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Caveolins and Caveolae

Roles in Signaling and Disease Mechanisms

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Caveolins and Caveolae

Roles in Signaling and Disease Mechanisms

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PREFACE

Caveolae are 50-100 nm flask-shaped invaginations of the plasma membrane that are primarily composed of cholesterol and sphingolipids. Using modern electron microscopy techniques, caveolae can be observed as omega-shaped invaginations of the plasma membrane, fully-invaginated caveolae, grape-like clusters of interconnected caveolae (caveosome), or as transcellular channels as a consequence of the fusion of individual caveolae. Caveolar domains are a subset of the well-described lipid raft structures that have been implicated in a variety of cellular processes such as endocytosis, cholesterol homeostasis, and signal transduction. Similarly, caveolae have been involved in the regulation of cellular cholesterol transport, the endocytosis of several proteins, such as albumin, and in pathogens entry, such as simian virus 40 (SV40) and *Escherichia coli*, among others. Caveolar domains have also been described as cellular platforms, which compartmentalize numerous signaling molecules such as endothelial nitric oxide synthase (eNOS), G-protein-coupled receptors, tyrosine kinases, and protein kinase C.

Caveolin (Cav) proteins represent the principal structural components of caveolae. The caveolin gene family consists of three distinct members, namely Cav-1, Cav-2 and Cav-3. Cav-1 and Cav-2 proteins are usually co-expressed and particularly abundant in epithelial, endothelial, and smooth muscle cells as well as adipocytes and fibroblasts. On the other hand, the Cav-3 protein appears to be muscle-specific and is therefore only expressed in smooth, skeletal and cardiac muscles. Caveolin proteins form high molecular weight homo- and/or hetero-oligomers and assume an unusual topology with both their N- and C-terminal domains facing the cytoplasm. Caveolin proteins, particularly Cav-1 and Cav-3, directly interact with most of the signaling molecules sequestered within caveolae through their scaffolding domains. Interestingly, interaction of the caveolin scaffolding domain with a caveolin-binding motif present within signaling molecules negatively regulates the activity of most of these associated proteins. For example, Cav-1 has been suggested to negatively regulate the activity of eNOS, G-proteins, protein kinase C, and epidermal growth factor receptor, among others. However, in some instances, Cav-1 has also been shown to promote the activation of receptor tyrosine kinases such as the insulin receptor.

Surprisingly, mice with homozygous deletion of the individual caveolin genes are viable and fertile. Nonetheless, caveolin knockout (KO) mice show numerous abnormal phenotypes. For instance, Cav-1 KO mice display reduced life-span, resistance to diet-induced obesity, decreased vascular tone secondary to eNOS hyper-activation, cardiomyopathy and pulmonary hypertension. Cav-2 KO mice show lung abnormalities with altered pulmonary structure and function. Finally, Cav-3 KO mice develop a progressive cardiomyopathic phenotype, insulin resistance and mild myopathic changes.

Importantly, the endogenous expression of caveolin proteins is altered in numerous human illnesses such as atherosclerosis, diabetes, breast and prostate cancers as well as heart, lung and infectious diseases. In addition, mutations of the caveolin genes have also been detected in human diseases such as breast cancer, lipodystrophy, familial hypertrophic cardiomyopathy, long Q-T congenital syndrome and limb-girdle muscular dystrophy.

Given the pleiotropic functions of caveolae and caveolin proteins outlined above, this volume was designed to present the role and function of caveolae and caveolins in cell signaling and human disease mechanisms.

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CONTENTS

SECTION I
IDENTIFICATION AND CELLULAR FUNCTIONS OF CAVEOLAE AND CAVEOLINS

1. LIPID RAFTS, CAVEOLAE AND GPI-LINKED PROTEINS 3

Valerie L. Reeves, Candice M. Thomas and Eric J. Smart

Abstract 3
Introduction: Lipid Rafts 3
Caveolae 4
Cholesterol 6
Caveolin-1 Role and Function 6
Caveolin-2 Role and Function 8
Caveolin-3 Role and Function 9
PTRF 9
Conclusion 10

2. CAVEOLAE AND THE REGULATION OF ENDOCYTOSIS 14

Anna L. Kiss

Abstract 14
Introduction 14
Caveolae at the Plasma Membrane 15
Internalization of Caveolae 16
Intracellular Route of Caveolae 19
Regulation of Caveolar Trafficking 23
Conclusion 24

3. CAVEOLIN-1: ROLE IN CELL SIGNALING 29

Cécile Boscher and Ivan Robert Nabi

Abstract.. 29
Introduction: Complexity of Caveolin Membrane Domains 29
Scaffolding Domain and Tyrosine Kinase Receptor Regulation............................... 33
Cav1 Regulation of Cell Proliferation, Survival and Differentiation.......................... 37
Cav1 Regulation of Signaling Pathways in Cell Migration....................................... 40
Conclusion: Cav1 or the Yin and the Yang of Signaling Regulation.......................... 42

