

CHARACTERIZATION OF PEPTIDES AND PHAGE THAT  
BIND GALECTIN-3 SELECTED FROM BACTERIOPHAGE  
DISPLAY LIBRARIES: A STUDY OF THE ROLE OF  
GALECTIN-3 IN METASTASIS-ASSOCIATED CANCER  
CELL ADHESION

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A Dissertation

presented to

The Faculty of the Graduate School

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Jun Zou

Dr. Susan Deutscher   Dissertation Supervisor

DECEMBER 2005

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

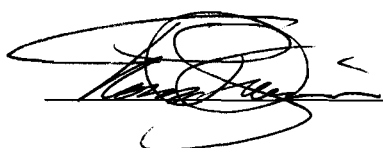
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Presented by Jun Zou

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.

  
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**CHARACTERIZATION OF PEPTIDES AND PHAGE THAT BIND  
GALECTIN-3 SELECTED FROM BACTERIOPHAGE DISPLAY  
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Dr. Susan Deutscher, Dissertation Supervisor

**ABSTRACT**

Intravascular cancer cell adhesion plays a significant role in the metastatic process. Studies indicate that galectin-3, a member of the galectin family of soluble animal lectins, is involved in carbohydrate-mediated metastatic cell heterotypic (between carcinoma cells and endothelium) and homotypic (between carcinoma cells) adhesion via interactions with the tumor-specific Thomsen-Friedenreich glycoantigen (TFAg). The adhesion may also be attributed to galectin-3 multivalent binding of carbohydrate ligands via formation of di/oligomeric galectin-3. We hypothesized that the specific molecular inhibition of the interaction between galectin-3 and its ligands would reduce metastasis-associated carcinoma cell adhesion. To test this hypothesis, we identified peptide antagonists of galectin-3 using combinatorial bacteriophage (phage) display technology. The peptides bound with high affinity to purified galectin-3 protein ( $K_d \cong 17\sim 80$  nM) and to cell surface galectin-3. Experiments with a series of recombinant serially truncated galectin-3 mutants indicated that the peptides bound the CRD of galectin-3. Furthermore, the peptides did not bind the CRD of other galectins and plant lectins. Synthetic galectin-

3 CRD-specific peptides blocked the interaction between galectin-3 and TFAg and significantly inhibited rolling and stable heterotypic adhesion of human MDA-MB-435 breast carcinoma cells to endothelial cells under flow conditions, as well as homotypic tumor cell aggregation. Moreover, our biochemical studies demonstrated that one of our peptides, G3-C12, inhibited the dimerization of galectin-3 and, thus, blocked aggregation of latex bead cross-linked by dimeric or oligomeric galectin-3, indicating that dimerization of galectin-3 also plays a crucial role in cancer cell adhesion. These results demonstrate that carbohydrate-mediated metastasis-associated tumor cell adhesion could be inhibited efficiently with short synthetic peptides, which do not mimic naturally occurring glycoepitopes, yet bind to the galectin-3 CRD with high affinity and specificity.

In addition to characterization of the galectin-3 binding peptide, we also investigated the possibility that galectin-3 specific phage would target to tumors highly expressing the protein in mice. The phage harboring galectin-3 binding peptide failed to exclusively target to human MDA-MB-435 breast tumor heterotransplanted in mice, perhaps due to the wide-spread distribution of the protein, especially in the reticuloendothelial system (RES) organ tissue. However, the pharmacokinetic properties of random phage peptide display fd-tet libraries, *in vivo*, were fully investigated in order to gain a better understanding of the *in vivo* behavior of phage (Chapter 3). This part of my study will be extremely useful in the design of *in vivo* application strategies, as more and more *in vivo* studies with phage display libraries have been conducted to isolate tumor-avid molecules with a priori optimum *in vivo* properties.

# CHAPTER 1

## INTRODUCTION

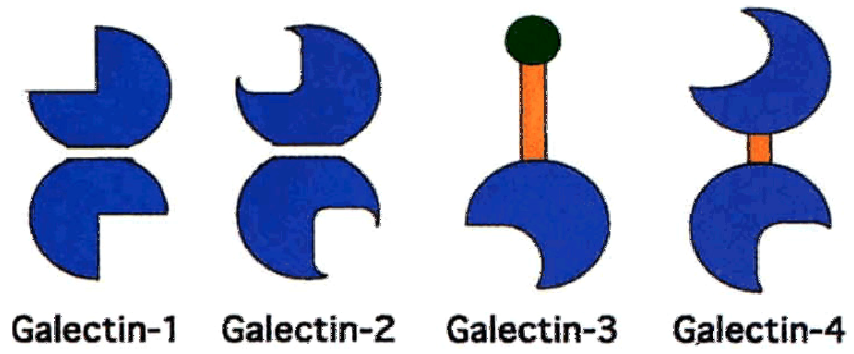
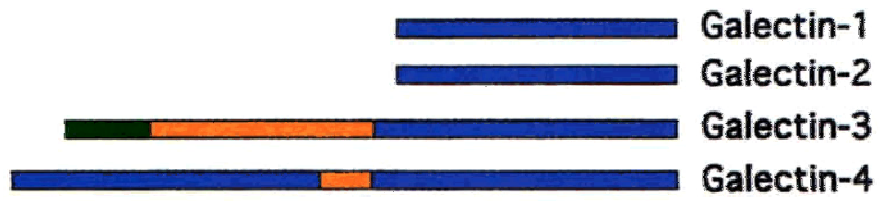
### *Galectin-3 Has Multiple Biological Functions*

#### **Galectin family**

Galectins are a family of animal lectins. Members of this family are defined by two properties: 1) they contain a highly conserved carbohydrate binding domain (Fig. 1, 2) and 2) they possess an affinity for  $\beta$ -galactosides. Broad distribution of homologous carbohydrate-binding proteins suggests they must play important biological roles. In the last few years considerable experimental evidence have been accumulated concerning their participation in different biological processes, such as cell adhesion, cell growth, inflammation, differentiation, apoptosis and metastasis.

The galectin family is growing: to date, fourteen members of this family have been identified (Kasai and Hirabayashi 1996; Perillo, Marcus et al. 1998; Cooper and Barondes 1999). The most recent galectins, such as galectin-5 and galectin-8, were identified as a result of immunoscreening cDNA libraries (Gitt, Wiser et al. 1995) (Hadari, Paz et al. 1995) or, as with galectin-9, by using degenerate oligonucleotide-based PCR cloning and homology searching (Wada and Kanwar 1997). In relation to their molecular architecture, galectins are classified into proto-, chimera-, and tandem-repeat types (Fig.2). These three types function differently due to their distinct structures. Proto-type galectins are non-covalent homodimers composed of two identical CRDs,

**Fig. 1. Schematic of the overall structures of galectin-1, -2, -3, and -4.** The proteins are shown schematically as linear diagrams corresponding to single peptide chains (*top*) and as assembled proteins (*bottom*). The carbohydrate-binding domains of about 130 amino acid residues are *blue*, the proline-, glycine-, and tyrosine-rich repetitive linker domain of galectin-3 (about 100 residues) and linker peptide of galectin-4 (about 30 residues) are *orange*, and the N-terminal leader domain of galectin-3 (about 30 residues) is *green*. (Barondes et Al., 1994 J Biol Chem)



**Fig. 2. Sequence of the carbohydrate-binding domain of galectin-1, -2, -3, and -4.**

The residues indicated with dots above the sequences directly interact with the carbohydrate (*light blue*) or contribute to the dimer interface (*purple*). Residues that are identical in all five of the carbohydrate-binding domains shown (and most other galectins) are indicated by *orange*. The sequences are for human galectin-1, galectin-2, galectin-3, and rat galectin-4 carbohydrate-binding domains I and II. (Barondes et Al., 1994 J Biol Chem)

Galectin-1 1 MACGLVASNLNLKPGECRLRVRGEVAPDAKS 30  
 Galectin-2 1 MTGELEVKNMDMKPGSTLKITGSIADGTDG 30  
 Galectin-3 115 IVPYNLPLPGGVVPRMLITILGTVKPNANR 144  
 Galectin-4-dom-I 16 TLPYKRPIPGGLSVGMSIYIQGIKADNMRR 45  
 Galectin-4-dom-II 193 PVPYVGTLQGGLTARRTIIIKGYVLPTAKN 222

Galectin-1 FVILNLG-----KDSNNLCLHFNPRFNAHGDAANTIVCN SKDGGAWGTEQREAVF -PFQPGSVAE  
 Galectin-2 FVINLG-----QGTDKLNLHFNPRF---SE-STIVCN SLDGSNWGQE QREDHL-CFSPGSEVK  
 Galectin-3 IALDF-QRGN-----D-VAFHFNPRFNE-NRRVIVCNTKLDNNWGREERQSVF-PFESGKPFK  
 Galectin-4-dom-I FHVNF-AVGQDEGAD-IAFHFNPRFDGW-D--KVVFNMQSGQWGKEEKKKS-MPFQKGHHFE  
 Galectin-4-dom-II LIINF-KVGS-TG-D-IAFHMNPRI-G--D--CVVRNSYMN GSWGSEERKIPYNPFGAGQFFD

Galectin-1 88 VCITFDQADLTIKLPDGYEFKFPNRLNLEAINYMAADGDFKIKCVAFE 135  
 Galectin-2 84 FVTTFESDKFKVKLPDGHELTFPNRLGHSHLSYLSVRGGFNMSFFKLKE 132  
 Galectin-3 200 IQVLVEPDHFKVAVND AHLQYNHRVKKNLEISKLGISGDIDLTSASYTMI 250  
 Galectin-4-dom-I 103 LVFMVMSEHYKVVVNGTFFEYEGHRL-PLQMVTHLQVDGDLEL--QSINFL 150  
 Galectin-4-dom-II 277 LSIRCCTDRFKVVFANGQHLDFSHRFQAFQRVDMLEIKGDITL---SYVQI 324

which consequently will cross link two glycoconjugates of a very similar nature. The best studied among them, galectin-1, can exert its biological function by cross-linking more than one sugar residue (Perillo, Marcus et al. 1998). As the only member in the chimera-type group, galectin-3 has one sugar-binding site and another unknown region, which might interact with biomolecules other than sugars. Therefore, galectin-3 can serve as a cross-linker between glycoconjugates and other biomolecules; in other words, as an adaptor molecule between different kinds of biomolecules. The third group, tandem-repeat galectins, includes proteins with two distinct CRDs, such as galectin-4. Because the specificity and binding strength of the two binding sites are not necessarily the same, they can form bridges between glycoconjugates of different types.

### **Secretion and expression of galectins**

Conservation of galectins throughout animal evolution strongly suggests they serve critical biological functions requiring carbohydrate recognition. However, null mutant mice for galectin-1 and -3 genes presented no major phenotype abnormalities, indicating that they are not crucial for life (Colnot, Fowles et al. 1998). It was proposed that other members of this family could potentially compensate for the absence of these proteins.

Galectins appear to play biological roles inside the cell, as they have acetylated N-termini, cysteine residues entirely in their free states (Hirabayashi and Kasai 1988), and no secretion signals; and are synthesized on free ribosomes (Wilson, Firth et al. 1989). However, most functions assigned to  $\beta$ -galactoside-binding proteins are restricted to the extracellular milieu or cell surface; few glycoproteins from cell extracts bind to galectins. One theory of this apparent discrepancy is that galectins are secreted with help from some



specific transmembrane carrier, like the secretion of some bacterial toxins. Another theory is that the plasma membrane evaginates and incorporates the galectins into vesicles that will be subsequently released (Hughes 1999).

The expression patterns of galectins vary among different adult tissues, change during development, and are dramatically altered upon neoplastic transformation or in other pathological conditions. Galectin-1 is found in many tissues in both normal and pathological conditions (Perillo, Marcus et al. 1998). Increased expression of galectin-1 has been observed to correlate with poorly differentiated and metastatic phenotypes (Skrincosky, Allen et al. 1993). It has also been suggested that the level of galectin-1 expression could be a diagnostic implication in malignant thyroid tumors (Xu, el-Naggar et al. 1995). Like galectin-1, galectin-3 is expressed in many tumors. Moreover, elevated galectin-3 expression significantly enhances tumor cell adhesion to common extracellular matrix (Calof, Campanero et al.) proteins, and has been shown to increase the incidence of lung metastases of fibrosarcoma cells (Raz, Zhu et al. 1990). Blocking galectin-3 expression in highly malignant human breast carcinoma MDA-MB-435 cells significantly reverses the tumorigenic phenotype and suppress tumor growth in nude mice (Honjo, Nangia-Makker et al. 2001).

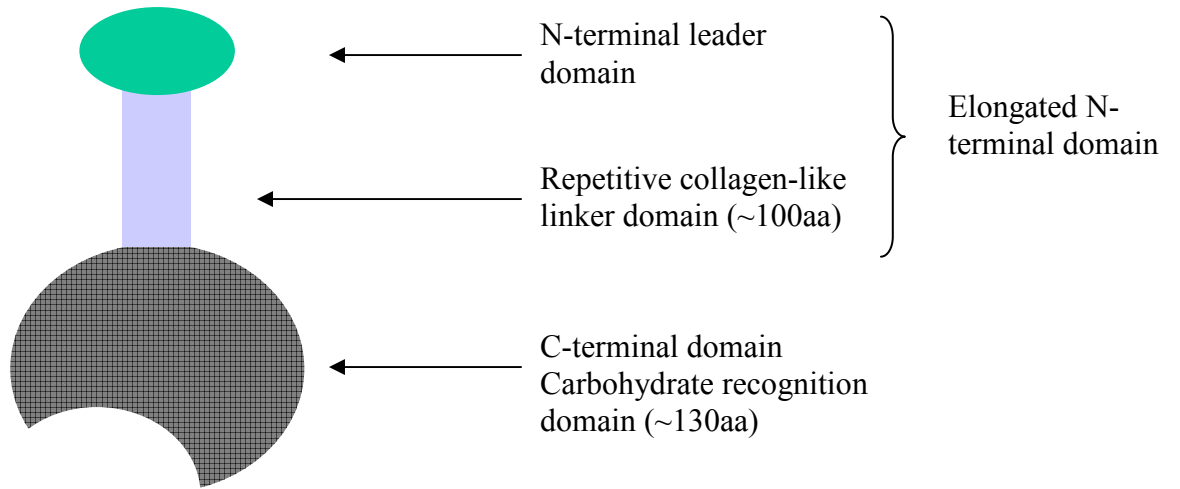
### **Subcellular distribution, structure and ligands of galectin-3**

Although there are conflicting reports regarding the expression pattern of galectin-3 in colon cancer, all studies using immunohistochemistry agree that galectin-3 is translocated from the nucleus to cytoplasm with colon cancer progression (Itzkowitz 1997). The cellular distribution of galectin-3 appears to be controlled by the N-terminal

leader motif of 12 amino acids of the galectin-3 protein, as deletion of the motif resulted in abolition of secretion and loss of nuclear localization.

Galectin-3, a chimeric gene product, consists of three distinct structural domains: a short N-terminal leader domain of 12 amino acids that controls its cellular compartmentalization; a repetitive collagen-like sequence rich in glycine, tyrosine, and proline, which serves as a substrate of matrix metalloproteinases (Brooks, Stromblad et al.) (Ochieng, Green et al. 1998); and, a globular C-terminal domain (Barboni, Bawumia et al.) that has a carbohydrate-binding site (Fig. 3). Like the CRD of other galectins, the galectin-3 CRD consists of approximately 125 amino acid residues arranged typically in a 12  $\beta$ -strand fold. Uniquely, galectin-3 has an extra long N-terminal domain consisting of a N-terminal leader motif and a linker domain with approximately 100-150 amino acids. Biophysical studies have shown that the N-terminal domain of galectin-3 is flexible with little secondary structure (Hsu, Zuberi et al. 1992). As mentioned before, galectin-3 cDNA with an 11 amino acid deletion in the N-terminus has been transfected into galectin-3 null-expression BT-549 human breast cancer cells. The truncated galectin-3 is no longer secreted and no nuclear deposition could be detected. None of the mice injected with cells expressing deletion mutants, or with parental BT-549 cells that have no galectin-3 expression, developed tumors within 4 weeks. In contrast, all of the mice injected with wild-type galectin-3 transfected BT-549 cells develop tumors (Gong, Honjo et al. 1999). Site-directed mutant studies in this group also showed that phosphorylation at the N-terminal Ser<sub>6</sub>, by casein kinase, dramatically reduced the binding of galectin-3 with its ligands and “may serve as an on/off switch for its sugar binding capabilities”

**Fig. 3. Schematic of the overall structures of galectin-3.** (Modified from overall structure figure of galectin-3 proposed by Barondes et. Al., 1994 J Biol Chem)



(Mazurek, Conklin et al. 2000). However, this phosphorylation seems to have no impact on the galectin-3 cellular distribution of the protein.

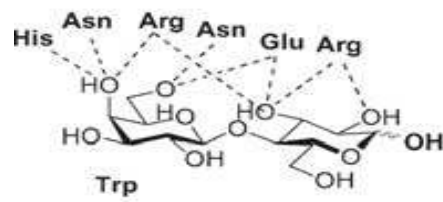
Exclusive of the N-terminal leader motif of galectin-3, the linker part also participates in its biological functions. The proline, glycine and tyrosine-rich domain consists of repeats of 7-10 amino acid with a consensus sequence of PGAYPG<sub>1-4</sub> (Bonifacino and Traub). The number of repeats varies with species; therefore the galectin-3 is a different size in different species (Barondes, Cooper et al. 1994). Interestingly, it has been demonstrated that recombinant galectin-3 is a novel substrate for metalloproteinases, particularly MMP-2 and MMP-9. These enzymes cleave the galectin-3 at Ala<sub>62</sub>-Tyr<sub>63</sub>, to create a 22kDa fragment with the intact CRD and a 9 kDa polypeptide comprising the amino terminal end of galectin-3. The 22kDa fragment of galectin-3 with the CRD showed a higher affinity for the carbohydrate ligands, but the self-association depending on the N domain had been abrogated (Ochieng, Green et al. 1998). The up-regulation of the expression of MMPs in malignant tumors has been suggested to facilitate tumor cell invasion, because of their unique ability to degrade all components of the extracellular matrix (Calof, Campanero et al.) (Coussens, Fingleton et al. 2002). Moreover, the 22kDa fragment of galectin-3 might still access or bind to carbohydrate ligands on the cell surface with higher affinity, and hence regulate the adhesive interaction between cells or between cells and extracellular matrix proteins.

Globular in shape, the galectin-3 CRD is typically arranged in 5-stranded (F1-F5) and 6-stranded (S1-S6a/6b)  $\beta$ -sheets with conserved carbohydrate ligand binding sites located in a shallow groove over S4-S5 (Seetharaman, Kanigsberg et al. 1998; Umemoto, Leffler et al. 2003). The two sheets are slightly bent with a convex side formed by F1-F5

strands and a concave side formed by S1-S6, in which the carbohydrate ligand is bound. Schematically, four binding subsites (A-D) (Fig. 4) are considered as the major binding sites. The most conserved feature of galectin binding activities is the binding of a galactose in the core binding sites C and B, which consists of His-158, Asn-160, Arg-162, and Asn-170 in galectin-3 (Fig. 5) (Seetharaman, Kanigsberg et al. 1998). The ability to accommodate carbohydrate at subsite B is the major factor for variation in the carbohydrate-binding specificity between galectins, this would have an effect on the linking to the 3-OH of the terminal galactose in subsite C, and therefore dramatically increase or decrease the sugar binding affinity (Leffler, Carlsson et al. 2004).

Like other galectins, galectin-3 is supposed to function through binding to its ligands, which are also called counter-receptors. As mentioned before, few intracellular counter-receptors with  $\beta$ -galactosides have been identified. The linker domain of galectin-3 has been shown to bind to ssDNA and RNA. This binding is not inhibited by lactose, indicating that galectin-3 may act as a RNA-binding protein in the nuclear matrix in a non-carbohydrate- dependent manner (Wang, Inohara et al. 1995). Galectin-3 is also reported to be required in pre-mRNA splicing, although the nuclear component that interacts with galectin-3 has not yet been identified (Dagher, Wang et al. 1995). The authors suggested that three classes of interactions are formally possible: galectin-3 CRD binding to nuclear glycoproteins, CRD interacting with non-glycosylated nuclear polypeptides, and protein-protein interactions via the N-terminal domain of galectin-3. Bcl-2 protein, a key regulator of apoptosis, is another intracellular ligand that has been reported to bind to galectin-3. Interestingly, although Bcl-2 is not a glycoprotein, this interaction is inhibited by carbohydrate, suggesting the CRD is involved in the binding

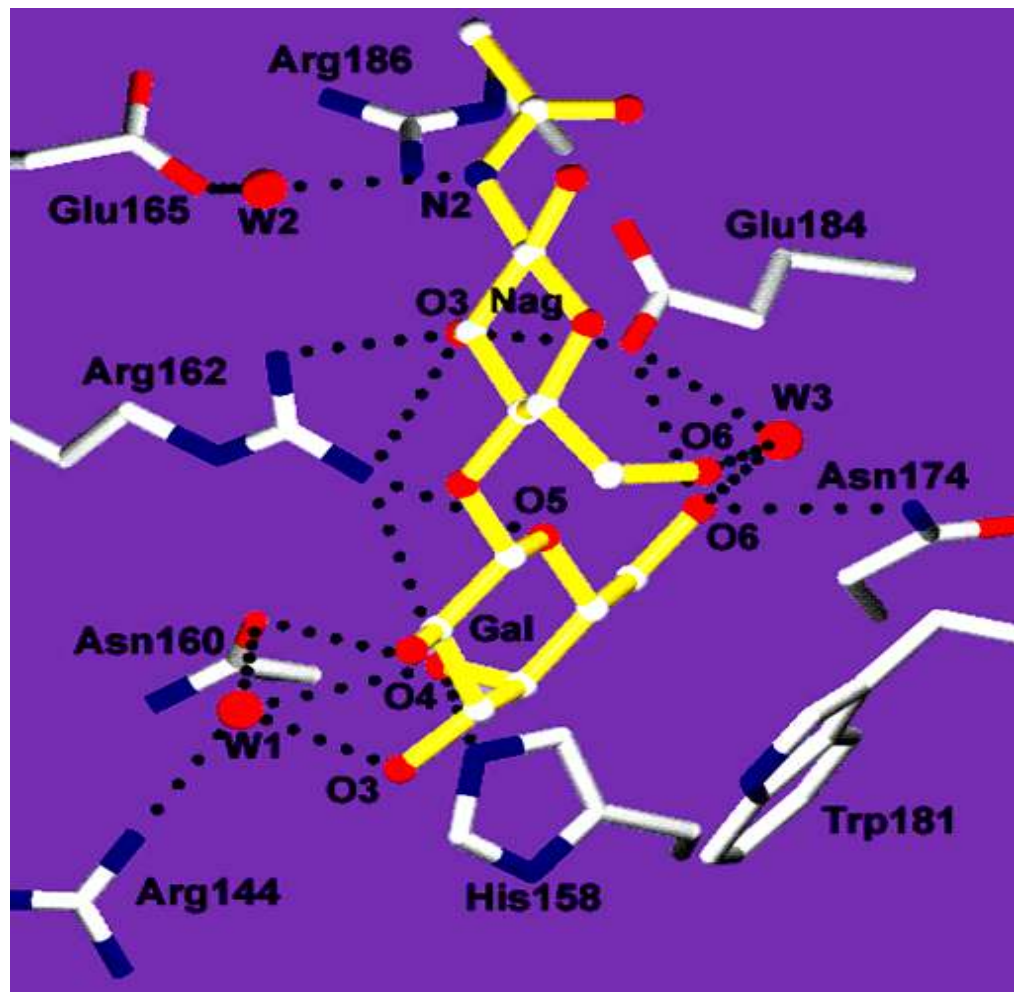
**Fig. 4. The galectin carbohydrate-binding site.** The galectin CRD is shown with the four established subsites indicated as A, B, C and D and a more loosely defined proposed site as E. The core binding site C and D is boxed and a close up view with bound lactose (Gal $\beta$ 1-4Glc) and named interacting amino acid side chains is shown in the middle. (Leffer et al., 2004 Glycoconj J.)



Gal $\beta$ 1-4Glc



**Fig. 5. The human galectin-3 carbohydrate binding site.** Residues interacting with the bound LacNAc moiety through direct and water mediated hydrogen bonds or through van der Waals contacts are shown. The bound LacNAc moiety is shown with yellow bonds. Oxygen and nitrogen atoms are colored *red* and *blue*, respectively. Water molecules are labeled *W1–W3*. Potential hydrogen bonds are shown as *dotted lines*. (Seetharaman et al., 1998 J Biol Chem)



(Yang, Hsu et al. 1996). While only a few ligands have been found inside the cell, there have been numerous extracellular ligands found, including glycosylated ECM components and glycoconjugates on the cell surface. The interactions between galectin-3 and these glycoconjugates play multiple roles in different biological processes, such as cell adhesion, cell growth, inflammation, differentiation, apoptosis and metastasis.

### **Galectin-3 in cell adhesion**

Among the functions of galectin-3, the action related to tumor procession is perhaps best studied. Although the exact role of galectin-3 in tumor progression is still debatable, galectin-3 expression has been associated with transforming and metastatic potentials (Konstantinov, Robbins et al. 1996). Ectopic expression of galectin-3 in the human breast cancer cell line BT549 resulted in the acquisition of a potent tumorigenic phenotype in nude mice (Akahani, Nangia-Makker et al. 1997). Further evidence was obtained when galectin-3 was introduced into weakly metastatic UV-2237-cl-15 fibrosarcoma cells, which resulted in these cells becoming highly metastatic (Raz, Zhu et al. 1990). The binding of galectin-3 to ECM glycoproteins such as laminin (Zhou and Cummings 1993) and fibronectin (Ozeki, Matsui et al. 1995) suggests that galectin-3 is involved in tumor cell adhesion.

Matarrese et.al, transfected the human breast cancer cell line Evsa-T with galectin-3 cDNA and picked up several clones that stably expressed different levels of galectin-3. Three of them were used to examine the possible role of galectin-3 in tumor cell adhesive properties. The number of cells attaching to the laminin, fibronectin and vitronectin were positively correlated to the expression level of galectin-3 (Matarrese, Fusco et al. 2000). However, there are conflicting reports on the role of galectin-3 in cell

adhesion. For example, addition of purified galectin-3 greatly reduced the adhesion of several cell lines to laminin, fibronectin and collagen IV (Ochieng, Leite-Browning et al. 1998). Thus, it appears that galectin-3 may have opposite effects on cell adhesion, depending on the cell types and other circumstances.

In addition to traditional ECM glycoproteins, the list of galectin-3 ligands is growing. Human colon cancer mucin, which is typically rich in polylectosamine chains, has been found to bind to galectin-3. Interestingly, mucin from a highly metastatic colon cancer cell line bound more galectin-3 than mucin from a less metastatic cell line (Bresalier, Byrd et al. 1996). Our previous studies showed that Thomsen-Friedenreich antigen (TFAg), a simple mucin-type disaccharide, mediates cancer cell adhesion by specifically interacting with galectin-3 (Glinsky, Glinsky et al. 2001). This TFAg mediated, tumor-endothelial cell interaction could be efficiently disrupted using synthetic compounds mimicking or masking this carbohydrate structure.

