DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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Studies on the functions of tumor-associated mucin-like leukosialin (CD43) in human cancer cells

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LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on the following original publications, which will be referred to by their Roman numeral in the text.

I. Kadaja-Saarepuu, L., Laos, S., Jääger, K., Viil, J., **Balikova, A.**, Lõoke, M., Hansson, G.C. and Maimets, T. (2008) CD43 promotes cell growth and helps to evade FAS-mediated apoptosis in non-hematopoietic cancer cells lacking the tumor suppressors p53 or ARF. *Oncogene* 27(12): 1705-1715.

II. **Balikova, A.**, Jääger, K., Viil, J., Maimets, T. and Kadaja-Saarepuu, L. (2012) Leukocyte marker CD43 promotes cell growth in co-operation with β-catenin in non-hematopoietic cancer cells. *Int J Oncol* 41(1): 299-309.

III. Kadaja-Saarepuu, L., Lõoke, M., **Balikova, A.** and Maimets, T. (2011) Tumor suppressor p53 down-regulates expression of human leukocyte marker CD43 in non-hematopoietic tumor cells. *Int J Oncol* 40(2): 567-576.

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My personal contribution to the articles referred to in this thesis is as follows:

Ref. I. participated in performing the experiments.
Ref. II designed and performed the experiments, analysed the data and wrote the manuscript.
Ref. III participated in designing and performing the experiments, and in data analysis.

ABBREVIATIONS

AP-1	activator protein 1
ARF	alternate reading frame
CBP	CREB-binding protein
CDK	cyclin-dependent kinase
DAG	diacylglycerol
DAXX	death-domain associated protein
ERK	extracellular signal regulated kinase
ERM	ezrin, radixin, moesin
FADD	FAS-associated death domain
GEF	guanine exchange factor
HDAC	histone deacetylase
HIPK2	homeodomain-interacting protein kinase 2
ICAM-1	intercellular adhesion molecule type 1
JNK	Jun-N-terminal kinase
LEF	lymphocyte-enhancing factor
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinases
MDM2	murine double minute 2
MeCP2	methyl CpG binding protein 2
MEK	mitogen-activated protein kinase kinase (also known as MAP2K)
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa B
NLS	nuclear localization signal
pAb	polyclonal antibody
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLCγ	phospholipase Cγ
PML-NB	promyelocytic leukaemia nuclear bodies
PTK	protein tyrosine kinase
SH	Src homology
SP1	specificity protein 1
SPN	sialophorin
TBP	TATA-binding protein
TCF	T cell factor
TCR	T cell receptor

INTRODUCTION

Cancer is a complex disease characterized as a malignant neoplasm. It may arise in all tissues composed of potentially dividing cells. Cancer cells proliferate uncontrollably forming malignant tumors and metastases in different parts of the body. They have acquired self-sufficiency in growth, unlimited proliferative capacity, insensitivity to signals mediating growth arrest and programmed cell death as well as ability to invade surrounding tissues. Apparently, cancerogenesis is a multistep process; the transformation of a normal cell into a malignant one requires accumulation of several mutations in the same cell. Inactivation of tumor suppressor and stability genes, as well as aberrant activation of oncogenes is the main cause of tumor cell formation. Transmission of accumulated genetic defects by a dividing tumor cell to daughter cells further facilitates tumor formation with a possible progression to malignancy. Although the number of genes mutated in cancer continues to grow rapidly, the acquisition of a transformed phenotype actually depends on alterations in several key signaling pathways that regulate cell proliferation and death. Proper understanding of the molecular mechanisms employed by cancer-associated proteins in the formation and development of the disease might be applied to the elaboration of novel therapeutic strategies.

In recent years, the glycoproteins of tumour cells gained significant attention in cancer research since they were found to be often abnormal, both in structure and in quantity. In particular, the *O*-linked oligosaccharides of mucins and mucin-type proteins have several cancer-associated features. In cancer cells, changes in the glycodynamics of mucins are common and result in new and unusual carbohydrate and peptide epitopes. Alterations in mucin structures have many biological and pathological consequences, because potential ligand-receptor pairings responsible for interplay between cancer cells and their microenvironment are changed. Aberrant interactions of the modified mucins with molecules on the surface of endothelium or of the immune system cells influence growth and survival of cancer cells, their ability to invade and metastasize.

In this thesis, the mucin-like glycoprotein CD43 was investigated, since CD43 was found to be aberrantly expressed in colorectal and several other types of cancer. Indeed, it has already been proposed in some works that this hematopoietic lineage-specific protein might be involved in formation of tumors of non-hematopoietic origin. However, the molecular mechanisms implicated in CD43-dependent tumorigenesis remain largely unknown. New insights into CD43 signaling in cancer cells are presented and evaluated in current study.

LITERATURE OVERVIEW

1. Background

Mucins are large glycoproteins with a "rod-like" conformation, which carry many clustered glycosylated serines and threonines in tandem repeat regions. The *O*-linked oligosaccharides (*O*-glycans) comprise 50–90 % of the mucin molecule by weight. The *O*-glycan cores are usually extended and substituted with other sugars and sulphate esters, resulting in many different structures. Secreted mucins function as a protective layer over the epithelium. The *O*-glycans of cell surface-bound mucins regulate antigenicity as well as interactions with the environment and binding to lectins. Depending on the structures of their *O*-glycan chains, mucins can be pro- or anti-adhesive. Membrane-bound mucins consist of a glycosylated extracellular domain, a hydrophobic transmembrane part and a cytoplasmatic tail which interacts with cytoskeletal adaptor proteins and signaling molecules indicating an involvement in cell adhesion and signaling.

Overexpression of mucins in cancers of epithelial origin (carcinomas) has been known for many years. In the normal polarized epithelium, mucins are expressed exclusively on the apical domain, toward the lumen of a hollow organ (Fig. 1). Likewise, soluble mucins are secreted exclusively into the lumen. However, loss of correct topology in malignant epithelial cells allows mucins to be expressed on all aspects of the cells, and soluble mucins can then enter the extracellular space and body fluids such as the blood plasma (1). In carcinomas mucins appear to be the major carriers of altered glycosylation. The changes in carbohydrate structures can alter antigenic and adhesive properties of cancer cells, as well as its potential to migrate. Apart from specific interactions of the *O*-glycans with endogenous lectins, the extended structures of the mucins and their negative charge are thought to prevent intercellular interactions and sterically obstruct other adhesion molecules such as cadherins and integrins from carrying out their functions. Therefore, in some instances, the anti-adhesive properties of mucins can promote displacement of a cell from the primary tumor during the initiation of metastasis. Evidence suggests that mucins might also physically block interactions with the host cytolytic cells such as natural killer cells. In addition, mucins may mask presentation of antigenic peptides by major histocompatibility complex molecules.

There is a growing body of evidence showing aberrant expression of mucin-like CD43 protein in human solid tumor cells (2–6). CD43 is a major glycoprotein on the surface of hematopoietic cells, and it was regarded as an exclusive leukocyte marker until the mid-'90s. The first evidence of CD43 association with cancer was provided by the colon carcinoma cell line COLO205 expressing high amounts of a CD43 glycoform that was more extensively glycosylated than in blood cells (5). Later observations showed CD43 expression already at early stages of colorectal tumors, but it was not

detectable in normal colonic epithelium (2, 7). Among multiple functions attributed to CD43, its ability to impair apoptotic cell response has been reported and accounts for its potential role in tumorigenesis (8, 9).

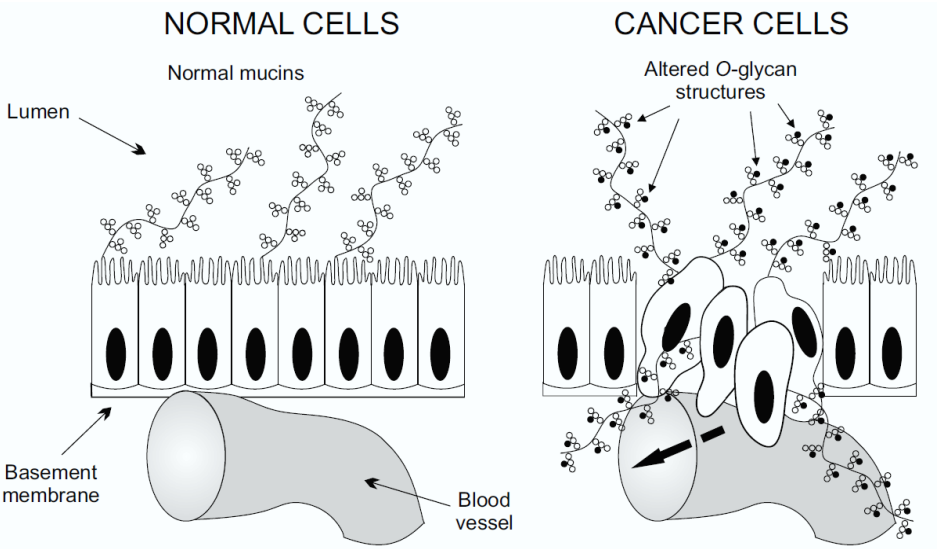

Figure 1. Role of mucins in cancers of epithelial origin. Altered mucin structures as well as loss of normal topology and polarization of epithelial cells in cancer results in secretion of mucins into the bloodstream and aberrant ligand-receptor interactions of the cells with each other and the microenvironment. Consequent alterations in adhesion allow displacement of cells from the primary tumor during the initiation of metastasis, transmigration and invasion of the tissues. Changes in mucin glycosylation also facilitate survival of cancer cells due to evasion of immune surveillance. Adopted from (1) with modifications.

2. CD43 in hematopoietic cells

CD43, also known as leukosialin, sialophorin, galactoglycoprotein, leukocyte sialoglycoprotein, gpL115, Ly-48, L-CanAg, is a mucin-like type I transmembrane protein. In humans it is ubiquitously expressed on cells of hematopoietic origin including T lymphocytes, monocytes, granulocytes, natural killer cells, platelets, and hematopoietic stem cells, but excluding mature erythrocytes and B cell subsets (10–16).

CD43 is an important contributor to immune homeostasis regulating a wide variety of cellular processes, e.g. activation, differentiation and motility. Dysfunction of CD43 accounts for several pathological conditions including immunodeficiency diseases: Wiskott-Aldrich syndrome (17) and the acquired immunodeficiency syndrome (18, 19). Abnormal CD43 expression and glycosylation also contribute to Alzheimer disease (20) and to formation of tumors of

hematopoietic and non-hematopoietic origin (2, 3, 6). However, CD43 knockout mice are reported to be fertile having almost normal development of T and B cells (mild anomalies of T cell activation and migration have been detected), which points to the existence of a compensatory mechanism (21, 22).

2.1. *SPN* gene and its transcriptional regulation

Human CD43 protein is encoded by a single gene on chromosome 16 (gene map locus 16p11.2). The CD43 gene (*SPN*, sialophorin) has an unusual genomic organization. Firstly, it consists of three exons, but the entire translation product is encoded by the third exon only (23, 24) (Fig. 2). Therefore no alternative splicing occurs (25). Secondly, the promoter region lacks canonical TATA or CAAT boxes, but contains only a degenerate CAAT box with the sequence CCACT (25). Moreover, the promoter is highly enriched (71 %) in G and C nucleotides and contains a number of short G- and C-rich repeats, which is typical of the promoter regions of 'housekeeping' genes (26). Thirdly, the unusually large number of Alu sequences associated with *SPN* suggests that retroposition may have played a role in the evolution of its structure: replacement of the bulk of an ancestral gene with a partially processed sialophorin transcript may account for the relative lack of introns (25). The lack of introns in the coding region, although unusual, is not unprecedented for integral membrane proteins (27).

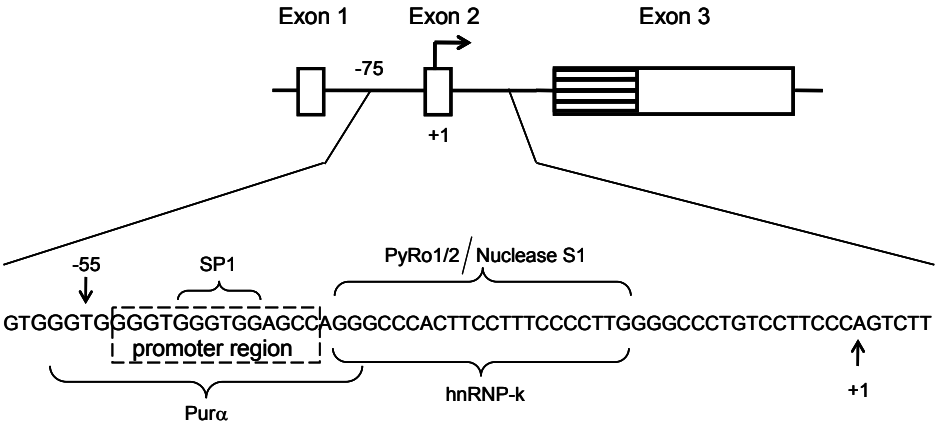

Figure 2. Schematic representation of *SPN* gene. The promoter region and exons of *SPN* gene are shown. The striped box within the exon 3 box corresponds to the translated region. Locations of the binding sites for known regulatory factors are indicated using braces and given relative to the second transcription start site (+1 and bent arrow). The first transcription start site is marked as –55.

There are two transcription initiation sites separated by 55 bp (25). In this work we refer to the second initiation site as a transcription start site. The *SPN* genomic sequence from the nucleotide –53 to –40 is an essential promoter region (28) containing a binding site for the transcription factor SP1 (specificity protein 1) which is critical for the activation of CD43 expression (29). However, it is believed that CD43 expression is regulated by transcriptional repression rather than activation of the basal transcription level. The activity of *SPN* promoter is inhibited by DNA methylation. The promoter is methylated in CD43 non-expressing cells and non-methylated in CD43 expressing cells. Moreover, CD43 expression can be restored in non-expressing cells by introducing a DNA methyltransferase inhibitor, 5-azacytidine, into the cells (30). The transcriptional repressor MeCP2 (methyl CpG binding protein 2) bound to methylated *SPN* promoter has been shown to inhibit the transactivating properties of SP1 (31) presumably by displacing it and recruiting the co-repressor SIN3A and histone deacetylases (HDAC) to the promoter (32, 33). In addition, transcriptional repression of *SPN* is achieved by binding and co-operation of transcription factors Purα and hnRNP-K (heterogeneous nuclear ribonucleoprotein K) on the promoter (34, 35). *SPN* promoter also contains a nuclease S1 cleavage site and binding sites for the nuclear factors PyRo1 and PyRo2. *SPN* transcription is upregulated by PyRo1 and PyRo2 interaction with this region, which prevents the nuclease cleavage (36).

