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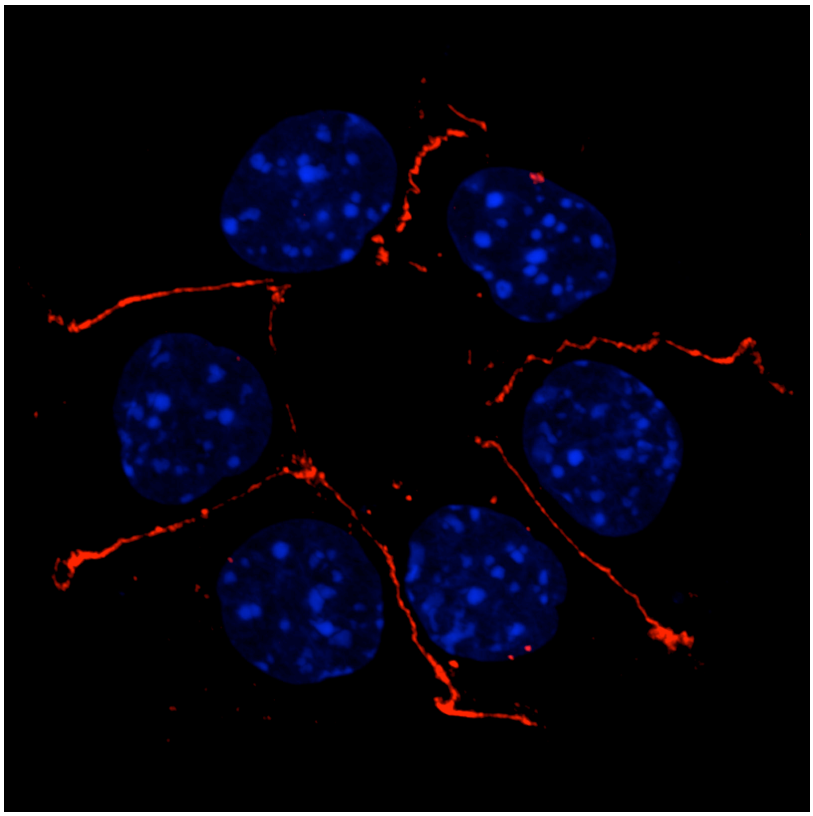
Role of Coxsackie- and Adenovirus Receptor (CXADR/CAR) in the regulation of cell plasticity in cancer and inflammation



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ROLE OF COXSACKIE- AND ADENOVIRUS RECEPTOR (CXADR/CAR) IN THE REGULATION OF CELL PLASTICITY IN CANCER AND INFLAMMATION

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To all the people whom I've learnt from, first and foremost my Parents.

‘’It always seems impossible until it is done’’

-Nelson Mandela

ABSTRACT

The coxsackie- and adenovirus receptor (CXADR) is a transmembrane protein, which localizes at tight junctions (TJ) in epithelial cells. As highlighted by its name, CXADR was initially identified as a receptor for type C adenoviruses and group B coxsackieviruses. Subsequently, CXADR has been shown to mediate cell-cell adhesion, immune cell activation and cellular signaling. Unlike other TJ components, CXADR is vital for the early stages of development. Deregulation of CXADR is frequently observed in pathological conditions including cancer and chronic inflammation. However, mechanistic insight into the role of CXADR in pathophysiology has been lacking. The overall aim of this thesis was therefore to study the role of CXADR in cancer progression and inflammatory diseases.

In Paper I, we show that CXADR regulates the capacity of breast cancer cells to undergo epithelial-mesenchymal transition (EMT) in response to the cytokine TGF-β1. The mechanism was traced to a previously unidentified role of CXADR in acting as a negative regulator of the AKT signaling pathway by forming a signalosome with, PTEN and PHLPP2. Through loss- and gain-of-function experiments we showed that by regulating the stability of the signalosome at tight junction, CXADR controls AKT activity and epithelial-mesenchymal plasticity in breast cancer cells. Moreover, we found that loss of CXADR correlated with loss of PTEN and PHLPP2, and poor prognosis in luminal A breast cancer.

In Paper II, we found that CXADR expression is significantly induced during the formation of atherosclerotic plaques in arterial walls. Macrophages were identified as a previously unknown cellular source of CXADR in both murine and human atherosclerotic plaques. A combination of gene expression profiling, mass spectrometric analysis and *in vitro* studies using human monocytes (THP1 cells), revealed that the induction of CXADR expression is linked to monocyte-macrophage differentiation and further polarization into M1 subtype, and foam cells. Intriguingly, we also found a significant correlation between CXADR and receptors for other viruses, associated with atherosclerosis in human plaques.

In Paper III, inspired by the results from Paper I, we show that CXADR also regulates the metabolic arm downstream of AKT. We found that CXADR controls glucose uptake in various types of cells by regulating the expression and localization of the glucose transporter GLUT-1. Further studies revealed that CXADR expression is upregulated in heart and liver tissues in a mouse model of type 2 diabetes (T2D). In line with this, we found that CXADR expression is induced by IL-6, an inflammatory cytokine which is known to play a role in T2D.

In conclusion, the results presented in this thesis provide a novel and mechanistic insight into the role of CXADR as a pathogenic factor in breast cancer progression and suggest that CXADR contributes to the progression of chronic inflammatory diseases including atherosclerosis and T2D. This may offer new possibilities for using CXADR as a target to develop novel diagnostic tools and therapeutic strategies in cancer and inflammation.

LIST OF SCIENTIFIC PAPERS

I. **CXADR-Mediated Formation of an AKT Inhibitory Signalosome at Tight Junctions Controls Epithelial–Mesenchymal Plasticity in Breast Cancer**
Azadeh Nilchian*, Joel Johansson*, Aram Ghalali, Sandra T. Asanin, Ana Santiago, Oskar Rosencrantz, Kerstin Sollerbrant, C. Theresa Vincent, Malin Sund, Ulla Stenius, and Jonas Fuxe
Cancer Research. 2019 January. 10.1158/0008-5472.

II. **Induction of the Coxsackievirus and Adenovirus Receptor in Macrophages During the Formation of Atherosclerotic Plaques**
Azadeh Nilchian, Estelle Plant, Malgorzata M. Parniewska, Ana Santiago, Aránzazu Rossignoli, Josefin Skogsberg, Ulf Hedin, Ljubica Mati and Jonas Fuxe
The Journal of Infectious Diseases. 2020 July. 10.1093/infdis/jiaa418

III. **The Coxsackie- and Adenovirus Receptor Regulates cellular uptake of glucose and is linked to type 2 diabetes via IL-6**
Azadeh Nilchian, Ana Santiago, Malin Peterson, Annelie. Falkevall, Eriksson Ulf, Malgorzata Parniewska, and Jonas Fuxe
Manuscript

*Equal contribution

Additional papers not included in this thesis:

Targeting mitochondria by α-tocopheryl succinate kills neuroblastoma cells irrespective of MycN oncogene expression
Björn Kruspig, **Azadeh Nilchian**, Ignacio Bejarano, Sten Orrenius, Boris Zhivotovsky and Vladimir Gogvadze
Cell Mol Life Sci. 2012 Jun. 10.1007/s00018-012-0918-4

Citrate kills tumor cells through activation of apical caspases
Björn Kruspig, **Azadeh Nilchian**, Sten Orrenius, Boris Zhivotovsky and Vladimir Gogvadze
Cell Mol Life Sci. 2012 Dec. 10.1007/s00018-012-1166-3

Different regulation of Glut1 expression and glucose uptake during the induction and chronic stages of TGFβ1-induced EMT in Cancer Cells
Azadeh Nilchian, Nikolina Giotopoulou, Wenwen Sun, Jonas Fuxe
Submitted Manuscript

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LIST OF ABBREVIATIONS

2-NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose
Ad5	Adenoviruses serotype 5
ADV	Adenovirus
AJ	Adherent junction
AKT/PKB	Protein kinase B
aPKC	Atypical protein kinase C
ATP	Adenosine triphosphate
BMI	Body mass index
C/EBPβ	CCAAT/enhancer-binding protein
CAF	Cancer-associated fibroblasts
CAR	Coxsackie- and Adenovirus receptor
CCR5	C-C chemokine receptor type 5
CLMP	CXADR-like membrane protein
CMV	Cytomegalovirus
CSC	Cancer stem cells
CTX	Cortical thymocyte marker in Xenopus
CVB	Coxsackie B virus
CXADR	Coxsackie- and Adenovirus receptor
DC	Dendritic cells
E-cad	E-cadherin
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
EPSC	EMT promoting Smad complexes
ERK	Extracellular receptor kinase
FDG	18F-fluorodeoxyglucose
FoxO	Forkhead box
GLUT	Glucose transporter
GOBO	Gene expression-based Outcome for Breast cancer Online

GSK-3β	Glycogen synthase kinase 3 beta
GTP	Guanosine-5'-triphosphate is a purine nucleoside triphosphate
HCV	Hepatitis C virus
HIF-1	Hypoxia-inducible factor 1
HIV	Human immunodeficiency viruses
IBD	inflammatory bowel disease
ICAM-1	Inflammatory cell adhesion molecule 1
IFN-γ	Interferon-ɣ
IgSF	Immunoglobulin superfamily
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
IRs	Insulin receptors
JAMS	Junctional adhesion molecules
LDL	Low-density lipoprotein
LdlR	LDL receptor
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
LNX	E3 ubiquitin-protein ligase
LPS	Lipopolysaccharide
MAGI	Membrane-associated guanylate kinase inverted
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal- epithelial transition
MMP	matrix metalloproteinases
mTORC	mammalian target of rapamycin complex
MUPP-1	Multi-PDZ domain protein 1
NK	Natural killer cells
NRP2	Neuropilin 2
NSAID	Non-steroidal anti-inflammatory drugs
OXPHOSE	Oxidative phosphorylation
PDK	PI3-K-dependent kinase
pEMT	Partial epithelial-mesenchymal transition

PET	Positron emission tomography
PHLPP	PH-domain leucine-rich-repeat-containing protein phosphatases
PI3K	Phosphoinositide 3-kinases
PIP2	phosphatidylinositol (3, 4)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PTEN	Phosphatase and tensin homolog
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
T2D	Type 2 diabetes
TAM	Tumor-associated macrophages
TF	Transcription factor
TGF-β	Transforming growth factor beta
TGF-βR	TGF-β receptor
TJ	Tight junction
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
ZEB	Zinc finger E-box-binding homeobox
ZO	Zonula occludens

1 REVIEW OF THE RESEARCH FIELD

1.1 CELL PLASTICITY

Cell plasticity is the ability of a cell to adapt to a new environment by changing its phenotype and identity through molecular reprogramming. While the importance of cell plasticity is well known for the development of an embryo, tissue regeneration and wound healing, there is increasing interest of cell plasticity in stem cell biology, regenerative medicine and cancer (1).