4. REGULATION OF eNOS IN CAVEOLAE 51

Chieko Mineo and Philip W. Shaul

Abstract.. 51
Introduction... 52
eNOS Localization in Endothelial Cell Caveolae... 52
Caveolin and the Regulation of eNOS.. 54
HDL Activation of eNOS 55
Estrogen Modulation of eNOS.. 56
CRP Modulation of eNOS 57
Conclusion 59

SECTION II
CAVEOLAE AND CAVEOLINS IN HUMAN DISEASES

5. RECENT DEVELOPMENTS IN THE INTERACTIONS BETWEEN CAVEOLIN AND PATHOGENS 65

Fabiana S. Machado, Nilda E. Rodriguez, Daniel Adesse, Luciana R. Garzoni, Lisia Esper, Michael P. Lisanti, Robert D. Burk, Chris Albanese, Koenraad Van Doorslaer, Louis M. Weiss, Fnu Nagajyothi, Joshua D. Nosanchuk, Mary E. Wilson and Herbert B. Tanowitz

Abstract.. 65
Introduction... 66
Viruses.. 66
Bacteria 69
Protozoan Parasites.. 70
***Trypanosoma cruzi*.. 72**
Fungi.. 76
Conclusion 77

6. CAVEOLIN-1 AND BREAST CANCER: A NEW CLINICAL PERSPECTIVE....................83

Isabelle Mercier and Michael P. Lisanti

Abstract........................83
Introduction........................83
Caveolin-1, a Potent Suppressor of Growth in Breast Cancer Cells........................84
Insights from the Caveolin-1 Knockout Mouse Model........................85
Caveolin-1 Mutations Found in Breast Cancer Patients........................85
Caveolin-1, a Regulator of Estrogen-Dependent Signaling and Proliferation........................86
Caveolin-1, a Novel Stromal Gate Keeper........................87
The Autophagic Tumor Stroma Model and Caveolin-1........................89
Conclusion........................91

7. CAVEOLIN-1 AND PROSTATE CANCER PROGRESSION....................95

Michael R. Freeman, Wei Yang and Dolores Di Vizio

Abstract........................95
Introduction........................95
The Role of Androgen in Prostate Cancer........................96
Caveolin-1 and the Androgen Receptor........................97
Evidence of Cav-1 Involvement in Prostate Cancer........................99
Cell-Associated Caveolin........................102
Secreted Caveolin........................103
Stromal Caveolin........................104
Conclusion........................106

8. CAVEOLINS AND CAVEOLAE, ROLES IN INSULIN SIGNALLING AND DIABETES....................111

Peter Strålfors

Abstract........................111
Introduction........................111
Insulin Signalling in Caveolae........................112
Caveolae Depletion and Insulin Signalling........................115
GLUT4 and Insulin-Regulated Glucose Transport in Caveolae........................117
Caveolae in Insulin Resistance and Type 2 Diabetes........................120
Conclusion........................122

9. ATHEROSCLEROSIS, CAVEOLAE AND CAVEOLIN-1 127

Stephanos Pavlides, Jorge L. Gutierrez-Pajares, Christiane Danilo, Michael P. Lisanti and Philippe G. Frank

Abstract... 127
Introduction... 127
Absence of Caveolin-1 Decreases Atherosclerosis Development 130
Conclusion 138

10. CAVEOLINS AND HEART DISEASES 145

Mathivadhani Panneerselvam, Hemal H. Patel and David M. Roth

Abstract... 145
Introduction: Caveolins in the Cardiovascular System... 145
Atherosclerosis 147
Arrhythmias 147
Cardiac Hypertrophy and Heart Failure.. 148
Myocardial Ischemia/Reperfusion Injury 150
Conclusion 152

11. CAVEOLINS AND LUNG FUNCTION 157

Nikolaos A. Maniatis, Olga Chernaya, Vasily Shinin and Richard D. Minshall

Abstract... 157
Introduction... 157
Caveolin in Lung Development.. 158
Caveolin and the Alveolar Epithelium 161
Caveolin and the Pulmonary Endothelium 162
Role of Caveolin in Pulmonary Arterial Hypertension 164
Role of Caveolin in Pulmonary Fibrosis 165
Role of Caveolin in Obstructive Airway Diseases 167
Caveolin Control of the Inflammatory Response... 169
Role of Caveolin in Lung Tumorigenesis 171
Conclusion 173

INDEX...181

SECTION I

IDENTIFICATION AND CELLULAR FUNCTIONS OF CAVEOLAE AND CAVEOLINS

CHAPTER 1

LIPID RAFTS, CAVEOLAE AND GPI-LINKED PROTEINS

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Abstract: Lipid rafts and caveolae are specialized membrane microdomains enriched in sphingolipids and cholesterol. They function in a variety of cellular processes including but not limited to endocytosis, transcytosis, signal transduction and receptor recycling. Here, we outline the similarities and differences between lipid rafts and caveolae as well as discuss important components and functions of each.