2.2. Structure of CD43 protein

Human CD43 protein consists of a 19-amino acid (aa) long signal peptide in its amino terminus (N-terminus), followed by 235 aa of an extracellular domain, a transmembrane domain of 23 aa, and an intracellular domain of 123 aa in the carboxy-terminus (C-terminus) (23, 24) (Fig. 3).

The mucin-like extracellular domain has an unfolded structure and protrudes about 45 nm from the cell surface. This is due to a high content of proline, alanine and glycine residues that make it rigid and inflexible (37). The extracellular domain is also rich in serines and threonines, which enables extensive *O*-glycosylation (70–80 polysaccharide side chains). However, there is only one potential *N*-glycosylation site located close to the transmembrane domain at position N239 (38). The CD43 region between residues 135 and 224 contains five imperfect repeating units of 18 aa of unknown function (23). The non-glycosylated precursor of CD43 is reported to migrate on SDS-PAGE at molecular weight of 54 kDa (10). The *O*-glycosylation pattern of CD43 varies between hematopoietic cell lines, which results in production of molecules with different sizes (5, 39). Two differentially glycosylated forms of CD43 have been described in hematopoietic cells. The 130 kDa isoform possesses mainly branched hexasaccharides, while the 115 kDa isoform contains almost exclusively tetrasaccharides (11). Resting T lymphocytes express mostly the 115 kDa form of CD43, while activated T cells, monocytes, neutrophils and

platelets express the 130 kDa form (10, 40). Several isoforms of CD43 can be coexpressed on the surface of the same cell (41, 42), suggesting that they are functionally distinct. CD43 has a negative net charge due to the abundance of sialic acid residues in its *O*-glycan structures (23).

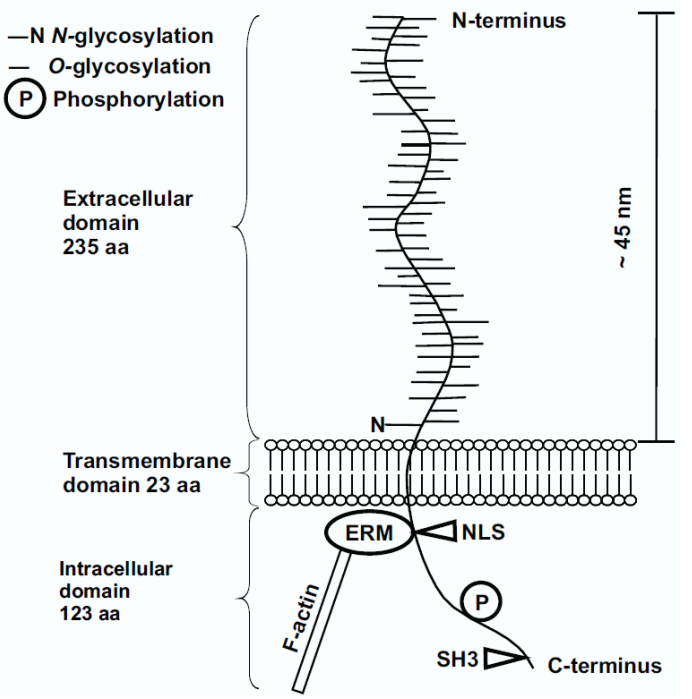

Figure 3. Structure of CD43 protein. The protruding extracellular domain, primarily regulating cell adhesion, contains multiple *O*-glycosylation sites and a potential *N*-glycosylation site. The intracellular domain, involved in signal transduction, can be phosphorylated and interacts with ERM adaptor proteins that link it to the actin cytoskeleton. The intracellular domain also contains a nuclear localization signal (NLS) and a proline-rich SH3 binding sequence.

The extracellular domain of CD43 is proteolytically cleaved from the cell surface and the soluble extracellular part can be detected in normal human sera (43). The shedding is mediated by metalloproteases and serine proteases in PMA (phorbol 12-myristate 13-acetate) stimulated granulocytes and lymphocytes (44), neutrophils (45), and mast cells (46). The cleavage site is suggested to be localized close to the transmembrane domain, between the residues F245 and R246 (43).

The relatively long intracellular domain of CD43 lacking catalytic activity is conserved among human, rat, and mouse species (38). It contains 6 threonines and 11 serines, which are potentially phosphorylated, but has no tyrosine

residues (23, 24). Five phosphorylation sites: S291 (47), T341 (48), S351 (49), S355 (50), and S368 (47) have been identified within the domain. At least protein kinase C (PKC) participates in CD43 phosphorylation (49); however, the involvement of protein kinase A and protein kinase G is also possible (50). A proline-rich region in the end of the C-terminus is homologous to SH3 (Src homology 3) binding domain (51).

2.3. Functions of CD43 protein

The functions attributed to CD43 remain contradictory, which reveals a complex nature underlying CD43 signaling. For example, CD43 has been shown to participate in both pro-adhesion and anti-adhesion, apoptosis and proliferation (52). Apart from regulating cell activation, differentiation, adhesion, and migration, CD43 is implicated in immune response by modulating cell growth, survival and apoptosis. Early expression of CD43 on hESC-derived hematopoietic progenitors may also indicate a possible role for CD43 in hematopoietic development (53).

2.3.1. Adhesion and migration

CD43 extends high above cell surface; therefore it is one of the first molecules that interact with the surface molecules of other cells. Moreover, it is abundantly expressed in blood cells, suggesting that CD43 regulates contacts between cells either by preventing or promoting cell adhesion (54). Cell adhesion is imperative for normal functioning of immune system, e.g. interactions between T cells and antigen presenting cells, leukocytes and endothelial cells, and between circulating blood cells.

During immune and inflammatory responses circulating leukocytes need to extravasate from the vascular system into the lymphoid or other tissues. This requires a cascade of coordinated adhesion and signaling events that allow recruitment, rolling, and transmigration of leukocytes (55). Leukocytes from CD43 knockout mice showed a significant impairment in binding to endothelium and ability to exit the circulation and infiltrate tissues (56, 57). Ligation of CD43 with specific antibodies support the findings that CD43 is involved in the regulation of leukocyte activation and adhesion to endothelial and extracellular matrix (ECM) ligands (57, 58). It is considered that CD43 promotes adhesion through the interactions with the lectins E-selectin (59), galectin-1 and galectin-3 (60), siglec-1 (61), M-ficolin (62), also integrins (63), cell surface nucleolin (64), and ICAM-1 (intercellular adhesion molecule type 1) (65). Interestingly, all ligands described for CD43 are ligands for other cell surface molecules, implying that by binding them, CD43 regulates their accessibility to their other cognate receptors (52). The interaction of CD43 with ICAM-1 and MHC (major histocompatibility complex) class I suggests that

CD43 induces association between T cells and antigen presenting cells and promotes T cell activation (65, 66).

On the other hand, CD43 prevents leukocyte aggregation in the blood flow. The anti-adhesive properties of CD43 are further proved by *in vivo* experiments. In transgenic mice expressing CD43 in peripheral mature B cells, which normally have very low levels of CD43, the interactions between T and B cells were impaired (67). CD43-deficient leukocytes showed increased homotypic adhesion and capacity to bind to different ligands (21, 68, 69). Furthermore, studies of leukocyte-endothelial cell interactions demonstrated that leukocytes from CD43 knockout mice had enhanced adhesive and rolling properties compared to those of leukocytes from wild type mice (56). The controversy in adhesional behaviour of CD43 can be explained by its variable post-translational modifications (e.g. glycosylation). It has been reported that among different CD43 isoforms on the surface of T lymphocytes only the 130 kDa protein displayed anti-adhesive properties (70). Besides, the bulky sialylated *O*-glycans without additional modifications are known to exhibit anti-adhesive properties. In contrast, the N-acetyl-galactosamine residues of the *O*-glycans on CD43 may express the sialyl Lewis^X (SLe^X) structure (i.e. a tetrasaccharide carbohydrate) in a differentiation-dependent manner (71), favouring cell-cell interactions. Since different CD43 isoforms can be simultaneously expressed on the cell surface (41, 42), it is supposed that they regulate cell-cell interactions through a very delicate interplay (52). It is important to mention here that the anti-adhesive properties of CD43 only partly depend on the negative charge and the protruding structure of the extracellular domain. The interaction of the intracellular domain of CD43 with the cytoskeleton is also indispensable (72).

The intracellular domain of CD43 participates in signal transduction and in relocalization of CD43 in the plasma membrane, e.g. during cell migration and immunological synapse (a tight and stable T cell/antigen presenting cell contact). It acts as a docking site for the ERM adapter proteins, ezrin, radixin, and moesin, which cross-link actin cytoskeleton with transmembrane proteins, such as CD43, CD44 and ICAM-2. The interaction region is proposed to be located within a cluster of positively charged amino acids in the juxta-membrane region of these transmembrane proteins (73). The ERMs are implicated in various functions that involve cytoskeletal and membrane remodelling, regulation of cell shape and migration. They favour CD43 redistribution on the cell surface in different cellular contexts. For example, CD43 was found to co-localize with the ERM proteins in the cleavage furrow of dividing cells (74) and in microvilli (75) that aid in the migration of leukocytes. Furthermore, by the agency of the ERM proteins, CD43 participates in establishment and maintenance of cell polarity, which is essential for migration, activation, and apoptosis of leukocytes. During T cell activation the ERM proteins together with F-actin translocate CD43 from the contact area between an antigen presenting cell (macrophage, B lymphocyte, dendritic cell) and a T helper cell

to the opposite pole of the T cell (76–78). Also, neutrophil and T cell polarization and locomotion were shown to be associated with the relocalization of CD43 by moesin to the uropod, i.e. an appendage at the posterior pole of a migrating cell that protrudes from the contact area with endothelial or ECM substrates (79, 80). Interestingly, the uropod is mostly involved in cell-to-cell interactions (in processes such as antigen transport, cytotoxicity, extravasation or in the foci of cell proliferation) and in such leukocyte activities as activation and apoptosis (81).

2.3.2. Signal transduction

Cumulative experimental evidence indicates that CD43 senses the extracellular environment through its interaction with a multitude of counter-receptors. Despite CD43 lacks an intrinsic catalytic activity, it transduces multiple signals to the intracytoplasmic milieu and regulates different aspects of immune and non-immune cells function. CD43-mediated signal transduction is studied using CD43-specific mAb since no CD43-specific ligand has been reported so far and the known ligands of CD43 interact with other receptors. CD43, as a co-receptor of TCR (T cell receptor), activates most of the signaling cascades triggered by the TCR contributing to T cell activation (52) (Fig. 4). Ligation of CD43 on the surface of T cells induced association between CD43 and the Src family non-receptor protein tyrosine kinases (PTKs) Fyn and Lck (51, 82, 83). The interaction is presumably mediated by the SH3 domain of PTKs and the proline-rich SH3 binding sequence in the C-terminus of CD43. This caused activation of the PTKs and phosphorylation of ζ chain as well as of the tyrosine kinase ZAP70 (84), the guanine exchange factor (GEF) Vav and of the adaptor protein Shc. As a result, macromolecular complexes containing Shc-GRB2-Vav and Vav-SLP-76 were formed through tyrosine-phosphorylated sequences and SH2, i.e. phosphotyrosine binding domains (83). These events led to activation of mitogen-activated protein kinases (MAPKs): extracellular signal regulated kinase (ERK) 1 and 2, p38, and Jun N-terminal kinase (JNK) (83, 85, 86). It was shown that activation of ERK pathway resulted in recruitment of transcription factors AP-1 (activator protein 1), NF-κB (nuclear factor kappa B) and ELK1 (E twenty-six-like transcription factor 1), whereas NFAT (nuclear factor of activated T cells) activation was independent of ERK (87, 88). In immature hematopoietic cells CD43 signaling was also found to activate ERK1 and ERK2 through a phosphorylation cascade starting from tyrosine kinases Syk and Lyn (89). The MAPK-dependent activation of transcription factors, in response to CD43 signaling, ultimately regulated gene expression (83, 87).

In addition, phospholipases Cγ (PLCγ) contain SH2 domains allowing them to interact with phosphotyrosines. Engagement of CD43 is known to activate PLCγ2, which induces generation of diacylglycerol (DAG), inositol 1,4,5-trisphosphate (IP3), and Ca^{2+} mobilization, leading to activation of the protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) (85, 88, 90). Opposed

to TCR-mediated signals, CD43 ligation results in the serine phosphorylation of the E3 ubiquitin ligase Cbl thus blocking Cbl negative effects on T cell activation. Cbl serine phosphorylation, MAPK activation, AP-1, NF-κB and NFAT activation, caused by CD43 ligation, are all PKCθ-mediated (52, 83, 88, 91). CD43 itself can probably be phosphorylated by PKC (49).