Among the ligands of galectin-3, the importance of integrins as regulators of a series of signaling events is often under-appreciated. Integrins not only integrate the extracellular matrix with the intracellular cytoskeleton to mediate cell migration and adhesion, but they also start integrin signaling to regulate cell cycle and growth (van der Flier and Sonnenberg 2001). The studies by Matarrese and his colleagues showed that breast cancer cells over-expressing galectin-3 exhibited enhanced adhesion to vitronectin via increased expression of specific integrin  $\alpha 4\beta 7$ ; also, the cells were characterized by a remodeling of cytoskeletal elements associated with cell spreading (Matarrese, Fusco et al. 2000). Galectin-3 has been demonstrated to bind to  $\alpha 1\beta 1$  through the CRD, as this interaction was inhibited by lactose (Ochieng, Leite-Browning et al. 1998). Galectin-3

transfected BT549 cells, 11-9-1-4, demonstrated a higher surface expression of  $\alpha 6\beta 1$ , compared to its galectin-3 null expression parental BT-549 cells. A significant up-regulation of  $\alpha v\beta 3$  has been observed on endothelial cells treated with exogenous galectin-3 (Nangia-Makker, Honjo et al. 2000). Integrins also control the cell cycle and death by activating signaling pathways, especially anoikis — a type of apoptosis attributable to detachment from the cell-matrix (Giancotti and Ruoslahti 1999). Galectin-3 plays an important role in regulation of the cell cycle and apoptosis, as well (Matarrese, Fusco et al. 2000), although the mechanism of its anti-apoptosis function is largely unknown.

### **Galectin-3 and apoptosis**

Galectin-3 may also enhance the metastatic potential in some cancers by protecting the tumor cell from apoptosis or anoikis, which is critical for anchorage-independent cell survival in the circulation during dissemination of tumor cells (Yoshii, Fukumori et al. 2002). The interaction observed between galectin-3 and a key apoptosis regulator, Bcl-2, is likely the reason for the galectin-3 anti-apoptosis effects (Yang, Hsu et al. 1996). Although galectin-3 does not belong to the Bcl-2 family, it contains the same four amino acid motif, NWGR, conserved in the Bcl-2 family. This NWGR motif is responsible for Bcl-2 anti-apoptosis function (Yin, Oltvai et al. 1994), as a substitution of the glycine in the motif abrogated the anti-apoptosis activity of Bcl-2 and its binding activity to another apoptosis regulator, Bax (Hengartner, Ellis et al. 1992; Yin, Oltvai et al. 1994). Similar to Bcl-2, substitution of the Gly<sup>182</sup> residue with Ala in the NWGR motif of galectin-3 also lead to a complete loss of its anti-apoptosis effect (Akhani,

Nangia-Makker et al. 1997), indicating that galectin-3 might interact with apoptosis-regulating molecules through the NWGR motif and replace Bcl-2 function.

Raz's group demonstrated that expression of galectin-3 leads to breast cancer cell line BT549's ability to respond to the loss of cell-ECM contact by growth arrest at G1 phase without detectable apoptotic cell death (Kim, Lin et al. 1999). Galectin-3 down-regulated expression of late G1/S phase cyclins and inhibited the cyclin dependent kinases (CDKs), which govern G1 to S progression of the cells. As mentioned before, the galectin-3 mutant, in which Gly182 of the NWGR motif was substituted with Ala, failed to modulate the expression of the cyclins and CDKs, reemphasizing that the NWGR motif is required for anti-apoptotic activity, as well as the ability to modulate cell cycle regulators. Two N-terminal domain mutation of galectin-3 constructed by this group, Ser6 to Ala and Ser6 to Glu, led to galectin-3 no longer being recognized as a substrate of casein kinase I. The non-phosphorylated galectin-3 mutants failed to protect cells from anoikis with G1 arrest (Yoshii, Fukumori et al. 2002), suggesting that both the N-terminal domain and the CRD are involved in the anti-apoptotic activity of galectin-3. It is possible that the two domains play different roles in this complex process, as the N-terminus controls the cellular compartmentalization and the CRD interacts with other intracellular apoptosis regulators. Inhibiting or competing with the interaction between galectin-3 and its intracellular ligands could possibly suppress galectin-3 anti-apoptotic activity.

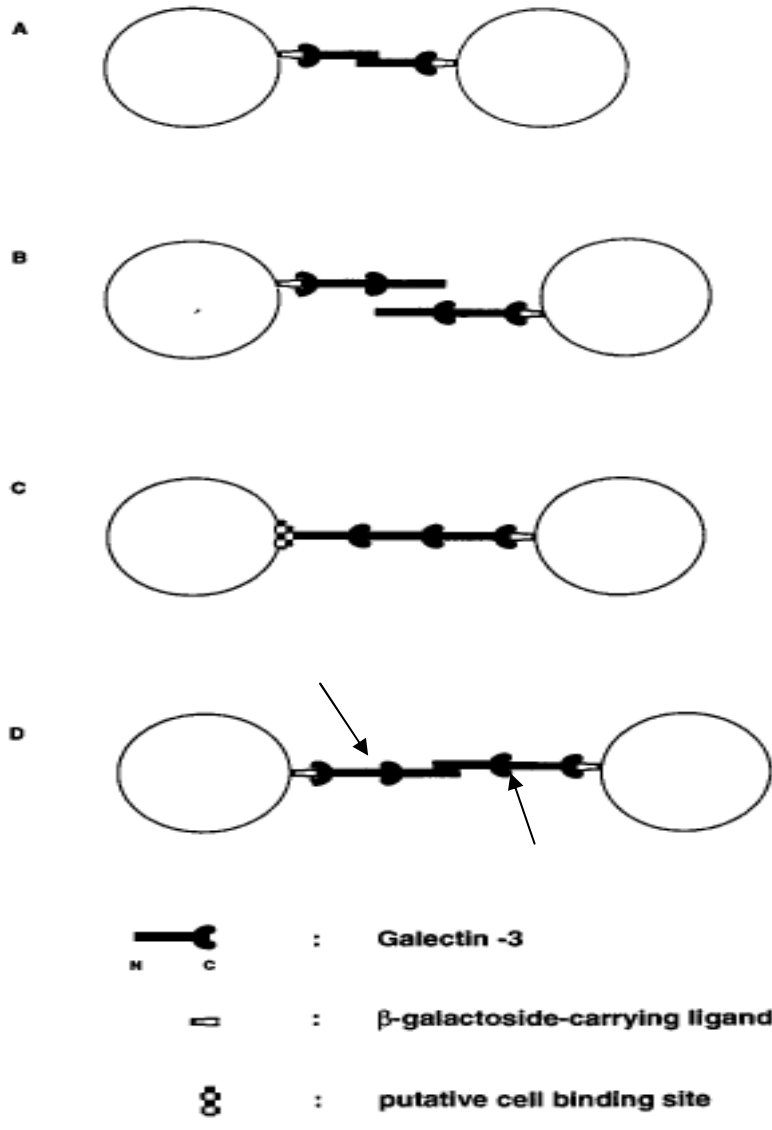
### **Self-association of galectin-3**

As described before, galectin-3 has only one CRD and an extra-long N-terminal domain that is flexible with little secondary structure (Hsu, Zuberi et al. 1992). Many of

galectin-3's biological activities have been attributed to the protein's multivalent binding of carbohydrate moieties (Frigeri, Zuberi et al. 1993; Yamaoka, Kuwabara et al. 1995). Since galectin-3 possesses a single carbohydrate-binding site, the observed cross-linking behavior may result from the formation of dimers or higher order multimers. Interestingly, galectin-3 is the substrate for human matrix metalloproteases 2 and 9 (Ochieng, Fridman et al. 1994). It has been suggested that the resulting cleaved C-terminus of galectin-3 serves an important functional role in that it exists under physiological conditions and binds to glycoconjugates with higher affinity than full-length galectin-3 (Ochieng, Green et al. 1998). However, the hemagglutination activity of galectin-3 was abolished by deleting the N-terminal domain, suggesting that self-association occurs via the N-terminal domain (Ochieng, Green et al. 1998). The carbohydrate-regulated positive cooperative binding of galectin-3 to IgE (Hsu, Zuberi et al. 1992) and laminin (Massa, Cooper et al. 1993) was only detectable with full-length galectin-3, not the CRD, suggesting again that the N-terminal domain is critical for full length galectin-3 to recruit free soluble galectin-3 and therefore form di/oligomers. On the other hand, it was demonstrated that radiolabeled galectin-3 binding to immobilized galectin-3 was inhibited by purified CRD, implying that in addition to the N-terminal domain, the CRD is involved in the self-association. However, it has been demonstrated that radiolabeled CRD did not bind to immobilized CRD (Kuklinski and Probstmeier 1998). Therefore, galectin-3 dimerization through intermolecular interactions appears to involve both the N- and C-terminal domains, and an N-C interaction could be a means by which galectin-3 binds to an adjacent galectin-3 (Fig. 6) (Kuklinski and Probstmeier 1998). Although dimeric galectin-3 appears to be unstable, it was determined that

**Fig. 6. A hypothetical model of the implication of the homophilic interaction of galectin-3 on cell-to-cell adhesion.** A: A galectin-3 dimer bridges two neighboring cell surfaces via noncovalent association of the N domains. B: N—C domain interactions alone do not lead to cell bridging unless a cell binding site other than the lectin binding site (C) exists. D: N—C domain interactions (arrows) in addition to N—N domain interactions may allow a greater flexibility and variability of lectin-bridged cellular complexes. (Kuklinski et al., 1998 J Neurochem)





approximately 45% of galectin-3 is in a dimeric form by using a chemical cross-linker (Yang, Hill et al. 1998). The di/oligomerization of galectin-3 may play a key role in the cross-linking of surface glycoproteins initiating several signal transduction cascades (Liu, Hsu et al. 1995; Yamaoka, Kuwabara et al. 1995) and promoting cell adhesion (Lotan, Belloni et al. 1994; Kuwabara and Liu 1996). Similarly, a study on P-selectin, a calcium-dependent lectin, revealed that dimerization of selectin and its ligand PSGL-1 (P-selectin glycoprotein ligand-1) stabilize cell rolling and enhance tether strength in shear flow, perhaps by establishing a bonding cluster. The authors also measured the dissociation kinetics and mechanical strength of transient tethers formed through the interaction of monomeric P-selectin and PSGL-1 or dimeric P-selectin and PSGL-1; the latter were more shear resistant and exhibited less fluctuation in rolling velocities (Ramachandran, Yago et al. 2001). A recent study from Sato and his colleagues showed that the direct cross-linking of neutrophils to the endothelium is partially dependent on galectin-3 di/oligomerization (Sato, Sexton et al. 2002).

Although the mechanism of di/oligomerization of galectin-3 remains unclear, inhibition of dimerization of galectin-3 appears to be an attractive way to modulate galectin-3 function. For example, self-association of galectin-3 is involved in the metastatic process as migration of human breast carcinoma cells through a laminin-rich layer could be mediated by cross-linking of carbohydrate ligands between the cell surface and extracellular matrix through a multivalent galectin-3 complex (Le Marer and Hughes 1996), inhibition of the formation of the galectin-3 di/oligomer could downregulate the metastasis potential of tumor cells. Moreover, galectin-3 perhaps interacts with Bcl-2 and forms heterodimers to participate in apoptotic events. Efficiently blocking the formation

of heterodimers may abrogate the galectin-3 anti-apoptosis activity. Taken together, a small molecule that inhibits dimerization of galectin-3 could play an important regulatory role in mediating the biological activity of galectin-3.

### ***Understanding the Role of Galectin-3 in Metastasis Using Galectin-3 Binding Peptides Isolated from a Phage Display Library***

#### **Phage display for discovery of tumor-targeting peptides**

Development of agents that specifically bind to tumor cells and their metastases for improved cancer detection and therapy is a modern day challenge. One of the most powerful strategies for novel anti-cancer agent discovery is the application of combinatorial chemistry and the creation of chemical or biological libraries (Cwirla, Peters et al. 1990; Salmon, Liu-Stevens et al. 1996; Ferrieres, Villard et al. 2000; Rademann and Jung 2000; Fernandes, Cohen et al. 2001; Walling, Peters et al. 2001; Smothers, Henikoff et al. 2002). In their simplest terms, chemical or biological libraries are collections of molecules that can be either chemically (Houghten, Pinilla et al. 1991; Kick and Ellman 1995; Salmon, Liu-Stevens et al. 1996; Villar and Koehler 2000; Walling, Peters et al. 2001; Rubio-Godoy, Ayyoub et al. 2002; Pinilla, Appel et al. 2003) or biologically (genetically) (Cwirla, Peters et al. 1990; Smothers, Henikoff et al. 2002) synthesized and screened for a desired function, specificity, stability or affinity. Chemical synthetic combinatorial libraries (SCL) consisting of millions of peptides have the advantage over genetically encoded libraries in that one can introduce modified, non-natural amino acids at desired positions, which can increase stability or desirable pharmacologic properties *in vivo* (Villar and Koehler 2000; Walling, Peters et al. 2001).

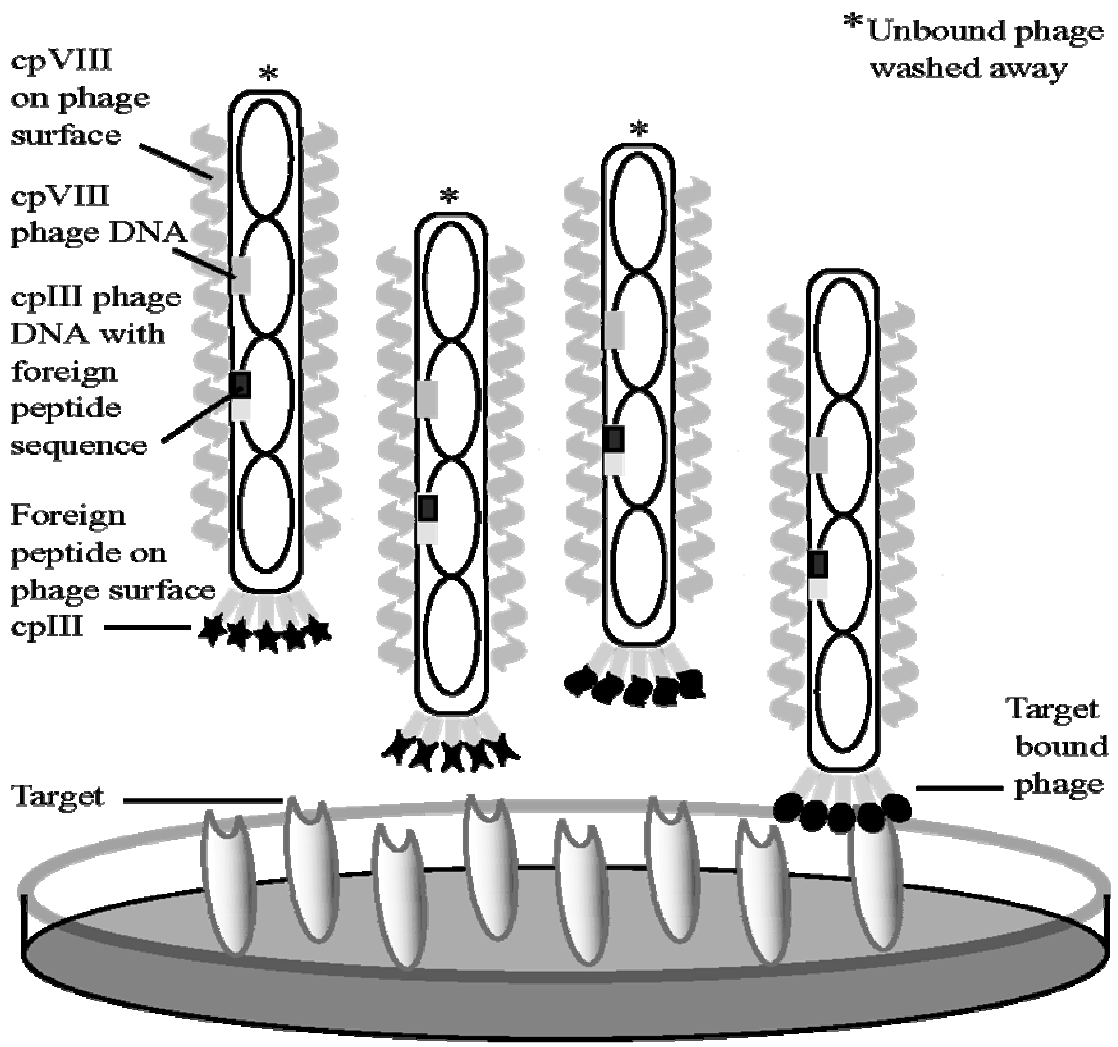
SCL have serious limitations, however, because given their synthetic nature, members in the library cannot be easily amplified and it is difficult to make complex libraries. For the purpose of basic cancer research and drug discovery, there are potential advantages in the use of genetically encoded combinatorial libraries (Smith 1985; Smith and Petrenko 1997; Brown, Modzelewski et al. 2000; Aina, Sroka et al. 2002; Yip and Ward 2002). These libraries can be displayed on phage (Smith 1985; Smith and Petrenko 1997), plasmids (Hertweck 2000; Naumann, Goryshin et al. 2002), or ribosomes (Bray, Bisland et al. 2001; James 2001; Johnston, Head et al. 2003). The genetic encoding of a library allows the re-synthesis and re-screening of molecules with a desired binding activity. The resulting amplification of interacting molecules in subsequent rounds of “affinity selection” can yield uncommon, specific binders from a vast collection of molecules. Genetically encoded libraries are somewhat limited in that they are composed of naturally occurring polymers; however, the power of selection coupled with amplification makes these libraries well-suited for the task of new target, as well as new ligand, discovery.

Of the combinatorial genetic approaches employed to date, phage display is the most widely utilized. George P. Smith at the University of Missouri initiated the field of phage display in 1985 (Smith 1985). A phage-display “library” is a mixture of phage with foreign peptides on their surface and the coding sequences for the peptides in the viral DNA. Typically, foreign peptides are incorporated into the N-terminus of minor coat protein III (cpIII) of fd phage so that at most five copies of the peptide are displayed (Smith and Petrenko 1997). Phage fd are long filamentous viruses that infect *E. coli* harboring the F-plasmid (Pratt, Tzagoloff et al. 1969). Foreign peptides have also been fused to the major coat protein VIII (cpVIII) of phage so that thousands of copies of the

peptide can be displayed (Smith and Petrenko 1997; Hoess 2001; Smith 2003). Each phage clone displays a single peptide of between 6-45 amino acids, but a library as a whole may represent billions of peptides altogether. The peptide libraries may be displayed as linear or constrained sequences. Constrained libraries have two cysteine residues flanking a random length of peptide, thus facilitating the formation of a disulfide bond. The vital advantage of surface exposure is that it allows phage-display libraries with vast numbers of peptides to be easily surveyed for clones whose displayed peptides bind specifically to any given molecular target – antibody, receptor, or tumor tissue. To accomplish the survey, the library is passed over immobilized target molecules; binding clones are captured, while non-binding clones are washed away (Fig. 7). This survey is often performed in a "high-throughput" manner using 96-well plates. The captured phage retain infectivity and can therefore be propagated and cloned at will by infecting fresh bacterial host cells. The primary structure of the foreign peptide can be elucidated easily by sequencing the peptide-coding sequence in the viral DNA. Affinity selection avoids the need to assay clones one by one. As a result, the number of structures that can be surveyed is enormous; billion-clone libraries are commonplace and much larger ones could be surveyed if they were available.

The expression of foreign sequences on phage coat proteins is not limited to small peptide sequences. Antibodies, receptors and enzymes have also been displayed (Matthews and Wells 1993; Barbas, Hu et al. 1994; Smith and Petrenko 1997; Harris, Peterson et al. 1998; Atwell and Wells 1999; Dreier, Beerli et al. 2001; Ting, Witte et al. 2001; Nixon 2002). Not only antibodies, but also peptides isolated from phage display libraries will likely develop into new drugs that target specific molecules. Thousands of

**Fig. 7. A schematic representation of phage display affinity selection using phage with foreign peptides on their surface and the coding sequences for the peptides in the viral DNA.** Surface display is accomplished by fusing the peptide coding sequence to a coat-protein gene cpIII (5 copies/virion). The library is passed over immobilized target molecules; binding clones are captured, while non-binding clones are washed away. The phage on the right represents a clone that binds the target. Bound phage are eluted and amplified for subsequent selections or analyzed by DNA sequencing of the foreign peptide coding sequence. Major coat protein cpVIII (thousands of copies/virion) is shown, as this protein can also be used for peptide display. (Landon et al., 2004 Current Drug Discovery Technologies)



papers have been published using phage display to isolate peptides that bind a myriad of targets (Smith and Petrenko 1997) (reviewed in (Ladner 1999; Brown, Modzelewski et al. 2000; Nielsen and Marks 2000; Nilsson, Tarli et al. 2000; Hoess 2001; Manoutcharian, Gevorkian et al. 2001; Aina, Sroka et al. 2002; Sehgal 2002; Work, Nicklin et al. 2002; Yip and Ward 2002)). Initial application of combinatorial genetic peptide libraries was for the isolation of “peptide mimics” that would cross-react with antibody molecules (Scott and Smith 1990; Simon-Haldi, Mantei et al. 2002; Kaufman, Kirby et al. 2003) (Reviewed in (Zwick, Shen et al. 1998)). These peptide mimics of antibody ligands are being explored as the basis for vaccine development. For example, peptides that bind an anti-mucin antibody, C595, illuminated potential epitopes for a cancer vaccine (Smith, Missailidis et al. 2002). While peptides serve a valuable role in vaccine development, their ability to functionally modulate disease is also being exploited for drug development. Dyax and Genzyme completed phase II clinical trials in March of 2003 on a phage display discovered peptide DX-88 that inhibits kallikrein, a protein involved in vascular permeability (BioPortfolio 2003; Dyax 2003). This trial confirmed the therapeutic potential of DX-88 to treat angioedema.

### **Phage display-derived anti-cancer peptides**

Far fewer studies have been published on the isolation of phage display peptides that may affect tumorigenic or metastatic processes in carcinoma cells. The difficulty in developing anti-cancer drugs, in general, and peptide-based cancer homing drugs in particular may be linked to the complex nature of cancer and metastasis. *In vitro*, *in situ* and *in vivo* phage display affinity selection procedures have been employed to isolate peptides that target molecules thought to be important at numerous stages of cancer



development and/or metastasis. Cancer-associated phage display targets have included receptors, carbohydrates, enzymes, and other proteins. Some of these peptides have clear prospective cancer therapy applications. For example, phage display-derived peptides were discovered by Böttger, *et al.* (Bottger, Bottger et al. 1996), which targeted the interaction between the p53 protein and the negative regulator of p53 function, the hdm2 protein, by binding to hdm2 and preventing its interaction with p53. Because tumor suppressor mutations in p53 are among the most common genetic lesions in human cancer (Campling and el-Deiry 2003), a therapeutic molecule that could relieve suppression of p53 function might well restore the control of P53 over cellular functions and reduce the frequency of transformation (Herbert, Pearce et al. 2003).

Almost twenty years ago, Rouslahti and co-workers demonstrated that ArgGlyAsp (RGD) containing peptides were potent integrin receptor antagonists (Pierschbacher and Ruoslahti 1984; Argraves, Suzuki et al. 1987; Gehlsen, Dillner et al. 1988). In 1996, Rouslahti and Pasqualini pioneered phage display applications by performing their selections in mice to obtain organ-targeting peptides (Pasqualini and Ruoslahti 1996; Arap, Pasqualini et al. 1998). Several peptides, particularly those that bind to tumor vasculature were discovered (reviewed in (Trepel, Grifman et al. 2000)). In particular, peptides containing the motif RGD predominated and targeted molecules on organ and tumor vasculature via the integrin  $\alpha_v\beta_3$  receptor. The RGD peptide sequences identified from phage display were used to deliver cytotoxic drugs to the vascular address of tumors (Arap, Pasqualini et al. 1998; Ellerby, Arap et al. 1999; Arap, Haedicke et al. 2002; Curnis, Arrigoni et al. 2002). When coupled to doxorubicin, the RGD integrin-targeting peptides increased the efficacy of doxorubicin in inhibiting tumor growth while

reducing non-specific toxicity to the animal (Arap, Pasqualini et al. 1998). Since then, numerous radiolabeled linear (Sivolapenko, Skarlos et al. 1998) and Cys-cyclized analogues (Janssen, Oyen et al. 2002; Su, Liu et al. 2002) of RGD-containing peptides have been examined in rodent models of human melanoma (Haubner, Wester et al. 2001) renal (Su, Liu et al. 2002), colon (Su, Liu et al. 2002), and ovarian cancers (Janssen, Oyen et al. 2002). Results of these studies are mixed showing some radioactive peptide accumulation in tumors (Haubner, Wester et al. 2001; Janssen, Oyen et al. 2002) but high levels of renal and liver accumulation (Haubner, Wester et al. 2001; Janssen, Oyen et al. 2002; Su, Liu et al. 2002). It has been speculated that the efficacy of RGD peptides as anti-cancer drugs may be compromised due to peptide small size, low affinity, and/or low  $\alpha_v\beta_3$  integrin receptor number (Su, Liu et al. 2002). *In vivo* selection has not been limited to selection of only RGD peptides. Numerous other vascular-targeting peptides have been identified by Pasqualini's group, including peptides with GSL and NGR motifs (Pasqualini, Koivunen et al. 1997). One peptide discovered, SMSIARL, was selected in transgenic adenocarcinoma of the mouse prostate (TRAMP) model and specifically targeted prostate tumor vasculature (Arap, Haedicke et al. 2002). An intriguing finding from this *in vivo* work in mice was that individual organs may have unique vasculature signatures. To test this theory in humans, a group of researchers studied the peptides selected from organs of a brain dead human injected with a disulfide constrained random phage library (Arap and Pasqualini 2001; Arap, Kolonin et al. 2002). Thousands of peptide motifs were identified many of which possessed structural and distribution similarities to known ligands for proteins differentially expressed on the tissues the peptides were selected from. A non-random frequency of selected peptide motifs in

relation to the organ(s) of origin was observed, which indicated that the peptides were homing to tissue in a specific manner (Arap, Haedicke et al. 2002).

Lectin-carbohydrate interactions have also been probed using phage display. Phage display has been used to identify peptides and antibodies specific for carbohydrates such as the Lewis X antigen (Dinh, Weng et al. 1996; Mao, Gao et al. 1999; Fukuda, Ohyama et al. 2000). Targeting molecules, such as these, can direct cytotoxic drugs to cancer cells (Bera and Pastan 1998) and inhibit cancer cell colonization of distal organs (Fukuda, Ohyama et al. 2000). Peletskaya, *et al.* (Peletskaya, Glinsky et al. 1996; Peletskaya, Glinsky et al. 1997), identified phage display-derived anti-TFAG peptides that were later observed to inhibit both homotypic aggregation between cancer cells (Glinsky, Huflejt et al. 2000) as well as heterotypic aggregation between cancer cells and endothelial cells (Glinsky, Glinsky et al. 2001; Glinskii, Huxley et al. 2003). One such peptide, P30, HGRFILPWWYAFSPS, bound specifically to carcinoma cells that were TFAG-positive, including those of the colon, breast and prostate (Peletskaya, Glinsky et al. 1996; Peletskaya, Glinsky et al. 1997). P30 was able to inhibit the adhesion of breast and prostate cancer cells to human bone marrow endothelial cells, suggesting TFAG-mediated interactions may be important in tumor cell adhesion to bone marrow vasculature (Glinsky, Glinsky et al. 2001). Conversely, peptides that bind to lectins could also be effective inhibitors of adhesion.