The reversible or irreversible nature of the plasticity process appears to be context-dependent. There are different forms which empower cells to adopt plasticity along a phenotypic spectrum. First mechanism is de-differentiation, in which a well differentiated cell reverts into a less specialized cell type while keeps the same cell linage; this can be a stem cell or a progenitor cell. On the contrary, trans-differentiation is the procedure in which conversion of a committed cell to another differentiated cell type, possibly from a distinct cell linage occurs. The third mechanism which results in a switch in lineage commitment in a stem or progenitor cell into another related pool of cells, is called trans-determination and forms the basis of metaplasia at the level of an entire tissue. Another form of cellular plasticity is the epithelial–mesenchymal transition (EMT), in which polarized epithelial cells take on mesenchymal characteristics and gain migratory and invasive properties (1-4).

1.1.1 Physiological Role of Cell Plasticity

Cell plasticity is crucial during embryogenesis and occurs mostly unidirectional from transformation of a totipotent zygote to a pluripotent stem cell and eventually to an unipotent differentiated cell state (2). The dynamic interplay between EMT and the reverse mechanism of mesenchymal to epithelial transition (MET), is necessary for the generation of mesenchymal and epithelium tissues during development (5, 6).

Formation of mesoderm and endoderm during gastrulation is a well-established example of EMT. During this process epiblasts lose their epithelial features -*i.e.* loss of apical-basal polarity and cell-cell junctions- and migrate through the primitive streak to form the basis of three embryonic germ layers. It is also shown that cell plasticity in the form of EMT is crucial for detachment, migration and final fate of neural crest cells and its differentiated descendant cells in heart, thymus, and melanocytes in the skin. The importance of epithelial mesenchymal plasticity is also known in the organogenesis of the liver and kidney among other tissues (2, 7, 8). EMT is coordinated at transcriptional, post-translational and epigenetic levels which will be discussed in detail under section **1.2**.

During adulthood, EMT and cell plasticity are rare incidents, but they can be reactivated under physiological and pathophysiological conditions such as tissue injury, inflammation, wound healing, and cancer (Fig. 1). A well-known demonstration of cell plasticity in adult tissue, is found in stem cells of the intestinal crypt. LGR5+ cells in the crypt are the result of either dedifferentiation of a common progenitor cell or a more differentiated secretory cell which are located in the higher position in the crypt (9, 10).

Wound healing is a complex and dynamic cellular process which initiates upon primary inflammation due to tissue injury. EMT and its reversed form MET, orchestrate de- and re-epithelialization during wound healing. The signals from the inflammation site result in activation of EMT transcription factors that cause alteration in gene expression depending on the microenvironment context and the injured tissue. The process starts by epithelial cells surrounding the wound losing their epithelial traits such as cell-cell junctions, polarity and loosen up their interaction to the extracellular matrix (ECM); all these events result in transformation of cells into a mesenchymal and migratory state. Later, cells which have undergone partial EMT, revert to a more epithelial phenotype regulated by signals transduction from the new microenvironment, and start to proliferate and differentiate into keratinocytes within the injured site (11, 12).

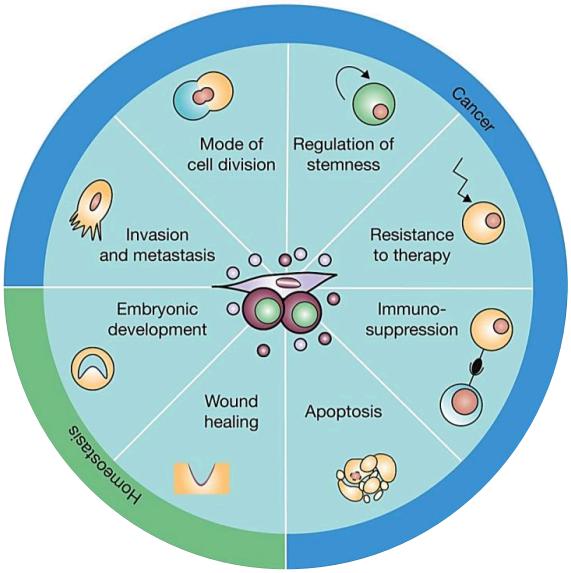

Figure 1. Cell plasticity plays a vital role in physiological process including embryonic development and wound healing. It also has pathological role in diseases associated with inflammation, including cancer. Epithelial–mesenchymal plasticity is the main form of cell plasticity during cancer progression and is believed to associate with therapy resistance, immune evasion and stemness. (Modified from (3))

1.1.2 Cell plasticity in Chronic inflammatory Diseases

Among immune cells, macrophages are divers set of cells playing crucial role at the first-line of defense against infection and inflammatory response. Plasticity is a signature of macrophages and provide them with a unique adaptability to polarize differently depending on the context and the microenvironment. Similar to epithelial cells undergoing EMT, macrophages may show a continuous spectrum of phenotypes in response to intrinsic and extrinsic stimuli. However, they are generally classified into two, yet heterogeneous but distinct subsets of either M1- (pro-inflammatory) or M2- (anti-inflammatory) polarized macrophages (13).

Within the inflammatory microenvironment, a complex network of signals is believed to govern macrophage plasticity, polarization, and function. In turn, different subsets of macrophages also are considered as potent drivers of inflammation, by secreting a wide range of cytokines, under pathological conditions such as cancer, atherosclerosis, and T2D.

Macrophages can be regarded as the most prevalent tumor infiltrating immune cells (14). Depending on the acquired phenotype, macrophages can play distinct and contrary roles at different stages of carcinogenesis. During early stages of tumor progression, M1-like cells act as protective killer cells and control tumor cell proliferation together with T cells. At later stages of cancer progression, M2 like macrophages secrete factors that can promote tumor growth and invasion (15). The notion that local inflammation is a hallmark of cancer, tumor-associated macrophages (TAMs) may be considered as the key inflammatory mediators of linking cancer progression to chronic inflammation. TAMs also contribute to angiogenesis, deconstruction of ECM and promoting of cancer cell invasion and migration in different type of carcinomas including breast, prostate and lung cancers (16).

Obesity is considered as a low grade, chronic inflammatory disease that is associated with increased risk of developing cancer, cardiovascular disease and T2D (17). As mentioned earlier macrophages have plastic nature and the shift between tow subtypes has been reported in several studies. For example, a switch from M2- to M1-like subtype in obese adipose tissue, results in production of proinflammatory cytokine such as interleukin-6 (IL-6), Intelukine-1β (IL-1β) and tumor necrosis factor- α (TNF-α) which eventually can lead to insulin resistance (18). In line with this, it is known that increased plasma levels of IL-6 is associated with increased body mass (BMI) and obesity while a reduction in BMI leads to decreased IL-6 levels (19). Moreover, inhibition of IL-6 has been shown to have a beneficial effect on adipose tissue inflammation and insulin resistance in mouse model (20); however, the precise cellular mechanism is not known well.

Atherosclerosis is a chronic inflammatory disease driven by impaired lipid metabolism and compromised immune response, which leads to plaque formation and restricted blood flow. During gradual progression of the disease, recruitment of monocyte and their differentiation into macrophages, occur as a result of sustained vascular walls inflammation, accumulated LDL, and its oxidized derivatives (21). Both M1 and M2 macrophages are found within atherosclerotic plaques. Indeed, has been shown that while foam cells, originate from M1 macrophages, found abundantly in the developed lipid core, M2 macrophages are present in the adventitia of normal arteries and contribute to plaque regression (22).

1.1.3 Cell Plasticity in Cancer

Cell plasticity is essential for normal homeostasis however, when excessive and aberrant can contribute to cancer. Signaling pathways that are involved in development, tissue regeneration or wound healing can be hijacked by cancer cells and promote tumor progression and chemoresistance (1, 3). In line with this, tumor considered as a wound that never heals (23). Heterogeneity and phenotypic adaptation are characteristics of cancer cells

in the mass of a tumor. EMT is considered as a central mechanism for cancer cell plasticity. A majority of adenocarcinomas are believed to originate from chronically damaged and inflamed tissues. Chronic inflammation is therefore considered a hallmark of cancer, and plays an important role during tumor initiation, progression, and metastasis (15). Infiltrating immune cells, such as macrophages and lymphocytes, fuel the tumor microenvironment (TME) by secreting various inflammatory cytokines and growth factors that contribute to tumor angiogenesis, fibrosis, and release of EMT signaling factors such as transforming growth factor β1 (TGF-β1), epidermal growth factor (EGF) and WNT ligands. TGF-β, which is the most potent inducer of EMT, signals through SMAD transcription factors that after translocation to the nucleus, interact with DNA-binding cofactors and induce expression of core EMT transcription factors, such as SNAIL, TWIST and zinc finger E-box-binding homeobox (ZEB) (24).

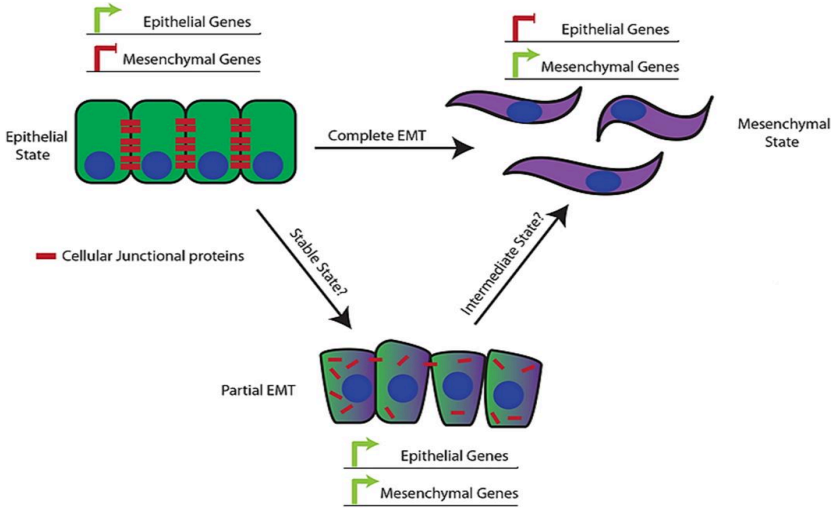

Figure 2. Epithelial–mesenchymal plasticity. Tumor cells undergoing EMT lose epithelial characteristics and gain mesenchymal properties. However, this process is not binary and partial-EMT cells are defined by dual expression of epithelial and mesenchymal genes. Relocalization of the junctional proteins is a crucial step during pEMT (Modified from (25)).