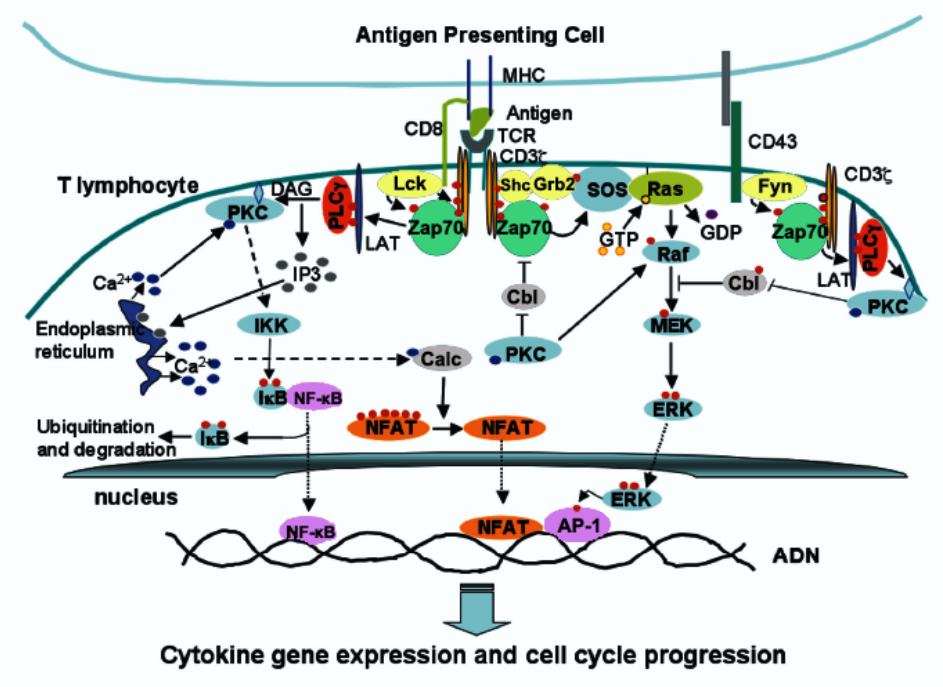

Figure 4. CD43-induced signaling pathways in T cells. The CD43 signaling pathways are complex and regulate important aspects of cell function such as cell growth, differentiation and apoptosis. The Src family of non-receptor protein tyrosine kinases, activated upon CD43 engagement by its ligand(s) on the APC, controls formation of the immunological synapse and T cells activation. Ligation of CD43 also triggers cell proliferation via PKC pathway. Presumably, CD43, as a TCR co-receptor, activates most of the signaling cascades triggered by the TCR, which provides a substantial amplification of the signal, sufficient to overcome the threshold for T cell activation (52). LAT – linker for activation of T-cells family member 1, IκB – inhibitor of nuclear factor kappa-B, IKK – inhibitor of nuclear factor kappa-B kinase, Calc – calcineurin. Reproduced by the permission of John Wiley & Sons, Inc. © 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

However, it is still unknown which small GTPase is activated in response to CD43 engagement and whether different CD43 ligands could activate different MAPK pathways by activating different GTPases. On the one hand, CD43 signaling increases the levels of DAG. Further, Ras guanyl nucleotide-releasing proteins (RasGRP), containing DAG-responsive C1 domain, are capable of activating Ras and other small GTPases by facilitating the exchange of GDP for GTP via their GEF domain (92, 93). Notably, DAG not only exerts a direct role in RasGRP activation through the C1 domain, but it also functions as an indirect regulator of RasGRPs through PKC-mediated phosphorylation (94). On the other hand, it is shown that upon T cell activation the macromolecular complexes containing phosphorylated Shc and the adaptor molecule GRB2, which recruits the GEF Sos, induce the GTPase activity of Ras, leading to activation of the Ser/Thr kinase Raf as well as Raf phosphorylation on serine residues (95). Yet, no changes in either Sos or Raf mobility on SDS-PAGE resulting from their phosphorylation were detected following CD43 cross-linking (83). Therefore, despite CD43 signaling induces cellular proliferation, a function that normally requires Ras activation, Shc-GRB2 complexes formed upon CD43 ligation are probably involved in the activation of other small GTPases. Alternatively, CD43 ligation favours cell proliferation by signaling downstream of Raf, e.g. through PKCθ-dependent Cbl serine phosphorylation, which prevents TCR-induced Cbl tyrosine phosphorylation, thereby allowing activation of MEK/ERK pathway (52, 96, 97). In addition, a yeast two-hybrid screen, identified the serine/threonine kinase HIPK2 (homeodomain-interacting protein kinase 2) as a molecule that interacts with the cytoplasmic domain of CD43. This kinase, localized to the cytoplasm and the nucleus of cells, is homologous to a yeast kinase regulator of the Ras pathway (98, 99). Finally, some reports indicate that Vav could be a GEF for Ras (100). Nonetheless, later studies provide evidence that Vav is most probably a GEF for Rac 1, another small GTPase that is also activated upon TCR cross-linking (101, 102). Differential activation of small GTPases may play a role in the different cellular responses mediated by CD43.

2.3.2.1. Cell proliferation and apoptosis

CD43 engagement results in production and secretion of cytokines, e.g. interleukin-2 (IL-2), which in turn stimulates proliferation and differentiation of cells (87, 103–105). However, in myeloid progenitors ligation of CD43 reduced DNA binding activity of AP-1 transcription factor, thereby causing down-regulation of gene expression and initiation of the BAD-dependent apoptotic pathway (106). Notably, cross-linking of CD43 induces apoptosis of human hematopoietic progenitor cells but not stem cells, indicating that CD43 might have opposing functions in different cells (41, 107).

The pro-apoptotic functions of CD43 are thought to be important for contraction of T cell-mediated immune response, once the infection is cleared.

FAS, a cell death receptor, the expression of which is upregulated by TCR-dependent signals (108), is involved in killing activated T cells by inducing Caspase-8-dependent apoptosis (109). CD43 signaling promotes FAS expression and T cell death (41, 110, 111). Apoptosis of T cells upon CD43 ligation was accompanied by repression of NF-κB activity (41, 110). In addition, galectin-1, a ligand for CD43, also upregulated during T cell activation (112), is involved in sensitizing T cells (particularly the ones bearing the 135 kDa CD43 isoform) to apoptosis (113, 114) by causing CD43 segregation into membrane microdomains (115, 116). Moreover, macrophage cell surface-expressed nucleolin was shown to specifically interact with the carbohydrate residues of CD43 concentrated in caps on early apoptotic T cells resulting in phagocytosis (64, 117).

In contrast, in activated B cells CD43-mediated signals contribute to cell division through a PKC-mediated mechanism (118, 119). In agreement with this, CD43 overexpression in mature B cells *in vivo* gave splenomegaly due to increased proliferation of these cells (120). CD43 also promoted survival of B cells as it reduced sensitivity to G1 arrest and apoptosis *in vitro* (121). Thus, uncontrolled proliferation and enhanced survival capacity, the two hallmarks of tumor cells, are positively regulated by CD43 in B cells. Moreover, expression of CD43 on B cell lymphomas correlates with a bad prognosis (122–124). Besides, tumor cells expressing abnormally high levels of CD43 have been proposed to escape FAS-mediated killing, thus providing a mechanism for better survival of cancer cells (9).

3. CD43 is a mucin-like cancer-associated glycoprotein

Mucins and mucin-like molecules are known to be expressed by many types of cancer, especially adenocarcinomas (epithelial cancers that originate in glandular tissue). Interestingly, these proteins are not often mutated in cancer cells, instead, alterations in mucin amounts and glycodynamics are commonly reported. Mucins contribute to cancer progression by modifying cell adhesion, migration, survival, proliferation and immune surveillance (125–127). The membrane-associated mucin MUC1 has been the focus of a considerable interest owing to its changing expression and glycosylation levels as well as modifications of *O*-glycans (often resulting in new and unusual carbohydrate and peptide epitopes) in different cancers (128). MUC1 expression has been reported to reduce cell adhesion due to the large and extended structure of its extracellular domain (129). On the other hand, MUC1 favours tumor cell adhesion to endothelium and subsequent invasion through the interactions with ICAM-1 (130), E-selectin (131) and sialic acid binding immunoglobulin-like lectins, siglecs (132). MUC1 carrying core 2 *O*-glycans has been shown to function as a molecular shield against natural killer cell attack, promoting

bladder tumor metastasis (133). Moreover, MUC1 participates in cell signaling via the Wnt pathway effector β-catenin and the EGFR (epidermal growth factor receptor) family of receptor tyrosine kinases which activate Ras-MEK-ERK2 pathway (134, 135). Therefore MUC1 plays an important role in tumor cell proliferation and differentiation. It is of note that the cytoplasmatic tail of MUC1 associates with β-catenin in the nucleus and co-activates transcription of Wnt target genes (136).

Another example of a cancer-related glycoprotein is the mucin-like type I transmembrane protein CD44. CD44 is a marker of "cancer stem" cells (137) being implicated, among others, in breast and colorectal cancers (138). The extracellular domain of CD44 binds numerous ECM components, including hyaluronan, and osteopontin, which affect cell motility and invasion (139, 140). Similar to CD43, CD44 has no intrinsic kinase activity; it induces signal transduction by recruiting intracellular kinases and adaptor proteins, e.g. ERMs (141). CD44 interacts with the EGF and HGF (hepatocyte growth factor) receptors and has been shown to activate a number of central signaling highways: Rho GTPases, the Ras-MAPK and the PI3K/AKT pathways which promote cell growth, survival, and invasion (138, 139, 142). In addition, CD44 serves as a docking site for matrix metalloproteases that degrade basement membrane and promote tumor invasion (141). The cytoplasmic tail of CD44 can be cleaved off and translocated to the nucleus where it mediates gene transcription (143).

Although most of the work investigating CD43-mediated signaling has been done in the context of hematopoietic cells and CD43 has long been considered a specific marker of immune cells, strong evidence supporting CD43 involvement in tumorigenesis start to emerge. A number of studies demonstrate CD43 expression in different tumors of non-hematopoietic origin, including lung, breast and colon, but not in normal tissues (2–4). CD43 expression is also detected in several cancer cell lines (5, 144, 145). Such an altered expression of CD43 has been associated with neoplastic transformation (3, 4, 7). CD43 signaling in tumor cells is suggested to promote oncogenesis by activating β-catenin, NF-κB, NFAT and AP-1, which are prosurvival transcription factors and contribute to a tumor phenotype when deregulated (87, 146).

Importantly, CD43 is glycosylated differently in cancer cells, e.g. in colon carcinoma cell line COLO205, the full length glycosylated CD43 possesses a molecular mass of over 200 kDa (5, 147). Moreover, tumor-specific glycoforms of CD43 are expressed in different carcinomas, but not in normal tissues from the same patients (148). Aberrant expression and glycosylation of CD43 are associated with immune deficiency (149, 150) and have been proposed to contribute to cancer progression (4, 125). Indeed, changes in the glycodynamics of CD43 might account for the oncogenic properties of the protein. It has been reported that abnormal expression level of certain *O*-glycan structures as well as occurrence of incomplete or truncated forms, precursors, or novel structures of *O*-glycans may affect ligand-receptor interactions (e.g. modulate binding to

alternative ligands) and thus interfere with regulation of adhesion, migration and signal transduction (127, 151). For example, the sialyl Lewis structures (present on CD43) are frequently overexpressed by cancer cells and could be found on both *N*- and *O*-glycans (152–155). Alterations in early branch points in the normal pathways of glycan biosynthesis can markedly affect the relative amount of one class of structure while allowing the dominance of another, promoting malignant transformation and tumor progression. Colon cancer cells simplify their *O*-glycan biosynthesis causing a relative increase of core 2 *O*-glycan structures, which are the main carriers of SLe^X. Moreover, in normal colonic mucosa, type 1 and type 2 carbohydrate chain extensions are formed, but only the terminal type 2 chain-repeat unit is the precursor for the SLe^X antigen. In adenocarcinomas, and especially in high-grade and advanced human colorectal cancers, type 2 chains are produced. Notably, the activity of glycosyltransferases involved in their synthesis is upregulated, and the activity of glycosyltransferases that synthesise other types of chains is inhibited (127). The sialyl Lewis structures are ligands for selectins that normally participate in the attachment of leukocytes to the endothelium. Therefore, cancer cells might use the SLe^X-selectin-binding mechanism during tumour invasion and metastasis (156). In fact, highly metastatic tumor cells have been reported to adhere more strongly to E-selectin than their poorly metastatic counterparts (157, 158). Also, CD43- and ICAM-1-mediated cell adhesion in several carcinomas was proposed to support metastasis (145). In addition, in human colon cancer metastasis, mucin-associated carbohydrate structures showed enhanced sialyation (159); and sialylated *O*-glycans were associated with an enhanced growth rate of mammary carcinoma cells in mice (160). Finally, proteolytic processing of certain proteins demonstrates a requirement for *O*-glycans at specific sites in order to prevent proteolytic cleavage which eliminates biological activity or prevents continued residence/activity of the intact protein at its designated subcellular location (161). Thus, abnormal glycosylation could modulate the proteolytic processing of CD43 and therefore interfere with CD43 functionality.

3.1. Regulated intramembrane proteolysis

The number of CD43 molecules expressed on the cell surface is tightly regulated by several mechanisms: changes in its transcription rate, redistribution on the plasma membrane or downregulation by proteolysis and shedding (52). Metalloproteases and serine proteases are known to be responsible for the proteolytical removal of the extracellular domain of CD43 from the plasma membrane (44–46, 162) thereby regulating its surface expression. On the other hand, the cleavage by metalloproteases in the extracellular domain of some transmembrane proteins has been shown to trigger a release of the intracellular domain, which is often mediated by γ-secretase complex.

While in blood cells CD43 is expressed predominantly in the cell membrane, in non-hematopoietic cancer cells, in contrast, CD43 is located primarily intracellularly and even nuclearly (7). Similarly to leukocytes, the cleavage in the extracellular domain of CD43 in cancer cells is well described (147) (Fig. 5). In addition, in several human carcinoma cell lines CD43 is known to be processed by γ-secretase proteins discharging its C-terminus (146). The resulting CD43 cytoplasmic tail (CD43ct) has been shown to localize into a subnuclear structure, known as PML (promyelocytic leukaemia) nuclear body, and is involved in the regulation of apoptosis (163). The cytoplasmic domain of CD43 contains a functional nuclear localization signal (NLS) and interacts with the nuclear transporter protein Ran, which offers an explanation for the nuclear localization of CD43 (164).