INTRODUCTION: LIPID RAFTS

The Singer-Nicholson fluid mosaic model is the classic textbook example for describing the cell membrane and the lipid-protein interactions within the membrane, yet it does not accurately describe the organized microdomains found in the membrane. These domains have a definite physical state and composition that are different from the neighboring membrane.[1,2] Sphingolipids and cholesterol are packed in the outer leaflet of the membrane. The abundance of saturated hydrocarbon chains of phospholipids and sphingolipids and the exclusion of unsaturated lipoproteins makes these microdomains more densely packed than other regions of the plasma membrane. This organization of lipids is termed liquid-ordered domain and lipid rafts are one example of these membrane domains.[1,3-6] Cholesterol and sphingolipids can interact with each other, as well as other lipids and proteins on both the inner and outer leaflets of the cell membrane to form lipid raft domains.[7] Sphingolipids associate with each other through their head groups and cholesterol interlocates between the sphingolipids in the outer leaflet of the membrane.[6] Although this mixture of lipids is found in the outer leaflet of the plasma membrane, it is not the same mixture of lipids found on the inner leaflet suggesting additional

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components on the inner leaflet are required or that outer leaflet molecules penetrate into the inner leaflet to help form the raft domain.[8] Investigation into the organization of the microdomains inner leaflet found that scaffolding proteins and acylated proteins often clustered on the inner leaflet.[9,10]

Lipid rafts are small, highly organized but mobile groupings of cholesterol and sphingolipids in the exoplasmic leaflet of the cell membrane involved in signal transduction.[2,4,11] Although these small domains cannot be viewed by light microscopy, they can be indirectly assessed using fluorescent techniques.[12] Because of their size, lipid rafts are too small to function on their own; however, clustering of rafts promotes function. Some resident scaffolding proteins, such as flotillin, caveolins and annexins, are involved in anchoring the inner leaflet of lipid rafts together, while other lipid raft-binding proteins, such as glycosylphosphatidylinositol-linked proteins, connect the rafts on the extracellular side of the membrane. These clustered rafts can then function as platforms in signaling processes.[13]

A large number of proteins are involved in signal transduction and many of these proteins preferentially reside in lipid raft domains either in the outer or the inner leaflet of the cell membrane.[14] Resident lipid raft proteins such as GPI-linked proteins are found in greater numbers inside lipid raft regions than nonraft regions of the membrane.[15] GPI-linked proteins are found on the outer leaflet of the plasma membrane and are attached by their carboxyl lipid additions. Src-family tyrosine kinases, as well as cholesterol-binding proteins, G proteins and other phospholipid-binding proteins also mediate lipid raft function.[4,16-18] Flotillins and annexins reside in lipid rafts on the inner leaflet of the membrane.

CAVEOLAE

Caveolae are described as flask-shaped invaginations with an approximate diameter of 70-120 nm on the plasma membrane and in the cytoplasm.[19] Caveolae are a subset of lipid rafts based on size and lipid content. However, not all lipid rafts are caveolae. These two microdomains have similar constituents and both overlapping and unique functions. Caveolae were described on the plasma membrane as early as 1953 by Yamada in the gall bladder epithelium and again by Palade in 1961 in blood capillaries and have since been described in nearly all cell types as nonclathrin coated membrane invaginations.[20,21] Caveolae are found in most cell types and are found in great numbers in endothelial cells and adipocytes.

Many proteins are known to be associated with the outer leaflet of the caveolae membrane such as GPI-linked proteins and a variety of transmembrane protein receptors such as GPCRs, insulin receptor and beta adrenergic receptors (Fig. 1). However, G proteins, cholesterol-binding proteins, such as caveolins and the Src-family of tyrosine kinases are attached to the inner leaflet of the plasma membrane.[22-24] After transport to the plasma membrane, GPI-linked proteins are sequestered in cholesterol rich caveolae. However, this idea is debated. Some research has shown that GPI-linked proteins reside near, but not in, caveolae and only move into caveolae after cross-linking. On the other hand, some say GPI-linkage is important for connecting proteins on the exoplasmic membrane to interior membranes and/or organelles such as the ER or Golgi.[25]

Caveolae function in endocytosis, transcytosis and also serve as signaling platforms and signal transduction.[26-28] Caveolae endocytosis is dependent upon dynamin-II. Dynamin-II resides along the neck of the invaginations and promotes caveolae budding.[29]