The importance of EMT in metastasis has been shown in different cancers including breast, colon, and liver carcinoma. During cancer dissemination epithelial cells lose their contact with neighboring cells. As a result of developing mesenchymal features, cells become migratory and invasive. These EMT like cells can intravasate into the lymphatic or blood system, reach to the secondary site and form a new distance colony (6, 26). It is still matter of debate whether tumor cells need to revert back to an epithelial state through MET in order to establish growth at secondary sites (27, 28).

It is important to note that EMT should not be regarded as a binary phenomenon, but it is rather a plastic process. Depends on microenvironment, metastatic tumor cells may display a spectrum of both epithelial and mesenchymal features (Fig.2). The concept of intermediate states and plasticity evolved the definition of EMT and proposed a new term in the cancer

field: partial EMT (pEMT). pEMT, intermediate or incomplete EMT is referring to a state(s), in which cells simultaneously express epithelial and mesenchymal genes (6, 25, 26). Interestingly, it has been shown that cancer cells expressing mesenchymal genes while still retaining some epithelial features, are highly efficient in metastasizing (29, 30).

Taken together our current knowledge, highlights the important role of epithelial-mesenchymal plasticity in different stage of metastasis; at the primary site, mesenchymal phenotypes stimulate migratory and invasive behavior, while epithelial characteristics promote outgrowth and the formation of secondary tumors at metastatic sites (31).

1.2 CANCER PROGRESSION AND METASTASIS

Metastasis is the major cause of mortality in humans with cancer. Unfortunately, there is no efficient way to combat metastasis mostly due to two major reasons: first, because of the limited understanding of the precise molecular mechanisms behind metastatic spread of tumor cells and second, the majority of existing therapeutic approaches have been evolved against tumor cell proliferation rather than tumor cell migration and invasion. Despite advanced cancer therapies including surgery combined with radiotherapy and/or chemotherapy, metastatic cancer cells are difficult to target and are frequently chemo-resistant (32). It is also important to understand that clinically detectable metastases is the end of a complex, multistep process, which was initiated by dissemination of cancer cells to the circulation long before diagnosis (Fig. 3) (33). Malignant tumor cells invading and migrating into the neighbor tissue may spread further by intravasating into blood or lymphatic vessels, and eventually infiltrating at distant sites (34).

1.2.1 Epithelial-Mesenchymal Transition (EMT)

The tumor mass is not only referred to a heterogeneous population of cancer cells, but it also consists of the tumor microenvironment including cancer stem cells (CSCs), cancer-associated fibroblasts (CAFs), and different types of immune cells, such as TAMs, dendritic cells (DCs), T and B cells (35). These cells produce and secrete proteases, growth factors and cytokines, which may trigger re-activation of developmental processes, such as angiogenesis, lymphangiogenesis, and EMT (15, 36).

Accumulating data from animal models of breast and lung carcinoma suggest that EMT promotes metastasis by providing cancer cells with migratory, invasive and stemness properties, as well as resistance to chemotherapy (37-39). Clinical studies have revealed EMT-like cells among malignant cells in the invasive front of numbers of tumor types such as breast, colon and lung cancer. Moreover, *in vitro* studies bring new insight into the mechanism underling EMT (40, 41).

Manifested EMT is the result of a series of events including loss of baso-apical polarization due to loss of cell junction components –e.g. E-cadherin and the Coxsackie- and Adenovirus Receptor (CXADR), single cell formation and decrease in cell–cell adhesion forces, gain of

motility and invasive properties that empower cells to migrate through the extracellular matrix and disseminate further (42, 43).

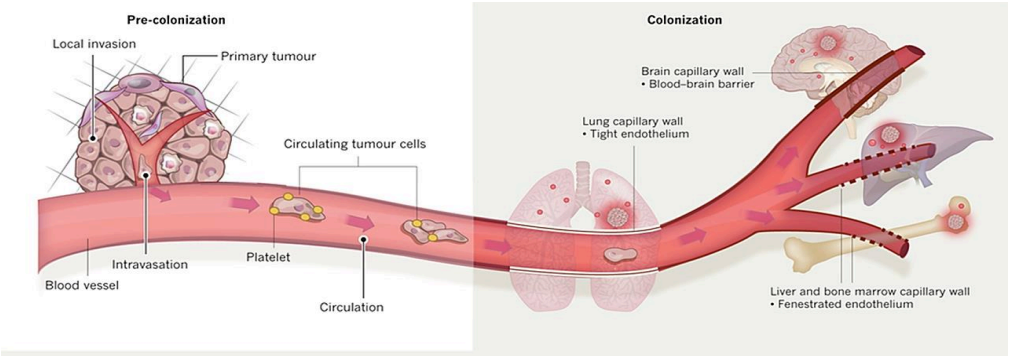

Figure 3. The metastasis processes. Metastasis is series of events that starts with invasion and migration of cancer cells from the primary tumor and ends in the formation of metastasis in distant organs (Modified from (33)).

1.2.2 EMT as a link between Cancer and Inflammation

It is shown that chronic inflammation increases the risk of tumor progression in different types of carcinomas such as breast and skin cancer (16). In line with this, long-term intake of non-steroidal anti-inflammatory drugs (NSAIDs) is reported to be associated with a decreased risk of developing metastatic disease in gastric, biliary and breast cancer (44, 45).

As chronic and local inflammation considered as a hallmark of cancer, infiltrated immune cells within tumor microenvironment, secrete wide array of inflammatory cytokines such as TGF-β1, TNFα and interleukins (23). Theses chemokine and growth factors can promote recruitment of additional innate immune cells, fuel the so-called ‘’cytokine storm’’ and effectively contribute to induction of EMT involving TGF-β and other cytokines (45).

Similar to TGF-β1, IL-6, a cytokine abundantly expressed during inflammatory condition and in cancer tissues, is capable of inducing EMT in breast cancer and other tumor cells (46). IL-6 is mostly considered as a pro-inflammatory cytokine, which contributes to cancer progression and relapse; however, recent studies propose homeostatic and anti-inflammatory effects of IL-6 depending on the context and source of the cytokine production (47, 48). Moreover, the cellular response to IL-6 can vary depending on the chronic or acute exposure to this cytokine (47). For example concerning the importance of IL-6 in metabolism, particularly glucose uptake in response to insulin, acute bioavailability of IL-6 after physical exercise is beneficial for whole body insulin response and lead to increased glucose uptake in muscle cells, whereas its chronic bioavailability may affect metabolism adversely and can induce insulin resistance in murine skeletal muscle (49).

1.2.3 TGF-β-induced EMT Signaling Pathways

Several signaling pathways, such as EGF, Wnt/beta-catenin, TGF-β as well as microenvironment stress (e.g. hypoxia, oxidative and metabolic stress) can induce EMT (50). Among those, TGF-β1 is considered as a potent inducer of EMT, which is frequently secreted by stroma and immune cells in the tumor microenvironment (51).

TGF-β is an evolutionarily conserved pleiotropic factor belongs to the TGFβ-superfamily, consist of more than 33 proteins, that regulates different biological processes in the cell such as cell cycle, differentiation, adhesion, apoptosis, and migration. While TGF-β plays a role in tissue growth and morphogenesis during embryonic development, it also important to maintain tissue homeostasis through its growth inhibitory effects (52). Perturbations in TGF-β signaling contribute to inflammatory diseases and tumor progression (53).

The same paradoxical concept of action of TGF-β can be found during different stage of tumorigenesis; TGF-β serves as a potent tumor suppressor at early stages but promotes EMT and invasion at later stages of tumor progression (54). An interesting example of the dual role of TGF-β was shown in metastatic mammary tumors expressing constitutive active TGF-β receptor I (TGF-βR1) that inhibited the growth of neu-driven mammary tumor in mice on one hand, but also simultaneously facilitated extravasation of tumor cells and formation of metastasis to lung parenchyma, in the same animal (55).

1.2.4 Smad-dependent TGF-β Induction of EMT

The dual role of TGF-β involves different levels of signaling and transcriptional regulation. TGF-β signaling is initiated upon interaction between TGF-β1 with the serine/threonine kinase TGF-β receptor II (TGF-β1RII), which in turn phosphorylates TGF-β1RI and subsequently forms a heteromeric ligand-receptor complex in the cytoplasm (56). It has been shown that occludin, a tight junction protein, contributes to recruitment of TGF-β1RI to the membrane (57). Activated kinase receptor-complex regulates signaling cascade through phosphorylation of the transcription factors and effectors Smad-2 and Smad-3, at their C-terminal serine residues. Later these elements form a trimeric complex with the cofactor Smad4. Smad-complexes can regulate expression of EMT associated core transcription factors like SNAIL1, ZEB1/2, TWIST, CCAAT/enhancer-binding protein (C/EBPβ) and β-catenin. Formation of the EMT promoting Smad complexes (EPSC) leads to induction of mesenchymal genes and simultaneously repression of epithelial genes expression (58-60).

1.2.5 Smad-independent TGF-β Induction of EMT

While the Smad pathway is regarded as the canonical TGF-β signaling pathway, non-Smad pathways including ERK-P38 MAPK, Rho-like GTPase and phosphatidylinositol 3-kinase/ protein kinase-B (PI3K/AKT) pathways are playing important roles during the TGF-β response (61).

The crucial role of PI3K/AKT pathway to promote EMT and cell invasion in epithelial cells has been reported in several studies (62, 63). AKT can regulate EMT through both direct and indirect mechanisms. AKT hinder the growth inhibitory effect of TGF-β1 by binding to Smad-3 and thereby disturbing its translocation to the nucleus, which leads to disruption of fork-head box (FoxO) activity and cell cycle arrest genes (64).

