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Transcriptional Control of Melanoma Metastasis: The Importance of the Tumor Microenvironment

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Abstract

The molecular changes associated with the transition of melanoma cells from radial growth phase (RGP) to vertical growth phase (VGP) and the metastatic phenotype are not very well defined. However, some of the genes involved in this process and their transcriptional regulation are beginning to be elucidated. For example, the switch from RGP to VGP and the metastatic phenotype is associated with loss of the AP-2 α transcription factor. AP-2 α regulates the expression of c-KIT, MMP-2, VEGF, and the adhesion molecule MCAM/MUC18. Recently, we reported that AP-2 α also regulates two G-protein coupled receptors (GPCR) PAR-1 and PAFR. In turn, the thrombin receptor, PAR-1, regulates the expression of the gap junction protein Connexin-43 and the tumor suppressor gene Maspin. Activation of PAR-1 also leads to overexpression and secretion of proangiogenic factors such as IL-8, uPA, VEGF, PDGF, as well certain integrins. PAR-1 also cooperates with PAFR to regulate the expression of the MCAM/MUC18 via phosphorylation of CREB. The ligands for these GPCRs, thrombin and PAF, are secreted by stromal cells, emphasizing the importance of the tumor microenvironment in melanoma metastasis. The metastatic phenotype of melanoma is also associated with overexpression and function of CREB/ATF-1. Loss of AP-2 α and overexpression of CREB/ATF-1 results in the overexpression of MCAM/MUC18 which by itself contributes to melanoma metastasis by regulating the inhibitor of DNA binding-1 (Id-1). CREB/ATF-1 also regulates the angiogenic factor CYR-61. Our recent data indicate that CREB/ATF-1 regulates the expression of AP-2 α , thus, supporting the notion that CREB is an important “master switch” in melanoma progression.

Keywords

Melanoma; metastasis; transcription; tumor microenvironment

1. Introduction

Melanoma progresses through a number of sequential events which must occur for establishment of metastatic lesions. Through genetic alterations, normal melanocytes are transformed into a benign nevus. Further molecular alterations lead to a premalignant lesion which grow and spread through the epidermis in a premalignant radial growth phase (RGP).

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The expression of basement membrane and matrix-degrading enzymes lead to local invasion through the dermis termed vertical growth phase (VGP), which allows for tumor cells to enter into the lymphatic and vascular networks [1,2]. The tumor cells must have the acquired ability to survive the circulation, arrest in the capillary bed of the distant organ, enter the parenchyma, grow within the organ microenvironment, and further initiate angiogenesis [1]. Only a small subset of cells from the primary tumor have the acquired molecular changes to complete the metastatic cascade and grow in select organs such as the lymph nodes, lung, liver, and brain [3]. Patient survival depends greatly on identifying the disease before the later events of the cascade can occur. Patients presenting with stage I and II disease, with no observable metastasis, have a five-year survival rate of approximately ninety and seventy percent respectively. Patients with lymph node involvement, stage III, have approximately a forty-five percent five-year survival; however, prognosis can worsen if metastasis has occurred in multiple lymph nodes. Once metastasis to distant organs occurs, the five-year survival rate significantly decreases to ten percent [4].

Melanoma has become an ideal model for identifying the molecular changes that occur during the metastatic transition. Multiple events have been identified during these well characterized sequential events from melanocyte to benign nevus, to the radial growth phase (RGP), then to the vertical growth phase (VGP), and finally metastasis [5]. The molecular changes occurring at specific stages of melanoma can infer that certain molecules are critical for the disease to initiate tumor development, angiogenesis, and survival. Others are required for the degradation of matrix membranes and entrance into the circulation. Finally, transcriptional regulation of certain molecules is required for transportation, extravasation, and homing to distant organs [1]. The deregulation of transcription factors during the transition from RGP to VGP, along with their downstream target genes can regulate the metastatic phenotype of melanoma. This review will summarize our data on the roles of two transcription factors, AP-2 α and CREB/ATF1 as major players determining the malignant melanoma phenotype.