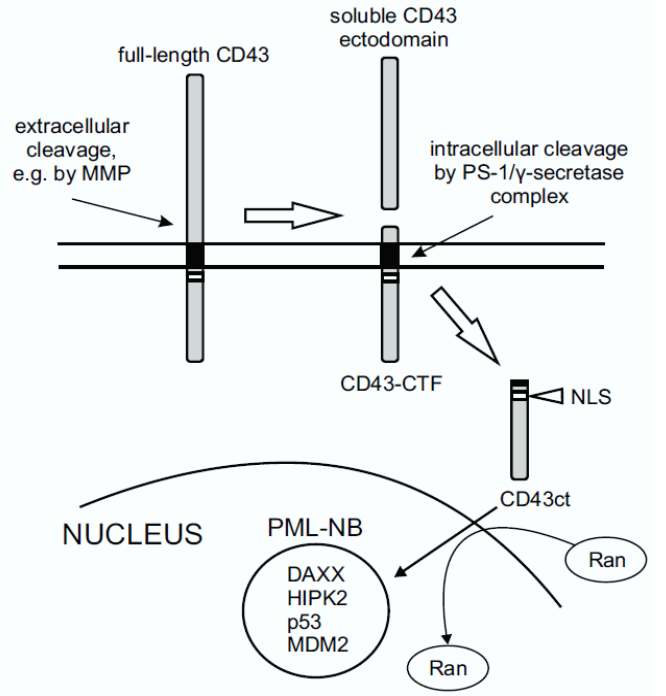

Figure 5. Processing of CD43 in the regulated intramembrane proteolysis (RIP) pathway. The proteolytic processing of the full-length CD43 protein starts with the primary cleavage that can occur in ER compartments or at the cell surface. The subsequent secondary intramembrane cleavage of the CD43 cytoplasmic tail fragment (CD43-CTF) by presenilin-1/γ-secretase complex releases the cytoplasmic tail (CD43ct) into the cytoplasm. CD43ct contains a nuclear localization signal (NLS) directing it into the nucleus, which is probably facilitated by RanGTPase. Nuclear CD43ct is involved in regulation of apoptosis by interacting with the proteins in PML nuclear bodies (PML-NB). Adopted from (164) with modifications.

Therefore, CD43 might be involved in the regulated intramembrane proteolysis (RIP) signaling pathway similarly to Notch-1 (neurogenic locus notch homolog protein 1) (165, 166), ERBB-4 (receptor tyrosine-protein kinase erbB-4) (167), CD44 (143, 168), E-cadherin (169), and amyloid precursor protein (170, 171). In RIP signaling the intramembrane cleavage does not take place until the bulk of the protein on the extracytosolic (luminal or extracellular) face has been removed by a primary cleavage. This primary cleavage can occur in the lumen of the ER, in a post-ER compartment, or at the cell surface. The secondary, intramembrane cleavage of type I transmembrane proteins, among which CD43 belongs, requires presenilin-1 (an important component of γ-secretase complex) that cleaves off the cytoplasmic tail together with a few amino acids from the transmembrane region. The generated cytoplasmic fragment has in some cases been shown to translocate into the nucleus where it triggers gene activation, e.g. acting as a transcription factor (172–175).

Interestingly, the NLS sequence of CD43 overlaps with the ERM-binding motif. For efficient nuclear transfer, CD43ct should be released from ERM proteins. Moreover, by structurally blocking the access of the protease complex to the cleavage site, the ERMs bound to the juxta-membrane region of CD43 may repress the release of the cytoplasmic tail. In either case, it is an interesting viewpoint of CD43 regulation that activation of CD43 processing proteases may be coupled with regulation of ERM proteins (176).

3.2. Interaction of CD43 with other signaling molecules and pathways

In accordance with the hypothesis that CD43 is involved in the RIP pathway, the CD43ct was found to translocate to the nucleus and interact with β-catenin, resulting in the up-regulation of the β-catenin target genes *MYC* and cyclin D1 in colon carcinoma cells (164). β-catenin is a multifunctional protein involved in embryonic development and renewal of adult tissue. It is also a potent proto-oncogene, the aberrant activation of which has been shown to play a critical role in the development of different cancers including colon (177, 178). β-catenin has dual functions: it belongs to the cell-cell adhesion apparatus and mediates Wnt signal in the nucleus (Fig. 6). Upon Wnt signaling β-catenin translocates to the nucleus where it interacts with T cell factor (TCF) and lymphocyte-enhancing factor (LEF) family transcription factors and with other transcriptional cofactors to form transcriptionally active complexes that regulate genes important for proliferation, differentiation, and apoptosis (177, 179). The functioning of β-catenin is controlled by a large number of binding partners that affect its stability and localization (180). Thereby, β-catenin is able to participate both in cell adhesion and in gene expression. The interaction between the cytoplasmatic domain of CD43 and β-catenin links CD43 to the Wnt/β-catenin signaling pathway which is often activated in colon cancer.

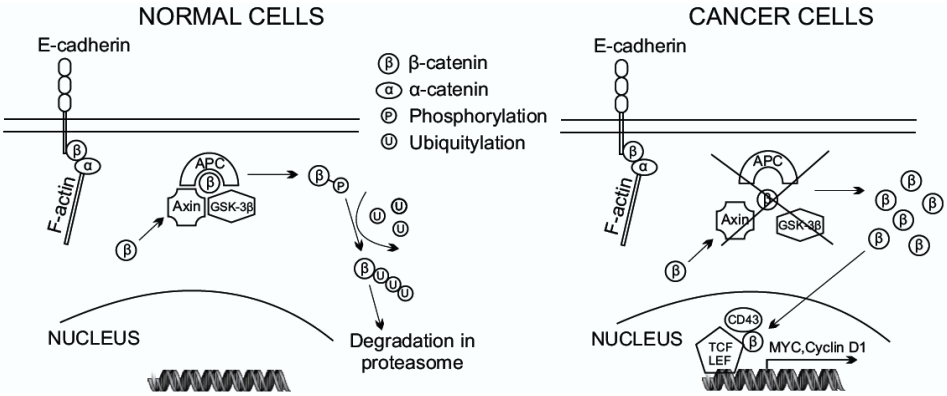

Figure 6. Role of β-catenin in cancer. β-catenin in adherens junctions links E-cadherin to α-catenin that interacts with the actin cytoskeleton. In normal cells the levels and localization of β-catenin are regulated by a complex of proteins that includes adenomatous polyposis coli protein (APC), glycogen synthase kinase-3 beta (GSK-3β) and axin. This complex phosphorylates β-catenin, which directs it to the ubiquitin-proteasome pathway. In most colorectal cancers the APC/GSK-3β/axin/β-catenin complex is unable to form due to mutations in one of its components. This results in accumulation of free cytoplasmic β-catenin and its translocation to the nucleus. Nuclear β-catenin interacts with TCF/LEF transcription factors and other cofactors to activate transcription of genes important for cell growth, e.g. *MYC*, *cyclin D1*, *JUN*, *CD44*. Adopted from (181) with modifications.

Furthermore, the proteolytically released CD43ct is shown to be SUMOylated and recruited into PML nuclear bodies (PML-NBs) (163) which increase in number during autoimmune and cancerous diseases (182). PML-NBs are implicated in key cellular processes such as transcriptional regulation, genome stability, response to viral infection, apoptosis, tumor suppression, senescence and stem cell self-renewal. PML-NBs are macromolecular substructures in the nucleus of mammalian cells organized by the PML protein that recruits various proteins including SP100, p53, DAXX, HIPK2, MDM2 (183). The only common feature of these proteins known to date is their ability to be SUMOylated (184). The CD43ct recruited into PML-NBs participates in cell homeostasis and apoptosis supposedly by interacting with PML-associated proteins (163). A yeast two-hybrid screening has revealed an interaction between the apoptotic regulator DAXX (death-domain associated protein) and the cytoplasmic domain of CD43 (106). DAXX has been shown to play a role in cellular functioning in the cytoplasm and in the nucleus mediating both pro- and anti-apoptotic signals (185–187). In addition, the intracellular domain of CD43 interacts with murine HIPK2 (alternative name STANK, sialophorin tail-associated nuclear kinase), a serine/threonine kinase, which shuttles between the nucleus and the cytoplasm (99). Its human ortholog activates the tumor

suppressor protein p53 by phosphorylating it at Ser46 (188). PML-NBs are proposed to fine-tune cellular processes through facilitation of partner protein post-translational modifications resulting in partner sequestration, activation or degradation. Importantly, p53-modifying enzymes (CBP, MDM2, HIPK2, and HAUSP) are concentrated within these NBs. PML-enhanced acetylation, sumoylation, and phosphorylation occurring in PML-NBs all appear to activate p53 (183). The data previously reported by our group indicates that CD43 overexpression in non-hematopoietic cancer cells leads to accumulation of active p53 (189). Therefore, one could speculate that CD43 is involved in these post-translational modifications of p53 via HIPK2. Alternatively, as provided by the example of hematopoietic cells, the membrane-bound CD43 might stabilize p53 through activating MAP kinases JNK, p38 and ERK, which have been shown to directly phosphorylate p53 at Ser15 (190). Supporting this hypothesis, an increase in the phosphorylation of p53 at Ser15 upon CD43 overexpression has been detected in non-hematopoietic cells, as well as subsequent induction of apoptosis (189). Activated MAPKs, functioning as effector protein kinases, phosphorylate a variety of substrates and affect cell growth, differentiation and apoptosis. In addition, CD43 interacts with other proteins that modulate the activity of p53, e.g. β-catenin (191) and nucleolin (192).

4. Tumor suppressor p53

p53 is considered to be a tumor suppressor protein with the most wide-ranging functions. The significance of p53 is illustrated by the fact that *TP53* gene is lost or contains inactivating mutations in about half of human cancers from different tissues (193, 194). It is worth mentioning that p53 was originally discovered in its mutated form and identified as a transformation associated protein (195–199). Since then p53 has become probably the most studied protein, and an immense amount of data concerning p53 has been gathered by now. According to the current understanding, p53 is a transcription factor that maintains genomic stability being involved in a wide range of cellular processes.

4.1. Functions of p53

p53 is the key player under stress conditions. It responds to various stress forms: genotoxic stress (UV and IR, cytotoxic drugs, carcinogens), non-genotoxic stress (hypoxia, temperature changes, nutrient deprivation) and oncogenic stress (193, 194). In order to prevent proliferation of damaged cells, p53 is capable of arresting the cell cycle or directing more injured cells to apoptosis (200–204). Being the "genome guardian" (205) p53 regulates DNA replication (206), DNA synthesis and repair (207–209), DNA damage response and gene expression. p53 is also implicated in cellular differentiation (210) and senescence (211).

Stress signals lead to the accumulation of p53 in the nucleus, where it modulates expression of its target genes acting as a transcriptional activator or repressor. For example, G2 arrest is mediated by p53-dependent transactivation of *GADD45B* (growth arrest and DNA damage-inducible, beta) and *SFN* (stratifin; also known as 14-3-3 sigma) genes and transrepression of cyclin B1, *CDC25C* (cell division cycle 25 homolog C) and *CDK1* (cyclin-dependent kinase 1) (212–216). Likewise, p53-mediated apoptosis requires both the activation of pro-apoptotic genes and the repression of genes that promote cell growth and survival. The overexpression of *BCL2* (B-cell CLL/lymphoma 2), *BIRC5* (baculoviral IAP repeat containing 5; alternative name survivin), *Mtap4* (microtubule-associated protein 4; human ortholog *MAP4*) and *Trp53bp1 (tumor protein p53 binding protein 1)* genes, which are transrepressed by p53, has been shown to inhibit induction of p53-dependent apoptosis (217–222). It is considered that p53-mediated transcriptional repression is first and foremost required for the normal progress of apoptosis.

In addition to the regulation of gene expression, p53 is able to induce apoptosis in a transcription-independent manner. In damaged cells a fraction of p53 protein translocates to mitochondria, where direct p53 signaling promotes cytochrome c release and Caspase-3 activation (223, 224). It is possible that the induction of p53-mediated apoptosis involves both p53-dependent regulation of gene expression and direct protein signaling in mitochondria.

4.1.1. Mechanisms of p53-mediated transcriptional activation and repression

Both transcriptional activation and repression involve p53 binding to a specific DNA response element in the promoter region of a target gene or its proximity and direct interaction of p53 with the basal transcriptional machinery. The consensus DNA-binding site for p53-mediated transactivation contains two copies of a 10-bp motif 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3', separated by a 0-13-bp spacer region (Pu and Py are purine and pyrimidine base containing nucleotides, respectively) (225, 226). In addition to the p53 consensus sequence, binding sites required for p53-dependent repression have been described (219, 227, 228), but p53 is also capable of transcriptional repression from the consensus binding site (228–230).

During p53-mediated transcriptional activation tetrameric p53 recognizes its consensus binding site and interacts with basal co-activators – acetyltransferases p300/CBP (CREB-binding protein) and PCAF (p300/CBP-associated factor) that enhance the activity of p53 (231–233). p300/CBP increases the sequence-specific DNA-binding activity of p53 by acetylating its C-terminus (234). The histone acetylase PCAF modifies p53, similarly to p300/CBP, and histones. The latter helps to convert the chromatin structure into an open form that facilitates the access of the transcriptional machinery to DNA (235, 236).

These events enable p53 to bind the basal transcription factor TFIIH that belongs to the RNA polymerase II preinitiation complex (208). p53 also interacts with the components of the transcription factor TFIID complex, TBP-associated factors dTAFII60, dTAFII40 and hTAFII31 (237, 238).

Three molecular models of the transcriptional repression by p53 have been proposed to date. p53 can obstruct binding of transcriptional activators to DNA or inhibit their activity by direct interaction (1), prevent assembly of basal transcription factors (2) and remodel chromatin structure (3).

Firstly, the binding of other transcriptional activators is obstructed by the interaction of p53 with their target sequences in DNA. For example, there is a partial overlap between p53 and transcription factor SP1 binding sites in the promoter of *POLD1* (polymerase (DNA directed), delta 1, catalytic subunit 125 kDa) gene (239). In the case of *AFP* (alpha-fetoprotein) gene p53 binds to its target sequence in *AFP* promoter region and prevents the binding of HNF-3 (hepatocyte nuclear factor 3) transcription factor (240). Also, the functioning of transcription factors can be disrupted by direct association with p53 protein. p53 is capable of interaction with SP1 bound to a promoter, which inhibits the transactivating properties of SP1. This mechanism is implicated in p53-dependent repression of cyclin B1 promoter lacking the p53-binding site (241). It has been demonstrated that the regulation of SP1 activity provides for p53-mediated transrepression of several other genes, such as telomerase reverse transcriptase, insulin receptor, vascular endothelial growth factor A (242–244). In addition to SP1, p53 binds estrogen receptor, hepatocyte nuclear factor 4-alpha-1 and glucocorticoid receptor, which results in transcriptional repression of their target genes (245–247).