Anti-cancer peptides derived from phage display libraries have been used with particular success in modulating the steps of transformation (Bottger, Bottger et al. 1996) and metastasis (Fukuda, Ohyama et al. 2000). Further, the whole phage bearing specific peptides that target a tumor marker could have the potential for use *in vivo* as a vehicle to

deliver an anticancer drug or imaging agent. In 1996, Rouslahti and Pasqualini pioneered phage display applications by performing their selections in mice to obtain organ-targeting phage and peptides (Pasqualini and Ruoslahti 1996; Arap, Pasqualini et al. 1998). A phage clone that binds the  $\alpha V$  integrin showed 40-80 fold greater tumor selectivity relative to control organs (Pasqualini, Koivunen et al. 1997). Pioneering work from the same group also successfully isolate a phage harboring a peptide that mimics a ligand of endothelium lectin, E-selectin, The injection of the phage itself significantly inhibited lung colonization of B16-FTHLM murine melanoma cells in mice, perhaps by down-regulating carbohydrate-dependent metastasis (Fukuda, Ohyama et al. 2000). Moreover, Frenkel et al., have successfully utilized filamentous phage that display anti- $\beta$  amyloid antibodies to monitor  $\beta$ -amyloid plaques in mice. It is reported that filamentous phage (f88) were able to penetrate the central nervous system without inducing toxic effects (Frenkel and Solomon 2002). As galectin-3 is highly expressed on MDA-MB-435 human breast carcinoma cell surfaces (Khaldoyanidi, Glinsky et al. 2003), it is reasonable to hypothesize that galectin-3 specific phage could target to mouse tumors heterotransplanted with MDA-MB-435 cells.

On the other hand, the practical application of tumor-avid phage and/or peptides will depend on their ability to target tumors *in vivo*. While most *in vivo* affinity selections and studies have targeted the molecules on the vasculature of organs and tumors, we believe that it would be of more value to select phage that actually target the cancer or specific receptors on tumor cell surfaces, not just the surrounding vasculature, especially if one wants to develop new cancer-specific imaging and/or therapeutic biomolecules. A systematic study of the biodistribution of phage in different tissues, therefore, is very

important for future quests for high affinity tumor-binding molecules. An understanding of phage clearance from the circulation as well as extravasation into the tissue would provide valuable insight for the design of *in vivo* selection or application schemes. Previous *in vivo* selection schemes by Pasqualini and her colleagues consisting of short incubation times of phage in the mice, may not have taken full advantage of the very strengths of combinatorial chemistry and library diversity. Their scheme may have favored very short motifs present in many independent clones in the initial random peptide library, rather than longer, higher-affinity and specific motifs, which would be very rare in the initial library (Smith and Petrenko 1997). It is envisioned that appropriate selections would be designed to optimize incubation times in animals while sampling the entire library population. To achieve this goal, one must gain a better understanding of the *in vivo* biodistribution, stability and rate of clearance of engineered phage libraries often used today in combinatorial affinity selections. In this work (chapter 3), we have investigated the pattern of distribution of phage in three common mouse models. Additionally, after establishing the optimum time point based on our preliminary information, we initiated a biodistribution study of our galectin-3 specific phage in tumor-bearing mice.

In this study, we isolated galectin-3 specific peptides from phage display peptide libraries to understand and perhaps modulate galectin-3 functions in the metastatic cascade. Besides exploring the effect of galectin-3 binding peptides on tumor cell adhesion, we also investigated if galectin-3 specific phage could target to tumors in mice heterotransplanted with MDA-MB-435 human breast carcinoma cells (in Chapter 3), as

galectin-3 is highly expressed on the MDA-MB-435 cell surface and increases its metastatic potential (Khaldoyanidi, Glinsky et al. 2003).

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## CHAPTER 2

### **Peptides Specific to the Galectin-3 Carbohydrate Recognition Domain Inhibit Metastasis-Associated Cancer Cell Adhesion**

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## CHAPTER SUMMURY

Metastasis is a complex process consisting of multiple steps. Metastasis-associated events initiate with release of cancer cells from a primary lesion, migration into the blood stream (Chambers, Groom et al. 2002) and binding to the endothelium in target organ microvessels (Al-Mehdi, Tozawa et al. 2000). Two forms of metastasis-related tumor cell adhesion, heterotypic adhesion between neoplastic and endothelial cells, and homotypic adhesion of tumor cells to each other, are involved in metastasis (Glinsky, Glinsky et al. 2003). Both heterotypic and homotypic cancer cell adhesion are mediated in part by specific interactions between cell surface lectins and their cognate carbohydrate ligands presented on glycoproteins and glycolipids (Inohara and Raz 1995; Inohara, Akahani et al. 1996; Kannagi 1997; Orr, Wang et al. 2000; Glinsky, Glinsky et al. 2003).

Galectin-3 is a predominant member of the galectin family of soluble mammalian lectins recognizing terminal  $\beta$ -galactopyranose. Galectin-3 may facilitate metastasis by promoting tumor cell adhesion (Raz and Lotan 1987; Inohara and Raz 1995), invasiveness (Le Marer and Hughes 1996), as well as by antagonizing tumor cell apoptosis (Akahani, Nangia-Makker et al. 1997; Matarrese, Fusco et al. 2000; Yu, Finley et al. 2002), and inducing endothelial cell proliferation and angiogenesis (Nangia-Makker, Honjo et al. 2000). Galectin-3 is prominently expressed in several types of cancer (Raz and Lotan 1981; Gillenwater, Xu et al. 1996); and, its expression correlates with tumor cell transformation and metastatic phenotype *in vivo* (Raz, Zhu et al. 1990). Elevated galectin-3 expression significantly enhances tumor cell adhesion to common extracellular matrix (ECM) proteins (Ochieng, Warfield et al. 1999), increases the incidence of lung

metastases (Raz, Zhu et al. 1990), and protects cancer cells from apoptosis (Akahani, Nangia-Makker et al. 1997; Matarrese, Fusco et al. 2000; Yu, Finley et al. 2002). Furthermore, pre-treatment of tumor cells with an anti-galectin-3 antibody reduces the incidence of metastatic lung colonies by up to 90% (Meromsky, Lotan et al. 1986). These data suggest that galectin-3 expression and interactions with its associated carbohydrate ligands could be important in tumor metastasis.

We previously demonstrated that the cancer-associated Thomsen-Friedenreich antigen (TFAg), a Gal $\beta$ 1-3GalNAc disaccharide (Springer 1984), exposed on up to 90% of human carcinomas (Springer 1984; Baldus, Hanisch et al. 1999), is one of the major galectin-3 carbohydrate ligands presented on cancer cells (Glinsky, Glinsky et al. 2001). Increased levels of TFAg correlate with metastasis in gastric (Chung, Yamashita et al. 1996), colorectal (Baldus, Hanisch et al. 1998), and breast carcinomas (Springer 1989). Recent work suggests that galectin-3 expressed on endothelial cells may interact with carbohydrate ligands such as TFAg on carcinoma cells to mediate tumor cell adhesion and metastasis (Lehr and Pienta 1998; Glinsky, Glinsky et al. 2001). Upon appropriate stimulation, endothelial galectin-3 is rapidly redistributed to the sites of tumor cell–endothelial cell contacts (Glinsky, Glinsky et al. 2003), suggesting that galectin-3/TFAg interactions might be important early steps in the formation of intravascular metastatic deposits. Inhibiting these steps could potentially modify metastasis-associated tumor cell adhesion and help control metastatic cancer spread. Traditionally, such inhibition could be achieved using carbohydrate-based compounds interacting with the galectins-3 carbohydrate recognition domain (CRD) (Glinsky, Price et al. 1996; Nangia-Makker, Hogan et al. 2002). In this study, we tested the hypothesis that metastasis-associated

heterotypic and homotypic adhesion of carcinoma cells mediated by interactions between galectin-3 and TFAg could be inhibited efficiently by small synthetic peptides, which specifically and selectively bind to the galectin-3 CRD.

Peptide antagonists of galectin-3 were identified using combinatorial bacteriophage display technology. The isolated peptides exhibited specific binding to cell surface galectin-3 and, as determined by fluorescence quenching, bound to the purified galectin-3 protein with high affinity ( $K_d \cong 17\sim 80$  nM). The selected peptides specifically bound to the carboxyl terminal CRD of galectin-3 but not to other lectins tested, suggesting that these peptides do not mimic naturally occurring galectin-3 carbohydrate ligands. Further, the galectin-3 CRD-specific peptides recognize galectin-3 expressed on tumor cells and inhibit homotypic adhesion of MDA-MB-435 human breast cancer cells and their heterotypic adhesion to endothelial cells, under physiological flow conditions. Our biochemical binding experiments indicated that G3-C12, one of the selected peptides, was able to inhibit galectin-3 dimerization. Combined with our data on the inhibitory effect of the peptide on galectin-3 mediated cell adhesion and aggregation, these studies suggest that the galectin-3 dimer is structurally and functionally involved in the carbohydrate-mediated cell adhesion process. These results demonstrate that  $\beta$ -galactoside-mediated metastasis-associated tumor cell adhesion could be efficiently disrupted by small synthetic peptides, which do not mimic naturally occurring glycoepitopes, but interact with the galectin-3 CRD with high affinity and specificity. Our findings may also provide insights into the understanding of cancer cell adhesion and the mechanisms of metastasis and may suggest a new novel therapeutic method for anti-adhesive drug design.

## INTRODUCTION

Metastases, rather than primary tumors, cause most cancer deaths. A better understanding of metastasis may facilitate development of new detection and therapeutic strategies for cancer. While the precise mechanisms of metastasis have yet to be fully defined (Marx 2001), it is clearly a multi-step process involving interactions between various adhesion molecules including carbohydrates, lectins and extracellular matrix proteins (Brockhausen, Schutzbach et al. 1998; Alper 2001). Metastasis is thought to begin with the release of malignant cells from the primary tumor. These cells then migrate into the circulation (Luzzi, MacDonald et al. 1998). An important contributing factor to increased metastatic potential in tumor cells is their tendency to aggregate homotypically to form multicellular aggregates (Lotan and Raz 1983; Meromsky, Lotan et al. 1986; Updyke and Nicolson 1986; Saiki, Naito et al. 1991). The aggregates are transported in the blood to distal sites where the cells adhere specifically to the endothelium, in a process similar to that by which activated lymphocytes adhere to endothelium (Luzzi, MacDonald et al. 1998; Al-Mehdi, Tozawa et al. 2000). Two forms of metastasis-related tumor cell adhesion, heterotypic adhesion between neoplastic and endothelial cells and homotypic adhesion of tumor cells to each other, are involved in metastasis (Al-Mehdi, Tozawa et al. 2000; Glinsky, Glinsky et al. 2003). Both heterotypic and homotypic cancer cell adhesion are mediated in part by specific interactions between cell surface lectins and their cognate carbohydrate ligands presented on glycoproteins and glycolipids (Inohara, Akahani et al. 1996; Kannagi 1997; Orr, Wang et al. 2000).

Galectin-3 is a member of a galectin family that is defined by two properties: 1) a highly conserved carbohydrate recognition domain (CRD, C-terminal domain for galectin-3) and 2) their affinity for  $\beta$ -galactosides. In the last few years considerable experimental evidence has accumulated concerning its participation in different biological processes such as cell adhesion, cell growth, inflammation, differentiation, apoptosis and metastasis. Galectin-3 is prominently expressed in several types of cancer (van den Brule, Buicu et al. 1995; Gillenwater, Xu et al. 1996), and its expression correlates with tumor cell transformation and metastatic phenotype *in vivo* (Raz and Lotan 1981). For example, the expression of galectin-3 has been suggested to be used as a diagnosis and/or prognosis maker in thyroid papillary carcinomas (Coli, Bigotti et al. 2002; Papotti, Volante et al. 2002). Elevated galectin-3 expression significantly enhances tumor cell adhesion to common extracellular matrix (ECM) proteins, increases the incidence of lung metastases (Raz, Zhu et al. 1990), and protects cancer cells from apoptosis (Akahani, Nangia-Makker et al. 1997; Matarrese, Fusco et al. 2000). A positive correlation has been shown between the galectin-3 levels and the stage of progress of breast, gastrointestinal, lung, and ovarian cancer (Iurisci, Tinari et al. 2000). Blocking galectin-3 expression in highly malignant human breast carcinoma MDA-MB-435 cells significantly reversed the tumorigenic phenotype and suppressed tumor growth in nude mice (Honjo, Nangia-Makker et al. 2001). Moreover, pre-treatment of B16 melanoma cells with an anti-galectin-3 antibody reduced the incidence of metastatic lung colonies by up to 90% (Meromsky, Lotan et al. 1986). More recently, it was reported that oral intake of galectin-3 specific polysaccharide, modified citrus pectin (MCP), suppressed tumor growth and spontaneous metastasis in nude mice injected with human breast



carcinoma MDA-MB-435 cells. *In vitro* studies also showed that MCP inhibited adhesion of MDA-MB-435 cell adhesion to human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner (Honjo, Nangia-Makker et al. 2001). These data suggest that galectin-3 expression and interactions with its cognate carbohydrate ligands could be important in tumor metastasis.

Although the precise role of galectin-3 in the metastatic process is not fully understood, much has been learned recently regarding its effect on the spread of cancer cells and adhesion. Studies from our laboratory (Glinsky, Glinsky et al. 2001; Glinsky, Glinsky et al. 2003; Khaldoyanidi, Glinsky et al. 2003) and others (Lehr and Pienta 1998) have shown that galectin-3 is key in initiating homo- and heterotypic adhesion of human breast and prostate cancer cells by specifically interacting with the cancer-associated Thomsen-Friedenreich antigen (TFAg), a Gal $\beta$ 1-3GalNAc disaccharide (Springer 1984). TFAg is a mucin-type disaccharide that is exposed on up to 90% of human carcinomas (Springer 1984; Baldus, Hanisch et al. 1999). Increased levels of TFAg correlate with metastasis in gastric (Chung, Yamashita et al. 1996), colorectal (Baldus, Hanisch et al. 1998), and breast carcinomas (Springer 1989). Recent work suggests that galectin-3 expressed on endothelial cells may interact with carbohydrate ligands such as TFAg on carcinoma cells to mediate tumor cell adhesion and metastasis (Lehr and Pienta 1998; Glinsky, Glinsky et al. 2001).

Upon binding to TFAg or other glycoconjugate ligands at the cell surface, galectin-3 molecules are thought to form dimers or oligomers which lead to cross-linking of surface glycoproteins. Because galectin-3 has only one carbohydrate-binding site, it is believed that this cross-linking between carbohydrate ligands results from the self-

association of two or more galectin-3 monomers through their N-terminal domains (Hsu, Zuberi et al. 1992; Massa, Cooper et al. 1993). Removing the N-terminal domain of galectin-3 abolished its hemagglutination activity without impairing its carbohydrate-binding ability (Hsu, Zuberi et al. 1992). Furthermore, carbohydrate-regulated cooperative binding to IgE (Hsu, Zuberi et al. 1992) and laminin (Massa, Cooper et al. 1993) is detectable with full-length galectin-3 but not with the CRD fragment lacking the N-terminal domain, strongly suggesting that self-association of galectin-3 through their N-terminal domain is critical for galectin-3 biological behaviors.

Efficient self-association requires a relatively high concentration of galectin-3 (Liu, Hsu et al. 1995). Accumulation of galectin-3 on the cell surface appears to be a rate-limiting step for its cross-linking ability. Our previous studies demonstrated a 3-fold increase in galectin-3 expression on endothelial cells upon TFAg treatment, implying that cell surface galectin-3 can be regulated and clusters at particular sites under the influence of certain stimuli, therefore providing the necessary concentration for dimerization (Frigeri and Liu 1992; Glinsky, Glinsky et al. 2003). Furthermore, localization experiments revealed that galectin-3 clustered at sites of heterotypic adhesion on endothelial cells and homotypic adhesion on tumor cells, indicating that dimeric or oligomeric galectin-3 could mediate cell adhesion via interactions with glycoproteins such as TFAg (Glinsky, Glinsky et al. 2003). In light of these findings, we hypothesized that specific molecular inhibition of the interaction between galectin-3 and its ligands and/or its self-association would lower the metastatic potential of carcinoma cells by reducing their homotypic and heterotypic adhesion capabilities.

Recently, extensive studies have been carried out using combinatorial chemistry to identify or isolate novel tumor targeting agents (Glinsky, Huflejt et al. 2000; Rademann and Jung 2000), including small molecules that bind specifically to tumor-associated carbohydrate antigens (Glinsky, Huflejt et al. 2000) as well as lectins (Fukuda, Ohyama et al. 2000). Self-replicating combinatorial libraries such as bacteriophage (phage) peptide display libraries do not require a protein target and the power of their selection coupled with amplification makes them well suited for the task of new target discovery (Smith and Petrenko 1997; Kay, Kasanov et al. 2000; Ryu and Nam 2000). A phage display “peptide library” is a mixture of filamentous phage with foreign peptides on their surface and the coding sequences for the peptides in the viral DNA. Surface display is accomplished by fusing the peptide coding sequence to a coat-protein gene—either the minor coat protein cpIII (5 copies/virion) or the major coat protein cpVIII (thousands of copies/virion) (Scott and Smith 1990). Each phage clone displays a single peptide, but a library as a whole may represent billions of peptides altogether. The vital advantage of surface exposure is that it allows phage display libraries with vast numbers of peptides to be easily surveyed for clones whose displayed peptides bind specifically to any given molecular target. We previously employed combinatorial chemistry with random phage display peptide libraries to obtain TFAg-targeting molecules that dramatically inhibited MDA-MB-435 carcinoma cells homo- and heterotypic adhesion. In this work, I set out to isolate galectin-3 specific peptides from phage display peptide libraries to understand and perhaps modulate galectin-3 functions in the metastatic cascade.

## MATERIALS AND METHODS

### ***Materials***

All chemicals and reagents, unless specifically noted, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

### ***Purification of Recombinant Galectin-3, Galectin-3C and Galectin-3 Mutants***

Full-length recombinant human galectin-3 was expressed in *E. coli* from the pET3 vector (a generous gift from Dr. Avraham Raz, Wayne State University, Detroit, MI) (Glinsky, Glinsky et al. 2001); The recombinant galectin-3 was purified using a  $\beta$ -lactose affinity chromatography matrix, as previously described (Ochieng, Platt et al. 1993) (Fig. 1). The C-terminal fragment, galectin-3C (aa 108-250), was produced by collagenase digestion of the full-length galectin-3 and re-purification on a  $\beta$ -lactose affinity chromatography matrix column, as previously described (Massa, Cooper et al. 1993).

Four truncated proteins with amino-terminal deletions were produced by cloning the full-length human galectin-3 cDNA into the pET15b vector (Novagen, Madison, WI), the mutant sequences are in Fig. 1. Mutants *Del1-28* and *Del1-63* were constructed by sequentially digesting galectin-3 plasmid DNA (*Del1-28*, *NdeI* and *BstXI*; *Del1-63*, *NdeI* and *XcmI*). The desired fragments were gel-purified and re-circularized using T4 DNA ligase. Mutants *Del1-12* and *Del1-118* were constructed by using primer-directed mutagenesis. A common downstream primer (5'-GGGTACCGA GCTCGAATTC-3') containing an *EcoRI* site was used. Distinct upstream primers introduced a unique *BamHI* restriction site (*Del1-12*, 5'-CGGGATCCGGGGTCTGGAAACCCAAACC-3');

*Del* 1-118, 5'- CGGGATCCGAA CCTGCCTTTGCCTGGG-3'). The PCR products were gel-purified, digested with appropriate restriction enzymes and ligated into the pET15b vector. All constructs were chemically transformed into the non-expression host strain, JM109. Plasmid DNA was isolated and the mutations were confirmed by DNA sequencing (University of Missouri DNA Core Facility). The mutants were expressed in *E. coli* BL21 and purified by using Ni-NTA columns (Qiagen, Valencia, CA).

The identity, purity and molecular weight of the recombinant proteins were analyzed using SDS-PAGE and immunoblotting with either a monoclonal antibody (MAb) TIB-116 (American Type Culture Collection), which binds an amino terminal epitope and recognizes full-length, *Del*1-12, and *Del*1-28 galectin-3 proteins, or an anti-galectin-3 polyclonal antibody (PAb) (Dr. A. Raz, Wayne State University, Detroit, MI), which recognizes all of these proteins. Protein concentration was determined using a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL), using BSA as the protein standard.

### ***Affinity Selection***

Two phage display libraries, f88/15 and f88/Cys6 (Dr. George Smith, University of Missouri-Columbia) (Smith and Scott 1993), each consisting of approximately  $2 \times 10^8$  phage clones displaying either a random 15-amino acid peptide or a random 16-amino acid peptide with 2 fixed cysteines, respectively, were used for the affinity selection against purified recombinant galectin-3, as previously described (Peletskaya, Glinsky et al. 1996). Briefly, 4 $\mu$ g biotinylated galectin-3 in 0.5 % v/v Tween 20-TBS (TTBS) was

**Fig. 1. cDNA and amino acid sequence of full-length galectin-3 and truncated galectin-3 mutants.**

ATGGCAGACAATTTTTTCGCTCCATGATGCGTTATCTGGGTCTGGAAACCCAAACCCTCAA  
**M A D N F S L H D A L S G S G N P N P Q**

▲ Del 1-12

GGATGGCCTGGCGCATGGGGGAACCAGCCTGCTGGGGCAGGGGGCTACCCAGGGGCTTCC  
**G W P G A W G N Q P A G A G G Y P G A S**

▲ Del 1-28

TATCCTGGGGCCTACCCCGGGCAGGCACCCCGGGGCTTATCCTGGACAGGCACCTCCA  
**Y P G A Y P G Q A P P G A Y P G Q A P P**

GGCGCCTACCATGGAGCACCTGGAGCTTATCCCGGAGCACCTGCACCTGGAGTCTACCCA  
**G A Y H G A P G A Y P G A P A P G V Y P**

▲ Del 1-63

GGGCCACCCAGCGGCCCTGGGGCCTACCCATCTTCTGGACAGCCAAGTGCCCCGGAGCC  
**G P P S G P G A Y P S S G Q P S A P G A**

TACCCTGCCACTGGCCCCTATGGCGCCCCTGCTGGGCCACTGATTGTGCCTTATAACCTG  
**Y P A T G P Y G A P A G P L I V P Y N L**

▲ Del 1-118

CCTTTGCCTGGGGGAGTGGTGCCTCGCATGCTGATAACAATTCTGGGCACGGTGAAGCCC  
**P L P G G V V P R M L I T I L G T V K P**

AATGCAAACAGAATTGCTTTAGATTTCCAAAGAGGGAATGATGTTGCCTTCCACTTTAAC  
**N A N R I A L D F Q R G N D V A F H F N**

CCACGCTTCAATGAGAACAACAGGAGAGTCATTGTTTGCAATACAAAGCTGGATAATAAC  
**P R F N E N N R R V I V C N T K L D N N**

TGGGGAAGGGAAGAAAGACAGTCGGTTTTCCATTTGAAAGTGGGAAACCATTCAAATA  
**W G R E E R Q S V F P F E S G K P F K I**

CAAGTACTGGTTGAACCTGACCACTTCAAGGTTGCAGTGAATGATGCTCACTTGTTCAG  
**Q V L V E P D H F K V A V N D A H L L Q**

TACAATCATCGGGTTAAAAAACTCAATGAAATCAGCAAACCTGGGAATTTCTGGTGACATA -  
**Y N H R V K K L N E I S K L G I S G D I**

GACCTCACCAGTGCTTCATATACCATGATA  
**D L T S A S Y T M I X**

immobilized on streptavidin-coated dishes. To this,  $10^{12}$  transforming units (Tu, Ho-Schleyer et al.) recombinant phages were added for 4 hour (h) at room temperature (RT). The dishes were extensively washed. Bound phage particles were eluted with 0.22 N HCl, pH adjusted to 2.2 with glycine, and neutralized with 1/8 vol 1 M Tris-HCl, pH 9.1. Eluted phage were amplified by infecting K91 Bluekan *Escherichia coli* bacteria cultured in LB medium. After 4 rounds of selection, eighty phage clones were randomly picked and propagated individually. Redundant phage clones were eliminated by using one-lane dideoxy DNA sequencing with a single termination mix (Haas and Smith 1993). The coding sequences of the unique clones were determined by using a modified dideoxy sequencing method, as described previously (Peletskaya, Glinsky et al. 1997).

#### ***Phage Membrane Binding Assay***

Phage membrane binding assays were conducted as described previously (Landon, Zou et al. 2003). Individual phage clones ( $2 \times 10^8$  virions) were spotted onto a nitrocellulose membrane and blocked. The membrane was incubated with 2 mg of biotinylated galectin-3. Bound galectin-3 was detected colorimetrically using a streptavidin-horseradish peroxidase (SA-HRP) conjugate.

#### ***Peptide Synthesis and Purification***

Peptides were chemically synthesized using solid-phase Fmoc chemistry on an Advanced ChemTech 396 multiple peptide synthesizer (Advanced ChemTech, Louisville, KY) and purified by high-pressure liquid chromatography. All masses were verified by mass spectroscopy analysis.

#### ***Peptide Binding and Immunoblot Analysis***

Purified full-length galectin-3, truncated galectin-3 proteins and a His6-tagged DNA-1 monoclonal antibody (Komissarov, Calcutt et al. 1996) (as a negative control) were separated by 12% SDS-PAGE (Laemmli 1970) and transferred to nitrocellulose membranes, which were subsequently blocked with 6% BSA for 1 h at RT and washed with TTBS. Next, the membranes were blocked and incubated either with 20  $\mu$ M solutions of biotinylated anti-galectin-3 peptides (10 mM Tris, pH 7.5, 2% BSA), anti-galectin-3 MAb (1/200 dilution) or anti-galectin-3 PAb (1/500 dilution) in TTBS for 1 h at RT, and washed extensively with TTBS. The membranes were then incubated with SA-HRP (1/1000 dilution) for peptides–or corresponding HRP-conjugated secondary antibody (1/1000 dilution) anti-rat for anti-galectin-3 MAb, or anti-rabbit for anti-galectin-3 PAb (Santa Cruz Biotechnology, Santa Cruz, CA). After extensive washing, binding was detected colorimetrically by using a 4CN membrane peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

To determine the binding specificity of the peptides, blotting experiments were performed with galectin-3, -1, and -4 (R & D Systems, Minneapolis, MN 55413), as well as with *Aleuria aurantia* lectin (AAL), *Vicia villosa* lectin (VVL) (Vector Laboratories, Burlingame, CA) and *Arachis hypogaea* lectin (PNA). Equivalent amounts of each lectin were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with biotinylated peptides, as described previously. Peptide binding was detected using an alkaline phosphatase (AP)-streptavidin conjugate (GibcoBRL, Gaithersburg, MD) and BCIP/NBT as substrate (Fisher Scientific, Pittsburgh, PA).