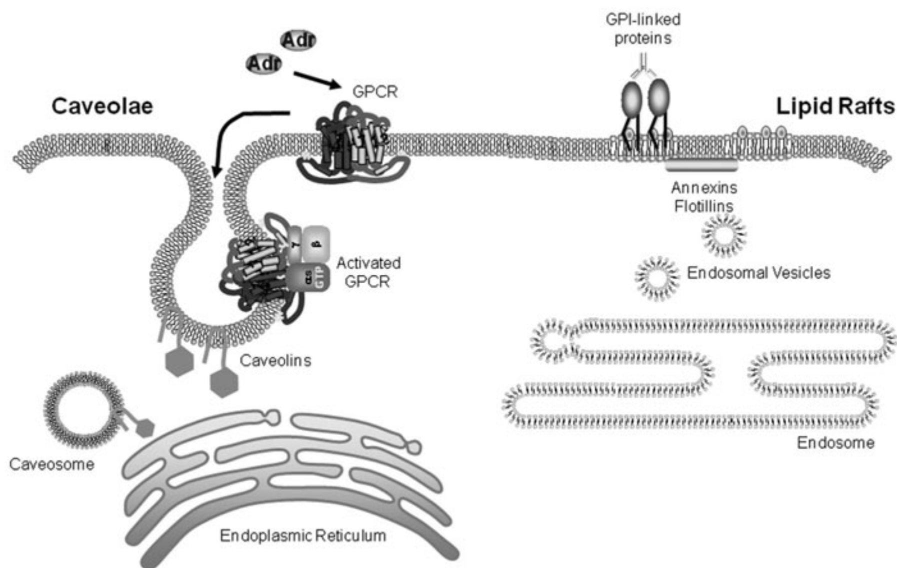

Figure 1. Membrane receptor regulation by caveolae and lipid rafts.

Research has shown that cross-linked GPI-anchored proteins move into caveolae and subsequently become endocytosed. Also, non-enveloped viruses, such as SV40, exploit the endocytocytic function of caveolae to gain entry into the cell. Recent research has shown that these internalized viruses are sent to endosome-like structures called caveosomes that are rich in caveolin-1, a cholesterol-binding scaffolding protein.[30] These caveosomes are different from the endosomes and are now recognized as distinct intracellular organelles. Caveosomes are thought to be regulators of receptor turnover (Fig. 1). Caveosomes fuse with caveolae vesicles that have pinched off from the membrane thereby regulating the downstream signaling pathways as well.[31] These vesicles usually contain receptors that can be recycled without using the endosome pathway. Many signaling molecules and/or their downstream targets move into or out of caveolae to initiate their activation, or to be held inactive.

Caveolae are enriched in important signaling lipids such as ceramide, phosphatidic acid, diglyceride and glycosphingolipids.[32,33] Ceramide is produced in caveolae in response to interleukin-1 beta. Caveolae and ceramide produced in caveolae may participate in blocking platelet derived growth factor stimulated DNA synthesis.[33] The enzymes required to make phosphatidic acid and diglyceride are found in caveolae. Both diglyceride and phosphatidic acid are important second messengers in many cellular signaling pathways including cytoskeletal arrangement and coordinated secretion.[34]

Endothelial cells are rich in caveolae and caveolae have a unique function in these cells. Caveolae are involved in mechanotransduction. Caveolae contain many receptors that modulate blood flow and vascular tone, such as VEGF and insulin, through downstream signaling cascades after ligand binds the receptor. However, endothelial cells with direct exposure to blood flow have been shown to activate the downstream signaling proteins without ligand binding to receptors. Therefore, endothelial cell caveolae have a distinct characteristic of sensing changes in blood flow and modulate vascular tone in

a cholesterol dependent manner by activating endothelial nitric oxide synthase and the MAPK pathways without ligand.[35]

Due to the organization of lipid rafts, the isolation of lipid rafts has proved challenging. The lipid composition of the rafts gives these domains a degree of insolubility in non-ionic detergents, a property that has been used to isolate lipid rafts from the rest of the plasma membrane.[36] Lipid rafts are resistant to cold Triton-X 100 solubilization however isolation using this method causes disruption of the native lipid raft states in the membrane.[37,38] Caveolae membrane fractions can be isolated from the plasma membrane by using Percoll and Optiprep gradient centrifugations instead of the detergent methods used to isolate lipid rafts.[39] This method preserves the resident proteins within caveolae allowing a comprehensive analysis of the role of caveolae and related caveolins in signal transduction.