Secondly, p53 specifically represses activity of promoters whose initiation is dependent on the presence of the TATA box, and the repression is mediated by an interaction of p53 with another component of the basal transcription factor TFIID complex, TBP (TATA-binding protein) (248–250).

Thirdly, p53 represses transcription via chromatin remodeling. The proline-rich domain of p53, which mediates p53-dependent apoptosis, is considered to be required for this phenomenon (251). The proline-rich domain recruits the co-repressor SIN3A that binds HDAC1 complex and thereby promotes the deacetylation of histones in the promoters of target genes (252). By the same token, the HDAC inhibitor TSA (trichostatin A) abrogates the ability of p53 to repress the expression of its target genes like of *Mtap4*, stathmin 1, alpha-tubulin and *BIRC5* (220, 252, 253). p53 has been shown to decrease acetylation of histone H3 in the *BIRC5* promoter (219, 220). In the case of *Mtap4* and *Myc* (myelocytomatosis oncogene; human ortholog *MYC*, v-myc myelocytomatosis viral oncogene homolog (avian)) an interaction between p53 and HDAC through the intermediary of SIN3A has been detected (252, 254). It is also known that hypoxia which induces mostly p53-dependent transrepression leads to increased formation of p53-SIN3A complexes (253).

4.2. Tumor suppressor ARF responds to oncogenic stress

The *CDKN2A* (cyclin-dependent kinase inhibitor 2A) locus encodes two tumor suppressor proteins, INK4a and ARF (alternate reading frame) that indirectly govern the activities of the retinoblastoma family proteins (RB, p107, and p130) and p53, respectively (255, 256). Since the inactivation of INK4a, ARF, RB, and p53 allows cells that sustain oncogenic insults to survive and proliferate, their loss of function is detected in most forms of human cancer. *CDKN2A* is the second most frequently inactivated gene in human cancers after *TP53*. Due to the organization of *CDKN2A* locus the two genes are induced by different stress signals and can be separately mutated, deleted or epigenetically silenced in tumor cells (257).

INK4a is a negative regulator of Cyclin D1-CDK4 and CDK6, and therefore it prevents cell cycle progression through maintaining RB-family proteins in their active hypophosphorylated state (258). In contrast, ARF antagonizes the p53 negative regulator MDM2 (murine double minute 2) (259, 260) (Fig. 7). MDM2 is known to inhibit p53-mediated transactivation (261) and promote degradation of p53 via its ubiquitylation (262) and shuttling to cytoplasmic proteasomes (263). ARF binds directly to MDM2, sequestering it in the nucleolus (259, 260) and enabling a p53 response that can lead to premature senescence or apoptosis depending on the biological context (257).

The ARF tumor suppressor acts as a sensor of hyperproliferative signals emanating from oncoproteins and inducers of S-phase entry, such as MYC (264), E1A (265), mutated Ras (266), E2F-1 (267), β-catenin (191) and viral ABL (268). Herewith, ARF is activated by abnormally elevated and sustained mitogenic signals triggered by oncogenes but not by physiologic signaling levels conveyed by their appropriately regulated proto-oncogenic counterparts (257). For example, ARF is not induced by MYC or Ras during normal cell cycle progression, but it is transcribed when proliferative signals are constitutively enforced through MYC translocation or oncogenic Ras mutation. However, ARF induction is mediated not only at the level of transcription. ARF is very unstable in normal human cells due to ubiquitylation and subsequent degradation (which is inhibited in cancerous cells), revealing the dynamic feature of the ARF-p53 pathway (269).

ARF serves as a fuse that gates mitogenic current, preventing abnormal cell proliferation in response to oncogene activation. However, the understanding how the promoter element of *ARF* distinguishes normal and abnormal signaling thresholds remains problematic. *Arf* is normally repressed by E2F complexes, but unlike many E2F-responsive genes that govern DNA synthesis and replication, *Arf* is not periodically expressed when cells enter S phase (271). Yet, when *Arf* is induced by oncogenes, transcription factors E2F 1, 2, and 3a replace repressive E2F complexes on the *Arf* promoter (272). Hence, other specificity factors must play a role in modulating this E2F response. An attractive candidate is the transcription factor DMTF1 (cyclin-D-binding Myb-like transcription factor 1) (273), which binds adjacent to an E2F site in the

proximal *Arf* promoter to activate the gene (274). DMTF1 mediates oncogenic Ras signals to ARF via Raf/MEK/ERK pathway (275). Ras-driven MAP kinase pathway also induces *MDM2* gene expression resulting in the degradation of p53, if ARF is absent (276). This reveals the mechanism how ARF protects p53 from inactivation by Ras and enables p53 response (266).

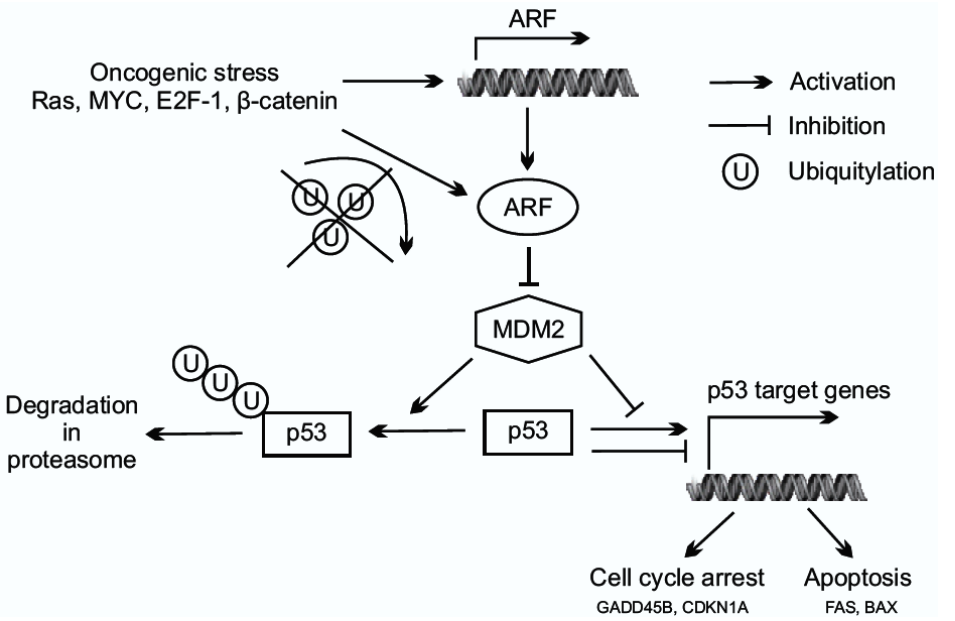

Figure 7. Activation of the ARF/p53 pathway in response to oncogenic stress. Abnormally elevated and sustained mitogenic signals induce transcription and stabilization of the ARF tumor suppressor. ARF binds MDM2, which acts as an E3 ubiquitin ligase of p53, confines it in the nucleus and prevents MDM2-mediated degradation of p53. Accumulation of active p53 leads to cell cycle arrest or apoptosis depending on the biological context. The p53 transcriptional activity results in repression of genes that promote cell growth and survival as well as activation of growth suppressing and/or pro-apoptotic genes. Adopted from (270) with modifications.

Although it is generally accepted that much of ARF tumor suppressor activity is mediated through p53, ARF also has p53-independent functions. Enforced expression of ARF can arrest the proliferation of p53-null cells, although much less efficiently than in cells that retain wild type p53 (277). Primary mouse fibroblasts and B lymphocytes lacking both ARF and p53 grow faster in culture than do cells lacking only one of the two genes (256, 278), and mice lacking ARF, MDM2, and p53 are much more prone to developing cancer than mice lacking MDM2 and p53 (279). ARF mediates p53-independent effects on gene expression by negatively regulating other transcription factors such as E2Fs (280–282), MYC (283, 284), and NF-κB (285). Surprisingly, the enforced

expression of ARF in mammalian cells promotes the sumoylation of several ARF-interacting proteins, e.g. MDM2, p53 (286, 287), implying that ARF has an associated catalytic activity. It has been suggested that the p53-independent effects of ARF on gene expression and tumor suppression might depend on ARF-induced sumoylation (273, 288).

AIMS OF THE STUDY

In recent years, the significance of glycoproteins in cancerogenesis came to light. The contribution of the mucin-like leukocyte marker CD43 to this process has found support in several studies. Namely, pieces of evidence concerning its aberrant expression and glycosylation in solid tumors and cancer cell lines of non-hematopoietic origin as well as involvement in p53 and Wnt/β-catenin signaling pathways emerged. However, a better understanding of CD43 functions in cancer cells is required to evaluate its role in the development of the disease.

During the studies on the activity of CD43 in cancer cells, specific aspects made up the goals of different projects that gave the content for the present thesis.

The precise aims of the study were as follows:

- examine subcellular distribution and potential function of different CD43 molecules described
- investigate the interconnection between CD43 and β-catenin signaling pathways in respect to tumorigenesis
- study the impact of CD43 overexpression on cell fate and means of CD43 action in the context of intact and disrupted tumor suppressor p53/ARF pathway
- assess the involvement of β-catenin in CD43-induced p53 response
- determine whether the expression of CD43, as a detrimental stimulus, is a subject to modulation by p53 and what could be the underlying molecular mechanism

RESULTS AND DISCUSSION

1. CD43 overexpressing cells gain growth advantage in the absence of either p53 or ARF

The tumor suppressor protein p53 has been found to accumulate in the early phases of different tumors, including colon adenomas (289, 290). According to our previous results CD43 overexpression in human cancer cells causes the accumulation of functionally active p53, which is dependent on the presence of the tumor suppressor protein ARF (189). To further investigate the potential role of CD43 in tumor development, the effect of CD43 overexpression on colony formation was tested in different mouse and human cell lines lacking either p53 or ARF tumor suppressors (Ref. I, Table 1). Initially, the cell lines express low levels of endogenous CD43 (Ref. I). To investigate the effect of increased CD43 expression on cellular response, we provoked the elevated levels of CD43 expression observed in colon adenomas by exogenous protein expression. In our experimental system, the overexpression of CD43 suppressed colony formation in all cell lines studied when both p53 and ARF proteins were present (Ref. I, Fig. 1, a–d). In contrast, in cells lacking either ARF or p53, CD43 overexpression increased colony formation compared to the control cells. This suggests that both p53 and ARF are required for the suppression of cell growth in response to CD43 overexpression. In addition, transient knockdown of CD43 by siRNA noticeably reduced colony formation confirming the involvement of CD43 in this process (Ref. I, Fig. 1, e and f). Furthermore, CD43 overexpression increased the growth rate of ARF-deficient (Ref. I, Fig. 3, a and c) and p53-deficient (Ref. I, Fig. 3, b and d) human and mouse cells. It appears that CD43 is capable of acting as a mitogenic stimulus and/or cell survival factor. Also, these observations provide the first evidence that aberrant CD43 expression in a certain cellular context may enhance the development of a transformed cell phenotype.

1.1. CD43 overexpression inhibits FAS-mediated apoptosis

Known oncogenes promote cell growth by affecting either cell cycle or apoptosis. Uncontrolled proliferation is nevertheless not sufficient for tumor formation, but it must be linked to impaired apoptotic signaling. This is confirmed by the observations that deregulated cell growth alone can lead to apoptosis (291). To study whether CD43 conveys proliferative signals, we performed cell cycle analysis by the bromodeoxyuridine incorporation assay. Upon CD43 overexpression in p53-defective or ARF-deficient cells there was no significant increase in the percentage of cells in S phase (Ref. I). Presumably, CD43 affects cell viability rather than proliferation, which is in good correlation with the published data. In transgenic mice the introduction of CD43 into

mature peripheral B lymphocytes (expressing very low levels of CD43 in normal physiological conditions) increased cell viability due to a reduced susceptibility to apoptosis (120, 121). It has also been speculated that tumor cells could escape FAS-mediated cell death by expressing CD43 (9). The FAS receptor is a transmembrane protein that belongs to the tumor necrosis factor (TNF) receptor family and mediates intracellular apoptosis signaling upon stimulation by its ligand, FASLG (292). The intracellular death domain of FAS interacts with the adaptor protein FADD (FAS-associated death domain), which recruits Caspase-8 and triggers the activation of caspase cascade (109). The FAS receptor is expressed at high levels in normal epithelial cells, e.g. in colon and breast, making these tissues sensitive to FAS-induced apoptosis. However, in colon cancer cells FAS expression is frequently downregulated leading to impaired FAS-mediated apoptosis, which has been shown to contribute to tumorigenesis (293–295). The decreased sensitivity to apoptosis triggered by FAS might provide for the ability of cancer cells to escape immune surveillance (294, 296). The mechanisms of acquiring resistance to FAS-mediated apoptosis by tumor cells are complex, and defects have been identified at several levels of FAS signal transduction.

In the first instance, we explored whether CD43 expression helps cancer cells to escape apoptosis triggered by TNF receptor family and FAS in particular. CD43 was overexpressed in human breast and colon cancer cell lines susceptible to FAS-mediated apoptosis and lacking either functional p53 of ARF. Cell death was provoked by TNFα or anti-FAS antibody, which imitates the attachment of FASLG to FAS. As expected, incubation with the anti-FAS antibody caused increase in apoptosis in the cells with uninduced CD43 expression (Ref. I, Fig. 4a). Importantly, after treatment with the antibody no induction of apoptosis was detected in the cells overexpressing CD43. TNFα treatment, however, did not cause elevated apoptosis in any of the cells. It was also observed that CD43 overexpressing cells were notably more viable when kept as non-adherent cells without anti-FAS antibody (Ref. I). In addition, CD43 slightly decreased the basal apoptotic level of the cells (Ref. I, Fig. 4b). Evidently, high expression levels of CD43 protect cancer cells from FAS-mediated apoptosis and may contribute to the immune failure of tumor elimination.