### ***Fluorescence Enhancement***



Galectin-3 was fluorescently labeled using the AlexaFluor 488 Protein Labeling Kit (Molecular Probes, Eugene, OR) according to the manufacturers' instructions; the number of fluorophores per galectin-3 molecule was about 4. High throughput fluorescence quenching (HTP-FQ) analysis of peptide binding affinity was performed, as described previously (Landon, Harden et al. 2004), using 1 nM AlexaFluor 488-galectin-3 as fluorophore and serial peptide dilutions as quenchers. The quantum fluorescence output (in cpm) was read on a Fusion Universal Microplate Analyzer (Packard Bioscience, Inc., Meriden, CT), using a  $485 \pm 20$  nM excitation filter and a  $520 \pm 20$  nM emission filter. The autofluorescence of empty wells was subtracted from each reading prior to analysis. Percent decrease from maximum  $[(1 - (Q_{\text{obs}}/Q_{\text{max}})) \times 100\%]$  was calculated. Binding affinity was calculated by using the Langmuir binding equation,  $Y = (B_{\text{max}} \cdot X)/(K_d + X)$  (Kim, Paulus et al. 1994) and the GraphPad Prizm 3.0 software, where  $K_d$  was the dissociation affinity constant,  $B_{\text{max}}$  was maximal binding and  $X$  was the total peptide molar concentration. All titrations were performed in triplicate.

### ***Cell Lines and Culture***

The human breast cancer cell lines BT549, which does not express detectable levels of galectin-3 (Akahani, Nangia-Makker et al. 1997), and BT549*trans*, transfected with the cDNA encoding galectin-3 (Dr. Avraham Raz, Wayne State University, Detroit, MI) were used as a model system of differential galectin-3 expression (Akahani, Nangia-Makker et al. 1997). The mouse monocyte/macrophage cell line J744A (American Type Culture Collection) (Andre, Kojima et al. 1999), was used as a positive control. The human breast carcinoma cell line MDA-MB-435 (National Cancer Institute) and human prostate carcinoma cell line PC-3M (Dr. Avraham Raz), both of which express moderate

levels of galectin-3 (Andre, Kojima et al. 1999), were also used. Cells were grown to confluence in RPMI 1640 media (GIBCO-BRL, Grand Island, NY) supplemented with L-glutamine, 10% fetal bovine serum, sodium pyruvate, and nonessential amino acids. The human bone marrow endothelial cell line, HBME-1 (26) kindly provided by Dr. Kenneth J. Pienta (University of Michigan, Ann Arbor, MI), which was used in the heterotypic adhesion assays, was cultured in DMEM media supplemented with L-glutamine, 10% FBS, and sodium pyruvate.

### ***Cell Surface Binding***

Laser scanning confocal microscopy was conducted as described previously (Landon, Peletskaya et al. 2003).  $1 \times 10^4$  cells of each cell suspension of dispersed cells (phosphate buffered saline, pH 7.4, 4% formaldehyde at a density of  $2$  to  $4 \times 10^6$  cells/ml) were dried onto a microscope slide and blocked. The slides were incubated with biotinylated peptide solutions (20  $\mu$ M in 10 mM Tris, pH 7.5, 1% BSA) or rat anti-galectin-3 MAb (1/20 dilution in 10 mM Tris, pH 7.5, 1% BSA) for 1 h at RT. Binding was detected by using 10  $\mu$ g/ml NeutrAvidin-Texas Red (Molecular probes, Eugene, OR) in 10 mM Tris, pH 7.5, 1% BSA for peptides and AlexaFluor 488 conjugated anti-rat secondary antibody (Molecular Probes, Eugene, OR) (10 $\mu$ g/ml in 10 mM Tris, pH 7.5, 1% BSA) for anti-galectin-3 MAb (incubation for 30 min at RT in the dark). Laser scanning confocal microscopy was performed using a Bio-Rad MRC 600 confocal microscope (University of Missouri Molecular Cytology Core Facility).

### ***Asialofetuin (ASF) Binding to Galectin-3***

Briefly, 10µg/ml biotinylated galectin-3 in TTBS was immobilized on streptavidin-coated dishes (BD Biosciences, NJ). ASF was fluorescently labeled using Molecular Probes' AlexaFluor 488 protein labeling kit according to the supplier's instructions. The wells were then incubated with 0.5 µg AlexaFluor 488-labeled ASF for 1 h in the presence or absence of competitors (N-acetyllactosamine (LacNAc), PNA or sucrose). The binding of ASF to immobilized galectin-3 was detected using a Fusion Universal Microplate Analyzer (Packard Instrument Co., Meriden, CT). The excitation wavelength was 495 nm and the emission wavelength was 519 nm. All the assays were performed in triplicate and the data was analyzed by using GraphPad Prism 3.0 software.

#### ***Inhibition enzyme-linked immunosorbant assay (ELISA)***

ASF (0.5 µg in 100µL) was pre-absorbed to a 96-well plate for 2 h at RT and blocked. Galectin-3 (0.5 µg in 100µL) in the presence or absence of increasing concentrations (1-50 µM) of anti-galectin-3 peptides or 'scrambled' galectin-3 control peptides (G3-A9s, DKFPPTLHPQSINPG; G3-C12s, PTHVTCKYCPAGNRDP) were added and incubated for 1 h at RT. Bound galectin-3 was detected colorimetrically by using a rat anti-galectin-3 primary MAb, an AP-conjugated goat anti-rat secondary antibody and developed with *p*-nitrophenyl phosphate. The absorbance at 405 nm was recorded. The assay was performed in triplicate. The data were normalized by using the GraphPad Prism 3.0 software package. The absorbance obtained without peptide was set as 100% and the inhibition of peptides was calculated as a decrease in the absorbance at 405 nm compared to the control (no peptide).

#### ***Homotypic Aggregation Assay***

The ability of the anti-galectin-3 peptides to inhibit homotypic aggregation of MDA-MB-435 breast carcinoma cells was determined as previously described (Glinsky, Price et al. 1996). Briefly, 500  $\mu$ l of a single cell suspension ( $5 \times 10^5$  cells) was mixed with 500  $\mu$ l serum-free medium in the presence of increasing concentrations (0-25  $\mu$ M final concentration) of anti-galectin-3 peptides or the control peptide, and agitated for 1 h at 37°C. Next, three 25  $\mu$ l aliquots from each sample were spotted onto a microscope slide, dried for 1 h at RT, and fixed overnight in formaldehyde vapors. Immediately prior to microscopic evaluation, 25  $\mu$ l of PBS was placed onto each spot to facilitate viewing. The total number of cells and the number of cells in aggregates were counted in 4 random fields on each spot. The percent of cells in aggregates (number of cells in aggregates / total number of cells  $\times$  100) and the average number of aggregated cells was calculated for individual samples ( $\geq 2$  adhesive cells were considered as aggregating cells). Each assay was performed in triplicate. The results, analyzed using GraphPad Prism 3.0 software, are presented as means  $\pm$  S.D.

### ***Carcinoma Cell Adhesion to Endothelial Cell Monolayer under Flow Conditions***

The effects of anti-galectin-3 peptides on rolling and stable adhesion of MDA-MB-435 human breast carcinoma cells to endothelial cells under conditions of physiological shear stress was performed in an *in vitro* parallel plate laminar flow chamber, as described previously (Glinsky, Glinsky et al. 2003). A human bone marrow endothelial cell line HBME-1 (Lehr and Pienta 1998) was used in parallel flow chamber experiments. HBME-1 cells were exposed to flow conditions (0.8 dyn/cm<sup>2</sup> shear stress) by perfusing warm media (RPMI containing 0.75 mM Ca<sup>2+</sup> and Mg<sup>2+</sup> and 0.2 % human serum albumin) using a constant infusion/withdrawal syringe pump KDS210 (KD

Scientific, New Hope, PA). Next, the flow chamber was perfused for 15 min with an MDA-MB-435 single cell suspension ( $1 \times 10^4$  cell/ml) in the presence or absence of 20  $\mu$ M anti-galectin-3 peptides or control peptide. Tumor cell interactions with HBME-1 monolayers were observed by using an inverted phase contrast Diavert microscope (Leitz Wetzlar, Germany) and video recorded for subsequent frame-by-frame analysis. The percent of rolling cells and the number of stably adherent cells per field was determined over 1 and 5 min time periods, respectively, in at least 3 different observation fields for each experimental setting. The data were presented as the means  $\pm$  S.D. of 3 observation fields from 1 representative experiment.

#### ***Binding of AlexaFluor-labeled Galectin-3 or Galectin-3C to Biotinylated Galectin-3***

Ten  $\mu$ g/ml biotinylated galectin-3 in TTBS was immobilized on streptavidin-coated dishes (Pierce, Rockford, IL). Galectin-3 was fluorescently labeled using Molecular Probes' AlexaFluor 488 protein labeling kit, as directed. The plates were blocked with 2% BSA in TTBS and washed extensively. The wells were then incubated with 2.5-40  $\mu$ g/ml AlexaFluor 488-labeled galectin-3 for 2 h in the presence or absence of G3-C12 or a control 'scrambled' peptide. The binding of AlexaFluor 488-labeled galectin-3 to immobilized galectin-3 was detected using a Fusion Universal Microplate Analyzer (Packard Instrument Co., Meriden, CT). The excitation wavelength was 495 nm and the emission wavelength was 519 nm. All the assays were performed in triplicate and data was analyzed using GraphPad Prism 3.0 software.

For the binding of AlexaFluor-labeled galectin-3C to biotinylated galectin-3 immobilized on streptavidin-coated plates, the procedure was identical to that described for binding of AlexaFluor-labeled galectin-3 to biotinylated galectin-3 immobilized on

streptavidin-coated plates. However, AlexaFluor-labeled galectin-3C was used in place of the AlexaFluor-labeled galectin-3.

### ***Latex Bead Aggregation Assay***

The latex bead aggregation assay was conducted as described (Kuklinski and Probstmeier 1998). Briefly, 50  $\mu$ l of latex bead suspension was incubated overnight at 4°C with 1ml 0.2mg/ml ASF. Beads were then washed twice with PBS and blocked with PBS containing 1% BSA for 4 h at RT. Thirty microliters of bead suspension was resuspended in 500  $\mu$ l PBS containing 1% BSA in the absence or presence of 20 mM lactose or 25  $\mu$ M G3-C12 after twice washing with PBS. Then, 50  $\mu$ g/ml of galectin-3 was added to the bead suspension and incubated for 1h at RT on a rotatory shaker. The resulting aggregates were analyzed by light microscopy.

## RESULTS

### *Isolation of Galectin-3 Binding Peptides*

Galectin-3 specific peptides were affinity-selected from both a random 15 amino acid and a cystine constrained library displayed on coat protein VIII. By the fourth round of selection, the output phage yield from both libraries was roughly 100 fold higher compared to the first round phage yield (data not shown), which indicated enrichment of galectin-3 binding phage clones in the eluted phage population. G3-A9 and G3-C12 were the predominant output clones (Table 1). Although selected from two very different libraries, peptides G3-A9 and G3-C12 shared common amino acids P-C-G-P-X-X-X-D-C-P that may stabilize an active binding structure.

In a phage membrane-binding assay, 6 immobilized clones from the final round appeared to bind to galectin-3 (Fig. 2A). Of these, G3-A9 and G3-C12 bound with the strongest intensity. Similar results were obtained from a phage capture ELISA, which detected phage bound by immobilized galectin-3 (data not shown).

### *Binding Affinity of Synthesized Peptides*

Four peptides were chemically synthesized. Three corresponded to the peptides displayed by the phage clones giving the strongest binding signals (G3-A9, G3-C9 and G3-C12) in the phage membrane-binding assay, and one corresponded to the predominant clone from the selection (G3-A3). Fluorescence spectroscopy titration (Landon, Harden et al. 2004) was used to determine the  $K_d$ , describing the binding of peptides to galectin-3. Nonlinear regression analysis of the dose-dependent increase in

**Table 1. Results of combinatorial selections against galectin-3: Deduced peptide sequences, percent occurrence of phage clones and affinity of peptides.**

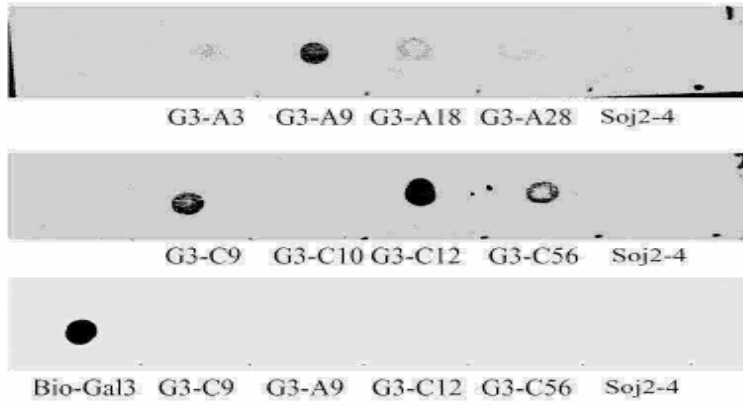
Name	Peptide sequence	Library	% of clones	Affinity K <sub>d</sub>
G3-A3	SMEPALPDWWKMFK	f88-15	8.8	K <sub>d1</sub> =17.7 ± 9.4nm K <sub>d2</sub> = 4.2 ± 2.3nm
G3-A4	DKPTAFVSVYLKTAL	f88-15	1.3	NA
G3-A9	PQNSKIPGPTFLDPH	f88-15	10	K <sub>d</sub> =72.2 ± 32.8nm
G3-A18	APRPGPWLWSNADSV	f88-15	1.3	NA
G3-A19	GVTDSSTSNLDMPHW	f88-15	1.3	NA
G3-A28	PKMTLQRSNIRPSMP	f88-15	1.3	NA
G3-A29	PQNSKIPGPTFLDPH	f88-15	1.3	NA
G3-A40	LYPLHTYTPLSLPLF	f88-15	1.3	NA
G3-C4	LTGTCLQYQSRCGNTR	f88-Cys6	1.3	NA
G3-C9	AYTKCSRQWRTCMTTH	f88-Cys6	1.3	K <sub>d</sub> =5.7 ± 2.2nm
G3-C12	ANTPCGPYTHDCPVKR	f88-Cys6	80.0	K <sub>d</sub> =72.2 ± 32.8nm
G3-C44	NISRCTHPFMACGKQS	f88-Cys6	1.3	NA
G3-C60	PRNICSRRDPTCWTTY	f88-Cys6	1.3	NA

\* The percentage of individual clones that were represented in the populations of 80 f88-15 and 60 f88-Cys6 libraries selected. G3-A9 represents the first 15 amino acid of a triple insert in the phage genome.

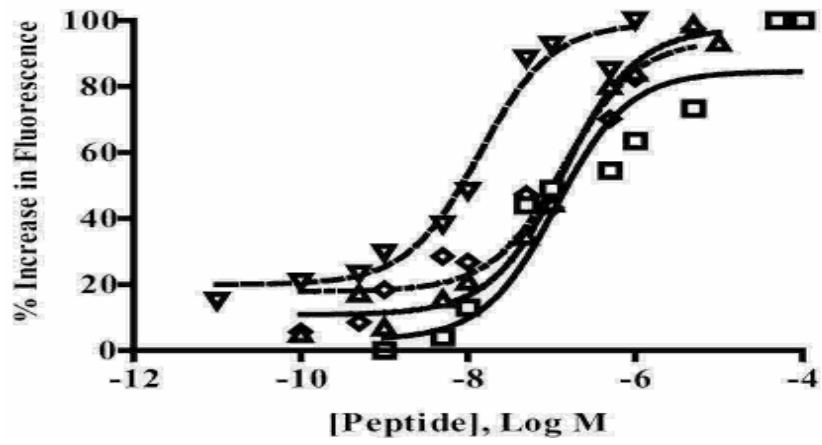


**Fig. 2. Phage displayed and chemically synthesized anti-galectin-3 peptides bind to galectin-3.** (A) Peptides displayed on immobilized phage bind to galectin-3 in solution. Individual galectin-3 peptide phage clones or negative control phage (Soj24) were spotted onto a nitrocellulose membrane and incubated with biotinylated galectin-3 (in solution) and streptavidin-horseradish peroxidase conjugate (first 2 panels) or streptavidin-horseradish peroxidase conjugate, only (3<sup>rd</sup> panel). (B) Dose-dependent quenching of AlexaFluor 488 fluorescence by galectin-3 binding peptides. Dose-dependent quenching of AlexaFluor 488 fluorescence by peptides was analyzed using the Langmuir binding equation and reported as  $K_d \pm \text{SEM}$ . Error bars were eliminated for clarity. G3-A3 (squares)  $K_{d1} = 17.9 \pm 9.4 \text{ nM}$ ,  $K_{d2} = 4.2 \pm 2.3 \text{ }\mu\text{M}$ ; G3-A9 (diamonds)  $K_d = 72.2 \pm 32.8 \text{ nM}$ ; G3-C12 (upward triangles)  $K_d = 88.0 \pm 23.0 \text{ nM}$ ; G3-C9 (downward triangles)  $K_d = 5.7 \pm 2.2 \text{ nM}$ .

**A**



**B**



fluorescence (Fig. 2B) indicated that G3-C9 had the highest affinity ( $K_d = 5.7 \pm 2.2$  nM) for galectin-3, with maximal binding observed at 0.5  $\mu$ M. Peptides G3-A9 ( $K_d = 72.2 \pm 32.8$  nM) and G3-C12 ( $K_d = 88.0 \pm 23.0$  nM) exhibited 10 fold lower affinity compared to G3-C9 (Table 1). Maximal binding of G3-A9 and G3-C12 to galectin-3 was observed at concentrations between 1 and 10  $\mu$ M. For all peptides but G3-A3, the range of 10% to 90% saturation fell within a 100-fold concentration range, indicating specific non-cooperative binding. Nonlinear regression analysis of fluorescence quenching by peptide G3-A3 indicated that the binding isotherm best fit a two-site binding algorithm ( $K_{d1} = 17.9$  nM,  $K_{d2} = 4.2$   $\mu$ M). Thus, G3-A3 was eliminated from further study.

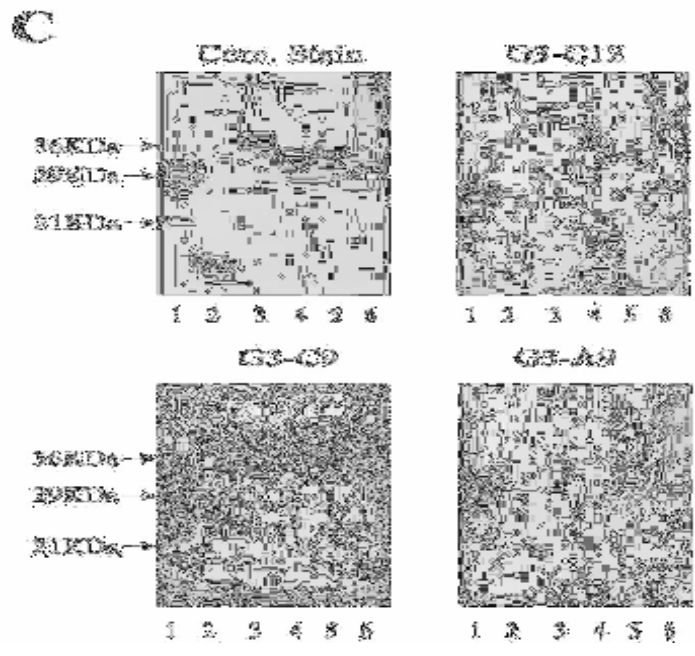
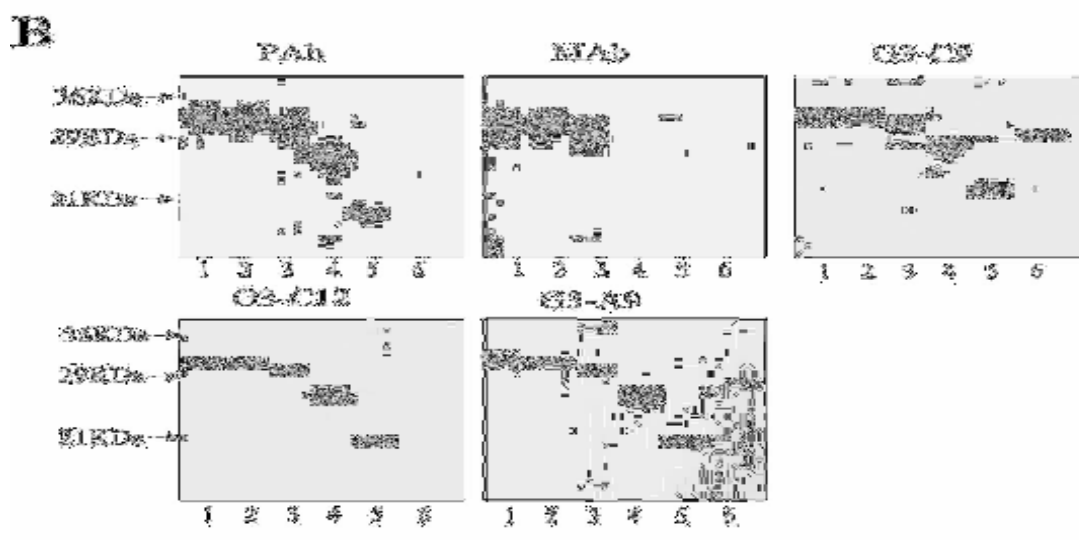
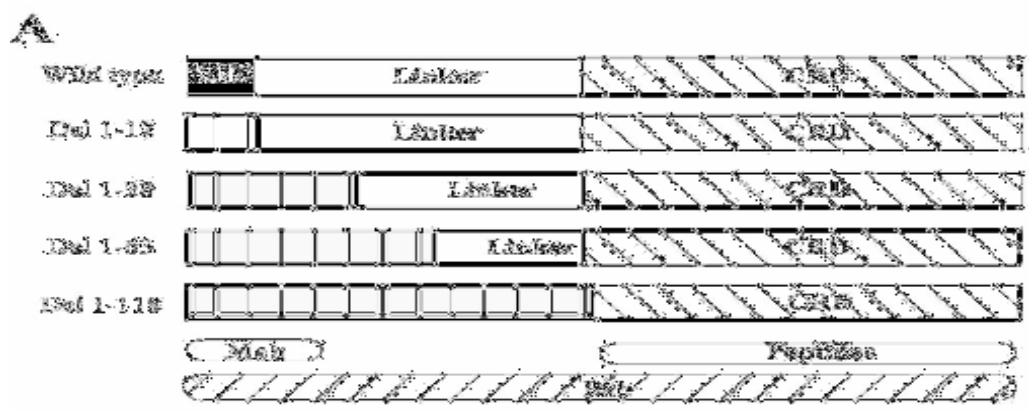
### ***Peptide Specificity***

Galectin-3 possesses a single CRD located at the carboxy-terminal end of the protein (Barondes, Cooper et al. 1994), which could be involved in tumor cell adhesion by interacting with cancer-associated cell surface carbohydrate ligands (Glinsky, Glinsky et al. 2001). Thus, we were interested in determining whether any of the selected peptides preferentially bound to the carboxy-terminal CRD region of galectin-3. To define the region of galectin-3 recognized by the synthetic peptides, a series of truncated galectin-3 proteins with deletions of 12, 28, 63 and 118 amino acids from the amino terminus of the protein were generated (Fig. 3A). Immunoblot analyses with anti-galectin-3 MAb and PAb permitted detection of full-length and truncated galectin-3 proteins (Fig. 3B). The PAb bound to all galectin-3 proteins, including *Del 1-118*, which consists of the CRD only, whereas the MAb recognized an epitope located at the amino-terminus of galectin-3. The chemically synthesized peptides G3-A9 and G3-C12 similarly bound to all immobilized galectin-3 proteins, including those lacking the entire amino terminal

domain and a linker region, but not to control proteins indicating that these peptides bound specifically to the CRD of galectin-3. In contrast, peptide G3-C9 bound all galectin-3 proteins, as well as the control protein (Fig. 3B), suggesting that its binding was non-specific.

The specificity of the three peptides was further examined. First, we analyzed peptide binding to other plant lectins with differing carbohydrate specificities, including VVL, AAL and PNA, which specifically recognize N-acetylgalactosamine, fucose, and Gal $\beta$ 1-3GalNAc (TFAg), respectively. No binding to these lectins was observed (Fig. 3C). Given this, the ability of the peptides to discriminate between the CRDs of other galectins was investigated. The galectin family of lectins is defined by a highly conserved CRD and affinity for  $\beta$ -galactosides (Barondes, Cooper et al. 1994). The sequence identity in the CRDs among galectins ranges from 20%~40% (Oda, Herrmann et al. 1993). In addition to galectin-3, two other galectins, galectin-1 and galectin-4, were used in these experiments. Structurally, galectin-1 protein is a prototypic galectin existing as a homodimer comprised of two identical CRDs (Barondes, Cooper et al. 1994). Galectin-4 has two distinct CRDs, one of which is similar to the galectin-3 CRD, and a repeating linker region similar to that of galectin-3 (Oda, Herrmann et al. 1993). As shown in Fig. 3C, both G3-A9 and G3-C12 peptides bound to galectin-3 only and did not recognize galectin-1 and galectin-4. In contrast, peptide G3-C9 clearly bound not only galectin-3, but also galectin-4 and VVL. These results suggest that G3-A9 and G3-C12 peptides specifically interact with the carbohydrate recognition domain of galectin-3 and do not recognize other lectins, even closely related galectins. One might question about whether those proteins may simply remain denatured on the blot and therefore incapable of

**Fig. 3. Mapping the binding site and specificity of peptides for galectin-3.** (A) Schematic diagram of the primary structure of the truncated galectin-3 proteins and the epitopes that are recognized by anti-galectin-3 antibodies (ovals below the proteins). The truncated proteins are shown in order, from smallest deletion to largest deletion. In each diagram, the vertically hatched areas indicate the deleted structures. (B) The binding of peptides to the truncated galectin-3 proteins was analyzed using immunoblot analysis. Purified recombinant galectin-3 (lane 1), truncated galectin-3 proteins (lane 2, *Del1-12*; lane 3, *Del1-28*; lane 4, *Del1-63*; lane 5, *Del 1-118*), and His6 tagged-DNA-1 monoclonal antibody (Komissarov, Calcutt et al. 1996) (lane 6, negative control) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The proteins were detected colorimetrically using biotinylated peptides or galectin-3 specific antibodies (indicated at the top of each panel). Molecular weight markers are labeled to the left. (C) Peptide specificity study. Purified recombinant galectin-3 (lane 1), galectin-1 (lane 2), galectin-4 (lane 3), *Aleuria aurantia* lectin (lane 4), *Vicia villosa* lectin (lane 5) and *Arachis hypogaea* lectin (lane 6) were detected colorimetrically using biotinylated peptides and alkaline phosphatase-streptavidin conjugate.



specific binding, further experiments are needed to do with native lectins, preferably immobilized chemically on amine-reactive plates under non-denaturing conditions.

