured uterine epithelial cells does not appear to require estrogen, the estrogen effects on SMC expression in the uterine luminal epithelium are likely indirect. Our hypothesis is that uterine stromal cells produce a factor(s) which modulates the expression of SMC in the luminal epithelial cell, but not glandular epithelial cell. The production or effectiveness of this factor is blocked by estrogen to allow uterine luminal cell expression in the normal animal, explaining the loss of expression in the ovariectomized animal and changes during the estrous cycle (Table 3). The estrogen effect can be antagonized by progesterone (54). One candidate for the stromal effector is TGF-b, which appears to directly downregulate SMC expression in uterine epithelial cells. TGF-b in the mouse uterus increases at the periimplantation period (58), possibly in response to increased progesterone (59). Thus, it is a candidate for downregulating SMC at the window of implantation. In support of this idea, a transgenic mouse overexpressing TGF-b in the uterus exhibits a loss of TGF-b receptors and delayed implantation (60). Our model provides a framework for the understanding the regulation of SMC expression in the uterine luminal epithelium during pregnancy and the estrous cycle and its role in implantation, but further studies are obviously needed to complete this complex picture.

7. SMC/MUC4 IN THE EYE

7.1. SMC/MUC4 of the Ocular Surface and Tear Film

SMC at the ocular surface was analyzed by Western and Northern blotting of isolated corneal and conjunctival tissues from the rat (11). A higher level of SMC protein expression (5-10 fold) was observed in the cornea than in the conjunctiva. Northern blot analyses of RNA isolated from the two tissues verified the greater expression in the cornea. The localization of SMC at the rat ocular surface was compared to that of MUC1 and "soluble ocular mucin" (goblet cell mucin) by immunofluorescence. The three mucins show different distribution patterns. SMC is present in both corneal and conjunctival epithelia, distributed throughout the stratified epithelia, but more abundant in the superficial layers. MUC1 is localized more to the basal layers. In contrast to both of those, the soluble ocular mucin is found predominantly in conjunctival goblet cells, which were not stained for either SMC or MUC1. These combined results suggest a model for the role of SMC at the ocular surface and in the tear film (Figure 8). We propose that SMC is a major component of the corneal surface glycocalyx, incorporated directly onto the surface from the corneal epithelial cells, by which it is synthesized. This glycocalyx covers the rough surface of the corneal epithelium (microvilli and microplicae) and may help to “smear out” that boundary transition at the ocular surface, a critical optical boundary. The soluble form of SMC in the glycocalyx is loosely bound, as we have demonstrated in

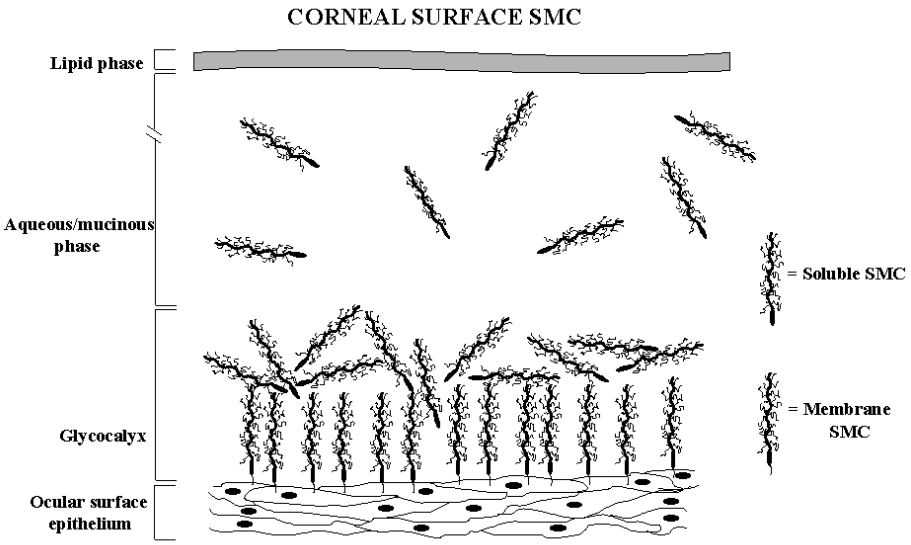

Figure 8: Model for SMC in tear film and epithelial surface of cornea.