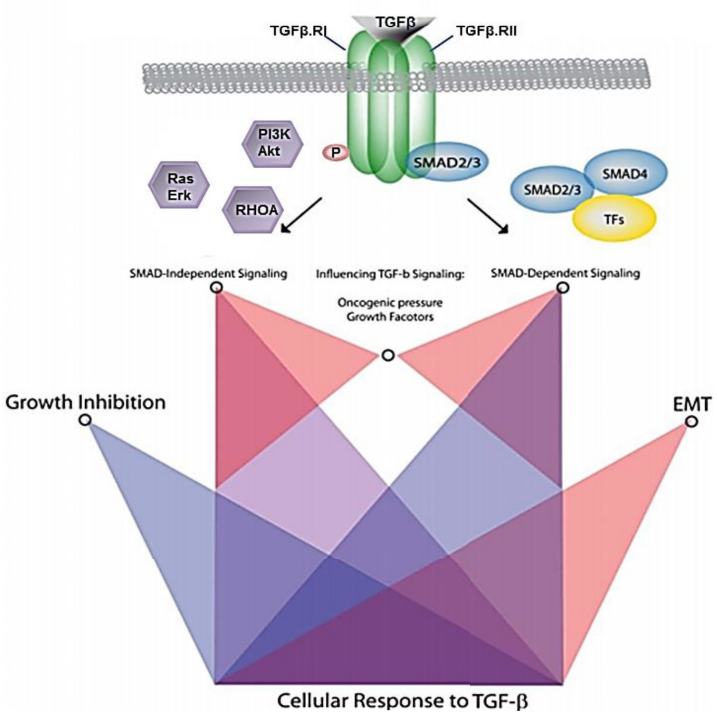

Figure 4. TGFβ-induced EMT. TGF-β1 induces EMT through Smad dependent and Smad-independent pathways. Both signaling cascades are under the influence of intrinsic and extrinsic factors that are altered in tumor cells. (Curtesy of Joel Johansson)

It has also been shown that activation of mammalian target of rapamycin complex (mTORC1), a downstream effector of AKT, is particularly important in cell survival, cell migration and invasion associated with TGF-β1-induced EMT (65). Moreover, PI3K/AKT pathway can positively regulates matrix metalloproteinases (*i.e.* MMP-9) expression which are proteases known to degrade E-cadherin during EMT (66). Importantly, active, phosphorylated AKT (p-AKT) inhibits the activity of glycogen synthase kinase-3 β (GSK-3β), a nodal enzyme, which negatively regulates EMT factors, such as SNAIL and TWIST (60).

1.3 GLUCOSE METABOLISM

Glucose is a primary source of energy for all cells and plays a central role in metabolism, cellular homeostasis, protein, and lipid synthesis. Glucose homeostasis is essential to provide energy for vital organs. Therefore, under normal conditions, glucose metabolism is tightly

regulated through different cellular processes including glycogenesis, glycogenolysis, glycolysis and gluconeogenesis.

Under aerobic conditions normal cells relay on mitochondrial oxidative phosphorylation (OXPHOS), which starts in the cytoplasm by production of pyruvate from glucose and then proceeds in the mitochondria to produce energy-storing molecules in form of ATP. However, under anaerobic conditions or hypoxia, normal cells shift their metabolism toward glycolysis.

Glycolysis is a procedure regulated by ten enzymatic reactions, initiated by processing glucose to pyruvate and ultimately to lactate merely in the cytoplasm. Final products of glycolysis are ATP, NADPH and pyruvate in aerobic settings and lactate in anaerobic conditions which eventually can enter the Krebs cycle and be used as precursor for other cellular reactions.

Altered energy metabolism is a hallmark of cancer cells (15). Despite a very low energy production efficiency of glycolysis compare to OXPHOS (2 vs. 36), most cancer cells use glycolysis even in the presence of sufficient oxygen levels. To maintain the elevated rate of glucose consumption for aerobic glycolysis, glucose uptake is accelerated up to around 30-fold in some cancer cells compare to the normal cells. The high rate of glycolysis in cancer cells provides substantial levels of intermediates that are essential for synthesis of macromolecules and fast growth of tumor cells. Aerobic glycolysis was initially discovered by Otto Warburg in 1926 and thus later termed the ‘’Warburg effect’’ in cancer cells (67).

1.3.1 GLUT-1 in Health and Disease

To make glucose available for the cells to use, it needs to be transported across the cell membrane into the cytoplasm. There are two main glucose transporters (GLUTs) control uptake of glucose into the cells: sodium dependent (SGLTs) and facilitative GLUTs. Among the later, GLUT-1 is the ubiquitous glucose transporter, which is present in most cell types and tissues and facilitates basal uptake of glucose (68).

It is known that GLUT-1 is widely expressed in endothelial cells that form blood–tissue barriers. Moreover, GLUT-1 is highly expressed during different stage of embryogenesis. In line with this it is shown that maternal diabetes with hyperglycemia, leading to GLUT-1 reduction and induction of apoptosis, is lethal and causes the death of murine embryo (69). However, GLUT-1 plays less important role in uptake of glucose in insulin-sensitive tissues, such as adipose tissue and muscle, where it is present in association with GLUT-4 (70).

Among other factors excessive hyperglycemia is considered a high-risk condition that predisposes individuals to development of T2D. Impaired clearance of glucose from the blood, is a hallmark of insulin resistance in T2D. This is due to weakened insulin-stimulated glucose flux into muscle and adipocytes and has been linked to altered expression and localization of GLUT-4 (71). In the context of diabetes mellitus, it has been shown that GLUT-1 overexpression play important roles in the pathophysiology of diabetic-induced kidney lesions and nephropathy (72).

Increased uptake and utilization of glucose have been reported in majority of human tumors. This was observed with a help of radiolabeled analog of glucose (18F-fluorodeoxyglucose, FDG) as a reporter by using positron emission tomography (PET) (73). Additionally, the correlation between enhanced glucose uptake, which is mostly mediated by overexpression of GlUT-1, with tumor invasion and metastasis to distant sites is documented by FDG-PET imaging and immunohistochemistry (74). Elevated expression levels of GLUT-1 is associated with poor survival in a variety of human tumors including lung and breast cancers (75, 76). In light of these observations, it is believed that a clearer understanding of glucose metabolism could provide better therapeutic approaches for cancer and T2D.

1.3.2 AKT Regulating Glucose Metabolism

The AKT signaling pathway plays an important role in regulating cellular metabolism and is often hyper-activated in tumors. AKT regulates glucose uptake and metabolism both at the transcriptional and protein levels. It has been shown that AKT modulates GLUT-1 mRNA translation indirectly through mTORC1 and hypoxia-inducible factor-1 (HIF-1α) (77). Moreover, one of the major effects of AKT is to promote trafficking of glucose transporters (GLUT-1/4) to the plasma membrane and increase glucose flux in favor of glycolysis, which is seen in cancer cells with an enhanced glycolysis rate (78).

Notably, malignant cells with hyperactive AKT show an elevated glucose uptake without an increase in total oxygen consumption, suggesting that activation of AKT signaling may contribute to stimulation of aerobic glycolysis in cancer cells (79). Besides the capacity of AKT to regulate glucose transporters, this serine-threonine kinase can also activate hexokinase 2 and phosphofructokinase 1, important glycolytic enzymes, and hence increase the rate of glycolysis (80).

However, hyperactivation of AKT signaling has been reported in many types of human cancer, impaired AKT signaling and reduced cellular uptake of glucose are considered as hallmarks of T2D (81).

1.3.3 Regulation of AKT Signaling

Upon binding of a growth factor to a tyrosine kinase receptor, such as insulin receptors (IRs) and/or IL-6R/JAK-STAT3, PI3K is phosphorylated and convert phosphatidylinositol (3, 4)-bisphosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 acts as a secondary messenger, which recruits AKT to the plasma membrane and facilitates its phosphorylation at threonine 308 (T308) and serine 473 (S473) residues in association with different protein kinases such as PI3-K-dependent kinase (PDK1/2) and mTORC2.

The PI3K/AKT pathway is negatively regulated by different cellular phosphatases including phosphatase and tensin homolog (PTEN), and the PH-domain leucine-rich-repeat-containing protein phosphatases (PHLPP1/2) (Fig. 5). PTEN prevents AKT activation by converting

PIP3 to PIP2, thus by counteracting PI3K (82). PHLPP2 inactivates AKT (AKT1/3) by dephosphorylating it directly on serine and threonine sites (83).

It is well known that PTEN, and more recently PHLPP2, function as a tumor suppressor in mammalian cells. PTEN is among the most inactivated phosphatases in different types of cancer such as breast, glioblastoma and prostate cancer (84, 85).

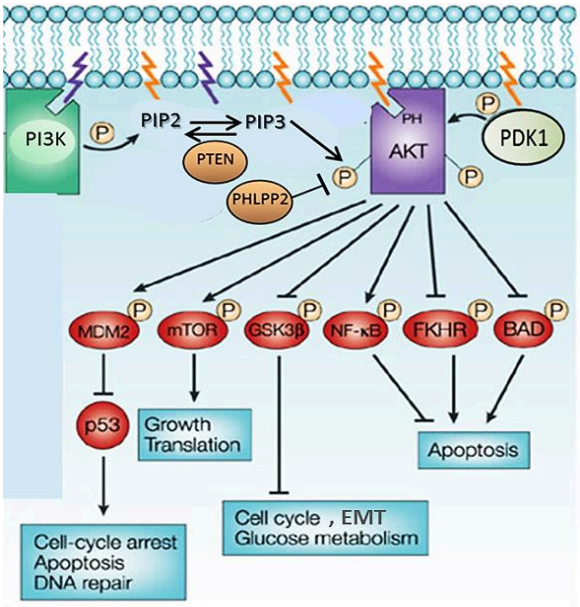

Figure 5. Schematic illustration of the AKT signaling pathway. PI3k/AKT has a central role a wide range of cellular signaling pathways including survival, proliferation, glucose metabolism and EMT. While AKT is phosphorylated and activated by PIP3, PTEN and PHLPP2 negatively regulate its activity by either preventing phosphorylation or dephosphorylating AKT. (Modified from (86))

In the case of sporadic breast tumors, less than 5% PTEN mutations have been reported, although loss of PTEN immunoreactivity is observed in about 40% of the same cohort, suggesting an alternative and post-translational inactivation of PTEN instead of merely mutation or gene deletion (87). In line with this, it has been shown that phosphorylation can regulate stability and activity of PTEN (88).