CHOLESTEROL

The shape of caveolae is most often described as flask-shaped; however this is dependent on the cholesterol content of the cell. Cholesterol is located in the outer leaflet of caveolae and depletion of cholesterol causes the caveolins to move to the endoplasmic reticulum and/or the Golgi apparatus.[40] Cholesterol is a major component of caveolae and depletion of cellular cholesterol, either by extraction using methyl beta-cyclodextrin or depletion using cholesterol oxidase, reduces the number of invaginated caveolae.[41,42] Importantly, cholesterol levels are not static and cholesterol moves in and out of caveolae which suggests that caveolae have a role in cholesterol transport.[43] Caveolin-1, one of the coat proteins that make up caveolae, has been shown to move from the caveolae membrane and to intracellular compartments. Located in caveolae, the scavenger receptor class B Type I is a receptor for HDL.[44] Caveolin-1 directly binds the cholesterol esters in the HDL particle and moves them into the cell without disrupting the HDL particle.[44]

CAVEOLIN-1 ROLE AND FUNCTION

Further investigation into the structure of caveolae revealed a striated pattern seen on the cytoplasmic surface indicative of the presence of a resident protein.[45] The resident protein was determined not to be clathrin when treatment with high salt, which strips the membrane of clathrin, did not alter the morphology of the striated coats found in caveolae.[46,42] VIP21 was positively identified as a scaffolding protein contained within the caveolae striated coat in human fibroblasts and was later called caveolin.[42,47] Caveolins and flotillin are similar in that both are scaffolding proteins that are involved in managing lipid rafts; however caveolins are found in caveolae, while flotillin are found in noncaveolae lipid rafts. Following the positive identification of caveolin as a caveolae coat protein, the 22kDa protein was sequenced and determined to be 178 amino acids long with a unique hydrophobic end.[48] Caveolins have the ability to form high molecular weight homo- and hetero-oligomers and can directly interact with many signaling molecules. Caveolins also can interact directly with many of the proteins in the lipid rafts through a unique protein sequence called the caveolin scaffolding domain.[49] The caveolin scaffolding domain is a 20 amino acid domain on the amino terminus end of caveolin. Proteins bound to the caveolin scaffolding domain are held in an inactive state, for example, caveolin-1 bound to endothelial nitric oxide synthase (eNOS) keeps eNOS in an inactive conformation.[50]

Therefore, caveolins, particularly the caveolin scaffolding domain, are thought to have an inhibitory effect on signal transduction when bound to proteins and only the release from caveolin allows the protein to become active. However, data contradicting this concept have arisen. In insulin and Ras signaling pathways, caveolin-1 plays an activating role.[51,52]

Since the sequencing of caveolin, many researchers have investigated the role of caveolin within the cell. Caveolins have the capacity to bind cholesterol and glycosphingolipids and are required to form caveolae.[53-55] Caveolin-1 is a resident protein of caveolae and is capable of forming invaginated caveolae in the plasma membrane of most cell types. This is just one role of caveolin-1; caveolin-1 also exists outside of the caveolae membrane on vesicles such as insulin granules and liposomes and in caveosomes.

Caveolin-1 directly binds to cholesterol and fatty acids and is involved in the transport of fatty acids through the caveolae membrane.[56,57] Cholesterol binds caveolin-1 and phospholipid liposomes which are required to form caveolae.[58] When cholesterol is depleted from the cell, caveolin moves out of the caveolae and to the endoplasmic reticulum. Upon cholesterol replenishment, caveolin moves back to caveolae independent of Golgi trafficking suggesting a role in cholesterol trafficking. Cholesterol is directly bound and trafficked by caveolin in a lipoprotein chaperone complex.[59,60] This complex contains caveolin, cyclophilin A, cyclophilin 40 and HSP56 and is used to deliver cholesterol to caveolae from the ER. HSP56 provides the specific drive to transport cholesterol from the ER to caveolae by exploiting acylated sites on caveolin that bind cholesterol.[60] Additionally, a lipoprotein chaperone complex can also be used to take up cholesterol into caveolae. This complex is comprised of caveolin, cyclophilin A cyclophilin 40 and annexin II where annexin II provides the specificity to move cholesterol from caveolae to the ER.[44,59,61]

Caveolae are capable of making tunnel like projections through the cell in order to allow movement of material from the apical side of the membrane to the basolateral side of the membrane in a process called transcytosis. Insulin, albumin and LDL are all known to be transported using caveolae and the transcytosis pathway. Transcytosis can be either receptor-mediated or constitutive; however, in both cases, transcytosis through caveolae is a specialized process.