the trachea (10) and uterus (54), and presumed to be in equilibrium with the aqueous mucin phase of the tear film above it. The aqueous mucin phase overlying the corneal and conjunctival epithelia contains both the gel-forming mucin(s), possibly other mucins and SMC. These mucins of the tear fluid are produced primarily by the conjunctival epithelium, either by the goblet cells (gel-forming mucins) or other epithelial cells (SMC and MUC4, possibly other mucins?) (61, 62), and mixed with tear fluid components from the lacrimal gland to form the aqueous mucin phase.

7.2. SMC/MUC4 from the Lacrimal Gland

One of the questions raised by the abundance of SMC in the rat and human tear fluid was whether the ocular surface epithelia were the only site of SMC production. The lacrimal gland is responsible for production of many of the tear fluid components, but is not usually considered a source of tear fluid mucin, though mucin-like components have been observed in the gland (63). The lacrimal gland of the female adult rat consists of serous acini connected by intercalated and interlobular ducts. Sialomucin complex (rat Muc4) was immunolocalized to secretory granules in acinar cells and the lumenal contents of intracalated and interlobular ducts (P. Li, unpublished observations). No other types of cells in the lacrimal gland were stained by mAbs to sialomucin complex. Western blot analysis of lacrimal tissue lysate detected both the mucin ASGP-1 and transmembrane ASGP-2 subunits of sialomucin complex. Sequential immunoprecipitation experiments showed that the mucin is produced in both soluble and membrane-bound forms in the rat lacrimal gland. These results clearly show that sialomucin complex is present in the rat lacrimal gland and is specifically associated with the acinar cells. Since sialomucin complex is also present in the ocular tear film, the rat lacrimal gland represents a second source of this mucin for the tear film, in addition to the corneal and conjunctival epithelia.

8. PERSPECTIVE

SMC/MUC4 is a unique gene product containing subunits proposed to be involved in two of the most important aspects of tumor progression: cell recognition and cell signaling through growth factor receptors. The anti-adhesive behavior in cells overexpressing SMC is a consequence of steric effects of the mucin related to its size (number of mucin repeats) (18). Interestingly, human MUC4 is considerably larger than SMC (rat) (6). Human MUC4 has a transcript whose size ranges (due to polymorphism) from 18-27 kb, compared to 9 kb for the rat Muc4. Thus, human MUC4 may be an even more potent anti-recognition molecule than rat SMC. The observation that SMC/MUC4 binds to ErbB2 in co-expressing cells has potential implications for the use of therapeutic antibodies, such as Herceptin, which target ErbB2. Their action could be blocked by overexpressed MUC4. The transmembrane subunit of SMC, through its EGF domain, has been shown to potentiate phosphorylation of ErbB2, the key receptor in heterodimer complex formation and signaling through class I (EGF receptor family) receptors (64, 65), and ErbB3, which has been implicated in phosphoinositol 3-kinase activation (65) and other pathways. We envision that this potentiation activates multiple signaling pathways in tumor cells and can contribute to tumor progression via autocrine growth responses, including repression of apoptosis. Undoubtedly, MUC4 contributes to the normal functions of the numerous epithelia in which it is found, though this subject has barely been broached, particularly in human tissues. It is clear from our studies that SMC is ideally situated to perform protective functions in epithelia, e.g. in the airway, uterus and eye. Its appearance at critical times in epithelial differentiation in different organs suggests a role in developmental processes, which has not been explored. Moreover, its unique dual anti-recognition and signaling functionalities suggest a potential role in the

maintenance of epithelia or in the repair of epithelial damage in the vulnerable epithelia in which it is found.

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