### ***Binding of Peptides to the Surface of Cultured Carcinoma Cells***

The galectin-3 binding peptides were selected against purified, immobilized recombinant galectin-3. Thus, we investigated whether the peptides would specifically recognize galectin-3 in its natural conformation present on cultured mammalian cells, and whether differential expression of surface galectin-3 would correlate with differential binding by the peptides. As evidenced by staining intensities in laser scanning confocal microscopy studies, peptides G3-A9 and G3-C12 bound most efficiently to J744A cells, expressing the highest levels of galectin-3. There was virtually no binding of these peptides to the galectin-3 negative cell line, BT549 (Fig. 4A). Heterologous expression of galectin-3 in BT549*trans* cells correlated with peptide binding efficiency. A moderate signal was observed for peptide binding to human breast (MDA-MB-435) and prostate (PC-3M) carcinoma cells, consistent with the levels of galectin-3 expression in these cell lines. In contrast to the other three peptides, G3-C9 peptide bound uniformly to all cell lines tested, affirming the results of the peptide blotting studies that suggested that this peptide was not specific for galectin-3. The immunocytochemical observations of the galectin-3 expression on the various cell lines were confirmed using laser scanning confocal microscopy of cells with an anti-galectin-3 MAb in (Fig. 4A), and immunoblotting analyses of cell lysates (data not shown). A similar pattern of MAb binding to that of the peptides was observed, suggesting that the degree of peptide binding correlated with the amounts of galectin-3 exposed on the carcinoma cell surfaces.

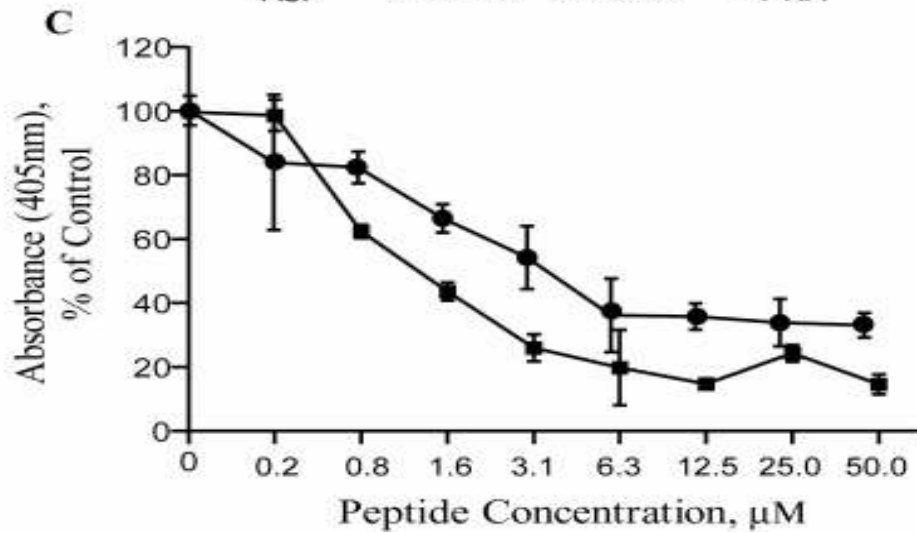
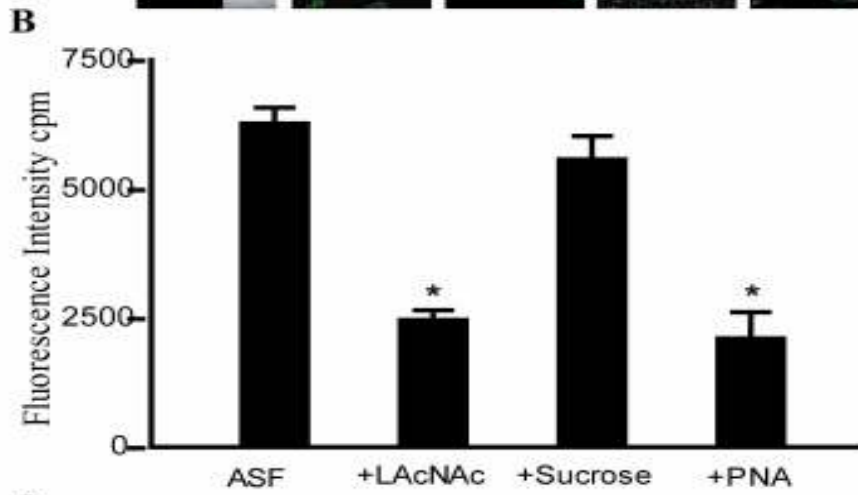
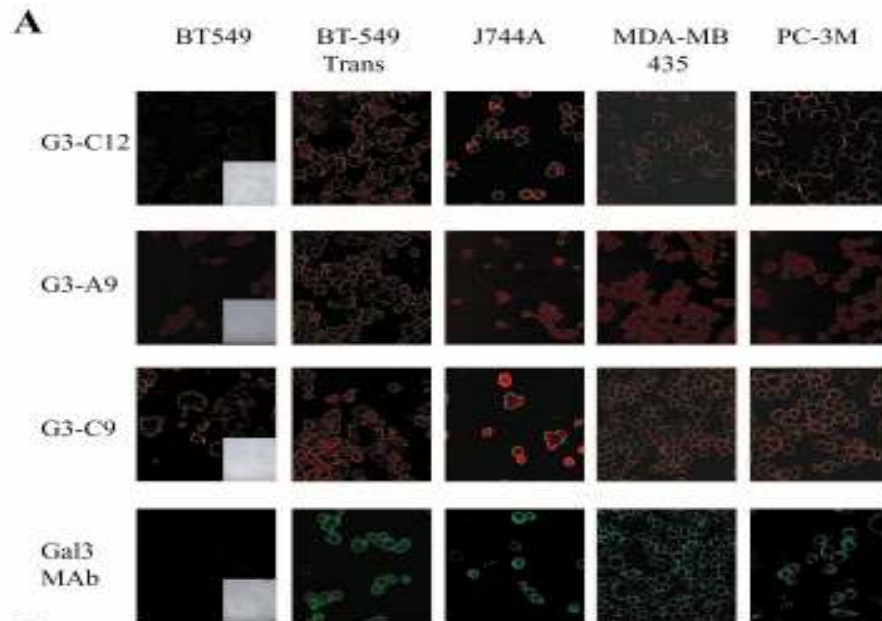
Other evidence might be needed to further confirm that peptides and antibodies are not able to access to intracellular galectin-3 during immunolocalization studies.

### ***Binding of Galectin-3 to TFAg is Inhibited by the Anti-Galectin-3 Peptides***

We then hypothesized that the selected galectin-3 CRD-binding peptides might act as inhibitors of galectin-3-TFAg interactions. To test this, we first examined the ability of recombinant galectin-3 to interact directly with TFAg in a solid-phase binding assay. AlexaFluor 488 labeled ASF, which possesses three O-linked de-sialylated-TFAg disaccharide moieties (Edge and Spiro 1987), bound efficiently to immobilized galectin-3 (Fig. 4B). Next, to determine carbohydrate-dependence and specificity, competition-binding experiments were conducted, using as competing ligands 10 mM LacNAc; 100  $\mu$ M TFAg-specific PNA lectin (Pereira, Kabat et al. 1976); and 500 mM sucrose. Both PNA and LacNAc were able to reduce ASF binding to immobilized galectin-3 by approximately 60%. Sucrose, even at concentrations as high as 500 mM, did not inhibit the binding of TFAg to galectin-3. Thus, the interaction of TFAg with galectin-3 is carbohydrate-dependent and the CRD domain is crucial for this interaction. We then tested whether the two most specific CRD-binding peptides, G3-A9 and G3-C12, could inhibit the interaction of galectin-3 with TFAg displayed on ASF. As revealed by inhibition ELISA, peptides G3-A9 and G3-C12 reduced the galectin-3 binding to immobilized TFAg in a dose-dependent manner (Fig. 4C). Both G3-A9 and G3-C12 peptides exhibited a similar maximal effect at concentrations of 6-12 $\mu$ M, which is consistent with the calculated binding affinities of these two peptides for galectin-3. Taken together, these data suggest that galectin-3 binds to TFAg through its CRD, and show that synthetic peptides specific to the galectin-3 CRD are capable of inhibiting



**Fig. 4. Galectin-3 peptides bind to carcinoma cells and inhibit binding of galectin-3 to immobilized TFAg.** (A) Binding of peptides to galectin-3 displayed on cultured human carcinoma cells, including MDA-MB-435 human breast carcinoma cells, PC-3M human prostate carcinoma cells, BT549 human breast carcinoma cells (negative control) and J744A mouse monocytes (positive control). Binding was detected by NeutrAvidin-Texas Red conjugate (10  $\mu\text{g/ml}$ ), or with rat anti-galectin-3 monoclonal antibody followed by AlexaFluor 488-conjugated anti-rat antibody and laser scanning confocal microscopy. (B, C) Peptides inhibit binding of galectin-3 to TFAg displayed on ASF. (B) Binding of AlexaFluor 488-labeled ASF in solution to immobilized galectin-3 is carbohydrate dependent. Immobilized galectin-3 was incubated with AlexaFluor 488-labeled ASF (in solution) in the presence or absence of 10 mM N-acetyllactosamine, 500 mM sucrose or 100  $\mu\text{M}$  *Arachis hypogaea* lectin. Binding was detected fluorometrically. All the assays were performed in triplicate and data was analyzed using GraphPad Prism 3.0 software. \* - Means are significantly different (unpaired t-test),  $P < 0.0001$ . (C) Peptides inhibit binding of galectin-3 (in solution) to TFAg displayed on immobilized ASF in solution. Immobilized ASF was incubated with galectin-3 in solution in the presence of different concentrations of either G3-C12 (1-50  $\mu\text{M}$ ) (squares) or G3-A9 (circles). Bound galectin-3 was detected colorimetrically. Wells with no galectin-3 were used as blanks. The assay was performed in triplicate. Percent inhibition was calculated as decrease in the absorbance at 405nm compared to controls (no peptide).



TFAg binding to galectin-3. Alternatively, because soluble galectin-3 binds to immobilized ligands in a positive cooperative manner by forming dimers or oligomers (Hsu, Zuberi et al. 1992; Massa, Cooper et al. 1993), it is possible that the peptides might block the dimerization of galectin-3, and therefore inhibit the recruitment of soluble galectin-3 into a galectin-3-TFAg complex.

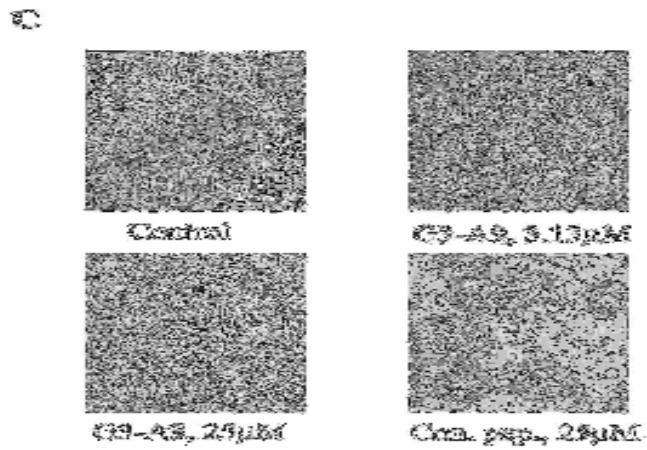
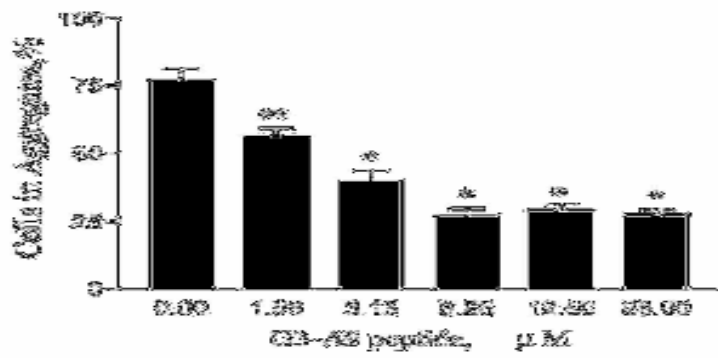
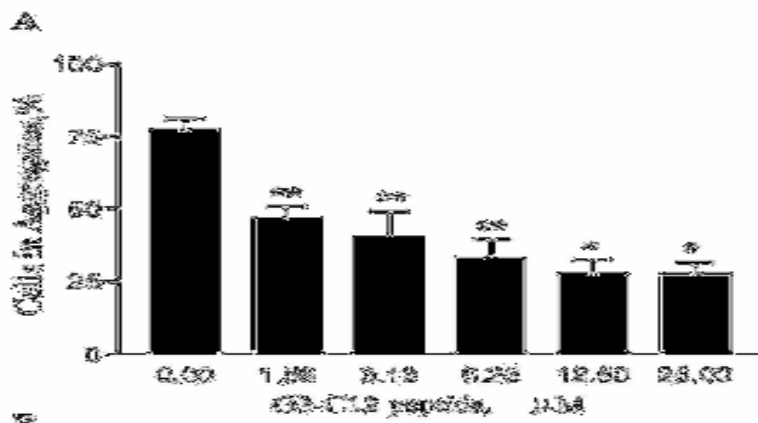
### ***Inhibition of Homotypic Adhesion of Metastatic Human Breast Carcinoma Cells by Galectin-3-Binding Peptides***

Metastatic cancer cells aggregate homotypically in a galectin-3-dependent manner to form multicellular intravascular clumps at sites of primary adhesion of tumor cells to the endothelium (Glinsky, Glinsky et al. 2003; Khaldoyanidi, Glinsky et al. 2003). Further, spontaneous carcinoma cell homotypic aggregation is mediated and promoted largely by interaction between cell surface TFAg and galectin-3 molecules (Glinsky, Huflejt et al. 2000; Glinsky, Glinsky et al. 2001; Glinsky, Glinsky et al. 2003). Since our peptides bound cell surface galectin-3 on MDA-MB-435 human breast carcinoma cells and inhibited TFAg-galectin-3 interactions, it was of interest to determine whether they would function to inhibit MDA-MB-435 homotypic aggregation. Both G3-A9 and G3-C12, but not an irrelevant control peptide, inhibited spontaneous MDA-MB-435 human breast carcinoma cell homotypic aggregation in a dose-dependent manner (Fig. 5A-C). Maximum reduction (~60 %) in homotypic aggregation was observed between 12.5-25  $\mu$ M for G3-C12 (Fig. 5A) and 6-12.5  $\mu$ M for G3-A9 (Fig. 5B).

### ***Anti-Galectin-3 Peptides Inhibit Adhesion of Human Breast Carcinoma Cells to Endothelium under Flow Conditions***

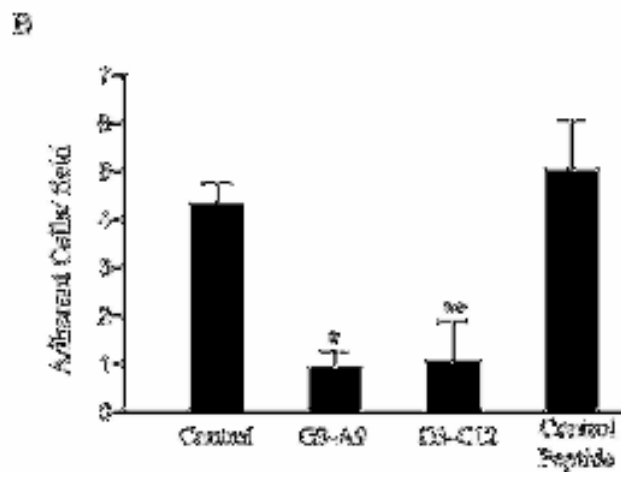
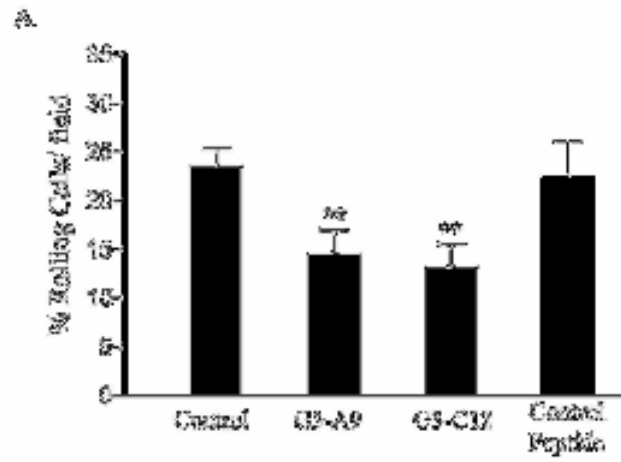
The metastatic cascade consists of coordinated, sequential steps (Glinsky, Huflejt et al. 2000; Glinsky, Glinsky et al. 2001; Glinsky, Glinsky et al. 2003), in which intravascular heterotypic adhesion between carcinoma cells and the endothelium precedes formation of intravascular carcinoma cell clumps. Heterotypic adhesion functions to slow the forward movement of blood-borne metastatic cells and facilitate their rolling and docking, which is necessary for more stable adhesive interactions to take place (Glinsky, Glinsky et al. 2001). Our model for involvement of galectin-3 in metastasis (Glinsky, Glinsky et al. 2001) suggests that endothelium-expressed galectin-3 may transiently interact with TFAg on carcinoma cell surfaces to slow the forward motion of tumor cells under flow conditions (Glinskii, Turk et al. 2004). Thus, we were interested in examining the effects of galectin-3-avid G3-A9 or G3-C12 peptides on carcinoma cell adhesion to human bone marrow endothelium (HBME) in a parallel plate laminar flow chamber system, which reconstructs *in vitro* the physical characteristics of *in vivo* microvascular blood flow (Orr, Wang et al. 2000). In our experiments, both rolling and stable adhesion of MDA-MB-435 human breast carcinoma cells on HBME-1 monolayers was inhibited significantly in the presence of anti-galectin-3 peptides, but not of the control peptide (Fig. 6, A,B). To rule out non-specific effects of peptide addition to the HBME-1 monolayer, in selected experiments, anti-galectin-3 and control peptides were added sequentially to the same HBME-1 monolayer. The test peptide (G3-A9 or G3-C12) was infused for ~30 min and inhibition of rolling and stable adhesion was recorded. The tumor cell infusion was halted and the HBME-1 monolayer was perfused with serum-free media for 20 min. After that, the infusion of cancer cells was resumed in the presence of the control peptide. As before, G3-A9 and G3-C12 inhibited MDA-MB-435 carcinoma

**Fig. 5. Galectin-3 binding peptides inhibit homotypic adhesion of MDA-MB-435 human breast carcinoma cells.** (A,B) MDA-MB-435 human breast carcinoma cell suspension was incubated with different concentrations (0-25  $\mu$ M) of G3-C12, G3-A9 or control peptide. Spontaneous aggregation was detected microscopically by fixing an aliquot of cells on a microscope slide. Four random fields from each slide were examined by transmission microscopy. Cells in aggregates in percent was calculated as described in “Materials and Methods” and plotted. The assay was performed in triplicate. \* - Means are significantly different (unpaired t-test),  $P < 0.0001$ . \*\* - Means are significantly different (unpaired t-test),  $P < 0.05$ . (C) Representative transmission photomicrographs.



**Fig. 6. Galectin-3 binding peptides inhibit rolling and adhesion of MDA-MB-435 human breast carcinoma cells to HBME-1 endothelial cells under flow conditions.**

(A) A single cell suspension of MDA-MB-435 cells was infused across HBME-1 cell monolayers in a laminar flow chamber in the presence or absence of 25  $\mu$ M of either G3-A9, G3-C12 or control peptide. (B) After stabilization, the number of rolling and adherent tumor cells was recorded. The data was presented as the mean  $\pm$  S.D. of three observation fields from one representative experiment of three independent experiments. (A, B) \* - Means are significantly different (unpaired t-test),  $P < 0.0001$ . \*\* - Means are significantly different (unpaired t-test),  $P < 0.05$ .





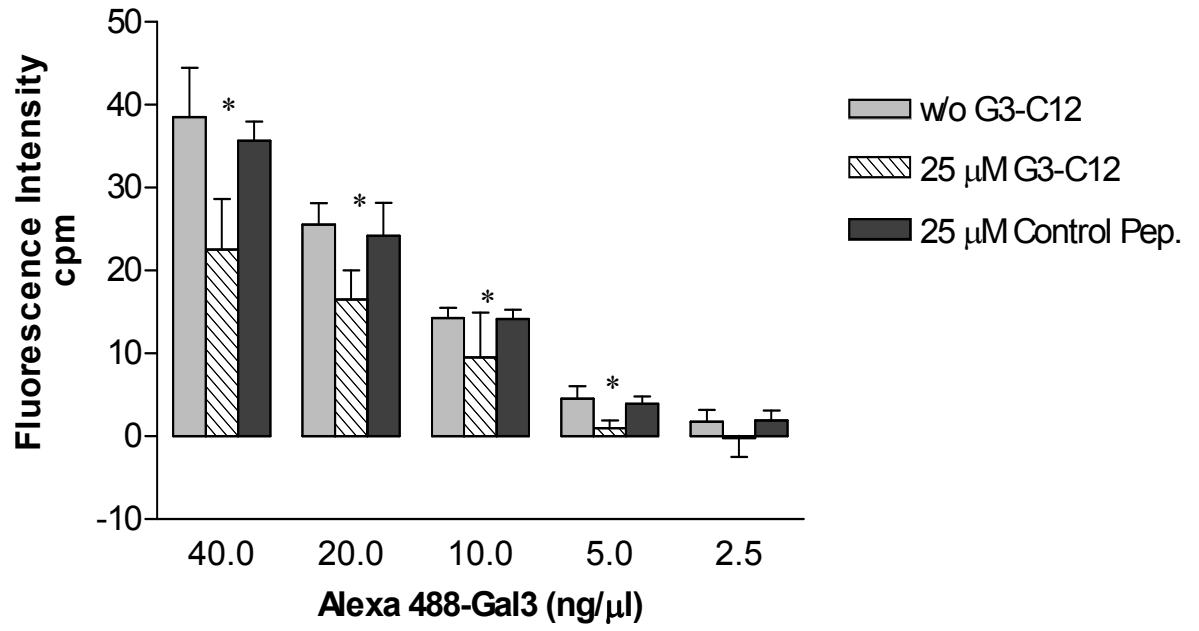
cell rolling and adhesion. However, once the anti-galectin-3 peptides were washed away from the system and the control peptide was introduced instead, the MDA-MB-435 carcinoma cells rolled and adhered (data not shown) as effectively as was observed for the fresh untreated, or control peptide-treated HBME-1 monolayers (Fig. 6A,B). These data demonstrate that, under physiologically relevant conditions, the anti-galectin-3 peptides inhibit metastasis-related heterotypic adhesion of carcinoma cells to bone marrow endothelium, in a manner dependent on transient galectin-3-mediated adhesive interactions.

### ***G3-C12 Inhibition of Dimerization of Galectin-3***

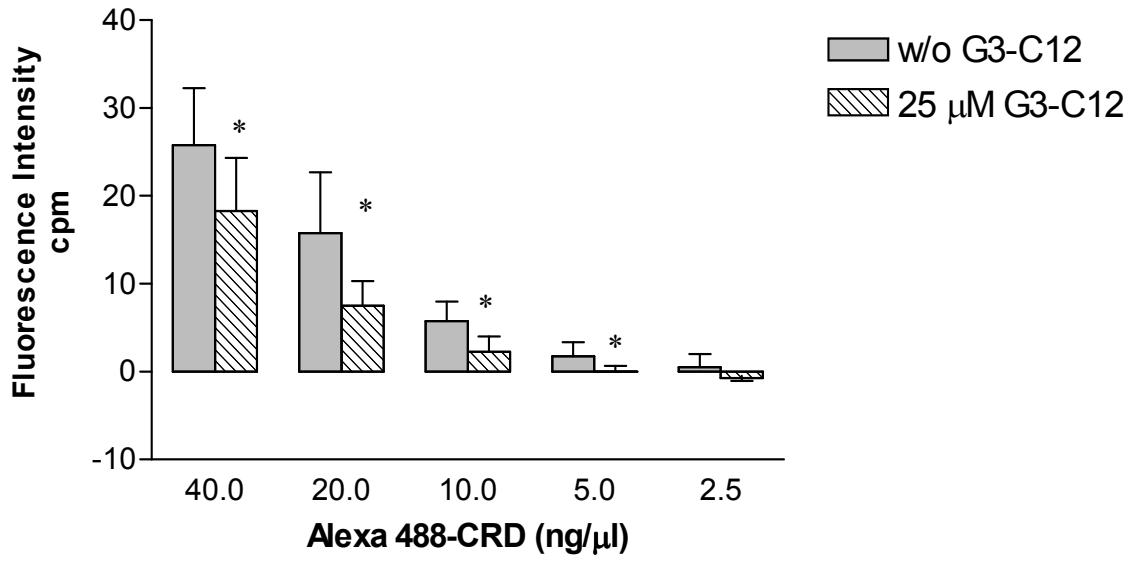
It is believed that galectin-3 could promote cell adhesion by forming dimers that cross-link the carbohydrate ligands on cell surfaces, such as TFAg (Kuklinski and Probstmeier 1998). Experiments with chemical cross-linkers demonstrated that approximately 45% of galectin-3 is in a dimeric form, which could cross-link cells and cause adhesion or aggregation (Yang, Hill et al. 1998). We conducted a self-association assay in the presence or absence of one of our galectin-3 binding peptides, G3-C12, to gain a better understanding of the role of galectin-3 in cell adhesion and how our peptides may modulate galectin-3 function. Galectin-3 self-association was previously studied by using biotinylated galectin-3 binding to immobilized galectin-3 (Yang, Hill et al. 1998). In our case, we tested the binding of AlexaFluor 488-labeled galectin-3 or galectin-3C, a CRD fragment created by collagenase cleavage, to immobilized biotinylated galectin-3 on streptavidin-coated plates. As shown in Figure 7, galectin-3 in solution bound to solid phase galectin-3 in a dose-dependent manner. We could not saturate the self-association even if the soluble galectin-3 concentration was as high as 40  $\mu\text{g/ml}$ , consistent with

**Fig. 7. Binding of AlexaFluor 488-labeled full-length galectin-3 (A) or galectin-3C (B) to immobilized galectin-3.** Immobilized galectin-3 was incubated with AlexaFluor 488-labeled full-length galectin-3 (concentration from 40 to 2.5 ng/ml) or galectin-3C (concentration from 40 to 2.5 ng/ml) in the presence of 25  $\mu$ M G3-C12. Binding was detected fluorometrically and the fluorescence detected from blank wells without immobilized galectin-3 (incubated with 40ng AlexaFluor labeled galectin-3 after blocking) were set as background. All the assays were performed in triplicate and data was subtracted background and then analyzed using GraphPad Prism 3.0 software. \* - Means are significantly different (paired t-test),  $P < 0.05$ .