Caveolae and caveolin-1 have roles in insulin secretion, insulin receptor mediated signaling and potentially in diabetes. As stated previously, GPCRs are partially localized to caveolae and one of these receptors, GPR40 is involved in insulin secretion. GPR40 is an orphan G-protein coupled receptor that binds long-chain fatty acids and stimulates insulin secretion.[62] Some ion channels involved in insulin secretion are partially localized to caveolae such as the Kv2.1 channel.[63,64] Caveolin-1 mediates the ATP dependent-potassium channels in pancreatic beta cells thereby regulating glucose stimulated insulin secretion.[64] Caveolin-1 also acts as a guanine nucleotide dissociation inhibitor blocking the exchange of GDP for GTP and activating cdc42, a small GTPase on the surface of the insulin granules.[65,66] Additionally, the docking/fusion proteins, VAMP2 and SNAP, that are required for insulin granule fusion and insulin secretion are also localized to caveolae.[67] Insulin receptors are found in caveolae membranes.[68] Caveolae can transduce insulin signals and recycle insulin receptors. Furthermore, both caveolin-1 and caveolin-2 are expressed in pancreatic islets however; caveolin-1 is only expressed in the beta cells while caveolin-2 is expressed in beta cells and non-insulin secreting cells.[64] Caveolin-1 null mice are thought to be hyperphagic, hypertriglyceridemic and have elevated free fatty acid levels in serum yet have normal blood glucose and insulin levels.[69]

In addition to its role in insulin signaling, caveolin-1 appears to play an important role in cardiac function. Caveolin-1 null mice develop cardiac hypertrophy resulting in a reduced life-span by as much as 50% compared to wild-type mice.[70] Echocardiography in twelve month old caveolin-1 null mice revealed a reduction in left ventricular systolic function evidenced by a decrease in fractional shortening, defined as end diastolic dimension minus end systolic dimension divided by end diastolic dimension.[70] Further, caveolin-1 null mice had an increase in wall thickness, indicative of concentric left ventricular hypertrophy. Similar to these findings, another group found that caveolin-1 null animals had depressed cardiac function measured by echocardiography showing a decrease in fractional shortening.[71] In contrast they documented that caveolin-1 null mice had dilated right and left ventricular chambers (eccentric hypertrophy) and thin posterior walls and septum compared to littermate controls as shown by histological examination. The dramatic difference in morphology of caveolin-1 null hearts between these two groups may be due to the use of different background strains.

Caveolin-1 has been studied in cancer development and has been shown to have varied effects in the progression of tumors. In breast cancer, caveolin-1 is suggested to be a tumor suppressor because it is down regulated in certain oncogenic cells.[72] Research has shown that approximately 16% of all breast cancer patients have a mutation in caveolin-1.[73] Mice lacking caveolin-1 have an increase in mammary tumors. Estrogen receptor beta, also involved in breast tumorgenesis, is partially localized to caveolae.[74] Estrogen receptors have elevated expression under tumorigenic conditions and overexpression is found in the majority of human breast cancers.[75,76] When caveolin-1 gene is inactivated in mammary epithelial cells, estrogen receptor and cyclin D1 are up-regulated leading to tumorigenesis.[77] In contrast to breast cancer, caveolin-1 expression is increased in prostate cancer.[78] Knocking out caveolin-1 expression in a prostate cancer mouse model showed that the lack of caveolin-1 reduced tumorigenesis, suggesting that caveolin-1 promotes the development of prostate cancer.[78]

CAVEOLIN-2 ROLE AND FUNCTION

Another caveolin protein, caveolin-2, was discovered in murine adipocytes when caveolin-enriched membrane domains were isolated and probed for protein components which were then microsequenced. It was determined that a 20 kDa protein, similar to caveolin-1, was present but it differed from caveolin-1 at several residues. When compared to human caveolin-1, caveolin-2 was determined to be roughly 38% identical and 58% similar with a conserved region of eight identical amino acids.[79] Caveolin-2 is expressed in white adipose tissue and lung tissue as well as endothelial cells, smooth muscle cells, skeletal myoblasts and fibroblasts as well as pancreatic islets. Caveolin-2 colocalizes with caveolin-1 and, interestingly, is unable to directly bind cholesterol without caveolin-1 interaction.[79,80] Unlike caveolin-1, caveolin-2 cannot form homo-oligomeric complexes and requires caveolin-1 to form stable hetero-oligomers.[81] In rat thyroid tissue, which expresses caveolin-2 but not caveolin-1, it was shown that caveolin-2 is retained in the Golgi complex. However, if caveolin-1 is expressed by transfection or adenovirus-mediated transduction in these cells, caveolin-2 redistributes to the plasma membrane indicating that caveolin-1 is required for caveolin-2 localization to the plasma membrane.[82] These combined results suggested that caveolin-2 acts only as an accessory protein to caveolin-1. However in caveolin-2 null mice, a severe pulmonary dysfunction was present indicating that caveolin-2 may have a role in lung function independent of caveolin-1.[83]