2. The Loss of the Transcription Factor AP-2 α Enhances the Metastatic Melanoma Phenotype

The acquisition of the metastatic melanoma phenotype can occur through the overexpression or repression of genes through transcription activators or repressors. The transcription factor AP-2 α is a 52 kDa protein first purified from HeLa cells [6]. The gene can be alternatively spliced to form the dominant-negative AP-2B protein that differs in its C-terminus, which inhibits AP-2 α transcriptional activity [7]. Immunohistochemistry analysis of melanoma patients identified that the loss of AP-2 α expression is correlated decreased p21 expression, and predicts poor survival [8]. The loss of AP-2 α also leads to the deregulation of c-KIT, MCAM/MUC18, MMP-2, MMP-9, PAR-1, VEGF, BCL-2, E-cadherin, and p21 [9–16]. Thus, it can be predicted that the loss of AP-2 α plays a key role during the transition from RGP to VGP in melanoma [2,16–19]. Indeed, re-expression of AP-2 α in highly metastatic melanoma cells (AP-2 α negative) resulted in inhibition of tumor growth and their metastatic potential *in vivo* [20].

2.1 AP-2 α Directly Affects Transcription of c-KIT, MCAM/MUC18, and PAR-1

The transition from RGP to VGP in cutaneous melanoma is associated with the loss of c-KIT expression and the gain of the melanoma cell adhesion molecule (MCAM/MUC18) [16,20–23]. Promoter analysis of the c-KIT gene identified three putative AP-2 α binding sites [24]. Transfection of the AP-2 α gene in the metastatic A375SM melanoma cell line induced c-KIT expression. Furthermore, after stable transfection of AP-2 α in A375SM cells,

the tumor size and the median number of experimental lung metastases were significantly decreased when injected in nude mice [20].

The adhesion molecule MCAM/MUC18 is commonly overexpressed in metastatic melanoma. However, increase of MCAM/MUC18 expression is not mediated by gene rearrangement or amplifications [25]. Interestingly, four AP-2 α binding sites are located on the MCAM/MUC18 promoter. We have demonstrated that AP-2 α directly binds to the MCAM/MUC18 promoter and inhibits transcription as confirmed by luciferase-driven promoter activity analyses and EMSA [16]. To further establish the regulation of MCAM/MUC18 by AP-2 α , the A375SM melanoma cell line was transfected with an AP-2 α expression vector and demonstrated a significant decrease in MCAM/MUC18 expression on the plasma membrane [16]. These results suggest that loss of AP-2 α during melanoma progression results in increased MCAM/MUC18 expression.

Tissue microarray and cell line studies have reported that the G-protein coupled receptor, Protease Activated Receptor-1 (PAR-1), is overexpressed during the transition from RGP to VGP, and its expression is inversely correlated with AP-2 α expression [18,26]. Interestingly, there are two AP-2 α /SP-1 complexes within the proximal 3' region of the PAR-1 promoter [2,26]. In highly metastatic melanoma cell lines, Sp1 is able to bind to the PAR-1 promoter and induce transcription. However, in less metastatic cell lines, AP-2 α predominantly binds to the promoter and inhibits PAR-1 transcription. As AP-2 α expression decreases in the more metastatic cells, Sp1 is then able to bind to the PAR-1 promoter and activate transcription [2,26]. Regulation of these genes by AP-2 α can play a critical role in invasion, apoptosis, and angiogenesis. The loss of AP-2 α expression and the deregulation of its downstream genes may underlie its contribution to the malignant melanoma phenotype.

3. G-Protein Coupled Receptors in Tumor Growth and Metastasis

3.1 Protease Activated Receptor-1 (PAR-1)

Melanoma utilizes the coagulation/platelet activation pathways by producing and responding to thrombin, tissue factor, fibrinogen, and platelet activating factor (PAF) [27–30]. Thrombin activates platelets which leads to the release of angiogenic factors such as VEGF, angiopoietin-1, PDGF, and lysophosphatidic acid [31–33]. Cleavage of the N-terminal domain of PAR-1 by thrombin reveals a new N-terminal which acts as a tethered ligand and binds to the body of the receptor, thus inducing downstream signaling to promote proliferation, invasion, and angiogenesis [27,30,34]. Thrombin has been reported to upregulate matrix metalloproteinases (MMP-1 and MMP-2) to enhance the invasive phenotype of melanoma cells *in vitro*, along with upregulating angiogenic factors such as IL-8, VEGF, PDGF and bFGF in cancer [35–38].