A



B



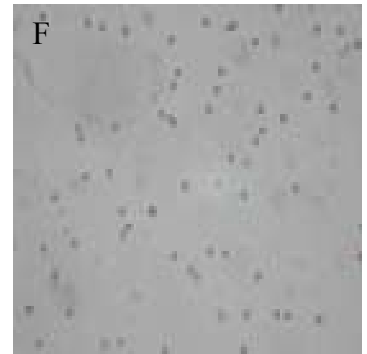
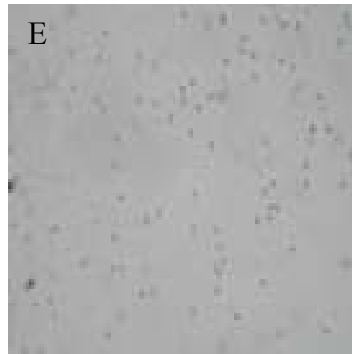
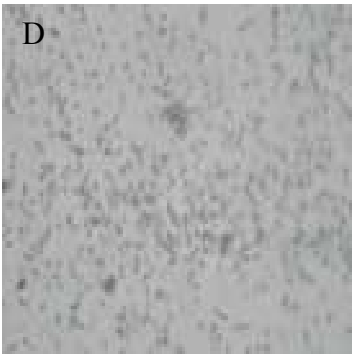
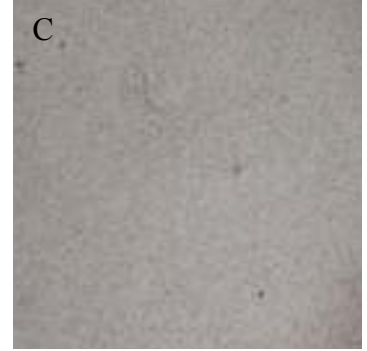
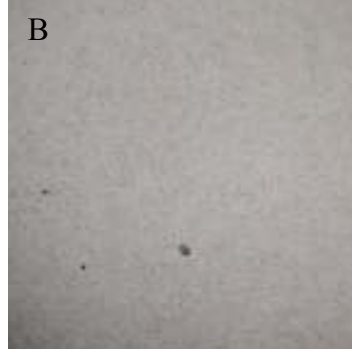
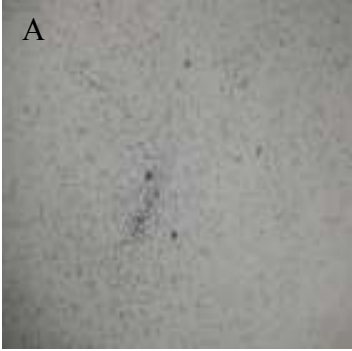
previous observation of positive cooperative binding of galectin-3 to its ligands. G3-C12 at 25  $\mu$ M could significantly inhibit the self-association of galectin-3 and galectin-3C bound to full-length galectin-3 (Fig. 7 A, B). Galectin-3C itself is not able to form a dimer, as described by Kuklinnski and his colleague (Kuklinski and Probstmeier 1998); however, it is still capable of binding to the full-length galectin-3, indicating that N-C interactions could be the mechanism by which that galectin-3 associates with itself. Since we have demonstrated that G3-C12 specifically binds to the CRD of galectin-3, it is possible that the peptide occupies or is in proximity to the site required for self-association. Of course, we cannot exclude the possibility that a conformational alteration elsewhere resulted from the peptide binding and thus disallowed the association.

#### ***G3-C12 Inhibition of Galectin-3/TFAg Mediated Aggregation***

As previously mentioned, galectin-3 but not galectin-3C can cause agglutination of erythrocytes in a lactose-inhibitable manner (Frigeri, Zuberi et al. 1993; Ochieng, Green et al. 1998). The cross-linking activity of galectin-3 could result from the bridging of two ligands on the cell surface, either by di/oligomeric galectin-3, or by a single galectin-3 with the N-terminal domain bound to some carbohydrate-independent ligands on one cell and a lectin domain bound to glycoconjugates on another cell surface. To avoid influences from other factors, Kuklinski et al devised a latex bead aggregation assay to test galectin-3 induced aggregation. In this assay, latex beads that mimic cells were coated with ASF. They demonstrated that ASF-coated beads aggregated in the presence of galectin-3, and this aggregation was inhibited by lactose but not by sucrose. Their data suggested that galectin-3 forms di/oligomers that mediate cell aggregation via a bridging of carbohydrate ligands on the cell surface. Similar to carbohydrate, the addition of the

peptide into this system resulted in a inhibition of aggregation, indicating that G3-C12 could disrupt the galectin-3 dimers that bridge glycoconjugates on the cell surface (Fig. 8). However, based on the inhibition ELISA results, it is reasonable to postulate that the peptide could work in a different way from lactose in this assay. Perhaps the peptide breaks the dimerization of galectin-3, whereas lactose competes off the galectin-3 ligands by occupying the CRD.

**Fig. 8. Aggregation assay using ASF- coated latex beads.** Latex beads coated with ASF were incubated for 1 h at room temperature with 50 $\mu$ g/ml galectin-3 in the absence (A, C) or the presence of 25 $\mu$ M G3-C12 (B, E) or 20mM lactose (C, F). The resulting aggregation patterns were analyzed by light microscopy with magnitude of 40 $\times$  (A, B, C) or 400 $\times$  (D, E, F).



## DISCUSSION

Development of intravascular secondary tumors, the precursors of metastatic lesions (Al-Mehdi, Tozawa et al. 2000), requires both heterotypic tumor cell adhesion to endothelia and homotypic adhesion between tumor cells (Glinsky, Glinsky et al. 2003; Khaldoyanidi, Glinsky et al. 2003; Glinskii, Turk et al. 2004). Previous observations from our laboratory demonstrated that TFAg is able to induce galectin-3 expression and its mobilization to the surfaces of cultured human endothelial cells *in vitro* (Balduis, Hanisch et al. 1999; Khaldoyanidi, Glinsky et al. 2003). Furthermore, galectin-3 cell surface translocation occurs intravascularly in well-differentiated microvessels of metastasis-prone tissues in response to metastasis-associated activation by TFAg expressed on circulating glycoproteins and tumor cells (Glinskii, Turk et al. 2004). Thus, endothelial galectin-3 might function to promote metastatic tumor cell adhesion to blood vessel walls by interacting with TFAg and/or other carbohydrate ligands displayed on tumor cells (Glinsky, Glinsky et al. 2001; Khaldoyanidi, Glinsky et al. 2003). Interactions between tumor cell-expressed galectin-3 and TFAg may also contribute to an increased metastatic potential by mediating homotypic adhesion between carcinoma cells (Glinsky, Huflejt et al. 2000). This line of homotypic intercellular communication between metastatic cells greatly increases their clonogenic growth and survival (Glinsky, Glinsky et al. 2003). Thus, blocking early intravascular galectin-3-mediated tumor cell adhesion has the potential to affect the entire metastatic process and control hematogenous cancer spread. Indeed, the efficient inhibition of breast (Glinsky, Price et al. 1996; Nangia-Makker, Hogan et al. 2002), colon (Nangia-Makker, Hogan et al. 2002), and prostate (Pienta, Naik et al. 1995) cancer metastasis has been achieved using carbohydrate-based



galectin-3 inhibitors, modified citrus pectin and lactulosyl-L-leucine (Glinsky, Price et al. 1996; Nangia-Makker, Hogan et al. 2002). Further, targeting  $\beta$ -galactoside-mediated interactions using truncated recombinant galectin-3 CRD also resulted in significant inhibition of spontaneous breast cancer metastasis (John, Leffler et al. 2003).

Using short synthetic peptides, which bind to the galectin-3 CRD with high affinity and specificity, may represent another attractive approach to inhibiting galectin-3 function and  $\beta$ -galactoside-mediated metastasis-associated tumor cell adhesion. Affinity and kinetic analysis suggested that the dissociation constant of the arbohydate-lectin interaction is in the mid-micromolar range (Mehta, Cummings et al. 1998; Nicholson, Barclay et al. 1998; Bachhawat-Sikder, Thomas et al. 2001). In contrast, peptide-protein interactions frequently occur with much higher affinity in the nanomolar range (Landon and Deutscher 2003; Landon, Zou et al. 2004). Thus, identifying peptides with high affinity that could block the corresponding CRDs of carbohydrate-binding proteins may represent a valuable new approach to modulating this protein function. In this study, we identified peptides that bind to the CRD of galectin-3 and thereby interfere with metastasis-associated carcinoma cell heterotypic and homotypic adhesion. The galectin-3 binding peptides were discovered using combinatorial phage display technology. We identified two peptides (G3-A9 and G3-C12) that bound to purified galectin-3 with high affinity ( $\sim 80$  nM; Fig. 2, Table 1) and recognized cell surface galectin-3 on cultured carcinoma cells and mouse monocyte cells (Fig. 4). Studies with full-length and N-terminal truncated galectin-3 proteins indicated that the peptides bound to the CRD (Fig. 5) and were capable of inhibiting the galectin-3 binding to TFAg displayed on glycoproteins (Fig. 4). Furthermore, the CRD-specific peptides were able to dramatically

inhibit both MDA-MB-435 human breast carcinoma cell homotypic and heterotypic adhesion (Fig. 5, 6). This indicates that small peptides are capable of blocking CRDs and thereby modulating their functions. Fukuda and co-workers previously reported that a peptide mimetic of an E-selectin ligand eliminated binding to sialyl Lewis X oligosaccharides, inhibited lung colonization by tumor cells, but cross-reacted with other selectins, including P- and L-selectin (Fukuda, Ohyama et al. 2000). In our study, both G3-A9 and G3-C12 showed remarkable specificity for the galectin-3 CRD and did not bind other galectins or plant lectins that were examined.

When both TFAg and galectin-3 are present on carcinoma cells, the interaction between the two is likely involved in metastasis-related homotypic tumor cell adhesion (Raz and Lotan 1987; Glinsky, Glinsky et al. 2003). Although the role of galectin-3 expressed on carcinoma cells is the subject of much debate (Lotz, Andrews et al. 1993; Schoeppner, Raz et al. 1995), previous observations from our laboratory have indicated that galectin-3 is clustered at the sites of homotypic contact. The results obtained in the present study suggest that the CRD of galectin-3 is exposed and available on the external cell surface, which correlates with other findings that galectin-3 is expressed both on cell surfaces and in the cytoplasm (Raz and Lotan 1987). The CRD-avid galectin-3 peptides bound to cell surfaces in a manner consistent with the varying levels of galectin-3 cell surface expression known to occur in these cell lines. The ability of the anti-galectin-3 peptides to block the galectin-3 CRD binding to its ligand was of further functional significance in that the peptides were able to interfere with homotypic carcinoma cell aggregation and adhesion (Inohara and Raz 1995; Khaldoyanidi, Glinsky et al. 2003).

Two of the peptides, G3-A9 and G3-C12, reduced carcinoma cell homotypic adhesion by over 50%. Taken together, these data suggest that galectin-3 is at least partially responsible for homotypic adhesion and that specific blockage of the CRD of cell surface galectin-3 is an effective anti-adhesive strategy. However, none of the galectin-3-binding peptides, TFAg binding-peptides or carbohydrate-based compounds that have been examined in this or previous studies (Glinsky, Glinsky et al. 2001) were able to inhibit homotypic adhesion completely, suggesting that there are probably several mechanisms involved in this type of adhesion.

Studies from our laboratory (Glinsky, Glinsky et al. 2001) and others (Lehr and Pienta 1998) suggest that endothelial cell localized galectin-3, may interact with  $\beta$ -galactosides exposed on carcinoma cells, to promote adhesion and metastasis. In support of this is the observation that PC-3 human prostate carcinoma cells (which do not express cell surface galectin-3), bind to endothelial cells in a manner that can be inhibited by an anti-galectin-3 MAb (Lehr and Pienta 1998). Similarly, an anti-galectin-3 MAb inhibited MDA-MB-435 human breast carcinoma cell rolling and adhesion to human umbilical vein endothelial cells under conditions of flow (Khaldoyanidi, Glinsky et al. 2003). We previously observed that exposure of endothelial cells to TFAg resulted in a 3-fold increase in galectin-3 expression on the endothelial cell surface (Glinsky, Glinsky et al. 2003) and that TFAg-binding peptides inhibited heterotypic carcinoma cell adhesion (Glinsky, Huflejt et al. 2000). These results imply that the galectin-3-TFAg interaction is involved in this process and that endothelial-derived galectin-3 may be able to significantly affect heterotypic carcinoma cell adhesion *in vivo* by creating adhesive “hot spots,” which may be at least partially responsible for the initial transient carcinoma cell

tethering to the endothelium (Glinsky, Glinsky et al. 2001; Khaldoyanidi, Glinsky et al. 2003; Glinskii, Turk et al. 2004). This event, in turn, may slow the forward movement of carcinoma cells sufficiently to allow other protein-protein interactions to participate in strong and irreversible binding of the carcinoma cells to the vascular endothelium.

We employed the galectin-3 CRD-binding peptides as inhibitors of galectin-3 adhesive function to analyze the importance of galectin-3 in metastatic cell adhesion to human bone marrow endothelium in an assay designed to replicate the *in vivo* environment of the microvasculature. The peptides dramatically inhibited heterotypic adhesion 60-70% and reduced the percentage of rolling cells in the floating cell population. These findings suggest that galectin-3 is involved in initial transient contacts, which mature eventually into tight adhesive bonds between cancer cells and endothelium. Adhesion of carcinoma cells to the endothelium *in vivo* is a complex process involving a variety of mechanisms. Endothelial selectins (Tozeren, Kleinman et al. 1995), integrins (Lafrenie, Gallo et al. 1994) and other cell surface molecules can promote the adhesion of tumor cells to endothelium in the presence of shear forces that are encountered within the microvascular environment. Recent observations from Al-Mehdi, *et al.*, demonstrate that endothelium-attached cells are required for development of secondary tumors, suggesting that early intravascular metastasis formation could be inhibited by intravascular drugs that reduce the attachment of tumor cells to the vascular endothelium (Al-Mehdi, Tozawa et al. 2000). Small galectin-3-specific peptides, such as those described here, open new avenues for the investigation of galectin-3 functions and the role of carbohydrate-lectin interactions in hematogenous cancer metastasis.

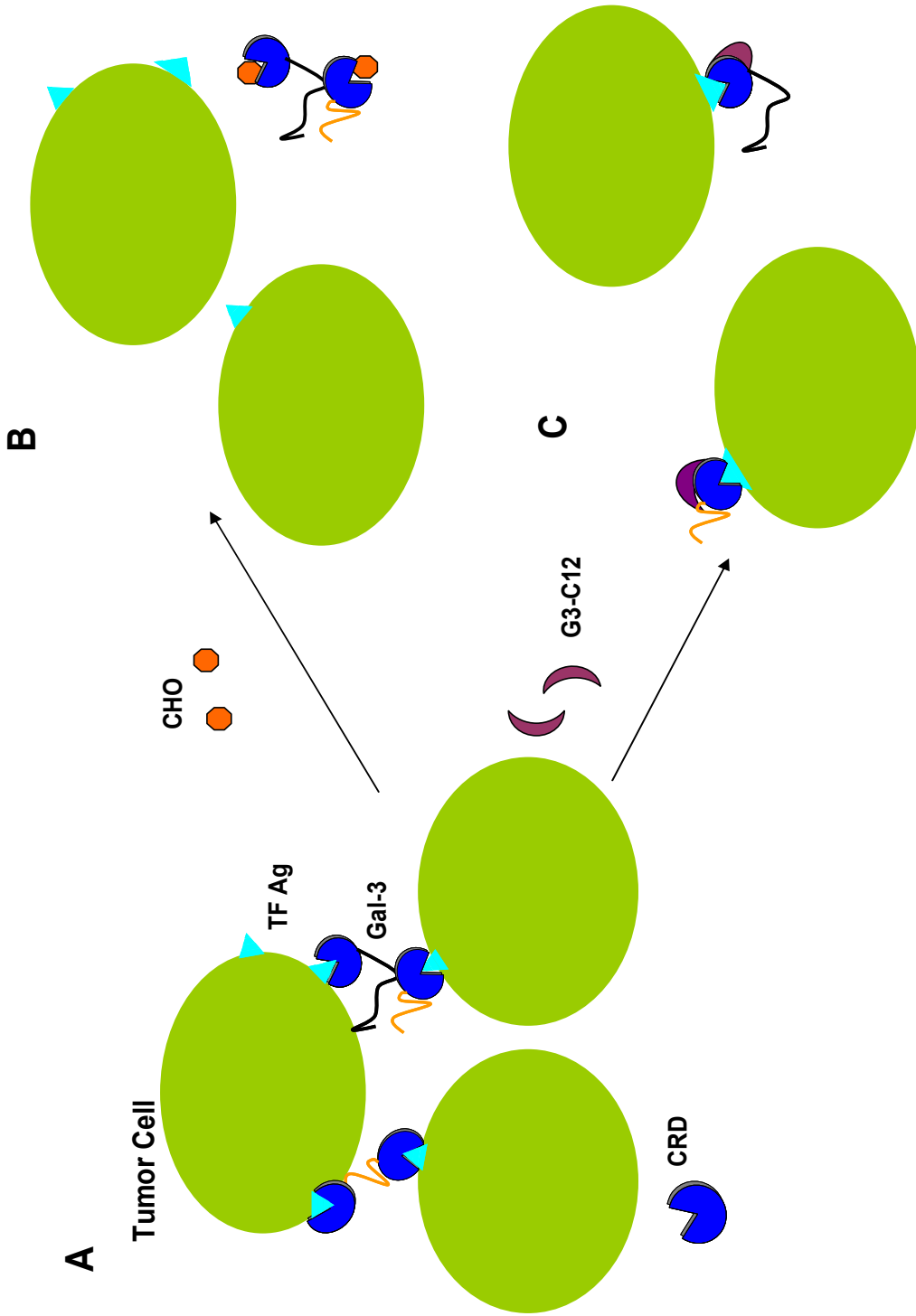
Galectin-3 is thought to be anchored into the cell surface via its CRD being bound to glycoconjugates on the cell surface. For example, Frigeri et. al., demonstrated that cell surface galectin-3 on macrophages and red blood cells was almost completely eluted off with lactose (Frigeri and Liu 1992). Furthermore, the amount of galectin-3 has been shown to correlate with the expression of certain glycoconjugates on the macrophage cell surface, and galectin-3 and its carbohydrate ligands have been found to co-precipitate in chemical cross-linking experiments (Sato and Hughes 1994). However, although the CRD bound to carbohydrate ligands on the cell surface, the galectin-3 CRD was still accessible or exposed. A rabbit antibody against a synthetic peptide of the CRD showed a similar binding pattern, on the cell surface, as was seen with another N-terminal specific antibody. Cell surface binding assays that we performed also demonstrated that CRD specific peptides showed a high degree of galectin-3 recognition on the cell surface similar to a galectin-3 mAb. Since galectin-3 is attached on the cell surface via its CRD, it was originally thought that only N-N domain interactions could lead to cell bridging, unless one postulated a cell binding site that could recognize the N-terminus of galectin-3, thus leaving the CRD available for other galectin-3 interactions (Fig. 6).

However, our investigations with the galectin-3 CRD specific peptides facilitate understanding of how galectin-3 mediates cell aggregation. The peptide G3-C12 binds to the CRD specifically and also blocks galectin-3 dimerization, indicating that the peptide binding site on the CRD may be at or close to the interface of dimerization. Obviously, further mutation and structural studies will be necessary to understand the dimerization properties of galectin-3 completely. Our biochemical binding experiments suggest that galectin-3C is able to bind to intact galectin-3 that has an N-terminal domain. However, it

has been demonstrated that galectin-3C does not bind to itself (Kuklinski and Probstmeier 1998). Collectively, we speculate that N-C domain interactions are the major mechanism by which galectin-3 forms dimers and cross-links cells (Fig. 9). If this assumption is correct then one would expect that cell surface galectin-3 monomers can recruit soluble galectin-3 or even free CRD fragments to form a cell surface multivalent complex, permitting stronger interactions with multi-glycosylated proteins. Alternatively, perhaps multivalent carbohydrate ligands bind to cell surface galectin-3, which then sequentially recruit soluble galectin-3 or free CRD, leading to a multivalent complex. This scenario could explain the observed positive cooperative binding of galectin-3 to a glycol-ligand (Hsu, Zuberi et al. 1992; Massa, Cooper et al. 1993). Our experiments have shown that soluble galectin-3 binding to immobilized ASF was inhibited by G3-C12, which could result from the blocking of di/oligomerization of galectin-3, eliminating the positive cooperative recruitment of soluble galectin-3. Furthermore, dramatic inhibition of G3-C12 on tumor cell adhesion and aggregation provides an insight into how important this di/oligomerization is in these adhesion events (Fig. 9).

Dramatic elevation of cell surface galectin-3 was notable after the endothelial cells were activated with certain stimuli (Frigeri and Liu 1992; Glinsky, Glinsky et al. 2003). Our previous studies demonstrate a 3-fold increase in galectin-3 expression on endothelial cells exposed to exogenous TFAg, suggesting that galectin-3 on the cell surface could accumulate and, therefore, provide the necessary concentration for dimerization. Furthermore, localization experiments revealed that galectin-3 clustered toward the sites of heterotypic adhesion on endothelial cells or to the homotypic cell-cell interfaces on tumor cells, indicating that concentration-dependent dimerization of

**Fig .9. A hypothetical schematic model for galectin-3 self-association in tumor cell adhesion.** A. Galectin-3 homodimer (two molecules of full-length galectin-3) or heterodimer (full-length galectin-3 + CRD) could bridge two neighboring tumor cells via binding to glycoconjugates on the cell surface, such as TFAg. N-C domain interactions may still allow two carbohydrate binding sites to be available, because the dimerization interface on the CRD of galectin-3 is far away from the carbohydrate binding site. B. The addition of exogenous carbohydrates could compete off the TFAg from galectin-3, and inhibit cell aggregation by occupying the sugar-binding site. Therefore, dimeric galectin-3 could be released from the cell surface. C. G3-C12 peptide inhibits cell aggregation in a different way: when bound to galectin-3, the peptide may divert the dimeric galectin-3 into monomer form by blocking the dimerization interface. In this case, monomeric galectin-3 could still bind carbohydrate ligands on the cell surface but would have no cross-linking ability.





accumulate and, therefore, provide the necessary galectin-3 could form and, thus, mediate cell adhesion via the interaction with glycoproteins such as TFAg (Glinsky, Glinsky et al. 2003). Taken together, these results suggest that self-association of galectin-3 may be able to mediate heterotypic or homotypic carcinoma cell adhesion by creating adhesive sites on the cell surface that, in turn, bind to glycoproteins or galectin-3 molecules on neoplastic cell surfaces. In addition to synthetic carbohydrates that occupy the carbohydrate binding sites, a small peptide that blocks the dimerization interface of galectin-3 would provide new insight for developing novel cancer therapies.

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## CHAPTER 3

### **Biodistribution of Filamentous Phage Peptide Libraries and Galectin-3 Binding Phage in Mice:**

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## CHAPTER SUMMARY

*In vivo* phage display is a new approach to acquire peptide molecules that bind stably to a given target. Phage peptide display libraries have been selected in mice and humans; and, numerous vasculature-targeting peptides have been reported. However, *in vivo* phage display has not typically produced molecules that extravasate to target specific organ or tumor antigens. Phage selections in animals have been performed for very short times without optimization for biodistribution or clearance rates to a particular organ. It is hypothesized that peptides that home to a desired antigen/organ can be obtained from *in vivo* phage experiments by optimization of incubation times, phage extraction and propagation procedures. To accomplish this goal, one must first gain a better understanding of the *in vivo* biodistribution and the rate of clearance of engineered phage peptide display libraries. While the fate of wild type phage in rodents has been reported, the *in vivo* biodistribution of the commonly used engineered fd-tet M13 phage peptide display libraries (such as in the fUSE5 vector system) have not been well established. Here, we report the biodistribution and clearance properties of fd-tet fifteen amino acid random peptide display libraries, in fUSE5 phage, in three common mouse models employed for drug discovery – CF-1, nude, and SCID mice.

Numerous peptides derived from phage display libraries have been demonstrated to have anti-tumor effects by inhibiting various steps of transformation (Bottger, Bottger et al. 1996) and metastasis (Fukuda, Ohyama et al. 2000). Moreover, directly utilization of phage bearing specific peptides that target a tumor marker could be a feasible approach to deliver anticancer drugs or imaging agents. We had success in the *in vitro* isolation of

phage that bind to galectin-3, which is reported to promote tumor cell adhesion and is highly expressed in some types of malignant cells. The synthesized peptides we isolated were able to specifically bind to galectin-3 on tumor cell surfaces and efficiently inhibit tumor cell adhesion (Zou, Glinsky et al. 2005), which makes it possible that galectin-3 binding phage might preferentially target to galectin-3 expressing tumor tissue, under appropriate *in vivo* selection condition. Based on our basic *in vivo* pharmacokinetic data of phage library, we therefore investigated the biodistribution of galectin-3 binding phage in mice heterotransplanted with MDA-MB-435 human breast carcinoma cells that has been found as a galectin-3 high-expression tumor cell line (Khaldoyanidi, Glinsky et al. 2003). Both normal tissues and tumor tissue were collected and the number of viable phage in each sample was determined. Not unexpectedly, high phage uptake was found in the reticuloendothelial system (RES), with fewer phage taken up in the tumor compared with that in the spleen and the liver. This could have resulted from the wide distribution of galectin-3 in the body, higher extravasation rates in organs (spleen and liver) with discontinuous basement vasculature (Yip, Hawkins et al. 1999), or non-specific adherence of phage themselves. Although galectin-3 binding phage were not able to exclusively target to tumor tissue, this study of the *in vivo* behavior of phage that will be valuable in the design of *in vivo* affinity selection.

## INTRODUCTION

Combinatorial technologies such as phage display provide a powerful approach to vaccine development and in discovery of new molecules that home to a desired target. In phage display, foreign peptides or antibodies are displayed on the surface of modified bacterial viruses, i.e. phage (Scott, Loganathan et al. 1992; Barbas, Hu et al. 1994; Smith and Petrenko 1997; Agris, Marchbank et al. 1999; Dreier, Beerli et al. 2001). Most studies have been based on the fUSE5 phage vector system originally developed some twenty years ago by George P. Smith, in which foreign peptides are incorporated into the N-terminus of minor coat protein III (pIII) of fd-tet phage so that at most five copies of the peptide are displayed (Smith 1985; Scott and Smith 1990; Smith and Petrenko 1997). Phage fd-tet are long filamentous viruses that infect *Escherichia coli* (*E. coli*) harboring the F-plasmid (Pratt, Tzagoloff et al. 1969; Smith and Petrenko 1997). Foreign peptides have also been fused to the major coat protein VIII (pVIII) of phage so that thousands of copies of the peptide can be displayed (Smith and Petrenko 1997; Hoess 2001; Smith 2003). Peptides corresponding to pathogenic, antigenic determinants including respiratory syncytial virus glycoprotein G (Bastien, Trudel et al. 1997), *Plasmodium falciparum*, glycoprotein G of herpes simplex virus 2 (Grabowska, Jennings et al. 2000), hepatitis B (Wan, Wu et al. 2001), and HIV-1 reverse transcriptase (De Berardinis, Sartorius et al. 2000) have been expressed on pIII and pVIII of fd-tet phage and elicit a strong immune response in mice, suggesting that phage may be vehicles for vaccine development (Willis, Perham et al. 1993; Gaubin, Fanutti et al. 2003).

A more widespread application of fd-tet phage in molecular biology and biotechnology involves the use of combinatorial phage peptide display libraries for novel

ligand identification. Phage display libraries can contain billions of random linear or cysteine constrained peptides ranging from ~6-35 amino acids in size. From these immense libraries, rare peptides that bind almost any given target including receptors (Johnson, Farrell et al. 1998), enzymes (Atwell and Wells 1999), nucleic acids (Agris, Marchbank et al. 1999; Dreier, Beerli et al. 2001) or carbohydrates (Peletskaya, Glinsky et al. 1997; Landon, Peletskaya et al. 2003) have been readily isolated by *in vitro* affinity selection. An underlying problem with *in vitro* selections of phage peptide libraries is that while the peptides often possess high affinity for the target antigen *in vitro* they may not effectively target the desired antigen *in vivo*. This may be due to low antigen expression *in vivo* as well as poor peptide affinity, specificity, and/or stability.