CAVEOLIN-3 ROLE AND FUNCTION

The identification of caveolin-2 gave rise to the caveolin family of genes. First described in 1995, caveolin-3 is a muscle specific member of the caveolin family of proteins, though caveolin-1 is also expressed in muscle.[84,85] Caveolin-3 is roughly 64% identical to caveolin-1 and similarly can form homo-oligomeric complexes with itself and does not require caveolin-1 to drive caveolae formation.[86] Caveolin-3 null mice have muscle fiber necrosis and regeneration, a phenotype similarly observed in human patients with muscular dystrophy.[86] Additionally, caveolin-3 deficient mice develop a severe cardiac phenotype as early as four months of age resulting in severe cardiac hypertrophy and fibrosis resulting in reduced fractional shortening with an increased activation of the Ras-p42/44 MAPK pathway.[87]

Extensive research has determined many roles of caveolin-3 in cardiac function mainly by regulating G-protein coupled receptors (GPCRs) function at the level of the myocyte. β_1 adrenergic receptors are found predominantly in noncaveolae lipid rafts and a small number can be found in caveolae. Contrastingly, β_2 adrenergic receptors are enriched in the caveolae membrane.[88] It has been determined that β_2 adrenergic receptors and downstream targets are localized to caveolin-3 enriched fractions at rest and translocate out of the caveolin-3 enriched membrane after treatment with isoproterenol.[89,90] Interestingly, a study by Insel et al raised the possibility that GPCR signaling components have different localization patterns in adult cardiomyocytes compared to neonatal myocytes suggesting that there are multiple subcellular microdomains involved in GPCR signaling in cardiac myocytes.[91] In a recent study, Calaghan et al disrupted the caveolae membrane in isolated cardiac myocytes and recorded the lusitropic response (myocardial relaxation) to β_2 adrenergic receptor stimulation using video edge detection.[92] Disruption of the caveolae membrane caused a decrease in relaxation in myocytes as well as a significant increase in the phosphorylation of phospholamban and Troponin I suggesting that the cAMP-dependent signal is no longer confined to the sarcolemma.[92] This study confirms the spatial orientation of the β_2 adrenergic receptors to the caveolae compartment and regulation by caveolin-3.

PTRF

Polymerase I and transcript release factor (PTRF), also known as cavin, is also localized to the cytosolic face of caveolae membrane. PTRF, a 60kDa protein, has the same abundance in a variety of cell types as caveolin-1. In a recent paper by Liu et al, mice deficient in PTRF did not form caveolae in lung epithelium, intestinal smooth muscle or skeletal muscle suggesting that PTRF is required for the formation of caveolae in these tissue.[93] However, in adipocytes, PTRF has a functional role beyond structure. In adipocytes, PTRF functions in the release of the polymerase complex from the transcript during rRNA transcription by dissociating the elongating complexes from the transcript.[94] Interestingly, mice lacking PTRF had elevated serum triglycerides, free fatty acids and insulin as well as decreased leptin and adiponectin expression suggesting that PTRF may also be involved in cholesterol and fatty acid transport as well as insulin signaling. PTRF is suggested to play a role in insulin signaling. After insulin exposure, PTRF moves from the cytosol to the nucleus along with hormone sensitive lipase.[95]

CONCLUSION

Lipid rafts and caveolae have similar components and functions. Both lipid rafts and caveolae are microdomains of the plasma membrane rich in sphingolipids and cholesterol. Both are involved in endocytosis. Caveolae are unique in that they also form intracellular structures, caveosomes. Caveolae are important in cholesterol uptake and transport across the cell membrane. The scaffolding proteins associated with each microdomain are unique to that domain. Lipid rafts use flotillins while caveolae employ caveolins to organize the membrane domain. GPI-linked proteins are found in both lipid rafts and caveolae. Cross-linked GPI-anchored proteins move into caveolae, are then endocytosed and enter the caveosome without going through the endosome recycling process. In lipid rafts, GPI-linked proteins are internalized and moved to the endosome for recycling.

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CHAPTER 2

CAVEOLAE AND THE REGULATION OF ENDOCYTOSIS

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Abstract: Although clathrin-mediated endocytosis constitutes the main pathway for internalization of extracellular ligands and plasma membrane components it has generally been accepted that other uptake mechanisms—caveolae-mediated and noncaveolar raft-dependent endocytosis—also exist. During the last 20 years many papers have been published about caveolar endocytosis. These studies have fundamentally changed our view about the endocytotic role of caveolae. Views that caveolae are permanently static structures[1] have been extensively considered and rejected. Although the initial steps leading to the pinching off of caveolae from the plasma membrane have been studied in details, there are still contradictory data about the intracellular trafficking of caveolae. It is still not entirely clear whether caveolar endocytosis represents an uptake pathway with distinct cellular compartments to avoid lysosomal degradation or ligands taken up by caveolae can also be targeted to late endosomes/lysosomes.