PTEN contains a functional PDZ domain-binding motif at its C-terminus. Phosphorylation of PTEN at the C-terminus, causes a conformation change termed as ‘’closed’’ and make PTEN less able to engage in PDZ domain-dependent interactions with other PDZ-containing proteins such as members of the family of membrane-associated guanylate kinase inverted (MAGI-1/3). Binding of PTEN to MAGI-proteins is believed to facilitate translocation of PTEN to the plasma membrane, specifically to the cell-cell junctions, where is the focal point for PiP3 regulation and recruitment of AKT (88-90). PHLPP2 also carries a C-terminal PDZ-

binding motif and can be modulated by binding to different scaffold proteins such as NHERF, however not much is known about the post-translational regulation of PHLPP2 (91).

1.4 TIGHT JUNCTION PROTEINS

Epithelial cell layers form a protective barrier against the outer environment stress, regulate the exchange of substances across the epithelium and can mediate secretion in glandular structures (92). Epithelial integrity is regulated by tight junctions, adherent junctions (AJ) and desmosomes. Tight junctions are found at the most apical side of the lateral membrane of polarized epithelial, and endothelial cells. Transmembrane proteins of tight junctions include occludin, claudins, and Ig-like proteins, such as the coxsackie- and adenovirus receptor (CXADR), CXADR-like membrane protein (CLMP), junctional adhesion molecules (JAMs), and intracellular scaffold proteins like zonula occludens (ZO) and MAGI-1 (93, 94).

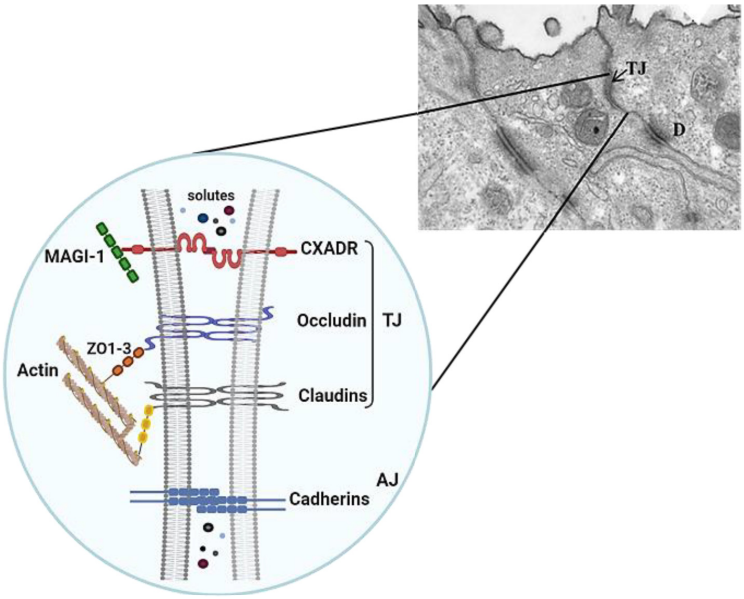

Figure 6. Transmission electron microscopy of junctional areas of an epithelial cell dyed with ruthenium red. Schematic presentation of the component of apical junctions; including tight junction proteins such as occludin, claudins and CXADR at the most apical side followed by AJs. TJs bind to scaffold proteins (e.g. ZO1-3 and MAGI-1) that in turn they interact with actin filaments. (Modified from (95))

Besides regulating epithelial barrier function, tight junctions control other cellular functions, such as cytoskeletal dynamics, cell polarity, morphogenesis, cell growth, proliferation, and cellular transformation (96). Transmembrane components of tight junctions bind to intracellular scaffold proteins that are anchored to the cytoplasmic proteins and actin filaments (Fig. 6). Together, this provides a functional protein network, which mediates transmission of different signals within the cells. For example MAGI-1, a PDZ domain-containing scaffolding protein concentrated at epithelial TJs, has been shown to interact with PTEN through its PDZ binding motif and facilitates its translocation to the membrane which

in turn can induce an inhibitory signaling cascade on AKT pathway (90). Therefore, TJs can transmit external signal and act as hubs for intracellular signaling networks controlling epithelial cell fate and function.

Considering the fact that more than 80% of human cancers originate from epithelial cells (carcinoma), it is not surprising that TJs play role in tumor progression and EMT (97). As EMT is a plastic and multi-step procedure, TJs can influence EMT and metastasis at several levels. Within a tumor, TJs preserve cell adhesion to prevent cancer cell dissemination, therefore they can be referred as the cellular "gatekeepers" to protect cells from undergoing EMT. Moreover, TJs maintain epithelial cell polarization in association with, an evolutionarily conserved signaling pathway, atypical protein kinase C (aPKC) (98).

Recently it was shown that components of TJs, such as occludin and ZO-1, regulate epithelial proliferation and differentiation (99). Interestingly, occludin has been shown to mediate recruitment of TGF-β1R1 to the membrane which means that the TGFβ signaling cascade, leading to EMT, starts at TJs (57). Moreover, TGF-β-PKCα-PTEN cascade is shown to play important role in metastasis and proliferation of pancreatic cancer (100). Although deregulation of TJ is considered as a hallmark of EMT, the precise signaling mechanisms connecting TJ to the induction of EMT are not well characterized.

1.4.1 CXADR – a TJ Protein with an Ambiguous Function in Epithelial Cells

CXADR is a 46-kDa transmembrane tight junction protein belongs to the immunoglobulin superfamily (IgSF). CXADR is a unique TJ protein that its genetic deletion leads to mouse embryonic lethality between E11.5-E13.5, due to impaired cardiomyocyte development and heart failure (101). CXADR-deficiency during development results in subcutaneous edema, which is associated with structurally abnormal and dilated lymphatic vessels (102). Moreover, conditional ablation of the CXADR gene (*CXADR*) in adult mice results in dilation of the intestinal tract and acinar-to ductal metaplasia of the exocrine pancreas (103).

As the name suggests, CXADR was originally discovered as a cellular receptor for group B coxsackieviruses (CVB) and type C adenoviruses (104). Thereafter CXADR was shown to be a component of epithelial tight junctions, which can interact with CXADR molecules on adjacent cells. The *CXADR* gene is located on chromosome 21q11.1 and is composed of 8 exons (105). CXADR produces different splice variants including three soluble isoforms and two transmembrane isoforms named human hCXADR1 (TVV) and hCXADR2 (SIV), which differ slightly in their intracellular domain and their expression in different tissues (106, 107).

Membrane-bound isoform of CXADR contain two Ig-like domains in its extracellular domain, which are responsible for homo- or hetero-dimerization to the extracellular matrix proteins (*e.g.* fibronectin) and other members of IgSF family such as JAM-L or JAM-C (108, 109). It has been shown that the CXADR and JAM-L interaction plays a role in immunity, inflammation, and tissue homeostasis. Upon binding of CXADR to JAM-L on the surface of γδ T-cells, PI3K is recruited and activated at the tight junction. This can subsequently lead to

increased proliferation and cytokine production of T-cell (110, 111). Moreover, It has been found that binding of CXADR on epithelial cells to JAM-L on neutrophils is important during neutrophil transmigration across TJs (112).

The intracellular domain of CXADR carries different motifs namely a class-I PDZ domain located at the extremity of the C-terminus (SIV or TVV). The PDZ motif is conserved between spices of human and mouse which enable CXADR protein to bind to the adaptor proteins contain PDZ domain such as ZO-1, multi-PDZ domain protein-1 (MUPP-1), E3 ubiquitin-protein ligase (LNX) and MAGI-1 (113, 114). This makes CXADR an interesting candidate to be involved in different intracellular protein complex and signaling networks, particularly in epithelial cells.

1.4.2 CXADR in Development, Inflammation and Cancer

CXADR shows developmental and tissue-specific expression. While it is highly expressed during murine embryogenesis particularly in brain, skeletal and heart muscles, CXADR expression is lowered significantly in adult mice tissues (115, 116). Despite developmental-dependent expression of CXADR in brain and heart muscle, it is found to be expressed constantly high in polarized epithelial cells of different organs such as gastrointestinal tract, male reproductive system, respiratory tract and mammary gland in adult mice and humans (107). CXADR is also present in the liver, lymphatic system, skeletal muscle, and myocardial cells (114, 117). However it is known that CXADR is not expressed in the endothelium of vessels in intact tissue, its expression has been detected in CD31+ (endothelial marker) cells in damaged areas of the heart (118).

The restricted expression pattern of CXADR in adult tissues suggests an exclusive role during development and tissue homeostasis. In adult heart tissue, CXADR is exclusively expressed in intercalated disks and plays a role in cardiac remodeling and electrical conductance between the atrium and ventricle (107). It has been shown that CXADR expression is deregulated under different pathological conditions such as cancer, chronic inflammation, and cardiac disease (119).

As mentioned earlier unlike other members of TJs, knockout of *CXADR* in mice is lethal between 11.5 and 13.5 embryonic days due to impaired heart and the lymphatic system development. Moreover, it was shown that cardiomyocytes had enlarged mitochondria and glycogen storage enriched in these cells (101, 102, 120). Several knockdown mouse models confirmed the unique role of CXADR for embryonic heart development and function (120-123). In one study it is shown that CXADR-deficient mice demonstrated over-proliferative and disorganized cardiomyocytes with poor differentiation causing hyperplasia of the left ventricle and death at embryonic day 12.5 (124).

It is worthwhile to note that, conditional inactivation of *CXADR* gene in adult mice results in multiple phenotypes including dilatation of the intestinal tract and atrophy of the exocrine pancreas (102). Moreover, recently it was reported that conditional knockout of CXADR in

mouse endothelial cells reduced the risk of disturbed flow-induced atherosclerosis as a result of decreased expression of the proinflammatory genes and endothelial inflammation (125).