To solve this dilemma, *in vivo* combinatorial chemistry methods are being developed. The *in vivo* application of combinatorial libraries has the advantage over *in vitro* selection strategies in that one can “select”, in the environment of the whole animal, peptides that bind with good stability to a tissue. Rouslahti and Pasqualini pioneered this approach using live mice to select for organ-targeting peptides (Pasqualini and Ruoslahti 1996; Arap, Pasqualini et al. 1998). The biodistribution of RGD-containing or random peptides in a Cys-X7-Cys constrained fd-tet phage library in the fUSE5 vector system was examined in brain, kidney, and tumors of MDA-MB-435 human breast cancer or melanoma-bearing nude mice (Pasqualini, Koivunen et al. 1997). The number of phage transducing units (Tu, Ho-Schleyer et al.) was determined after very short post-injection incubation times (2 and 4 min). They found ten-fold more RGD phage versus library phage in the tumors compared to the examined organs. Immunohistochemical staining of the phage in tumors and organs after 24 h showed RGD-phage but not library staining of

the tumors. These *in vivo* selections (Pasqualini and Ruoslahti 1996; Pasqualini, Koivunen et al. 1997; Arap, Pasqualini et al. 1998; Pasqualini, McDonald et al. 2001; Arap, Haedicke et al. 2002; Arap, Kolonin et al. 2002; Curnis, Arrigoni et al. 2002; Essler and Ruoslahti 2002; Pasqualini and Arap 2002) as well as those reported by others (Asai, Fukatsu et al. 2002; Asai, Nagatsuka et al. 2002) resulted in a plethora of peptide sequences with integrin-avid motifs, including RGD, GSL, or NGR that targeted organ or tumor vasculature (Pasqualini, Koivunen et al. 1997; Arap, Pasqualini et al. 1998; Arap, Haedicke et al. 2002; Curnis, Arrigoni et al. 2002). Because these results indicated that each organ might contain vasculature with unique features that may be targeted through combinatorial chemistry approaches, a consortium of researchers initiated a project to map tissue-specific vascular addresses in humans, using similar *in vivo* phage display techniques. Thousands of peptide motifs were identified that were unique to various human tissues including prostate and skin (Arap, Haedicke et al. 2002; Arap, Kolonin et al. 2002). However, the reported *in vivo* experiments may have preferentially selected for small and common motifs such as the  $\alpha_v$  integrin-targeting RGD motif, because of their short selection times (15 min or less) and propagation of only a miniscule portion of the entire phage library (Pasqualini, Koivunen et al. 1997; Arap, Kolonin et al. 2002). Furthermore, the optimal time for phage to distribute to and clear from organs and tissues was not reported.

Thus, a scheme that favors selection of longer, higher-affinity and specific motifs, which would be very rare in the initial library (Smith and Petrenko 1997), would have benefits over an experiment designed to select very short motifs, present in many independent clones in the initial random peptide library. In addition, these experimental



conditions may yield peptide-bearing phage that can migrate from tumor vessels, which are known to be particularly leaky to large macromolecules including phage (Hobbs, Monsky et al. 1998; Hashizume, Baluk et al. 2000; Pasqualini, McDonald et al. 2001; McDonald and Baluk 2002), to target specific cell surface or intracellular antigens. We hypothesized that *in vivo* selection schemes that are optimized for incubation time in the animal while sampling the entire library population will more effectively exploit the power of combinatorial chemistry and, further, may yield phage-bearing peptides that migrate from the vasculature and target specific cell surface or intracellular antigens (Landon and Deutscher 2003). However, the successful *in vivo* application of tumor avid phage will be improved from a better understanding of the *in vivo* biodistribution, stability, and rate of clearance of the engineered fd-tet phage libraries often used today in combinatorial affinity selections. To gain this knowledge, we set out to examine organ and tissue biodistribution and clearance of the phage in three common mouse models for drug discovery – CF-1, nude, and severe combined immune deficiency (SCID) mice.

The knowledge of distribution and extravasion into interstitial compartment is tremendously helpful for the design *in vivo* affinity selections; also, it enables us to further our *in vivo* studies of tumor-specific phage with maximal beneficial outcomes. We had success in the *in vitro* isolation of phage that bind to galectin-3. The synthesized peptides we isolated were able to specifically bind to galectin-3 on tumor cell surfaces and efficiently inhibit tumor cell adhesion (Zou, Glinsky et al. 2005). While most *in vivo* applications of tumor-avid phage have been focused on targets inside of the cancer vasculature, we hypothesized that under the appropriate conditions galectin-3 binding phage might preferentially target to tumor tissue with high expression levels of galectin-3.

To investigate this, we performed a preliminary biodistribution study of galectin-3 binding phage in SCID mice heterotransplanted with MDA-MB-435 human breast carcinoma cells. Unquestionably, the former studies of biodistribution of phage libraries provide information about optimum extravasation time and binding background levels of phage injected.

## MATERIALS AND METHODS

### *Animals*

CF-1 normal mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Athymic nude mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). CF-1 mice possess a healthy immune system, whereas nude (athymic) mice are T-cell deficient, but have normal B-cell and natural killer cell production (). The ICR SCID (Icr:Ha(ICR)-*Prkdc*<sup>scid</sup>) were purchased from Taconic Farms, Inc. (Germantown, NY). These mice are an outbred stock and are homozygous for the SCID gene (Bosma 1992; Nonoyama and Ochs 1996; Szabo, Zhao et al. 1998). Thus, they lack both T and B cells due to a defect in recombination V(D)J variable region of the immunoglobulin heavy chain ((Bosma 1992), Taconic Farms, Inc.) The backcrossing of SCID mice with ICR mice reduces observed leakiness in the original SCID strain that can result in B cell production and an antibody response (Bosma 1992). All mice were acquired at 4-5 weeks of age and were used within one month, following initial acclimation. The CF-1 mice were housed in regular cages and conditions, while the nude and SCID mice were housed in microisolators in a temperature and humidity controlled room. All mice had food and water *ad libitum*. All experiments were conducted according to protocols approved by the University of Missouri Animal Care and Use Committee and the Institutional Animal Care and Use Committee at the Harry S Truman Memorial Veterans Hospital.

### *Establishing Human Tumor Xenografts in SCID Mice*

For the biodistribution study of galectin-3 binding phage, we used SCID mice that were xenografted with MDA-MB-435 human breast carcinoma cells. 4-5-week-old SCID female mice were anesthetized with isoflurane to alleviate any pain or discomfort. Once the animal was under anesthesia, it received bilateral subcutaneous inoculations (using a 26 gauge needle) of 1 million MDA-MB-435 human breast cancer cells per flank in a 100 $\mu$ l cell suspension in PBS/Matrigel. The growth of tumors was monitored every 3 days. Galectin-3 binding phage was injected 3-4 weeks after inoculation when the tumor size is about 1-1.5 cm in diameter.

#### ***Phage library propagation, tissue processing, and biodistribution***

Cesium chloride purified filamentous fd-tet fUSE5 random peptide display library ( $2 \times 10^8$  primary phage clones, a generous gift from George P. Smith) was used in these studies. In a sterile hood using sterile equipment, the library was dialyzed against sterile phosphate buffered saline (PBS) before injection into mice. Mice were injected in the tail vein with  $1.5 \times 10^{10}$  TU fUSE5 in a total volume of 150  $\mu$ l in PBS. The phage were allowed to circulate in the mice for 5, 15, 30, 60 min and 24, 48, and 72 h. This sample collection strategy is similar to those described for pharmacokinetic analysis of high molecular weight liposomal drug formulations (Krishna, Webb et al. 2001; Gabizon, Shmeeda et al. 2003; Pastorino, Brignole et al. 2003) and monoclonal antibodies (Yang, Corvalan et al. 1999; Yang, Shenoy et al. 2003). The mice were then sacrificed by cervical dislocation. Blood was collected and the mice were perfused with 90 ml of sterile PBS prior to organ and tissue retrieval. The organs and tissue were removed, weighed, and stored at  $-80^{\circ}\text{C}$ . A portion of the tissue was formalin fixed. The distribution of the phage in the organs and tissues was determined after chopping the frozen tissue

with a razor blade followed by douncing in a 2 ml Kontes dounce homogenizer in 500  $\mu$ l of Dulbecco's Modified Eagles Medium + protease inhibitor + 0.25% BSA (DMPB). The tissues were washed three times with 1 ml DMPB. The final tissue pellet was weighed and 500  $\mu$ l of DMPB containing 0.25% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was added to facilitate phage extraction. The samples were placed on a rotator at 4°C for 1 h. These mixtures were used to infect K91BlueKan *E. coli* cells concurrently with appropriate controls to account for variability in phage infectivity. The amount of infectious phage particles in the blood was determined by incubating 50  $\mu$ l of blood with 500  $\mu$ l DMPB containing 0.25% CHAPS, rotating at 4°C for 1 h, prior to infecting the *E. coli*.

For the galectin-3 binding phage biodistribution study, the protocol was the same as above with only minor modification. Briefly, tumor-bearing mice were injected in the tail vein with  $1.0 \times 10^{12}$  TU/ml fUSE5 in a total volume of 100  $\mu$ l in PBS. The phage were allowed to circulate in the mice for 60 min. The sample collection strategy was the same with the exception that tumor tissue was also collected.

### ***Pharmacokinetic analyses***

Phage titer in the blood was analyzed at several time points by determining the titer of phage in the blood at each time point and then the plotting the normalized titer against circulation time. The highest titer value for each strain was used as 100% for that strain. Pharmacokinetic parameters describing phage elimination were estimated by nonlinear regression analysis (Steimer, Mallet et al. 1984; Motulsky and Ransnas 1987; Lindstrom and Bates 1990). Computerized fitting was conducted using Graphpad Prism 3.0 software. Half-life was estimated using the formula:  $t_{1/2} = \ln 2/k_{el}$ , where  $k_{el}$ , is the

elimination constant, which was derived from the nonlinear regression analysis. Volume of distribution ( $V_d$ ) was estimated using the formula:  $V_d = C_t/C_0$ , where  $C_t$  is the total number of phage particles injected ( $1 \times 10^{11}$  TU/ml) and  $C_0$  is the phage titer in the blood at time zero, as estimated by using nonlinear regression analysis. The magnitude of phage accumulation in organs was estimated by using Graphpad Prism 3.0 software to calculate the area under the curve (AUC) for a graph of tissue:blood ratio versus time.

### ***Immunohistochemical staining***

Tissue was embedded in Tissue-Tek O.C.T compound (Sakura Finetek, Torrance, CA) and was cut at  $-20^\circ\text{C}$  to a thickness of  $5 \mu\text{m}$  before applying to glass slides. Slides were air dried overnight at RT, treated with acetone for 10 min at  $4^\circ\text{C}$ , and air dried at RT. The slides were stored at  $-80^\circ\text{C}$  prior to use. For immunohistochemistry, slides were brought to RT, rinsed 2 times in PBS, and treated with 4% paraformaldehyde for 15 min. Slides were then rinsed 3 times in PBS, blocked with PBS containing 2% bovine serum albumin (BSA), and incubated for 1 h at RT with a 1:100 dilution of a rabbit anti-phage polyclonal antibody (gift of George P. Smith). Slides were rinsed for 2 min in 3 changes of PBS, blocked with PBS containing 2% BSA for 30 min at RT and a 1:200 dilution of an AlexaFlour 488 conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR) was added at RT for 30 min. Slides were again rinsed for 2 min in 3 changes of PBS, cover slips applied, and laser scanning confocal microscopy was performed on the sections using a Bio-Rad MRC 600 confocal microscope (University of Missouri Molecular Cytology Core Facility).

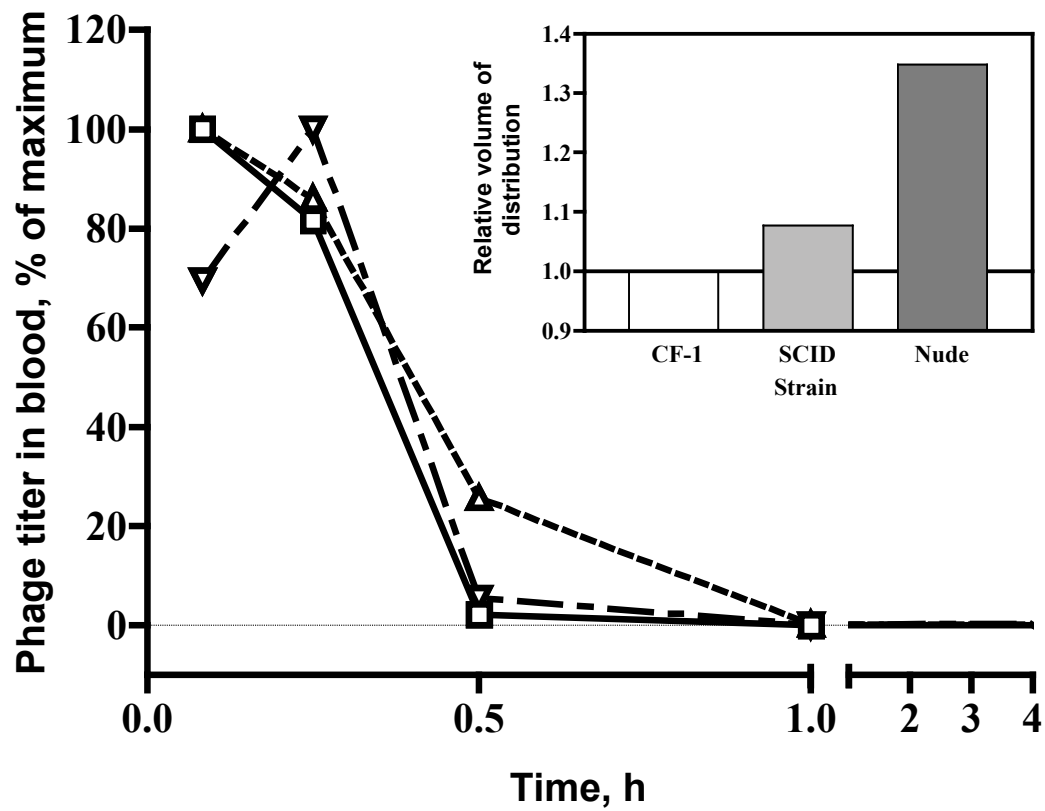
## RESULTS

### *Time course of phage elimination from blood*

The biodistribution pattern of the commonly employed fUSE5 phage random peptide library was evaluated in three laboratory strains of mice. CF-1, nude, and ICR SCID mice were chosen because they are among the most commonly used laboratory strains employed in biology, oncology, drug discovery and combinatorial chemistry and because they represent a range of immune system function backgrounds. The time course of elimination of infectious phage particles from the blood was analyzed (Fig. 1). In all experiments, this analysis defined only the concentration of infectious phage particles in the blood but did not quantify the reduction in blood concentration due to inactivation or break down of phage. Prior to 4 h, no differences in phage pharmacokinetics were observed between the mouse strains. As shown, the highest phage titer detected in blood occurred at 5 min for both CF-1 and SCID mice whereas phage titer in the blood of nude mice did not reach a maximum until 15 min of circulation time. The number of infectious phage in the blood was reduced after 15 min in all mouse strains. The half-life of phage in the blood of the mice was determined from nonlinear regression analyses. In CF-1 mice, the serum half-life of the phage was ~12 min, while it was 16 and 18 min in SCID and nude mice, respectively. These values are consistent with the slightly larger volumes of distribution found for phage in the SCID and nude mice (Fig. 1 inset).

**Fig. 1. Time course of phage elimination from blood.** The immunocompetent mouse strain (CF-1) was observed to more efficiently eliminate phage from the blood than did the immunocompromised strains (SCID, nude). The concentration of phage per ml of whole blood was determined as described in “Materials and methods.” The phage concentration in blood expressed as a percentage of the maximum titer observed in blood was plotted against time (in h) after injection. Pharmacokinetic parameters (half-life,  $t_{1/2}$ ; volume of distribution,  $V_d$ ) were estimated by using nonlinear regression analysis. Calculated half-life values were CF-1 (open squares),  $t_{1/2} = 12.1$  minutes; SCID (open upward triangle),  $t_{1/2} = 16.1$  minutes; nude (open downward triangle),  $t_{1/2} = 17.9$  minutes. Relative  $V_d$  (inset panel) was calculated by dividing the calculated  $V_d$  values for the SCID and nude strains by the calculated  $V_d$  value for the CF-1 strain.



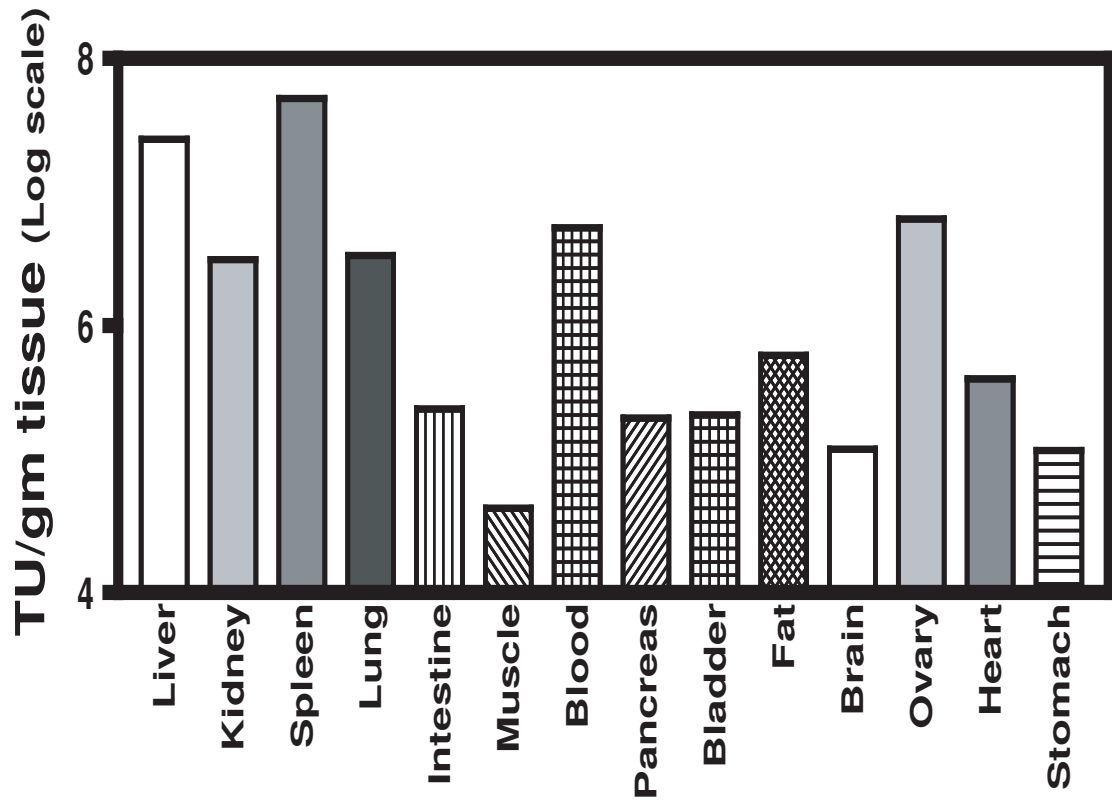


### ***Biodistribution of phage in blood and tissue***

The number of infectious phage particles (transducing units, TU) in organs and tissues at various times post-injection was determined from frozen tissue samples. Control experiments were conducted to determine that freezing did not significantly change the phage titer in the tissues and that 0.25% CHAPS was the optimal detergent concentration to maximize phage extraction with no reduction in infectivity (data not shown). The biodistribution of intact, infectious phage in all major organs and tissues in CF-1 mice was evaluated at 15 min (data not shown) and 1 h. As shown in Figure 2, there was a marked difference among organs in the amount of phage uptake per gram of tissue after 1 h, with liver and spleen retaining the most phage and muscle, pancreas, and brain harboring the least. These results are consistent with fd-tet phage preferentially accumulating in organs of the RES, as was also observed for MCO1 phage displaying Fab (Yip, Hawkins et al. 1999).

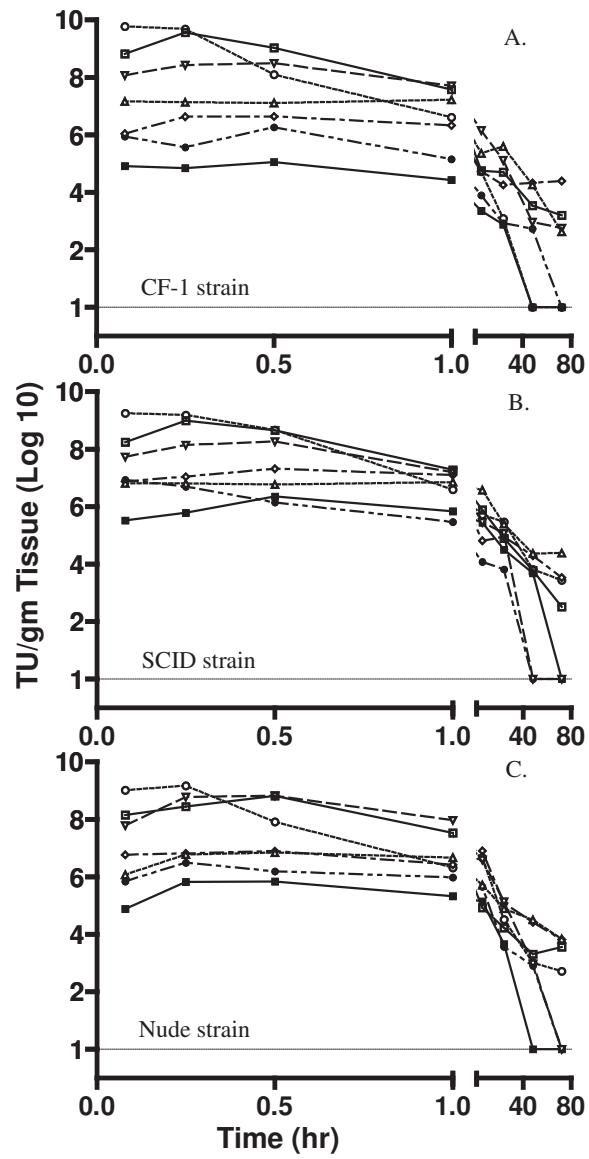
Subsequent comprehensive time course analysis in the three strains of mice focused on fd-tet phage library uptake in major organs (intestine, kidney, liver, lung, spleen and muscle) (Fig. 3). Depending on the study, up to three independent replicates of the pharmacokinetic experiments were performed with similar patterns of results observed among the replicates. For these studies, CF-1, nude and SCID mice were injected with phage, which were allowed to distribute for 5, 15, 30, 60 min and 24, 48, and 72 h. The titer of infectious phage particles in each organ was determined and plotted against time. Prior to 24 h, the differences between the strains in the phage detected per gram of tissue were subtle. At 5 and 15 min, the highest numbers of phage per gram

**Fig. 2. Organ distribution of phage in CF-1 mice at 1 hr post-injection.** After one hour of in vivo incubation, the mouse was sacrificed, the organs were excised and weighed, and phage remaining in the tissue were isolated and titered, as described in “Materials and methods.” Phage TU/gm tissue (Y-axis) was plotted against the organs (X-axis) from which tissue was collected. Bar shading is varied for clarity but does not correlate with individual organs. The data is derived from a single representative CF-1 mouse.



**Figure 3. Comparison of changes in distribution of phage with time in CF-1, SCID and nude mouse strains.** Phage were injected into the tail vein of CF-1, SCID and nude mice and then incubated for varying time lengths ranging from 5 min to 72 h. At the end of each incubation, animals were sacrificed, organs were excised and weighed and phage remaining in the tissue were isolated and titered, as described in “Materials and methods.” Log phage TU/gm tissue (Y-axis) was plotted against time in h (X-axis).

Symbols: Liver, □ , solid line; kidney, △ , dotted line; spleen, ▽ , dashed line; lung, ◇ , dash-dot line; intestine, ● , dash-dot-dot line; muscle, ■ , solid line; blood, ○ , dotted line. The values shown are the means ± range of up to three independent replicates with similar patterns of results observed among the replicates. Range bars were eliminated for clarity. (A) Phage distribution in CF-1 mice. (B) Phage distribution in SCID mice. (C) Phage distribution in nude mice.



tissue were detected in the blood in all three strains (Fig. 3). By 15 min, phage accumulation in the liver and spleen began to rise in all three mice strains. At 30 min, the phage concentration was beginning to decrease in all organs, in all three strains, with the highest number of phage per gram tissue, in all three strains, remaining in the liver and spleen. CF-1 and SCID mice, but not the nude mice, appeared to be retaining phage in the blood and kidney, as well. By 24 h of circulation, the CF-1 strain had the lowest number of phage per gram tissue in the blood. It was only at longer time points (48 and 72 h after injection) that significant differences in phage pharmacokinetics were observed between the strains. In the immunocompetent CF-1 mice, phage were detected only in the spleen, kidney, liver and lung but, most significantly, not in the blood. In contrast, infectious phage particles were not identified in the spleen of the immunocompromised SCID and nude mice by 72 h, although these strains still harbored phage in the liver, kidney, and lung. The blood of both the nude and SCID mice also contained infectious phage particles at 72 h.

### ***Tissue: blood ratio analysis***

Accumulation of intact infectious phage particles in the organs was analyzed by using tissue: blood ratios (as opposed to the concentration of infectious phage, used in the earlier analyses) (Table 1). In all tissues examined, the tissue: blood ratio of phage was much less than one at 15 min, which indicated the phage remained primarily in the blood at this time. At later time points, the circulation time to obtain optimum extravasation of phage to specific organs appeared to be an organ and mouse strain-specific characteristic that varied with the combination of mouse strain and organ. There was no overall pattern

Table 1. Tissue:blood<sup>1</sup> ratio at all time points in all mouse strains

Time <sup>2</sup>	Spleen			Liver			Kidney			Lung		
	CF-1	Nude	SCID	CF-1	Nude	SCID	CF-1	Nude	SCID	CF-1	Nude	SCID
5 min	0.011	0.008	0.012	0.026	0.040	0.035	0.001	0.000	0.004	0.000	0.005	0.008
15 min	0.021	0.507	0.038	0.350	0.082	0.369	0.002	0.002	0.004	0.001	0.005	0.011
30 min	0.844	11.783	0.495	2.520	<b>7.224</b>	2.319	0.058	0.024	0.046	0.021	0.197	0.246
1 h	9.321	<b>15.314</b>	<b>1.427</b>	<b>4.617</b>	1.788	<b>2.775</b>	0.578	0.739	1.165	0.621	0.816	<b>3.289</b>
6 h	34.578	5.112	0.595	0.343	0.075	2.214	<b>2.387</b>	0.429	<b>10.989</b>	0.995	<b>8.862</b>	0.091
24 h	<b>59.770</b>	6.021	0.385	0.779	0.823	0.272	2.172	<b>3.515</b>	0.625	<b>11.730</b>	5.349	0.158
AUC <sup>3</sup>	962.0	159.3	14.5	24.3	16.0	36.7	49.1	38.0	135.2	118.5	152.3	12.1

Time <sup>2</sup>	Intestine			Muscle		
	CF-1	nude	SCID	CF-1	nude	SCID
5 min	0.000	0.000	0.000	0.000	0.000	0.000
15 min	0.000	0.001	0.005	0.000	0.001	0.001
30 min	0.015	0.057	0.005	0.001	0.017	0.026
1 h	0.044	<b>0.552</b>	<b>0.059</b>	0.008	0.094	0.190
6 h	0.123	0.123	0.025	0.049	0.174	<b>0.814</b>
24 h	<b>0.295</b>	0.028	0.013	<b>0.489</b>	<b>0.203</b>	0.113
AUC <sup>3</sup>	3.9	2.8	0.3	4.5	4.4	10.7

<sup>1</sup>Tissue:blood ratio was calculated by dividing the titer (in TU/gm tissue) in a given organ at a given time by the titer in blood (in TU/ml) at that time.