Tissue microarray analysis show weak staining of PAR-1 in melanocytic nevi, and strong staining in melanoma and metastatic lesions [18,39]. Furthermore, cell lines with a more metastatic phenotype express significantly higher amounts of PAR-1 than less metastatic melanoma cells [40,41]. This indicates that PAR-1 expression correlates with the transition of RGP to VGP and the metastatic phenotype of melanoma. To study the role of PAR-1 in melanoma, we silenced its expression by means of lentiviral shRNA in the highly metastatic A375SM and C8161 melanoma cell lines, and subsequently analyzed their tumor growth and metastatic potential *in vivo*. Injection of these cells in nude mice significantly reduced subcutaneous tumor growth and decreased the number of experimental lung metastasis [42].

3.2 Liposomal Delivery of PAR-1 siRNA Targeting Melanoma Metastasis

We next wanted to investigate whether PAR-1 can be silenced *in vivo* in mice bearing melanoma tumors. To that end, PAR-1 siRNA was packaged into neutral 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) liposomes, as DOPC liposomes have been used effectively *in vivo* [43–45]. Liposomal delivery effectively transported siRNA into melanoma tumors, and twice weekly injections of 10ug PAR-1 siRNA efficiently decreased PAR-1 expression. This in turn significantly decreased tumor growth and experimental lung metastasis *in vivo* [42]. Immunohistochemistry analysis of subcutaneous tumors identified a significant decrease in the expression of VEGF, IL-8, and MMP-2 within the tumor and surrounding microenvironment after treatment with PAR-1 siRNA. Furthermore, the role of PAR-1 in tumor angiogenesis was further established as the number and size of blood vessels significantly decreased after delivery of PAR-1 siRNA [42]. Collectively, this data represents the therapeutic potential of PAR-1 siRNA delivery to reduce tumor growth and melanoma metastasis.

3.3 Regulation of Connexin 43 and Maspin by PAR-1

In addition to the proangiogenic factors mentioned above, whose secretions are induced by PAR-1 activation, we next wanted to investigate other potential target genes regulated by PAR-1 which contribute to melanoma growth and metastasis. Gene expression analysis comparing PAR-1-silenced melanoma cells and non-targeting (NT) transduced melanoma cells revealed the differential expression of novel downstream target genes regulated by PAR-1. Among the differentially expressed genes, we have identified that the expression of the gap junction protein Connexin-43 is downregulated following transduction of PAR-1 shRNA in metastatic melanoma cells [46]. Although the loss of gap junctions occurs during early stages of cancer, connexins are known to have adhesive properties [47]. Expression of Connexin-43 has been observed in gliomas, breast cancer, hepatocellular carcinoma, and melanoma [48–51]. It has been reported that Connexin-43 can mediate tumor cell adhesion to endothelial cells and extravasate into the metastatic site [46,52,53]. Furthermore, downregulation of Connexin-43 in breast cancer decreased tumor cell attachment to the pulmonary endothelium [54]. Silencing of connexin-43 in metastatic melanoma cells resulted in a significant decrease in their invasive potential through matrigel [46]. Validation of our cDNA microarray analysis demonstrated that the protein expression of Connexin-43 is decreased after PAR-1 silencing. Analysis of Connexin-43 promoter after PAR-1 silencing, showed a significant decrease in luciferase promoter activity demonstrating that PAR-1 regulated Connexin-43 expression at the transcriptional level. We further demonstrated that the decreased expression of Connexin-43 is mediated by the differential binding of Sp-1, c-Fos, and c-Jun to the connexin-43 promoter, as chromatin immunoprecipitation assays show decreased binding after induction of PAR-1 shRNA [46]. Thus, we propose that PAR-1 signaling increases the binding of SP-1, c-Fos, and c-Jun to the Connexin-43 promoter which results in the upregulation of Connexin-43.

Another target gene regulated by PAR-1 is the tumor suppressor maspin, as it was increased following cDNA microarray analysis of PAR-1-silenced melanoma cells. PAR-1 silencing decreased the activation of p38 MAPK, and activated the histone acetyltransferase CBP/p300. This increased the binding of ETS-1 and c-Jun to the Maspin promoter and induced maspin expression. Furthermore, transduction of the Maspin gene in metastatic melanoma cells decreased their invasive potential by decreasing MMP-2 activity (unpublished data). Thus, PAR-1 contributes to the malignant melanoma phenotype in part by up regulating Connexin-43 and inhibiting the expression of Maspin. Moreover, we have recently shown that the regulation of another g-protein coupled receptor, platelet-activating factor receptor (PAFR), which is overexpressed during melanoma progression, is mediated by PAR-1 [55].