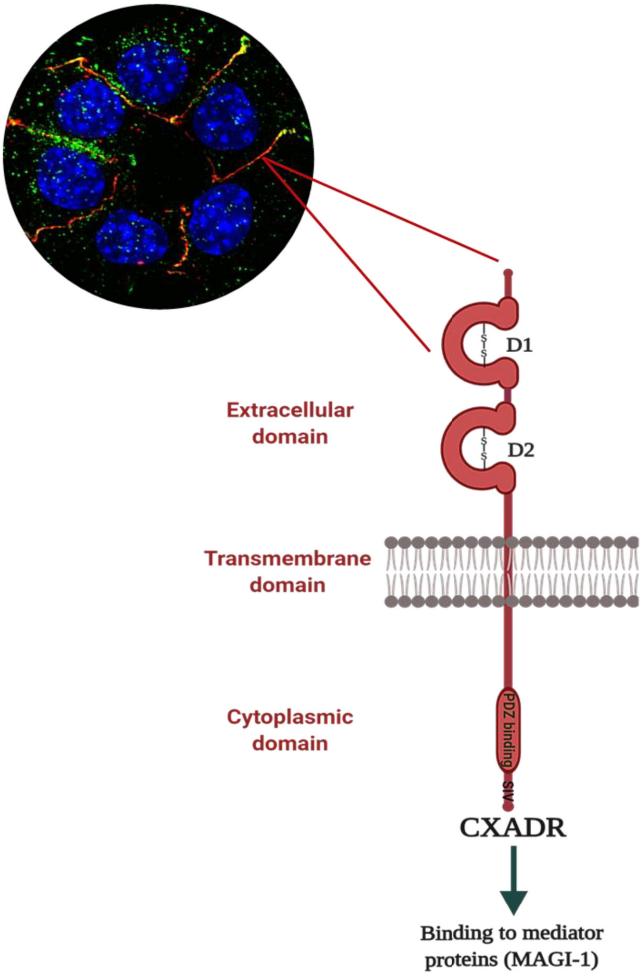

Figure 7. CXADR localization and structure. Immunofluorescent image demonstrating co-localization of CXADR (red) with MAGI-1 (green) in EPH4 mouse mammary epithelial cells. The scheme represents the structure of the membrane bound isoform of CXADR. D1-2 are two Ig-like domains. Residues (SIV) at the C-terminus involved in protein interaction via PDZ binding domain to scaffold protein MAGI-1.

On the other hand, overexpression of CXADR has been reported in different pathological conditions such as ischemic cardiomyopathy, pancreas of type 1 diabetes patients, coxsackievirus B3 (CVB3)-induced myocarditis and in animal model of autoimmune myocarditis (116, 126-128). In the latter study, re-expression of CXADR in myoblast of rat model, was reported to be due to inflammatory cytokine induction such as IFN-γ, TNF-α and IL-1β (128). Interestingly it has been shown that CXADR overexpression has a protective

effect in the pathogenesis of inflammatory bowel disease (IBD) by preventing TNF-α-induced inflammation and save the epithelial integrity in human colon cells (129).

CXADR -as a virus receptor- contributes to induction of inflammation upon virus infection through activation of the MAPK, JNK, and NF-κB pathways (130). Interestingly it has been shown that CXADR expression itself -independent of viral infection- can also induce inflammation in the myocarditis animal model. CXADR-cardiac inflammation was reported to be as a result of CXADR-induced stress-activated MAPK signaling pathway followed by infiltration of mononuclear cell (mostly NK cells and macrophages) and induction of inflammatory cytokine such as IL-1β and IL-6 in the heart tissue of transgenic mCAR+/αMtTA+ mouse model (131).

With the establishment of gene therapy, particularly with oncolytic adenoviruses (e.g. Ad5) for battling cancer cells, and the fact that CXADR has high binding affinity to adenoviruses, multitude of cancer cells have been examined for CXADR expression levels (132). Impaired expression and localization of CXADR is reported in many cancer cells as well as in a number of different human tumors, particularly in those displaying loss of differentiation in advanced disease stages. Among them are breast cancer, lung cancer, human endometrial adenocarcinoma, primary and metastatic colon cancer (108, 133, 134). These studies can suggest a tumor-suppressor role for CXADR, which was confirmed in studies demonstrating a growth inhibitory effect of CXADR overexpression in CXADR-deficient and tumorigenic prostate and bladder cancer cell lines (132, 135).

Moreover, loss of cell-cell contacts in primary tumor cells enable cancer cell to migrate and initiate tumor spread and metastasis. In this context CXADR -as cell-cell adhesion molecule- has been reported to be lost or downregulated during metastatic outgrowth in several carcinomas (108, 136). With the help of gain- and loss function studies, CXADR has been shown to be downregulated early upon induction of EMT with TGF-β1 in a SNAIL-Smad-dependent fashion (136-139). In line with this, CXADR (together with E-Cadherin), is lost at the invasive front of human ductal breast cancer, as well as in the invasive area of the xenografts subcutaneously-grown mouse breast carcinomas (137). However, contradictory reports showing either decreased, increased, or not significantly changed CXADR level in human breast cancer (132, 140-142).

Taken together these findings highlight the importance of CXADR in organogenesis, regulation of inflammation, tissue homeostasis and its function under normal and pathological conditions. Considering the fact that CXADR is a multifunctional protein and appears to play role in different disease, the novel approaches to regulate CXADR can shed light on therapeutic potential of this tight junctional protein in future.

2 OVERALL AIMS

Based on the essential role for CXADR during development, and the documented deregulation of CXADR in various cancer and inflammatory diseases, we hypothesized that CXAR might play a, yet uncharacterized, role in regulation of signaling mechanisms controlling cellular fate and function. The overall aim of this thesis was therefore to study whether CXADR could be linked to changes in cell plasticity in breast cancer and chronic inflammatory diseases including atherosclerosis and type 2 diabetics.

2.1 Specific Aims

The specific aims of the thesis were:

1. To determine whether CXADR regulates tumor cell plasticity in a model of TGF-β-induced EMT (Paper I).

2. To elucidate whether changes in CXADR expression is associated with atherosclerotic plaque formation (Paper II).

3. To investigate the role of CXADR in regulating cellular uptake of glucose and its possible link to T2D (Paper III).

3 RESULTS AND DISCUSSION

3.1 PAPER I.

CXADR-Mediated Formation of an AKT Inhibitory Signalosome at Tight Junctions Controls Epithelial–Mesenchymal Plasticity in Breast Cancer

Azadeh Nilchian, J. Johansson, A. Ghalali, S. Asanin, A. Santiago, O. Rosencrantz, K. Sollerbrant, T. Vincent, M. Sund, U. Stenius, and Jonas Fuxe

Rationale

Loss of CXADR and other TJ proteins is a hallmark and an early event of EMT during carcinoma progression. However, it is not known whether CXADR plays a role in regulating the EMT process. In this study we explored the role CXADR in regulating TGF-β1-induced EMT in breast cancer cells.

CXADR regulates epithelial–mesenchymal plasticity in mammary tumor and human breast cancer cells

To explore the role of CXADR in EMT, we employed an established model system of TGF-β-induced EMT in mouse mammary tumor cells and performed a series of loss- and gain-of-function experiments. Results from western-blot and immunofluorescence analysis, measuring EMT markers (E-cadherin, occludin and vimentin), in EpRas cells treated with TGF-β1 showed more robust EMT response in cells transfected with CXADR siRNA compared to control cells transfected with scrambled siRNA. In contrast, overexpression of CXADR in EpXT cells, which display a persistent EMT phenotype due to an autocrine TGF-β1 loop, resulted in partial MET, which was evident by increased levels of E-cadherin and occluding, and decreased levels of vimentin. Similar results were observed using human MCF-7 and T-47D breast cancer cells, which are CXADR-negative and CXADR-positive, respectively. Moreover, shRNA-mediated CXADR knockdown led to increased capacity of EpRas cells to migrate towards a source of TGF-β1 in invasion assays. The opposite results were observed in EpXT cells overexpressing CXADR compare to the controls. These results implicated that CXADR regulates epithelial–mesenchymal plasticity in breast cancer cells.

CXADR mediates epithelial–mesenchymal plasticity via the AKT pathway

While the Smad pathway is the canonical signaling pathway for TGF-β, non-Smad pathways including ERK, p38 MAPK, and PI3K/AKT pathways are also important for TGF-β-induced EMT. As a next step, we therefore studied which of these signaling pathways that were involved in CXADR-mediated regulation of EMT. The results revealed that stable knockdown of CXADR in EpRas cells did not affect TGF-β1-induced activation of Smad3, ERK1/2 or p38 MAP kinase. However, AKT was already activated by knocking down of CXADR (without TGF-β1) and further enhanced after treating with TGF-β1. In line with these results, we found that the levels of phosphorylated GSK-3β, a downstream substrate of AKT, increased after knockdown of CXADR. Moreover, knockdown of CXADR also resulted in enhanced nuclear

localization of the core EMT factors SNAIL and TWIST, which are inhibited by GSK-3β. Conversely, overexpression of CXADR in EpXT cells resulted in decreased levels of p-GSK-3β. Altogether, these results suggested that CXADR regulates epithelial–mesenchymal plasticity by inhibiting the AKT/GSK-3β-Snail/Twist signaling axis.

In contrast to cancer cells, most normal epithelial cells are resistant to TGF-β1-induced EMT. Moreover, integrity of cell-cell contact in confluent cells, is a potent inhibitor of TGF-β1-induced EMT and proliferation (contact inhibition) (143). On this notion we highlighted the important role of CXADR locating at tight junction, where numbers of stimuli (*e.g.* TGF-β) can trigger AKT hyperactivation, to act as a break on AKT signaling and maintain the epithelial fate of cell.

Direct and indirect control of AKT via CXADR though PHLPP2 and PTEN

AKT serves as a central player for a wide range of biological processes, thus its activity is tightly regulated through phosphorylation. While various growth factors including TGF-β, promote activation of AKT, it is negatively regulated by phosphatases including PTEN and PHLPP2 (83). Based on our results, we were interested to examine whether CXADR regulated AKT via PTEN and PHLPP2. Indeed, knock down experiments in EpRas and T-47D cells showed that loss of CXADR resulted in decreased expression of both PTEN and PHLPP2. In contrast, overexpression of CXADR in EpXT cells and MCF-7 cells induced expression of PTEN and PHLPP2.