Secondly, we asked whether CD43 could affect the expression of FAS on cancer cells, since the downregulation of cell surface FAS is a common mechanism for cancer cells to decrease the sensitivity to FAS-induced apoptosis (297, 298). In the cancer cell lines that were used in previous experiments FAS receptor was found to be expressed on the surface of wild-type cells (Ref. I, Fig. 5a). However, in the cells overexpressing CD43 the level of FAS expression on the cell surface was decreased (Ref. I, Fig. 5, b and c). Interestingly, the overexpression of the cytoplasmic tail of CD43, which participates in signal transduction, did not affect the cell surface expression of FAS (Ref. I, Fig. 5d), indicating that the full length glycosylated CD43 is required to reduce the level

of FAS receptor in the membrane. These results are in good correlation with the observations that CD43ct had no impact on cell growth and activation of ARF–p53 pathway (Ref. I).

To study the possibility that CD43 might have affected the expression of other cell surface proteins, we analysed the MUC1 mucin. This molecule is similar to CD43, but has a longer extended highly glycosylated extracellular domain. No alterations in the amounts of cell surface MUC1 were observed upon CD43 overexpression (Ref. I, Fig. 7a). Another possibility is that CD43 by its large extracellular domain shields smaller molecules like integrins. However, detection of the integrins α6 and β1 was not altered upon CD43 overexpression (Ref. I, Fig. 7, b and c). This indicates that the shielding effect of CD43 is not involved in the CD43-mediated interference with the cell surface FAS expression.

The mechanism by which CD43 affects FAS-mediated apoptosis is currently unknown, but several speculations can be made. Firstly, CD43 overexpression has been shown to inhibit DNA-binding activity and target gene expression of the NF-κB p65 (299), one of the transcription factors necessary for FAS expression (300, 301). Moreover, CD43 has been reported to interact with β-catenin (164), which suppresses activation of NF-κB and thereby reduces FAS expression in colon and breast tumors (302). This raises a possibility that CD43 could be involved in transcriptional regulation of *FAS*. However, in our experiments the overexpression of CD43 reduced the cell surface level of FAS, but the total amount of receptor was not altered (Ref. I, Fig. 6). Although the mRNA levels of *FAS* in CD43-overexpressing cells have not been assessed yet, it is more likely that CD43 interferes with FAS rather at the post-translational level. Secondly, CD43 possibly interferes with FAS signaling and modulates sensitivity to apoptosis through the interaction with DAXX protein (106) (Fig. 8). The FAS death domain is capable of binding DAXX instead of FADD, which activates the JNK pathway and induces apoptosis (185). Together, these results support the hypothesis that high CD43 expression levels, as found in cancer cells, may provide a mechanism for increased cell survival and enable the evasion of FAS-dependent apoptosis in cancers.

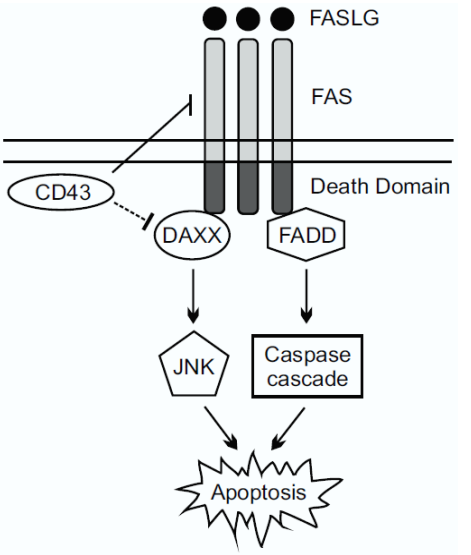

Figure 8. Potential role of CD43 in the FAS-mediated apoptotic pathway. Engagement of FAS receptor by FASLG triggers FAS trimerization and recruitment of FADD adaptor protein to its intracellular death domain. FADD in turn activates the cascade of caspases that cleave vital substances in cells resulting in apoptosis. Besides FADD, the FAS death domain is capable of binding DAXX that enhances FAS-driven apoptosis by inducing the JNK kinase cascade. In addition, DAXX, localizing both in the cytoplasm and in the nucleus, can act as a transcriptional repressor or operate within the PML nuclear bodies. Adopted from (270) with modifications.

1.2. CD43 and β-catenin co-operate in promoting colony formation

The growth-promoting properties of CD43 in human cancer cells indicate that CD43 may have a function in an oncogenic pathway. The understanding of signaling pathways activated by CD43 in cancer cells is rather poor. The cytoplasmic domain of CD43 was found to interact with the oncogene β-catenin and increase expression of proto-oncogenes MYC and Cyclin D1 that are activated by the β-catenin/TCF-4 complex (164). Therefore, it is possible that CD43 is involved in tumorigenesis via the Wnt/β-catenin pathway. Many works have shown that aberrant activation of β-catenin signaling plays an important role in colon cancer development. Similarly to CD43 silencing, inhibition of β-catenin expression reduces growth of colon cancer cells (303). We applied siRNA-mediated gene silencing to explore whether CD43 promotes colony formation via β-catenin signaling. The ability to form colonies was tested in ARF- or p53-deficient human cancer cells. CD43 and β-catenin overexpression increased the number of colonies, which is consistent with the previous results. However, the cell growth-promoting property of CD43 overexpression was

considerably reduced by the downregulation of β-catenin (Ref. II, Fig. 1). Also, the cells overexpressing β-catenin formed fewer colonies when CD43 expression was inhibited. These results suggest that CD43 promotes cell growth in co-operation with β-catenin. Therefore, CD43 has a potential role in Wnt/β-catenin signaling pathway, which is often deregulated in human cancers.

2. Full length CD43 localizes to the nucleus and binds chromatin

By this moment we have already presented a few pieces of evidence indicating that the full length CD43, but not the cytoplasmic tail of CD43, facilitates malignant transformation. The CD43ct, as opposed to the full length CD43, was not able to affect cell growth, evade FAS-mediated apoptosis and activate ARF-p53 pathway. To further investigate the role CD43 plays in cancer formation we analysed different CD43 proteins in detail. A variety of CD43 molecules have been described in the cell: the mature full length glycosylated CD43, the non-glycosylated CD43 precursor, the CD43-specific cytoplasmic tail fragment (denoted as the CD43-CTF), and the CD43 cytoplasmic tail (designated here as CD43ct). The CD43-CTF, which is suggested to be formed by the proteolytic removal of the ectodomain, includes a small part of the extracellular domain, the intact transmembrane and intracellular domains. The CD43ct is released as a result of the intramembrane γ-secretase cleavage that follows the cleavage in the extracellular domain (146).

Since localization of a protein indicates its function to some extent, we studied distribution of CD43 molecules in different subcellular fractions. We used human colon cancer cell line COLO205 that expresses high levels of endogenous CD43. CD43 protein was visualized with the mAb anti-CD43-4D2 which reacts with all CD43 molecules of interest because the epitope is located near the C-terminal end of CD43 intracellular domain (aa 337–343). Surprisingly, the full length glycosylated CD43 was detected in the soluble nuclear fraction and even at a more significant level in the chromatin-bound nuclear fraction (Ref. II, Fig. 4A). The precursor CD43 was found in the both nuclear fractions as well. The membrane fraction contained the highest amount of the precursor CD43 and the mature CD43. The precursor CD43 was absent from the soluble cytoplasmic fraction, which indicates that it is compartmentalized in the cell apparently being incorporated into the endoplasmic reticulum (ER) and the Golgi apparatus for glycosylation. A considerable amount of the precursor and the mature CD43 proteins was detected in the cytoskeletal protein fraction. This is consistent with the previous findings showing that the cytoplasmic domain of CD43 associates with the cytoskeleton via ERM family adapter proteins (304). The CD43-CTF was observed in the soluble cytoplasmic fraction, in the membrane fraction and very poorly in the soluble nuclear fraction.

When cells were treated with the proteasome inhibitor MG132, the amount of the CD43-CTF increased as previously described (146), and two shorter CD43 fragments became detectable in the soluble cytoplasmic fraction, in the membrane fraction and in the soluble nuclear fraction (Ref. II, Fig. 4B). We presume that the smallest fragment is the cytoplasmic tail of CD43, because it is an expected position for the CD43ct relative to the CD43-CTF (43, 146). Both of the fragments are produced from the cytoplasmic part of CD43 because the epitope of the anti-CD43-4D2 mAb used for detection is located in the distal C-terminus of CD43. In our experiments with MG132 proteasome inhibitor we have noticed that the intracellular domain of CD43 is cleaved in multiple sites (Ref. II), which explains the appearance of the other fragment, but the biological meaning of this phenomenon is still unclear. The effect of the proteasome inhibitor implies that these CD43 molecules have a short protein half-life and are quickly degraded in the proteasome pathway. It has also been previously proposed that CD43ct is quickly metabolized (146). None of these CD43 fragments was found in the chromatin-bound nuclear fraction, they appeared only in the fractions where CD43-CTF accumulated. This is somewhat contradictory to the published data indicating that CD43ct binds β-catenin to activate the expression of β-catenin target genes *MYC* and cyclin D1 (164). However, the role of CD43 in β-catenin-mediated transcriptional activation is not fully understood. Still, we do not know whether CD43 acts as nothing but a chaperone/stabilizer for β-catenin and this way contributes to its transcriptional activity, or CD43 belongs to the protein complex that binds to the promoter regions of β-catenin target genes (Fig. 9). For example, in human colorectal cancer cells, pp60, a Src family PTK, is responsible for β-catenin tyrosine phosphorylation and protein stabilization (305). By the same token, in chronic myeloid leukaemia cells, the PTK ABL activates β-catenin (306). In both cases, the tyrosine-phosphorylated β-catenin (and serine/threonine unphosphorylated) binds to the TCF-4 transcription factor, thus representing a transcriptionally active pool. Moreover, β-catenin tyrosine phosphorylation prevents its cytosolic/membranous retention as well as axin/GSK-3β binding to free β-catenin and subsequent β-catenin serine/threonine phosphorylation that leads to its degradation (305, 306). It can be proposed that the CD43ct is involved in β-catenin stabilization through the modulation of its post-translational modifications. The incorporation of the CD43ct into the PML-NBs is consistent with this hypothesis. Furthermore, it cannot be excluded that the mature CD43 localizes to PML-NBs as it is also SUMOylated (discussed in Ref. I). Nevertheless, the presence of the mature CD43 in the chromatin-bound nuclear fraction and the failure of the CD43 cytoplasmic fragments to accumulate in this fraction correlate well with our results showing that the overexpression of the full length CD43, and not the CD43ct, helps the cells with defective ARF-p53 signaling to evade FAS-mediated apoptosis and promotes cell growth (8). Besides, β-catenin is also known to inhibit FAS expression on the cell

surface (302). These results indicate that the mature CD43 might have a role in regulating gene expression in the nucleus together with β-catenin.

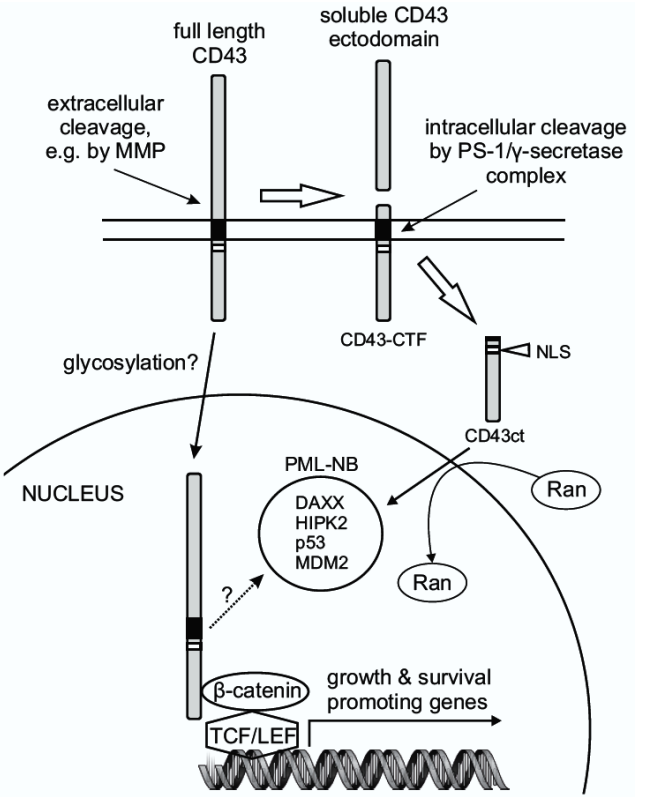

Figure 9. Potential role of CD43 in the nucleus. According to our hypothesis, the CD43ct and the mature CD43 could have distinct functionality in the nucleus. The CD43ct might participate in cell homeostasis primarily by interacting with PML-associated proteins, which supposedly stabilize β-catenin by post-translational modifications, and this way contributes to β-catenin transcriptional activity. Whereas, the mature CD43, capable of binding chromatin and β-catenin, apparently belongs to the protein complexes on the promoters of β-catenin target genes. The translocation of the full-length CD43 into the nucleus might depend, in addition to NLS, on its glycosylation, which is often abnormal in cancer cells.

The mechanism for the translocation of the full length glycosylated CD43 into the nucleus can be based on the presence of the NLS in the cytoplasmic domain of the protein (164) or on the reversible attachment of *O*-linked N-acetyl-glucosamine (*O*-GlcNAc) which is known to play an important role in the modulation of the biological activity of intracellular proteins. Interestingly, it has been shown that only the glycosylated form of the cytoplasmic transcription

factor STAT5 (signal transducer and activator of transcription 5) could be found in the nucleus and bind co-activator CBP that is essential for the STAT5-mediated gene transcription (307). The involvement of *O*-GlcNAc in the NLS-independent nuclear transport of cytosolic proteins was first sustained by studies on bovine serum albumin showing that the protein could be actively carried to the nucleus when it was modified with sugars (308). One of the mechanisms through which *O*-GlcNAc might act as a nuclear localisation signal is by counteracting the function of phosphorylation (308, 309). Phosphorylation has been shown to affect nuclear translocation of cytosolic proteins (309, 310). The localization of CD43 might be regulated by the same mechanism because CD43 is phosphorylated and extensively *O*-glycosylated carrying core 2 *O*-glycan structures which contain GlcNAc (161).