3.4 Platelet Activating Receptor (PAFR) and its Ligand PAF

The proinflammatory and proangiogenic platelet-activating factor (PAF) was the first bioactive lipid ever identified [56]. The biolipid PAF induces signaling by binding to the seven transmembrane-spanning G-protein-coupled receptor PAFR [57]. Several studies have identified a number of inflammatory molecules that are up regulated by cellular stimulation with PAF including IL-6, IL-8, COX-2, VEGF, and IL-10 [27]. It has been reported that bFGF enhances human umbilical vein endothelial cell proliferation by stimulating PAF production [58]. Furthermore, PAF induces the activation of MMP-2 and enhances endothelial cell invasion and migration [59]. The role of PAF in melanoma metastasis was first observed in the B16F10 murine melanoma cells as pulmonary metastasis was enhanced with a single intraperitoneal injection of PAF [60]. Injections of the PAFR antagonist BN50739 decreased B16F10 lung colonization [60].

Release of PAF by both tumor cells and the microenvironment seem to play an important role in stimulating endothelial cells and angiogenesis. However, the expression of its receptor PAFR is not limited to endothelial and inflammatory cells. Tumor cells can also overexpress the PAF receptor [61,62]. Our lab has previously shown that PAFR is expressed in melanoma cell lines regardless of their metastatic potential [61]. The PAFR antagonist PCA4248 inhibits the growth of A375SM human melanoma cells in vitro. Furthermore, incubation with the nonhydrolyzable PAF analog cPAF revealed increased phosphorylation of the transcription factor cAMP-response element-binding protein (CREB). Interestingly, neither PAF nor cPAF increased CREB phosphorylation in the less metastatic SB-2 melanoma cell line even though it expresses PAFR [61]. Intermediate signaling is most likely absent in the SB-2 cell line which occurs in the more aggressive melanoma cells. Two of these intermediate signaling proteins are PKA and p38 MAPK. Phosphorylation of p38 and PKA by PAFR stimulation in turn phosphorylates CREB, and inhibition of p38 or PKA with the inhibitors SB202190 or H-89 inhibited PAF mediated phosphorylation of CREB [61]. Moreover, the stimulation of PAFR by cPAF in melanoma cells also leads to increased expression of the invasive molecules MMP-2 and MT1-MMP [61]. Interestingly, PAR-1 and PAFR seem to cooperate together during melanoma metastasis, as PAR-1 induces the expression of PAFR. The induction of PAFR resulted in the activation of CREB which leads to increased transcription of genes such as the adhesion molecule MCAM/MUC18 and downregulated p21 (Figure 1) [55,61]. Since thrombin and PAF are secreted by stromal cells, these studies emphasize the role of the tumor microenvironment in regulating the melanoma metastatic phenotype.

4. Adhesion Molecules and Melanoma metastasis

4.1 Adhesion Molecules

Cell adhesion molecules play critical roles in normal biological functions such as membrane integrity and leukocyte tethering [63,64]. They are cell surface molecules which can form homophilic or heterophilic interactions on surrounding cells. In the case of melanoma and other cancers, these interactions allow for the aggregation of cancer cells to each other (homotypic adhesion) or to the host stroma and cells within the circulatory system (heterotypic adhesion) [65]. These interactions contribute to invasion, migration, intravasation, survival during circulation, and extravasation into the parenchyma of distant organs (Reviewed in [65,66]). There are numerous adhesion molecules that are sub-grouped based on their structural similarity and are categorized as integrins, cadherins, immunoglobulins, or selectins (Figure 2) [65–67]. Multiple cell adhesion molecules have been reported to play a role in melanoma progression. In melanoma patients, overexpression of the integrin $\alpha 4 \beta 1$ (VLA-1) is correlated with tumor stage and contributes to experimental lung metastasis [68,69]. The cadherin switch from E-cadherin to N-cadherin

results in disassociation of melanoma cells from keratinocytes and promotes melanoma cell invasion through the dermis [70,71]. Multiple adhesion molecules from the immunoglobulin family are associated with melanoma metastasis such as ICAM-1, L1CAM and MCAM/MUC18 [72–74].