CXADR forms an AKT-inhibitory signalosome at TJ

CXADR is known to interact with cytoplasmic scaffold proteins containing PDZ domains, such as MAGI-1. Similar to CXADR, PTEN and PHLPP2 contain a PDZ binding domain in their C-terminus (144, 145). On this note, we hypothesized that PTEN and PHLPP2 might form a protein complex with CXADR at TJs. To test this, we performed co-immunoprecipitation experiments by pulling down endogenous CXADR, PHLPP2, and PTEN with an antibody against MAGI-1 in EpRas cell lysates. Further analysis revealed that PHLPP2 and CXADR interact with the same PDZ domain (#3) of MAGI-1. In addition, we found that CXADR co-localized with PTEN and PHLPP2 in proximity ligation assays. Moreover, through a set of overexpressing experiments in CXADR-negative COS-7 cells, we observed that CXADR expression controls function of both phosphatases, evident by further inactivation of AKT in cells overexpressing CXADR together with either PHLPP2 or PTEN compare to the control cells.

Hyperactivation of AKT signaling is found in practically all types of human cancer and in some cases, correlates with high incidence of PTEN mutations. However, in breast cancer, loss of PTEN due to mutagenic events is rare (only 5% of breast tumors). Yet, PTEN immunoreactivity is lost at a significantly higher degree (40% of breast tumors), suggesting that inactivation of PTEN primarily occurs at the protein level in breast tumors (146). Based on this, we think that our identification of CXADR as a regulator of PTEN stability and

function as an AKT inhibitor, adds a significant level of new knowledge into the mechanisms by which PTEN is lost and AKT hyperactivated in breast cancer.

Correlation of CXADR expression with loss of PHLPP2 and PTEN, and poor survival in luminal A breast cancer

To study the expression of CXADR in human breast cancer we used the GOBO breast cancer database, which is an open access database of 1881 samples of human breast cancer and 51 human breast cancer cell lines (147). Data analysis showed that *CXADR* mRNA expression negatively correlated with poor survival in luminal A tumors (P=0.002), but not in basal tumors or when all tumors were included. To get more insight into whether loss of CXADR correlated with loss of the other components of the signalosome, we performed co-staining of CXADR and PHLPP2, PTEN, or MAGI-1 in 14 human breast tumors. The data showed heterogenous expression of CXADR in the tumors, and a significant correlation between CXADR with PTEN, PHLPP2 and MAGI-1 in different tumor regions.

These observations imply that loss of CXADR might play a significant role for tumor progression and survival in luminal A breast cancer because it leads to loss of PTEN and PHLPP2 at TJs and thereby hyperactivatino of the AKT signaling pathway (Fig. 8). However, in basal tumors, which are poorly differentiated, loss of CXADR expression has less of an impact. This indicates that in more differentiated tumors, the existence of CXADR at TJ contributes to maintaining epithelial differentiation and preventing induction of EMT, and thereby increases the overall survival.

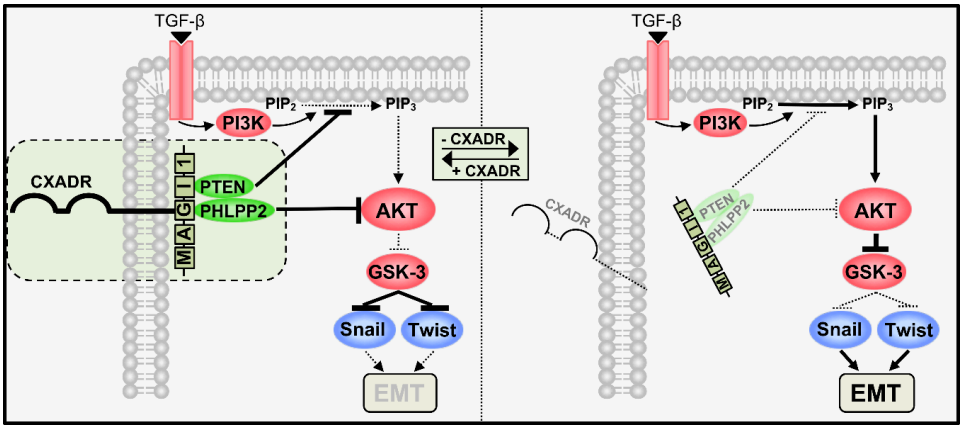

Figure 8. CXADR-signalosome mediates TGFβ-induced EMT via regulation of AKT signaling pathway

Metastasis has been considered as the final stage of the cancer which leads to death in more than 90% of patients. Yet, most traditional, and novel therapies target tumor cell proliferation rather than tumor cell migration and invasion. Our result which identified CXADR as a novel regulator of AKT and EMT, may propose possibilities to target CXADR for therapeutic intervention in future.

3.2 PAPER II.

Induction of the Coxsackievirus and Adenovirus Receptor in Macrophages During the Formation of Atherosclerotic Plaques

Azadeh Nilchian, E. Plant, M. Parniewska, A. Santiago, A. Rossignoli, J. Skogsberg, U. Hedin, L. Matic, and Jonas Fuxe

Rationale

Enteroviruses including coxsackievirus B (CVB) have been linked to atherosclerosis, but the mechanisms are not clear (148). We hypothesized that the formation of atherosclerotic plaques could be linked to changes in the expression of CXADR, which is the main high-affinity receptor for CVB and other subtypes of enteroviruses.

CXADR is induced during plaque formation

To investigate the possible expression of CXADR in atherosclerotic plaques, large-scale microarray analysis was performed on carotid arteries obtained from patients with carotid stenosis (n = 127). In comparison to the normal arteries, CXADR expression was significantly upregulated in carotid arteries with atherosclerotic plaques ($P < 0.001$). Additionally, CXADR protein levels were also significantly increased in the plaques compared to the adjacent control arterial tissue, as determined by proteomic data. To obtain a better insight of CXADR expression during plaque formation, we immunostained aortic walls from transgenic *Ldlr*−/− Apob100/100 mice, which is an animal model of atherosclerosis, at 10 weeks (prior to plaque formation) and 50 weeks (with an established plaque) of age. In line with the human data, CXADR was upregulated in the plaque-invested aortic walls of 50-week old mice but not in 10-week old ones. Taken together, the data demonstrated that CXADR expression is induced during plaque formation in both human carotid arteries and in a mouse model of atherosclerosis.

Identification of plaque resident macrophages as a cellular source of CXADR

As a next step, we studied which cell type(s) that might express CXADR in plaques. Immunohistochemical staining demonstrated that CXADR was co-expressed with CD68 (a common marker of macrophages), both in mouse model and human carotid plaques. Interestingly, not all macrophages in the human carotid plaque were positive for CXADR expression. In line with this, further analysis of human samples revealed a significant correlation between CXADR and CD68 mRNA, but not at the protein level. Taken together, the data indicated that a subpopulation of CD68-positive cells represent a cellular source of CXADR in plaques.

CXADR expression correlates with M1 and foam cell markers

The fact that CXADR was highly expressed in a subpopulation of macrophages led us to investigate the type of macrophages that are commonly express during plaque formation.

Macrophages have an especially plastic nature and can shift their phenotype in response to specific microenvironmental stimuli. There are different subpopulations of macrophages in plaques which, by compromise, can be classified as M1 (pro-inflammatory) and M2 (anti-inflammatory) (13). As their names suggest, M1 is more correlated to plaque formation while M2 contributes to plaque regression and can be found in adventitia of normal arteries.

Analyzes of the RNA and proteomic datasets revealed that CXADR expression significantly correlated with CD11b and CD11c (markers of M1 and dendritic cells) at both mRNA and protein levels. A weaker correlation was found between CXADR and the M2 marker CD163.

An important part of the atherosclerotic process is that macrophages accumulating in plaques take up oxidized low-density lipoproteins (LDL) and thereby turn into foam cells (149). CD36 a scavenger receptor, shown to mediates cellular uptake of oxidized LDL and contributes to foam cell formation. Our results revealed that CXADR correlated strongly with CD36 at mRNA and protein levels, suggesting that CXADR is expressed in foam cells within plaques. In line with this, we also observed co-staining of CXADR and CD36 carotid plaques by immunohistochemistry.

To elucidate more about the induction of CXADR expression during M1 and M2 polarization, we took advantage of a frequently used *in vitro* model of monocyte-to-macrophage differentiation in human THP-1 monocytes. THP-1 cells were first exposed to phorbol-12-myristate-13-acetate (PMA) to induce M0 macrophages. M0 macrophages were subsequently incubated with either IFN-γ and LPS for 24 hours, or with IL-4 and IL-13 for 72 hours, to induce polarization into M1 or M2 macrophages, respectively. Western blot analysis and immunofluorescent staining showed that CXADR expression was induced upon monocyte-macrophage differentiation and further during polarization into a M1 phenotype.

CXADR correlates with receptors for other viruses linked to atherosclerosis

To learn more about the CXADR expression in plaques, and its association with other inflammatory markers, we performed further analysis of the gene expression data. CXADR correlated strongly only with a few additional markers including inflammatory cell adhesion molecule 1 (ICAM-1), CCR5 and neuropilin-2 (NRP-2). These inflammatory markers have been reported to contribute to atherosclerosis initiation, progression and/or mediating macrophage recruitment and plaque formation. Interestingly, ICAM-1, CCR5, and NRP2 have also been shown to act as receptors for viruses that have been linked to atherosclerosis including rhinoviruses, HIV, and cytomegalovirus respectively (150). In addition to the role of CD36 in mediating uptake of oxidized LDL and foam cell formation, it is linked to atherosclerosis through its role as a receptor for HCV (151). In line with recent findings from our group showing that the transcription factor C/EBP-β is a potent inducer of CXADR expression (152), we found a significant correlation between CXADR mRNA with C/EBP-β in human plaques. Interestingly, C/EBP-β is also a well-known transcription factor for playing key roles in macrophage differentiation and polarization, as well as expression of CCR5 and CD36 (153).

Since atherosclerosis is considered as a chronic inflammatory disease, it is enticing to speculate that CXADR expression in the plaques and infection with common enterovirus eventually can

increase vulnerability of macrophages for co-infection with other viruses and triggering of the inflammatory response. This in turn could ultimately lead to phagocytosis and development of a necrotic core, plaque disruption and myocardial infarction. Future studies are needed to elaborate to what extent macrophages in atherosclerotic plaques are prone to infections and co-infections with viruses associated with atherosclerosis.