<sup>2</sup>Time after injection.

<sup>3</sup>Area under the curve. AUC was calculated by using the GraphPad Prism software package.



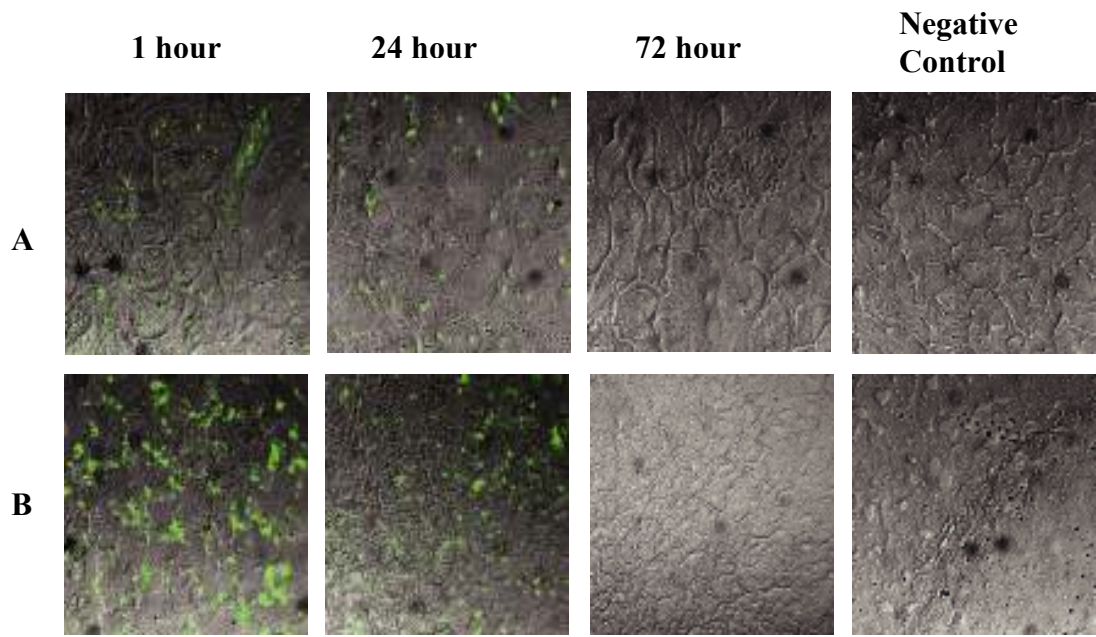
of peak tissue to blood ratios with respect to circulation time, although the peak tissue:blood ratios tended to occur slightly earlier in the SCID (i.e., peak lung ratio at 1 h) and nude (i.e., peak lung ratio at 6 h) mice than in CF-1 mice (i.e., peak lung ratio at 24 h). Furthermore, strain-specific differences in the magnitude of accumulation of phage in specific organs were observed, which were estimated by calculating area under the curve (AUC). In the CF-1 and nude strains, the AUC values indicated that over the 24 h study period, phage accumulated and remained infectious in the spleen and lung. In the SCID strain, however, phage appeared to pass through and then disappear from organs. In the kidney, a peak ratio (ratio = 10.99) occurred at 6 h although, at 1h and 24 h (ratio  $\cong$  1.0), there was no difference between the phage concentrations in tissue and blood (AUC = 38). Thus, preferential, but transient, phage accumulation was observed in the SCID kidneys. The data also indicated that over a long incubation period the immunocompetent CF-1 strain might be able to move phage, in terms of both time and magnitude, from the blood into the organs of the RES more efficiently than the other strains.

### ***Immunohistochemical analysis of phage biodistribution***

The localization of the phage in organs at various times post-injection was also analyzed using immunohistochemistry. These studies were done to visualize phage protein components and/or catabolites that may or may not represent functional infectious particles. Thin sections of tissue were analyzed for the presence of fd-tet phage using a rabbit anti-phage polyclonal antibody and detected using an AlexaFlour 488 conjugated fluorescent goat anti-rabbit antibody. Representative results are shown for kidney and liver sections from SCID mice. As shown in Figure 4, phage accumulated maximally in

**Figure 4. Histological detection of phage particles in excised SCID mouse organs.**

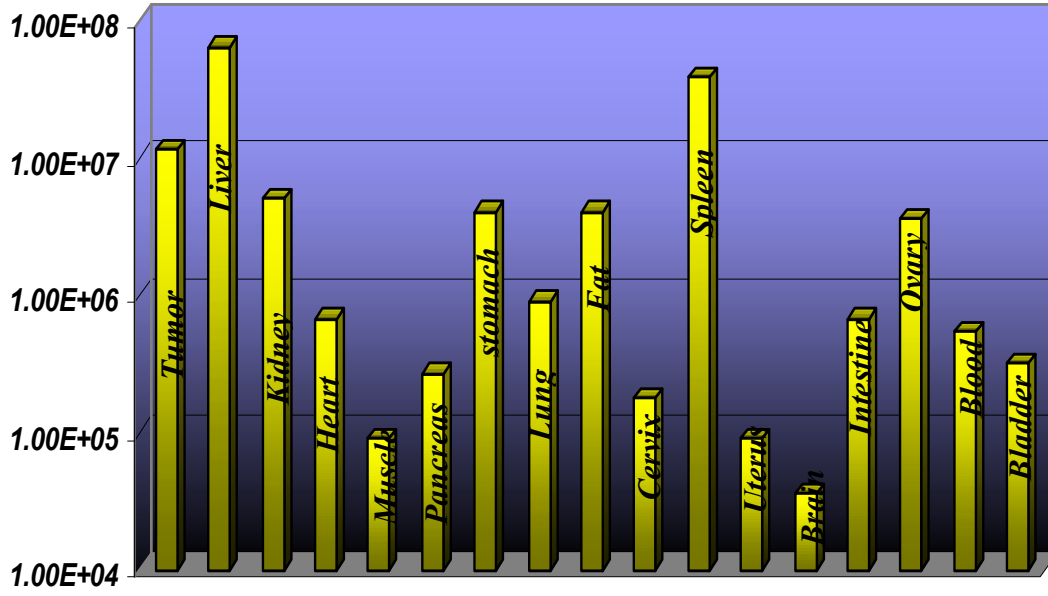
Phage particles remaining in (A) kidney and (B) liver at various times (shown above photographs) were detected using a polyclonal anti-phage antibody and an AlexaFluor 488 conjugated secondary antibody, as described in “Materials and methods.” The negative controls were tissue sections incubated with the secondary antibody only.



**Figure 5. Distribution of phage G3-A9 (A) and G3-C12 (B) in tissues at 1 h time point.** Values expressed as phage transducing unit (TU) per gram of tissue.

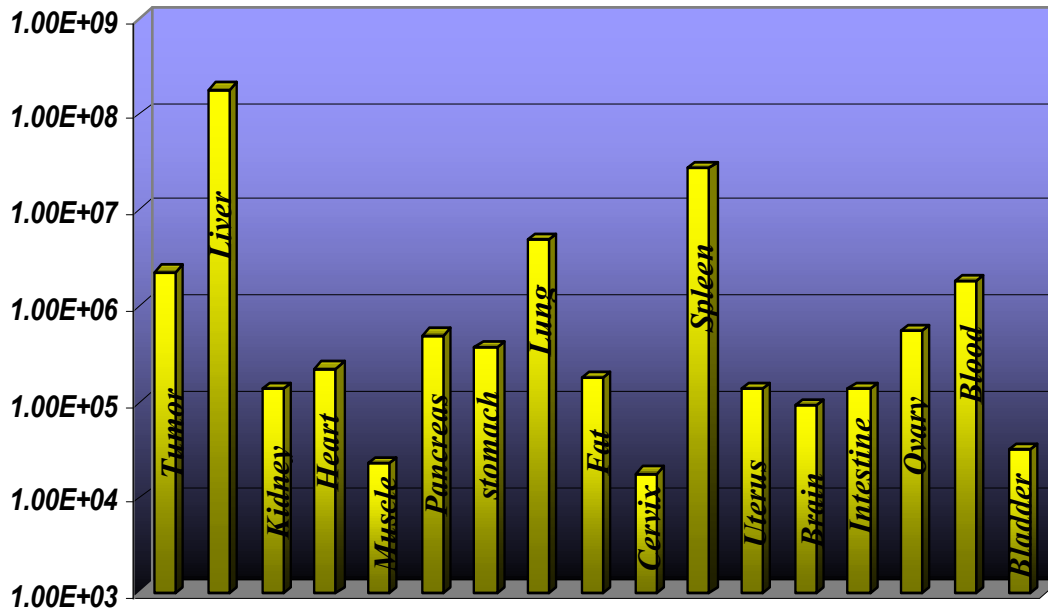
A

Tissue distribution of phage G3-A9



B

Tissue distribution of phage G3-C12



the SCID mice in the kidney and liver at 1 h. Phage could still be immunohistochemically detected at 24 h, but not by 72 h in all three strains of mice (data not shown). The inability to detect immunoreactive phage proteins in the nude and SCID mouse tissue sections at 72 h implies that this method of phage detection is not as sensitive as phage titering experiments (Fig. 3).

### ***Biodistribution of galectin-3 binding phage in tumor-bearing SCID mice***

From the biodistribution study of the phage library, the peak tissue:blood ratios tended to occur around the 1 h time point in SCID mice. Therefore, we studied the distribution of galectin-3 binding phage (Zou, Glinsky et al. 2005) 1 h after injection into tumor-bearing SCID mice. The mice were inoculated with MDA-MB-435 human breast carcinoma cell. Around 4 weeks after the inoculation, the tumors were approximately 1-1.5cm in diameter. Two of the galectin-3 binding phage, G3-A9 and G3-C12, were injected into the tail veins and rescued phage per gram was quantified. These two phage showed similar distribution patterns, with the liver and spleen having the highest phage uptake in both instances (Fig. 5 A, B). The phage uptake in liver and spleen was approximately  $1 \times 10^7 \sim 1 \times 10^8$  Tu/Gram tissue for both phage injections. For phage G3-A9, phage retained in the tumor was found to be higher than most organs, other than the liver or spleen, and was approximately 5-6 fold less than the phage found in the liver. Compared to the phage uptake in brain, ~300 fold more G3-A9 phage can be recovered from the tumor. G3-C12 uptake in tumor is less than that of G3-A9, and approximately 80 fold less than phage taken up by the liver. The variation of phage uptake in individual organs is unlikely due to the difference of perfusion in a given tissue. For example, phage uptake in tumor tissue is usually much higher than that in brain, although fewer blood

vessels are located in the tumor (Pasqualini, Koivunen et al. 1997). However, the ultrastructure of vasculature in liver and spleen might influence the distribution of phage, as discontinuous basement membrane in these organs could facilitate the extravasation of phage from circulation. Preferential retention in the RES, as seen in both phage library and galectin-3 binding phage injections, appears to be a property of phage in general (Geier, Trigg et al. 1973). Still, galectin-3 expressed on macrophage and kupffer cells in the RES organs could enhance preferential retention even further.

## DISCUSSION

The *in vivo* biodistribution properties of the commonly used engineered fd-tet phage peptide display libraries in the fUSE5 vector system have not been well established. However, the results, *in toto*, of studies, in which various phage types, including lambda (Geier, Trigg et al. 1973), T2,  $\phi$ X 174 (Uhr and Weissman 1965), T4 (Inchley 1969; Inchley and Howard 1969; Inchley 1970), and M13 (Ivanenkov, Felici et al. 1999) phage, were injected into animals have provided specific hints of the *in vivo* pharmacokinetic behavior of phage. Thirty years ago, Merrill and co-workers (Geier, Trigg et al. 1973) reported the fate of lambda phage in germ-free mice. They examined the number of plaque forming units in blood, kidney, liver, peritoneum, and spleen at various times post injection and found the spleen to contain the highest phage titer. Phage were detected in the spleen for up to 7 days. An antigen trapping mechanism in the spleen was thought to account for this long retention time. In contrast, the other organs examined showed little residual phage after 48 h. Merrill's work, as well as that of others, suggested the large phage particles possess biodistribution and clearance properties similar to colloidal particles that are processed through the RES, (Inchley and Howard 1969; Inchley 1970; Geier, Trigg et al. 1973). This analysis was echoed in a recent study by Molenaar, *et al.*, (Michon, Penning et al. 2002) who reported predominate uptake of <sup>35</sup>S-methionine/cysteine radiolabeled M13 phage by the RES and a serum half-life of 4.5 h. Importantly, the Molenaar study advanced the notion that phage were able to extravasate to subendothelial tissue and that such phage could be readily selected from a diverse random phage display library. Specific uptake of phage by parenchymal cells was analyzed and a small proportion (8%) of the wild-type phage were found to be



extravasated. Finally, a study with phage engineered to display antibody fragment (Fab) molecules indicated that not only the type of phage but also the nature of the displayed polypeptide might affect phage biodistribution *in vivo* (Yip, Hawkins et al. 1999). The serum half-life of these phage was found to be ~3.6 h. Predominant phage uptake occurred in the lungs, kidney, and liver as well as the spleen (Yip, Hawkins et al. 1999), which is in contrast to the lambda phage studies of Merrill (Geier, Trigg et al. 1973) where the spleen was, by far, the major organ for phage accumulation. Taken together, these findings lend further support to the notion that biodistribution of different types of phage – whether wild type or engineered – must be empirically determined in animals to optimize/facilitate discovery of organ or tumor cell specific-targeting phage and corresponding peptides.

In 1996, Rouslahti and Pasqualini pioneered phage display applications by performing their selections in mice to obtain organ-targeting peptides (Pasqualini and Ruoslahti 1996; Arap, Pasqualini et al. 1998). Several peptides, particularly those that bind to tumor vasculature, were discovered (reviewed in (Trepel, Grifman et al. 2000; Trepel, Grifman et al. 2000))). In particular, peptides containing the motif RGD predominated and targeted molecules on organ and tumor vasculature via the integrin  $\alpha_v\beta_3$  receptor. Since the peptides were selected with an RGD motif that targeted integrin molecules on the vasculature of organs and tumors, they collected the phage 2-4 min after injection. Nevertheless, we believe that it would be of far more value to be able to select peptides that actually target the cancer cell, not just the surrounding vasculature, especially if one wants to develop new cancer-specific imaging and/or therapeutic biomolecules. It is our supposition that previously employed *in vivo* selection schemes

consisting of short incubation times in the mice and propagation of a miniscule portion of the entire phage library did not take advantage of the very strengths of combinatorial chemistry and library diversity. We set up our time points at 5, 15, 30, 60 min and 24, 48, and 72 h, which would effectively yield phage-displaying peptides that target to the organs but not the vasculature. These data may be insufficient to detect the alpha phase of phage distribution in the blood of CF-1 and SCID mice. However, since our study focused on the kinetics of the extravasation process, which occurs at later time periods, the lack of data at time points prior to 5 minutes does not detract from our conclusions. Nevertheless, if one were interested in the kinetics of the phage distribution in the circulation, addition of one or more time points prior to 5 minutes might prove useful. The observed half-lives for the fd-tet phage library in all three strains of mice were shorter than the 4.5 h half-life reported for wild-type M13 phage in C57BI/6KH mice (Ivanenkov, Felici et al. 1999; Molenaar, Michon et al. 2002). These differences may be due to the use of engineered phage versus wild-type phage and/or the mouse strains utilized. However, modification of the wild-type M13 phage by lactosamination reduced the serum half-life of the phage to 18 min (Ivanenkov, Felici et al. 1999; Molenaar, Michon et al. 2002). One might surmise that modifications to the phage coat may drastically alter its half-life and/or clearance *in vivo*. This notion is further supported by results of the biodistribution properties of filamentous phage displaying Fab on pIII in nude mice. A blood half-life of 3.6 h was observed with predominant uptake in the lung, kidney, spleen, and liver (Yip, Hawkins et al. 1999). The long half-life was consistent with modification of the phage with large antibody fragments (~50 kDa) (Yip, Hawkins et al. 1999).

In general, the elevated tissue: blood ratios in RES organs suggested that clearance and catabolism of the phage in all three strains of mice occur via the major organs of the RES. Rapid blood clearance and high spleen uptake of macrophage phagocytosed phage were previously observed for T4 phage in normal mice (Inchley 1969; Inchley and Howard 1969; Inchley 1970). Interestingly, the catabolism of T4 phage via macrophages led to high levels of anti-T4 antibodies due to macrophage-lymphocyte interactions in the spleen (Uhr and Weissman 1965; Inchley 1969; Inchley and Howard 1969; Inchley 1970). It was suggested that RES capturing is independent of the peptide insert and could be prevented by co-injecting with non-infective fuse 5 phage (Pasqualini, Koivunen et al. 1997). In this study, phage particles were not detectable in the spleen of immunocompromised SCID and nude mice by 72 h, which suggested that phage extravasation to target organs might be more efficient in these animals. Alternatively, the spleen in the CF-1 mice may act to increase the specificity of phage extravasation by reducing the level of nonspecific phage binding in the target organs.

In order to investigate if galectin-3 binding phage could preferentially target to tumors that highly express galectin-3 proteins on the cancer cell surface, a biodistribution study of galectin-3 binding phage was conducted on tumor bearing mice. As also reported by Pasqualini and her colleagues, high phage uptake was found in RES organs, which is known to capture circulating phage in normal (Geier, Trigg et al. 1973) or immunocompromised mice (Pasqualini, Koivunen et al. 1997). Not only Kupffer cells or macrophages in RES organs capture the phage particles, but the vasculature ultrastructure in the liver and spleen also facilitates the extravasation of phage. The discontinuous basement membrane in the vasculature of the spleen and liver could allow phage to

penetrate quickly to tissue (Clough 1991). Another reason that could lead to this high uptake by the RES is that galectin-3 is highly expressed on macrophages and other antigen presenting cells (APC) (Rabinovich, Riera et al. 1999), which would attract the phage to these APC rich organs. Thus, overcoming the limitation that high phage uptake by the RES would be a challenge for the future application of phage targeting or delivery and/or *in vivo* selection.

Taken together, these results suggest that implementation of a short *in vivo* incubation time to allow phage to circulate may not be the best approach for isolation of organ-targeting phage-displayed peptides. Previous studies (Pasqualini and Ruoslahti 1996; Arap, Pasqualini et al. 1998; Arap, Haedicke et al. 2002) with phage libraries have allowed the phage to circulate for as little as 3 min and, at most, 15 min – times that are insufficient for phage to distribute to most organs, particularly in the immunodeficient mice, which are often used for drug discovery. The time it takes for optimal phage localization and clearance is dependent on the organ that is targeted and which strain of mice is utilized. Based on our findings, as well as other studies, biodistribution patterns are likely to vary between types of phage (fd-tet, MCO1, M13, lambda) and the nature of the displayed foreign molecule (Pasqualini, Koivunen et al. 1997; Ivanenkov, Felici et al. 1999; Yip, Hawkins et al. 1999; Molenaar, Michon et al. 2002). Moreover, prevention of high uptake of phage in RES organs would be a new challenge for utilization of phage in targeting or delivering imaging or toxic agent to tumor tissue. Our results may have widespread application because the phage libraries utilized in these studies are among the most commonly employed libraries used today in drug discovery and in *in vivo* combinatorial approaches.

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## CONCLUSION AND PERSPECTIVES

Metastasis consists of a series of sequential steps, including detachment of malignant cells from the primary site, migration to the distant secondary site and adhering to the endothelium (heterotypic adhesion) and each other (homotypic adhesion). A better understanding of the process of the metastatic cascade may facilitate the development of new strategies for cancer detection and treatment. Numerous molecular factors have been demonstrated to contribute to metastasis, including endogenous lectins-cell surface carbohydrate interactions. Malignant cells are characterized by various changes in cell surface carbohydrates as well as the levels of lectins. Likewise, lectins derived from normal cells might interact with carbohydrates on tumor cells during tumor cell metastasis. Galectin-3, a member of the  $\beta$ -galactoside specific lectin family, has been shown to promote hetero- and homotypic tumor cell adhesion by interacting with glycoconjugates with  $\beta$ -galactose termini, such as TFAg. Additionally, galectin-3 could cross-link the tumor cell to endothelial cells or other tumor cells by forming dimers or oligomers. It is logical, therefore, that the specific molecular inhibition of the interaction between galectin-3 and its ligands and/or its self-association may lead to the development of new effective antiadhesive therapies of cancer metastases.

The goal of this study was to examine the involvement of galectin-3 in carbohydrate-mediated metastatic heterotypic and homotypic cancer cell adhesion via interactions with the tumor-specific Thomsen-Friedenreich glycoantigen (TFAg). We hypothesized that blocking the galectin-3 with synthetic peptides would reduce metastasis-associated carcinoma cell adhesion. We first identified peptide antagonists of galectin-3 using combinatorial bacteriophage display technology. Not only did the

synthesized peptides bind to the purified galectin-3 with high affinity ( $K_d \cong 80$  nM), the peptides also specifically recognized cell surface galectin-3. Experiments using serial truncated galectin-3 proteins indicated that the selected peptides bound to the CRD of galectin-3. The galectin-3 CRD-specific peptides inhibited soluble galectin-3 binding to TFAg, and significantly down-regulated homotypic adhesion between human MDA-MB-435 breast carcinoma cells. We employed the galectin-3-homing peptides as inhibitors to analyze the importance of galectin-3 in metastatic cell adhesion to human bone marrow endothelium in an assay designed to replicate the *in vivo* environment of the microvasculature. The peptides dramatically inhibited heterotypic adhesion and reduced the percentage of rolling cells in the floating cell population. These findings suggest that galectin-3 may be involved in the initial transient contacts, which might eventually lead to the integrin-mediated stable adhesion to occur (Glinskii, Turk et al. 2004). These results are quite important in that they established for the first time a direct role of galectin-3-TFAg interactions in carbohydrate-mediated cancer cell adhesion and suggested that peptide binding of the galectin-3 CRD may ultimately serve as a functional modulator of metastasis. Moreover, we investigated the possible mechanism by which the peptides modulate galectin-3 function. Our biochemical studies demonstrated that one of our peptides, G3-C12, inhibited the dimerization of galectin-3 and blocked aggregation of TFAg-coated latex beads by dimeric or oligomeric galectin-3, indicating that dimerization of galectin-3 may also play a crucial role in cancer cell adhesion. Our studies on galectin-3 CRD specific peptides demonstrate that tumor cell adhesion is mediated by the interaction of galectin-3 and its ligands and/or its self-association. Blocking these steps with synthetic peptides derived from phage display

library may be a powerful approach to the development of new antiadhesive therapies of cancer metastases.

While cancer-targeting peptides have been widely used in research and clinical work, phage bearing specific peptides that target a tumor marker have also been successfully utilized to a certain extent. We thus investigated if our galectin-3 peptides could target to tumors that highly express galectin-3. Phage harboring galectin-3-binding peptides failed to exclusively target to human MDA-MB-435 breast tumors in mice, perhaps due to the extensive distribution of this protein, especially in the RES organs. However, the preliminary pharmacokinetic properties of random phage peptide display fd-tet libraries *in vivo* were fully investigated, which will be extremely useful in the design of future *in vivo* selection strategies. We examined organ and tissue biodistribution and clearance of the phage in three common mouse models for drug discovery – CF-1, nude, and severe combined immune deficiency (SCID) mice. Phage uptake studies suggested that phage remained primarily in the circulation at 15 min after injection, and for most organs notable extravasation occurs after 1 h. The information we learned from this *in vivo* biodistribution study will help us more effectively exploit the power of combinatorial chemistry and, further, may yield phage-bearing peptides that migrate from the vasculature and target specific cell surface or intracellular antigens (Landon, Peletskaya et al. 2003).

The current study provides insight into the role of galectin-3 in metastasis. Furthermore, it opens several new doors for future studies, including ongoing structural and *in vivo* studies with the peptides, which are a further exploration of the mechanism of lectin - carbohydrate mediated tumor cell adhesion and the therapeutic intervention

before metastatic adhesion. Structural and biochemical investigations of the galectin-3 CRD and full-length galectin-3 upon peptide and disaccharide (*N*-acetylactosamine) binding are being conducted in collaboration with Dr. Lixin Ma (University of Missouri-Columbia). One of the galectin-3-specific peptides, G3-C12, has been observed to bind to an apolar site on the CRD, which Seetharaman, et al. (Seetharaman, Kanigsberg et al. 1998) suggested is a possible site of monomer-monomer interaction during homodimerization of galectin-3. Both biochemical and NMR relaxation analyses of the full-length galectin-3 protein and its complex with the peptide have revealed that the binding of the peptide induces a transition from dimer to monomer. Moreover, the studies with a cleaved galectin-3 fragment implied a novel dimerization mechanism by which galectin-3 forms dimers through N-C interactions, such that the cell surface full-length galectin-3 would recruit free galectin-3 or CRD in solution to form multivalent carbohydrate-binding proteins. These studies suggest that galectin-3 homodimers may be involved in the carbohydrate-mediated cell adhesion process and that addition of the peptides might interfere with this process. (Manuscript in preparation)

Other ongoing work with galectin-3 peptides will be focus on investigating the role of galectin-3's anti-apoptosis function. Galectin-3 contributes to anchorage-independent cell survival during carcinoma cell dissemination by protecting cancer cells from apoptosis or anoikis (Kim, Lin et al. 1999). Cytoplasmic galectin-3 may sequester proapoptotic proteins through heterodimerization and thus may shift the cellular homeostatic balance away from apoptosis toward enhanced cell survival (Yang, Hsu et al. 1996; Akahani, Nangia-Makker et al. 1997). We speculate that the anti-galectin-3 peptides will antagonize the anti-apoptotic functions of galectin-3 by interfering with

heterodimer formation. Cell penetrating forms (Tat uptake sequence) of the anti-galectin-3 peptides have been constructed and we are examining their effects on apoptosis in BT549 breast carcinoma cells heterologously-expressing galectin-3 (Kim, Lin et al. 1999). Dr. Raz and coworkers have reported that BT549 cells expressing galectin-3 are resistant to staurosporine-induced apoptosis (Yu, Finley et al. 2002). Flow cytometry, fluorescence caspase activation assays, and structural analyses are currently being conducted to clarify the possible pro-apoptotic effect of the galectin-3 peptides.

Finally, we are conducting *in vivo* assays to analyze the ability of the galectin-3 peptides to inhibit cancer cell adhesion and deposition, in mice heterotransplanted with MDA-MB-435 human breast carcinoma cells. Our early observations indicate that carcinoma cells pre-incubated with peptide prior to implantation take longer to form palpable tumors than do peptide-free cells. PET imaging and histological analyses of the tumors are our future directions.

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## VITA

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