4.2 MCAM/MUC18 Enhances the Metastatic Phenotype of Melanoma

MCAM/MUC18 is a 113 kDa transmembrane glycoprotein that was originally cloned and sequenced from a melanoma cDNA library, and is expressed on melanomas but rarely on premalignant lesions [22,75,76]. Tissue array analysis have identified MCAM/MUC18 as an independent prognostic marker for decreased patient survival [77]. Expression of MCAM/MUC18 in MUC18-negative primary cutaneous melanoma cells increases homotypic adhesion along with increased attachment to endothelial cells [78]. Furthermore, MCAM/MUC18 expression increased MMP-2 activity and *in vitro* invasion through matrigel [78]. The connection between MCAM/MUC18 and MMP-2 was further corroborated as silencing of MCAM/MUC18 expression with lentiviral based shRNA in metastatic melanoma cells decreased MMP-2 activity and the cells invasive potential through matrigel. Injection of these cells *in vivo* significantly reduced tumor growth and experimental lung metastasis (unpublished data).

Currently no ligand has been identified for MCAM/MUC18 beyond its homophilic interaction. However, its cytoplasmic domain contains several protein kinase recognition motifs, which implies that MCAM/MUC18 can induce downstream signaling [76,79]. To elucidate downstream signaling of MCAM/MUC18, cDNA microarray analysis comparing MCAM/MUC18-silenced cells and non targeting transduced cells identified the inhibitor of DNA binding protein, Id-1, as a downstream target of MCAM/MUC18. Id-1 was found to be downregulated following MCAM/MUC18 silencing. Interestingly, one of the known Id-1 inhibitors, ATF-3, was found to be upregulated following MCAM/MUC18 silencing (unpublished data). Furthermore, Id-1 has been previously shown to induce MMP-2 expression in several cancers including melanoma [80–82]. Based on these data, we hypothesize that MCAM/MUC18 expression is required to mediate downstream signals that leads to the inhibition of ATF-3 transcription and up regulation of Id-1, thus leading to up regulation of MMP-2 and melanoma cell invasion.

4.3 Targeting MCAM/MUC18 with Fully Human Antibodies to Inhibit Melanoma Growth and Metastasis

As MCAM/MUC18 is expressed on both vascular endothelial cells and melanoma cells and enhance melanoma metastasis, it can be hypothesized that targeting MCAM/MUC18 could be a promising therapeutic agent. We thus developed A fully humanized IgG2 monoclonal antibody (ABX-MA1; in collaboration with Abgenix, Inc., Fremont, CA) was developed to target the extracellular domain of MCAM/MUC18. *In vitro*, treatment of highly metastatic melanoma cell lines with ABX-MA1 decreased homotypic aggregation and heterotypic adhesion to HUVECs [83]. Decreased promoter activity of MMP-2 was also observed when melanoma cells were incubated with ABX-MA1. Following inoculation of subcutaneous tumors with the metastatic melanoma cell lines A375SM and WM2664, treatment with ABX-MA1 (100 µg/week intraperitoneally for 5 weeks) significantly decreased tumor growth, angiogenesis, and reduced the expression of MMP-2 as compared to mice treated with IgG alone [83]. Importantly, treatment with ABX-MA1 significantly decreased the formation of experimental lung metastasis [83]. These studies utilizing antibodies to target MCAM/MUC18 indicate that this could provide a potential treatment modality, either alone, or in combination with conventional chemotherapy or other antitumor agents for patients with stage II or III melanoma to inhibit the formation of distant metastatic lesions.

5. Activation of CREB Acts as a “Major Regulator” of Metastatic Melanoma

Several growth factors that increase melanocyte proliferation and differentiation can also upregulate cAMP [84,85]. This has led to the investigation of cAMP-responsive element binding (CREB) protein and its role in melanoma. CREB is a member of the bZIP superfamily, and binds to the cAMP-responsive elements (CRE's) located on the promoter sequences of various genes. Phosphorylation at Ser133 of CREB leads to its activation and association with its co-activators CBP and p300 [86,87]. CREB can also heterodimerize with members of its subfamily which includes ATF-1 and CREM [88]. Indeed, upregulation of activating transcription factor-1 (ATF-1) and CREB in melanoma are associated with the transition from RGP to VGP, and the inhibition of CREB activity with the dominant-negative form, KCREB, in metastatic melanoma cells decreased their tumorigenicity and metastatic potential *in vivo* [89–92].