3.3 PAPER III.

The Coxsackie- and Adenovirus Receptor Regulates Cellular Uptake of Glucose and is Linked to Type 2 Diabetes via IL-6

Nilchian Azadeh, Peterson M, Falkevall A, Eriksson U, Parniewska MM, Fuxe Jonas

Rationale

Based on our identification of CXADR as a negative regulator of AKT signaling and the induction of EMT in breast cancer cells (paper I), we hypothesized that CXADR might also regulate the metabolic arms of the AKT signaling pathway. We therefore set out to analyze whether CXADR could regulate glucose uptake in cells.

CXADR regulates cellular uptake of glucose by regulating GLUT-1

A series of loss- and gain-of function experiments were performed to elucidate whether CXADR levels affected AKT signaling and glucose uptake in various cell types. SiRNA-mediated knockdown of CXADR in Namru mouse Mammary Gland (NMuMG) epithelial cells, which express high levels of CXADR, resulted in increased AKT activity and significantly enhanced capacity of cells to take up 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose)), a fluorescent derivative of glucose. Conversely, we found that overexpression of CXADR resulted in inhibition of AKT and significantly reduced uptake of 2-NBDG in mouse MS1 endothelial cells, which express very low levels of CXADR. The results prompted us to investigate whether CXADR affected downstream targets of AKT, in particular GLUT-1, which is a major glucose transporter in epithelial and endothelial cells. In line with the results from glucose uptake studies, we found that GLUT-1 expression increased upon knockdown of CXADR in NMuMG cells and conversely, decreased in MS1 cells after overexpression of CXADR. Immunofluorescence staining confirmed these results and furthermore, indicated that CXADR hindered translocation of GLUT-1 from the cytoplasm to the plasma membrane.

Elevation of CXADR expression in T2D mouse model tissues

Unlike cancer, in which AKT signaling is shown to be overtly active and playing role in the glycolytic switch in tumors, inactivation of AKT considered as pathogenic signature of T2D development (81). Based on our results showing that CXADR is a negative regulator of glucose uptake in cells, we hypothesized that changes in CXADR levels might associate with development of T2D. To study this, we employed db/db mouse a spontaneous T2D model. Western blot analyses showed significantly increased levels of CXADR in both heart and liver tissues from db/db mice compare to control db/+ mice. These results suggested a role for a systemic signal as an inducer of CXADR expression during the T2D development.

IL-6 induces CXADR expression via C/EBP-β

Accumulated results suggest that local chronic inflammation (e.g. in obesity), is a strong risk factor for T2D development. In line with this, inflammatory cytokines including IL-6 may play

a role in the pathophysiology of T2D (154). Considering that C/EBP-β is known as key transcription factor of the IL-6 pathway, and the recent identification of C/EBP-β as a transcriptional activator of CXADR, we hypothesized that IL-6 might be a systemic factor that induces CXADR expression during the development of T2D.

Western blot and immunofluorescent analysis demonstrated a significant induction of CXADR expression in a dose and time dependent fashion in MS1 cells after exposure to IL-6. We also observed a temporary peak in glucose uptake, measured by 2-NBDG upon short term (40 minutes) IL-6 treatment followed by a significant decrease during longer exposure time points (48 to 96 hour), in MS1 cells. This observation was in line with other studies showing a dual role for IL-6 in regulation of insulin sensitivity in murine cells (49). Upregulation of CXADR in MS1 cells was observed from 48 h and onwards and thus was associated with inhibition of glucose uptake, as well as inhibition of AKT.

In the next step, MS1 cells were transfected with either siRNA against C/EBPβ or scrambled control. As expected CXADR expression was induced in control cells treated with IL-6, however this induction was blocked in the C/EBP-β-knockdown cells; confirming that IL-6-induced CXADR expression is dependent on C/EBP-β in MS1 cells.

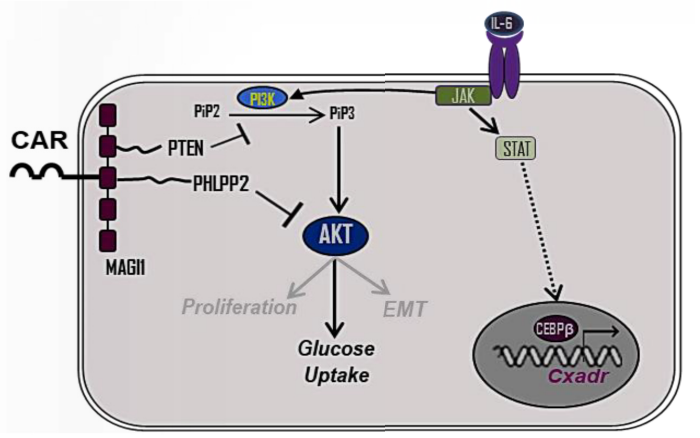

Figure.9. Schematic suggesting the role of CXADR in regulating glucose uptake via controlling AKT signaling. IL-6 induces CXADR expression through C/EBP-β.

Taken together our results demonstrate an important role for CXADR in regulating glucose homeostasis via affecting GLUT-1 expression and localization, downstream of AKT signaling pathway (Fig. 9). Further studies are needed to elucidate the precise role of CXADR in induction of insulin resistance and T2D development and possibly suggesting CXADR as a new therapeutic target in T2D.

4 CONCLUDING REMARKS

Considering the complexity of human physiology and disease, there is a high demand for interdisciplinary studies. Modern medical research cross over classical boundaries and provide opportunities to identify and apply new treatment strategies, primarily intended for a specific pathological condition, in a different context. Under this light, the results extracted from this thesis demonstrates the important role of CXADR, as a multifunctional TJ protein, in bridging the seemingly unconnected fields: cancer metastasis, atherosclerosis and T2D.

The results from our first paper revealed that CXADR regulates EMT via controlling AKT activity in breast cancer cells. Deregulation of TJ is a hallmark of EMT, which is known to contribute to cancer progression and metastasis. We found that CXADR forms a functional TJ-based signalosome with two AKT-inhibitory phosphatases PTEN and PHLPP2. Our results demonstrated that loss of CXADR sensitizes cells to undergo TGFβ-induced EMT. Conversely restoration of CXADR promotes epithelial differentiation by recruiting and stabilizing the AKT-inhibitory signalosome at the TJ. Further investigation revealed that loss of CXADR significantly correlated with loss of PTEN and PHLPP2 and poor prognosis in luminal A breast cancer. The results from this study indicate that CXADR might be used as a prognostic marker and therapeutic target in luminal breast cancer.

In the second study presented in this thesis, we showed that CXADR expression is induced in macrophages during the formation of atherosclerotic plaques. CXADR is a high affinity binding receptor for subtypes of enteroviruses that is expressed in the epithelial cells. These results, for the first time, identified macrophages as the cellular source of CXADR. However not all CD68-positive macrophages were expressing CXADR. This led us to identify foam cells and M1 macrophages with a specific correlation with CXADR, accumulating in the inflammatory, lipid-rich microenvironment in atherosclerotic plaques. The observed significant correlation between CXADR expression and a cluster of receptors for other viruses in the plaque, might make the macrophages prone to be infected with several viruses (e.g. enteroviruses). This underlies the likelihood of an intense inflammatory trigger leading to phagocytosis and cell death which are the risk factors associated with the development of a necrotic core, plaque disruption and myocardial infarction. Future studies poised to understand that how the biological manipulation of CXADR, affecting the atherosclerotic plaque formation and progression.

The steppingstone to the third paper was the results in paper I, identifying CXADR as novel regulator of AKT. Here, we demonstrated that CXADR regulates the capacity of cells to take up glucose. Loss of CXADR in epithelial cells resulted in hyperactivation of AKT, upregulation of GLUT-1 and increased glucose uptake. On the other hand, overexpression of CXADR in endothelial cells inhibited AKT and restricted glucose uptake. Further studies showed that CXADR expression was significantly induced in the liver and heart tissues of mice developing T2D. Moreover, our results identified IL-6 as an inducer of CXADR expression in a C/EBPβ-dependent manner. Similar to paper I and II, advancement in methods to manipulate

CXADR expression in *in vivo* models are needed to study the importance of CXADR for disease induction and progression, however our research shed a light on the potential of CXADR as a possible new therapeutic target in T2D treatment.

5 FUTURE PERSPECTIVES

The conclusions of the three studies presented in this thesis rise the quest for further investigation to obtain better understanding of CXADR causal effect under different pathological condition.

For example, studies using larger cohorts may provide information on whether loss of CXADR could be used as a marker to predict malignant progression in luminal breast and/or other types of carcinoma. Study on the precise molecular mechanism by which PHLPP2 and PTEN are recruited to the CXADR-mediated signalosome at TJs may provide a possible strategy to prevent PTEN and PHLPP2 inactivation or malfunction. Since epithelial-mesenchymal plasticity is associated with chemoresistance, it would be interesting to elucidate the effect of CXADR expression on re-sensitization of cancer cells to commonly used chemotherapeutic drugs.

Considering our observation that CXADR expression was upregulated in a mouse model of T2D, it would be interesting to study whether CXADR affects insulin resistance in skeletal muscle and adipocyte. In addition to the AKT-dependent GLUT-1 expression mediated by CXADR, it should also be valuable to investigate other molecular mechanisms by which CXADR regulates GLUT-1, for example a possible molecular competition between CXADR and GLUT-1 for binding to the same PDZ domain of the MAGI-1 molecule.

Based on our results from Paper I and III, it is enticing to speculate that overexpression of CXADR in macrophages may inhibit glucose uptake as a consequence of AKT inhibition, and instead promote the increase in fatty acid uptake - a mechanism known as Randel cycle (155). Randel cycle is cellular process involving the competition between glucose and fatty acids cellular flux.

Furthermore, taking advantage of conditional and tissue specific knockout mouse model can provide a new insight on the role of CXADR in induction and/or progression of atherosclerosis and T2D *in vivo*.

Finally, our attempts to understand more about the contributing role of CXADR in different types of disease may open up possibilities to target CXADR, in specific cell types (e.g. macrophages and endothelial cells) for AdV-based gene therapy and/or targeting CXADR by small molecules as a new therapeutic approach in chronic inflammatory diseases and cancer.

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- Unknown author

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