2.1. Chromatin-bound CD43 interacts with β-catenin and enhances the reporter gene expression regulated by β-catenin

Having established that the glycosylated CD43 is translocated to the nucleus (Ref. II, Fig. 4A) we were interested if the mature CD43, similarly to CD43ct (164), promotes transcriptional activity of β-catenin. First of all, we clarified whether the mature CD43 interacts with β-catenin in the chromatin-bound nuclear fraction. CD43 was immunoprecipitated using the mAb anti-CD43-1G10 which recognizes only the full length glycosylated CD43 protein. β-catenin was found to be co-immunoprecipitated with CD43 from all fractions isolated (Ref. II, Fig. 4C). The interaction between the mature CD43 and β-catenin in the chromatin-bound nuclear fraction suggests that the full length CD43 might indeed modulate β-catenin/TCF/LEF-mediated transcription and contribute to impaired Wnt signaling in colon cancer.

The additional evidence supporting the role of the mature CD43 in β-catenin-mediated signaling comes from our next finding obtained by the employment of the reporter assay. We used TOPflash luciferase reporter vector which reflects activation of TCF/LEF-sensitive transcription (311). The overexpression of the full length CD43 enhanced the reporter gene expression regulated by β-catenin but not as much as the overexpression of β-catenin (Ref. II, Fig. 3A). The coexpression of both exogenous CD43 and β-catenin showed more pronounced luciferase activity relative to exogenous β-catenin alone (Ref. II, Fig. 3A). On the other hand, when CD43 expression was silenced, the overexpression of β-catenin did not increase the transcription level of its reporter gene, indicating important cross-talk between the CD43- and β-catenin-dependent pathways (Ref. II, Fig. 3C). These results demonstrate that in our experimental system the presence of both mature CD43 and β-catenin is required for the TCF/LEF-mediated transcription.

3. CD43 overexpression induces p53/ARF-dependent apoptosis

Our observations indicate that CD43 promotes cell growth and survival upon the disruption of ARF/p53 tumor suppressor pathway. Induced ARF expression has been described in response to oncogenic stress. The main role of ARF activation is the stabilization of p53 by MDM2 inhibition and activation of p53-dependent growth suppression. If cells lack ARF, they fail to inhibit oncogene-induced hyperproliferation (312). As elevated CD43 expression led to an increase in the level of ARF and accumulation of transcriptionally active p53, it is clear that in our case p53 activation operates via ARF tumor suppressor protein (189). Moreover, CD43 overexpression in human cancer cells and mouse embryonic fibroblasts increases the ratio of apoptotic cells when both ARF and p53 are present (Ref. I, Fig. 2). Hence, the described reduction in colony formation upon CD43 overexpression in the cells expressing both p53 and ARF occurs due to induction of apoptosis. Besides, CD43 not only co-operates with β-catenin in promoting colony formation but also requires β-catenin to activate p53. In the cells with silenced β-catenin, the induction of the transcriptional activity of p53 in response to CD43 overexpression was inhibited (Ref. II, Fig. 2, A and B). Thereby, the synergistic stimulatory effect of CD43 and β-catenin on cell growth leads to the activation of tumor suppressors ARF and p53 that in turn results in cell death (Fig. 10).

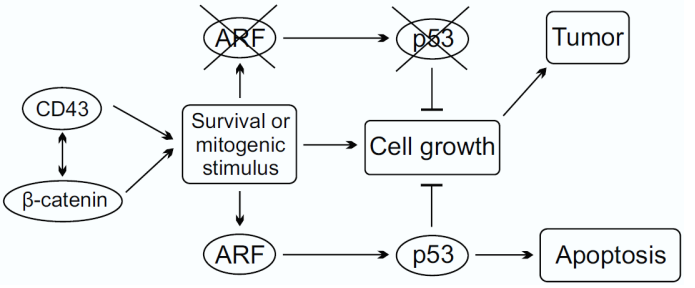

Figure 10. Role of CD43 in cell fate depending on the cellular context. Synergistic activity of CD43 and β-catenin provides a pro-survival/mitogenic stimulus, which causes activation of ARF, subsequent stabilization of p53 and a p53-depended apoptotic response. In cells lacking either functional ARF or p53, CD43 enhances cell growth, presumably via Wnt/β-catenin pathway, revealing its potential role in tumorigenesis.

4. p53 downregulates CD43 expression in cancer cells

p53 is known to trigger ARF-dependent apoptosis in response to oncogene activation (257), and CD43 overexpression induces death in cells expressing p53 and ARF. Furthermore, p53-dependent downregulation of cell survival-related genes is an important mechanism in apoptosis regulation (313). Thus, we proposed that p53 could negatively regulate CD43 expression in cancer cells. Indeed, p53 specifically suppresses CD43 expression both at the levels of mRNA and protein (Ref. III, Fig. 1 and Fig. 2). p53 reduces the expression of only endogenous and not exogenous *SPN* mRNA (Ref. III, Fig. 2B), therefore p53 does not affect the stability of *SPN* mRNA, and the observed effect is due to a downregulated *SPN* gene transcription. At the protein level wild type p53 decreases the expression of both precursor and fully glycosylated CD43 (Ref. III, Fig. 1). We also confirmed that the lower levels of CD43 protein observed in p53-expressing cells were not caused by apoptosis during first 24 h (Ref. III), as p53 and CD43 coexpression might induce apoptosis of the cells (189). At the same time, ARF alone did not considerably affect neither mRNA nor protein levels of CD43 (Ref. III, Fig. 2C), despite the fact that ARF has both p53-dependent and p53-independent tumor-suppressive activities (314). Our results confirm that *SPN* gene expression is regulated by the tumor suppressor p53 and that p53 affects CD43 expression at both transcriptional and protein levels.

Similarly to CD43, p53 has been shown to negatively regulate transcription of presenilin-1 gene (315). Presenilin-1 (PS-1), as a part of the γ-secretase complex, mediates the intramembrane cleavage of type I transmembrane proteins (e.g. CD43 (146) and CD44 (168)). Interestingly, the downregulation of PS-1 expression resulted in cell death and tumor suppression, and the overexpression of PS-1, on the contrary, protected cells from apoptosis (315). One can speculate that the facilitated release of the CD43ct accounts for the described survival of PS-1 overexpressing cells. Therefore, p53 might indirectly inhibit the pro-survival functions of CD43ct (e.g. in PML-NBs) by decreasing PS-1 expression level.

Since our previous studies demonstrate that CD43 overexpression activates p53 and leads to programmed cell death (189), we suggest the existence of a negative feedback loop between p53 and CD43, where the overexpression of CD43, as of an oncogene, induces p53 and results in the downregulation of CD43.

4.1. p53-mediated transactivation and transrepression are required for the downregulation of CD43

As a transcription factor, p53 regulates cell fate in response to cellular stress through activating and repressing the transcription of downstream target genes. Using various p53 mutants (Ref. III, Fig. 3A), we demonstrate that lower levels of CD43 protein correlate with DNA binding and transactivation abilities of p53

(Ref. III, Fig. 3, B and C). This suggests that p53-dependent downregulation of CD43 protein requires transactivation of a subset of target genes. Moreover, an examination of the CD43 turnover by the pulse-chase method revealed that p53 significantly reduces the half-life of CD43 protein (data not shown) confirming that other proteins participate in p53-mediated post-translational regulation of CD43 expression.

Data from several studies show that p53-mediated transrepression is mostly required for apoptosis induction. Since high levels of CD43 trigger p53-dependent apoptosis, we explored whether p53 regulates CD43 expression by transrepression as well. The regions of p53 protein responsible for the down-regulation of *SPN* mRNA are N-terminal transactivation domain, oligomerization domain, proline rich domain and DNA-binding domain (Ref. III, Fig. 4). A number of studies have described the domains of p53 required for its transrepression activity. Among these were the N-terminus, the proline-rich domain and the C-terminus (313). Moreover, it has been reported that tetrameric p53 binds DNA more efficiently than monomeric (316), while residues 339–346 in the oligomerization domain are required for p53 transrepression activity (317). In our study, the p53 protein with the deletion of the proline-rich region failed to downregulate *SPN* mRNA level (Ref. III, Fig. 4). It has been shown that the proline-rich domain of p53 is important for transcriptional repression involving recruitment of histone deacetylases (HDAC) to target promoters (313). The proline-rich domain interacts with the co-repressor protein SIN3A (251, 252, 318) which tethers p53 into a repressor complex containing histone deacetylases (252). In the presence of trichostatin A (TSA), an inhibitor of HDAC, the ability of wild type p53 to reduce *SPN* mRNA level was disrupted (Ref. III, Fig. 5, A and B), elucidating the molecular mechanism underlying p53-mediated repression of *SPN* transcription. In addition, p53-dependent acetylation of histone H3 is known to contribute to the promoter regulation of several genes (319). We ascertained that the repression of CD43 expression is accompanied by a reduced acetylation at histone H3 K14 in *SPN* gene promoter region (Ref. III, Fig. 5C). Our findings suggest that the ability of p53 to repress *SPN* mRNA level occurs via transrepression of the *SPN* gene itself. Obviously, the histone deacetylation mechanism is involved in p53-mediated transcriptional repression of *SPN*, suggesting that CD43 expression is inhibited by the HDAC-SIN3A-p53 complex.

4.2. The CD43 promoter region contains a p53 response element and the promoter activity is repressed by p53

There are different mechanisms of p53-mediated transrepression. p53 can interfere with the functions of either basal transcriptional machinery or specific transcriptional factors, and it is capable of repressing promoters that lack p53-binding site or act in a sequence-specific manner. Thus, our next goal was to

determine whether the promoter region of *SPN* gene contains p53-binding sequences.

We identified three putative p53 response elements in proximity to *SPN* gene (Fig. 11). Two sites with a similarity to the consensus sequence (225) lie ~1.5 kb upstream and 1.7 kb downstream of the transcription start site and one sequence for transrepression (227, 228), spanning –75 to –48 bp, is adjacent to the minimal promoter region of *SPN*. The *SPN* genomic sequence from –53 to –40 bp is an essential promoter for the expression of human CD43, and it contains a binding site for the SP1 transcription factor which is indispensable for the transcriptional activation of *SPN* (28, 29). Notably, the p53-binding site for transrepression partially overlaps with the SP1- and Purα-binding sequences (–57 to –37 bp) and lies almost adjacent to hnRNP-K-binding site (–38 to –17 bp). The transcription factors Purα and hnRNP-K have been shown to repress transcription from the *SPN* promoter (34, 35).

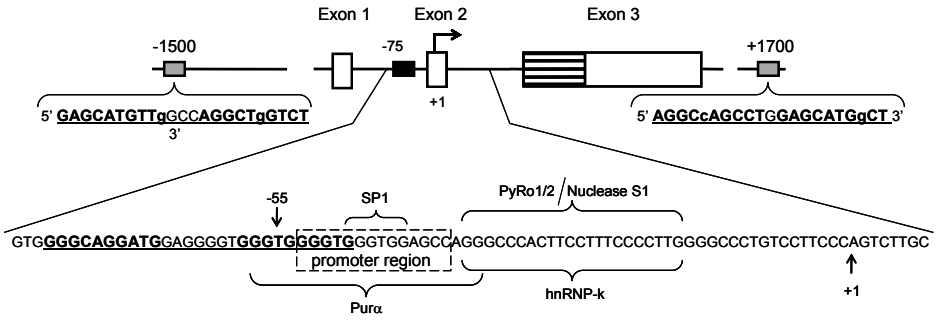

Figure 11. Schematic representation of p53 response elements near *SPN* gene. The promoter region and exons of *SPN* gene are shown. The striped box within the exon 3 box corresponds to the translated region. Three potential binding sites for p53 are indicated schematically and by underlined sequences. The black box represents the p53 binding site known to be necessary for p53-mediated transrepression. p53 consensus sites are indicated with grey boxes. Mismatches to the consensus sequence are shown by lowercase letters. Locations of the p53-binding sequences are given relative to the second transcription start site (+1 and bent arrow). The first transcription start site is marked as –55. The locations of the binding sites for known regulatory factors are indicated using braces.

A transient reporter gene assay revealed that the sequence from –91 to +439 bp is sufficient for p53-mediated repression of *SPN* promoter activity (Ref. III, Fig. 7, A and B). These facts imply that apart from the recruitment of histone deacetylases p53 regulates CD43 expression by other molecular mechanisms. One plausible explanation is that p53 physically displaces SP1 from its binding site as provided by the repression of *POLD1* gene (239). Alternatively, p53 could interact with SP1 bound to a promoter producing a complex that is

transcriptionally inactive. In this case the presence of a p53-binding site is not imperative (241). It is possible that p53-SP1 interaction interferes with the downstream recruitment of the TFIID general transcriptional machinery complex or other transcriptional modulators. In the latter case the p53-SP1 complex could be a signal for the recruitment of repressor complexes to a promoter. Transcriptional repression of *BIRC5* gene demonstrates that the p53-SP1 complex acts as a platform for the engagement of transcriptional repressors such as DNA (cytosine-5-)-methyltransferase-1 (DNMT1), histone methyltransferase G9a and HDAC1 onto the promoter. Subsequent methylation of histone H3 and DNA enforces transcriptional silencing of the gene (320). Thus SP1, although believed to be a transcriptional activator, may act as a mediator of p53-driven transrepression in co-operation with other transacting protein factors (320). In the regulation of *SPN* gene the transactivating properties of SP1 are inhibited by the methylation-specific repressor MeCP2 (31). Transcriptional repressor MeCP2 bound to methylated DNA recruits the SIN3A co-repressor and histone deacetylases to repress transcription (32, 33). It is tempting to speculate that p53 is involved in this process, since p53 reduces acetylation at histone H3 K14 in *SPN* promoter and a repression complex including p53, DNMT1, HDAC1 and MeCP2 has been shown to silence gene expression in human cancer cells (321).