CREB activation leads to the expression of invasive, inflammatory, and survival molecules including MCAM/MUC18, MMP-2, HER-2, IL-8, and BCL-2 [5,17,93,94]. CREB activation through PAFR has been demonstrated to be required for the expression of MMP-2 and MT1-MMP when stimulated with PAF [61]. Interestingly, silencing of CREB in melanoma cells leads to down regulation of MCAM/MUC18 and up regulation of p21 [11,16,17]. Silencing of CREB in metastatic melanoma cells also identified the cysteine-rich protein 61 (CYR61) to act as a tumor suppressor gene in melanoma [95]. Silencing of CREB in these cells has also resulted in the upregulation of AP-2 α expression. This led our lab to further investigate the inverse correlation between CREB and AP-2 α during melanoma progression, and elucidate the mechanism which controls this switch. Targeting CREB in two metastatic melanoma cell lines by means of lentiviral based shRNA, significantly up regulated the expression of AP-2 α [17]. Chromatin immunoprecipitation (ChIP) analyses identified CREB binding to the AP-2 α promoter. CREB shRNA increased the promoter activity of AP-2 α , and rescue of CREB expression in CREB-silenced cells downregulated AP-2 α expression. Silencing of CREB also decreased E2F-1 expression which can also bind to the AP-2 α promoter. The decreased binding of these two transcription factors in metastatic melanoma cell lines leads to AP-2 α expression after CREB silencing. Downstream targets of AP-2 α were also affected, as this up regulated p21 and downregulated the adhesion molecule MCAM/MUC18 [17]. These data suggest that down regulation of AP-2 α during melanoma progression is attributed by the activation of CREB. Since AP-2 α regulates several genes involved in melanoma progression, these studies identify CREB as a “major regulator” of melanoma progression.

6. Conclusion

Recent work has elucidated the relationship between AP-2 α and CREB along with the molecular changes regulated by these two transcription factors during melanoma progression. The change in CREB and AP-2 α activities leads to the downregulation of c-KIT, and overexpression of MMP-2, MCAM/MUC18, and two G-protein coupled receptors, PAR-1 and PAFR. Further signaling through these molecules enhances the expression of invasive, angiogenic, and survival factors, thus emphasizing the roles of the tumor microenvironment in determining the metastatic phenotype of melanoma. Since CREB regulates the expression of AP-2 α , our studies support the notion of CREB being a “master switch” in melanoma progression. Moreover, targeting these genes with fully human antibodies or liposome-encapsulated siRNA could provide potential treatment modalities.

Abbreviations

CREB	cAMP response element binding protein
AP-2α	activator protein-2 α
RGP	radial growth phase
VGP	vertical growth phase
PAR-1	protease activated receptor-1
PAFR	platelet activating factor receptor
PAF	platelet activating factor
MCAM/MUC18	melanoma cell adhesion molecule
MMP-2	matrix metalloproteinase-2
ATF-1	activating transcription factor
CYR-61	cysteine-rich protein 61

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Fig. 1. A proposed model for PAR-1 and PAFR cooperation during melanoma progression

PAR-1 activation in melanoma cells leads to PAFR expression, presumably through transcriptional activation. PAFR signaling activates protein kinase A (PKA) and p38 MAPK which enhances phosphorylation of CREB. CREB binding to the MCAM/MUC18 promoter, along with CREB-dependent Sp1 recruitment results in MCAM/MUC-18 expression during melanoma progression.

Fig. 2. Cell adhesion molecules (CAM) and their representative structure within each family
 Integrins, composed of an α and β subunit heterodimer, form heterophilic interactions with several other adhesion molecules and ECM proteins. Selectins bind to glycosylated proteins through their lectin domain. Cadherins contain five extracellular segments which are necessary for cell adhesion. The Ig superfamily (IgSF-CAM) mediate both homophilic and heterophilic interactions.