Our reporter assay also revealed that along with the transactivation and the proline-rich domains, sequence-specific DNA-binding and oligomerization domains of p53 are required for exogenous CD43 promoter repression (Ref. III, Fig. 7C). C-terminal amino acids 321–363, which include the oligomerization domain of p53 protein, are required for the physical interactions with SP1 (322). Besides, there is a strong binding affinity between SP1 and p53 (323). These observations support the idea that p53 might interact with SP1 to suppress *SPN* promoter activity. Interestingly, the C-terminal regulatory domain was completely dispensable for inhibition of CD43 transcription. The C-terminal domain of p53 has been shown to bind DNA without sequence specificity, but truncation of this entire basic domain activates p53 binding to its consensus DNA (324). These data suggest that p53 directly binds to the *SPN* promoter. Additionally, in some cases the promoters of downregulated target genes contain one or more potential p53 response elements which do not participate in p53-mediated regulation, but functional DNA-binding domain of p53 is still required for the inhibition (325).

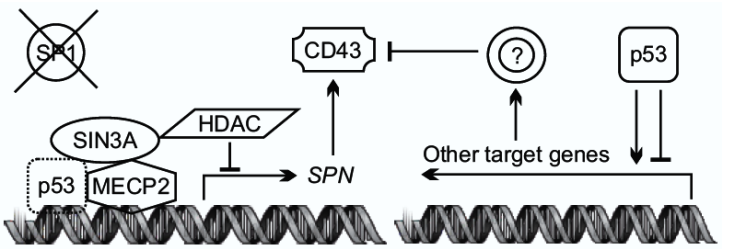

Figure 12. Mechanisms of p53-dependent regulation of CD43 expression. p53 modulates CD43 expression at both transcriptional and protein levels. p53-dependent transcriptional activation of other genes is necessary for the downregulation of CD43 protein. The p53-mediated decrease in *SPN* mRNA level occurs via direct trans-repression of *SPN* gene. Possibly, p53 inhibits the transactivating action of the SP1 transcription factor on *SPN* promoter. The histone deacetylation mechanism is involved in p53-mediated transcriptional repression of CD43, suggesting that p53 facilitates DNA binding by the transcriptional repressor MeCP2 which recruits the SIN3A co-repressor and histone deacetylases to inactivate *SPN* promoter.

Altogether, we demonstrate that CD43 expression is downregulated at transcriptional and protein level following activation of wild type p53 in human cancer cells and that p53 directly inhibits *SPN* transcription at least partly by initiating the deacetylation of histones near *SPN* promoter (Fig. 12). In the same manner, expression levels of the mucin-like protein CD44 are negatively regulated by p53 (326). Moreover, in breast tissue from p53-null mice CD44 was expressed at high levels, and restoration of p53 expression to this tissue resulted in decreased CD44 expression. The loss of p53 affected *CD44* at the mRNA level, suggesting transcriptional control. Using a luciferase reporter vector containing the *CD44* promoter sequence the authors found that p53 required a functional DNA binding domain to repress *CD44* expression. Further, gel-shift assays and chromatin immunoprecipitation experiments showed that p53 is able to interact with the *CD44* promoter both *in vitro* and *in vivo* by binding to a non-canonical p53 consensus sequence. This interaction of p53 with the *CD44* promoter enables an untransformed cell to respond to stress-induced, p53-dependent cytostatic and apoptotic signals that would otherwise be blocked by the actions of CD44. In the absence of p53 function, the resulting derepressed CD44 expression is essential for the growth and tumor-initiating ability of mammary epithelial cells (326). Thus, the relations between p53 and CD43 provide another example of existing negative feedback loops between p53 and growth promoting proteins.

CONCLUSIONS

Tumor suppressor protein p53 becomes activated in response to various detrimental stimuli, including inappropriate cell growth, followed by elimination of such cells. Proceeding from our previous studies demonstrating that CD43 overexpression activates p53 and leads to programmed cell death, the existence of a negative feedback loop between p53 and CD43 was proposed. Particularly, the overexpression of CD43, representing elevated levels of CD43 in colon adenomas, induces p53, as does the oncogenic stress, and results in the down-regulation of CD43 expression, displaying the tumor suppressive role of p53.

The mechanisms underlying these reciprocal relations were studied in current thesis and the following conclusions can be drawn:

- The full length glycosylated CD43 might have a role in regulating gene expression, because it binds chromatin, interacts with β-catenin and affects the TCF/LEF-mediated transcription. The capacity of CD43 to promote cell growth in co-operation with β-catenin in the absence of the tumor suppressors p53 and ARF is a possible implication of the CD43-driven changes in gene expression. In any case, we affirm the involvement of CD43 in Wnt/β-catenin signaling pathway, which is often deregulated in human cancers.
- Another aspect of the pro-oncogenic CD43 activity is the ability of the mature CD43 to protect cancer cells lacking the tumor suppressors from FAS-mediated apoptosis. This is achieved due to reduced cell surface expression of FAS receptor, which in turn may contribute to the failure of tumor immunosurveillance. We propose that CD43 interferes with FAS expression at the protein level.
- The present work shows that the full length glycosylated CD43, as opposed to the CD43ct, is capable of binding chromatin, reducing the level of FAS receptor in the membrane and activating the ARF-p53 pathway. This endows the mature CD43 protein with the tumor-promoting properties which have so far been assigned to the cytoplasmic tail of CD43.
- The synergistic stimulatory effect of CD43 and β-catenin on cell growth leads to the activation of tumor suppressors ARF and p53 that in turn results in cell death.
- p53 affects CD43 expression at both transcriptional and protein levels. p53-dependent downregulation of CD43 protein requires transactivation of a subset of target genes. p53-mediated decrease in *SPN* mRNA level occurs via direct transrepression of *SPN* gene. The histone deacetylation mechanism is involved in transcriptional repression of *SPN* by p53, suggesting that CD43 expression is inhibited by the HDAC-SIN3A-p53 complex. Possibly, p53 inhibits the transactivating action of the SP1 transcription factor on *SPN* promoter.

Taken together, these findings suggest that CD43 expression in a certain cellular context may enhance the development of a transformed cell phenotype. In accordance with this, the p53-dependent downregulation of CD43 expression is a part of the negative feedback loop we have determined between p53 and CD43. Our case provides another example of mutual asymmetrical relations existing between p53 and growth promoting proteins.

SUMMARY IN ESTONIAN

Kasvajaseoselise mutsiini-sarnase leukosialiini (CD43) funktsioonidest inimese kasvajarakkudes

Kasvajates on rakud kaotanud kontrolli jagunemise üle, paljunedes valel ajal ja vales kohas sõltumata välistest signaalidest. Kasvajarakkude piiramatu jagunemine on tingitud onkogeenide aktivatsioonist, mis põhjustavad liigseid kasvusignaale, ja samaaegsest tuumorsupressorite ja stabiilsusgeenide inaktivatsioonist. Vähkkasvaja tekke on mitmeastmeline protsess, mille käigus rakkudesse kuhjuvad geneetilised muutused, ning lisaks kontrollimatule kasvule omandavad rakud metastaseerumise võime. Kasvajaseoseliste geenide hulk kasvab pidevalt, kuid normaalse raku transformeerumine kasvajarakuks toimub tänu muutustele peamistes signaaliülekande radades, mis vastutavad raku surma ja kasvu eest. Samas, kasvajaseoseliste valkude identifitseerimine ja nende funktsioonide uurimine on keskse tähtsusega vähivastaste ravistrateegiate väljatöötamisel.

Viimastel aastadel on vähkkasvate uuringutes palju tähelepanu pööratud mutsiinidele tänu nende muutunud ekspressiooni tasemele ja struktuurile vähirakkudes. Need on jäiga konformatsiooniga kõrgelt *O*-glükosüleeritud valgud raku pinnal, mis vahendavad interakteerumist teiste rakkude pinnamolekulidega või ümbritseva keskonna komponentidega. Kasvajarakkudes on mutsiinide ja mutsiini-sarnaste molekulide glükodünaamika sageli ebanormaalne, mille tulemusena muutub nende glükosüleerituse tase ning *O*-glükaanide struktuur. Sellel on palju bioloogilisi ja patoloogilisi tagajärgesid, kuna muutuvad potentsiaalsed ligand-retseptor paarid ning seega ka rakkudevahelised interaktsioonid. Tulemuseks võib olla kasvu ja ellujäämise soodustavate signaaliradade aktiveerimine, invasiivsete omaduste ja metastaseerumise võime omandamine ning immuunsüsteemi kontrolli alt pääsemine.

Järjest enam vihjeid koguneb selle kohta, et mutsiini-sarnane transmembraanne valk CD43 võiks käituda soodustava faktorina mitte-verepäritolu kasvajate tekkes. CD43 peeti pikka aega leukotsüütide-spetsiifiliseks markeriks, kuid mitmed hilisemad tööd on näidanud kõrge tasemelist CD43 ekspressiooni erinevates kasvajarakuliinides ja -kudedes, sealhulgas käärsoole adenoomides, aga mitte normaalsetes kooloni rakkudes. Samuti on näidatud CD43 seost erinevate signaaliradade komponentidega, mis rakkude elulemust või kasvu mõjutavad. Näiteks lokaliseerub CD43 PML tuuma kehakestesse ning interakteerib β-kateniiniga soodustades tema sihtmärkgeenide MYC ja Cyclin D1 ekspressiooni. Paljude kasvajate nagu ka käärsoolekasvaja üheks peamiseks tekkepõhjuseks on häired β-kateniini signaalirajas, mille tulemusena β-kateniin stabiliseerub ja aktiveerib rakkude kasvu stimuleerivate geenide ekspressiooni. Need algsed andmed vaid vihjavad molekulaarsetele mehhanismidele, mis on CD43 kasvajasoodustavate omaduste aluseks. Seega, põhjalikumad teadmised CD43 funkt-

sioonidest kasvajarakkudes on vajalikud hindamaks CD43 rolli kasvajatekkel ja arengus.

Normaalsetes rakkudes aktiveeritakse vastusena onkogeensele stressile tuumorsupressor ARF/p53 rada, mis on oluline kasvajate ärahoidmisel. p53 käitub transkriptsioonifaktorina, reguleerides mitmete geenide ekspressiooni ja indutseerides seeläbi erinevaid nn stressi vastusprogramme, nende hulgas rakutsükli peatumist ja apoptoosi. Kuna meie varasemas töös leidsime, et vastusena CD43 üleekspressioonile aktiveeritakse ARF/p53-rada, siis oletasime negatiivset tagasisidet CD43, kui potentsiaalse kasvu soodustava faktori, ja p53 vahel, mis võiks omada tähtsust kasvajatekke ärahoidmisel.

Nendest andmetest ja spekulatsioonidest tulenevalt vaadati käesolevas uurimistöös, kuidas mõjutab CD43 rakkude jagunemist sõltuvalt ARF/p53 kontekstist ning kas CD43 mõju rakkude jagunemisele võiks olla seotud onkogeense β-kateniini-rajaga. Töö tulemused näitavad, et CD43 koostöös β-kateniiniga soodustab rakkude ellujäämist ja kasvu, mis indutseerib ARF/p53-sõltuva apoptoosiraja, kui peamist mehhanismi kasvaja tekke takistamisel. Rakkudes, kus ARF/p53 rada on rikutud, võib CD43 kõrgendatud ekspressioon läbi β-kateniini signaaliraja viia rakkude kontrollimatu paljunemiseni, mis on aluseks kasvaja moodustumisele. Leidsime, et p53 on võimeline spetsiifiliselt vähendama CD43 valgu ja mRNA taset rakus. Eeldatavalt aktiveerib p53 kolmandate geenide ekspressiooni, mis CD43 ekspressiooni valgu tasemel reguleerivad, kuna eksogeense CD43 valgu taseme alandamiseks oli vajalik p53 transaktivatsioonivõime. CD43 mRNA tase alaneb p53-sõltuvalt tänu transkriptsiooni vähenemisele CD43 geenilt. p53 takistab transkriptsiooni CD43 geenilt kutsudes esile promootori läheduses olevate histoonide atsetüleerituse vähenemise. Võib arvata, et p53 mõjutab endogeense CD43 ekspressiooni nii transkriptsiooni reguleerimise kui ka valgu tasemel. Kokkuvõttes näitavad töö tulemused uusimaid aspekte seoses CD43 võimega rakukasvu soodustada ning seeläbi kasvajat tekitada ning varem kirjeldamata tagasisidet CD43 ja p53 poolt vahendatud signaaliradade vahel, mis võiks olla olulise tähtsusega kasvajate tekke vältimisel.

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PUBLICATIONS

CURRICULUM VITAE

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Education and professional employment

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Special courses and conferences

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Scientific work

My research is focused on cancer-associated molecular mechanisms. The main emphasis is on the contribution of the proto-oncogenes leukosialin (CD43) and β-catenin to cancerogenesis. Another important part of my study concerns programmed cell death which is triggered in response to oncogene activation and is mediated by tumor suppressors p53 and ARF.

List of publications

Balikova, A., Jääger, K., Viil, J., Maimets, T. and Kadaja-Saarepuu, L. (2012) Leukocyte marker CD43 promotes cell growth in co-operation with β-catenin in non-hematopoietic cancer cells. *Int J Oncol* 41(1): 299–309.

Kadaja-Saarepuu, L., Lõoke, M., Balikova, A. and Maimets, T. (2011) Tumor suppressor p53 down-regulates expression of human leukocyte marker CD43 in non-hematopoietic tumor cells. *Int J Oncol* 40(2): 567–76.

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Teadustöö

Minu teadustöö keskendub kasvajaseoseliste molekulaarsete mehhanismide uurimisele. Põhitähelepanu on pööratud proto-onkogeenidele leukosialiin (CD43) ja β-kateniin, mis soodustavad kasvaja arengut läbi rakkude ellujäämise ja kasvu stimuleerimise. Samuti minu töö oluliseks suunaks on programmeeritud rakusurma uurimine, mis käivitub vastusena onkogeenide aktivatsioonile ning mida vahendavad tuumorsupressor valgud p53 ja ARF.

Teaduspublikatsioonid

Balikova, A., Jääger, K., Viil, J., Maimets, T. and Kadaja-Saarepuu, L. (2012) Leukocyte marker CD43 promotes cell growth in co-operation with β-catenin in non-hematopoietic cancer cells. *Int J Oncol* 41(1): 299–309.

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Ühiskondlik tegevus

Osalesin Tallinna Haridusameti poolt organiseeritud Tallinna keemiaviktoriinide ja Tallinna keemiaolümpiaadide läbiviimisel.

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