In this chapter, we summarize the data available about caveolar endocytosis focusing on the intracellular route of caveolae and we provide data supporting that caveolar endocytosis can join the classical endocytotic pathway.

INTRODUCTION

Caveolae—flask-or omega-shaped plasma membrane invaginations with a diameter of 50-100 nm—are abundantly present in many but not all eukaryotic cell membrane.[2] Biochemical studies have revealed that caveolae are detergent resistant, highly hydrophobic membrane domains composed of mainly cholesterol and sphingolipids. These detergent-resistant liquid-ordered membrane domains are currently referred to as lipid rafts.[3-6]

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The main structural proteins of caveolae are members of the caveolin protein family. Morphologically defined caveolae can be formed by the expression of caveolin-1, which is a small integral membrane protein inserted into the inner leaflet of the membrane bilayer in a special hairpin-like structure. The cytosolic N-terminal region of caveolin-1 has a special amino acid sequence functioning as a scaffolding domain that has been suggested to be important for caveolin binding to cholesterol and sphingolipid-rich membrane domains.[7-8] This domain has also been implicated in the binding of signaling molecules.[2] The C terminus aligns along the inner leaflet of the bilayer by multiple palmitoylations.[9] Individual caveolae were estimated to contain 144 molecules of caveolin.[10]

Caveolae have been implicated in numerous functions including cell signaling, lipid metabolism and vesicular transport via their endocytosis. They have also been suggested to play a role in a variety of diseases including cancer, diabetes and viral infection. In this chapter, we focus on the function of caveolae in endocytosis.

CAVEOLAE AT THE PLASMA MEMBRANE

Expression of caveolin-1 has been described to be necessary for the formation of morphologically defined caveolae.[11-12] The function of caveolin-2 has not yet been defined in details and was thought to be accessory to the formation of caveolae. Recent result suggests that in addition to caveolin-1, caveolin-2 is necessary for the formation of deep plasma membrane-attached caveolae.[13]

At the level of endoplasmic reticulum, caveolin-2 interacts with caveolin-1 to form high molecular mass hetero-oligomeric complexes. The model of caveolar biogenesis suggests that caveolae form in the Golgi complex by association of caveolin-1/caveolin-2 hetero-oligomers with cholesterol. Interaction of caveolin-1 with caveolin-2 renders caveolin-2 detergent insoluble and targets Golgi-localized caveolin-2 to the plasma membrane. Exit from the Golgi complex is accelerated by cholesterol[14] and inhibited by glycosphingolipid depletion.[15] The small caveolae “precursors” (also called “exocytic caveolar carriers”)[16] traffic to the plasma membrane.[17] Thus, caveolae do not form de novo at the cell surface but their appearance at the plasma membrane results from the fusion of previously assembled caveolar vesicles with the cell surface.[18] Once small caveolar carriers are inserted into the plasma membrane, caveolae become relatively immobile structures.

Based on ultrastructural studies, it seems likely that caveolae formation also requires proteins other than caveolins. Overexpression of caveolin-1 in endothelial cells does not lead to an increased number of caveolae[19] indicating that additional structural components may be necessary for the formation of caveolae. PTRF-cavin (p-cavin, cavin), a protein initially identified as polymerase I and transcript release factor, was identified as a prominent caveolar protein. It was found to be required for caveolae formation in mammalian and zebrafish cells.[20-21] Recently, four members of the cavin family have been described[22-25] and were found to be soluble cytosolic proteins that are abundantly present at the cytosolic face of caveolae.[26-27] Binding of cavin to the membrane domain containing oligomerized caveolins, cholesterol and phosphatidylserine stabilizes the membrane curvature to produce the classical flask shape of caveolae. If the level of cavin expression decreases, caveolin-1 diffuses from the plasma membrane and becomes internalized into the endosomal/lysosomal system.[28] Biochemical and morphological data suggest that cavin associates with mature caveolae at the plasma membrane but does not associate with noncaveolar caveolin present in the Golgi complex.[27-28]

Caveolae are anchored to the plasma membrane by cytoskeletal components.[29] An F-actin binding protein, filamin, is one of the proteins identified as a ligand for caveolin-1.[30] However, the molecular mechanism for binding of caveolae to the cytoskeleton has not yet been established. Liu and Pilch[31] have provided experimental data suggesting that cavin may serve as a direct connection between caveolar components and the cytoskeleton.

Cholesterol itself is essential for caveolae formation and caveolin transcription.[32-33] Cells treated with agents that remove cholesterol (filipin, methyl-β-cyclodextrin or nystatin) lose caveolin and caveolae, resulting in flattened plasma membranes.[34]

INTERNALIZATION OF CAVEOLAE

Are Caveolae Stable and Highly Immobile Invaginations at the Plasma Membrane?

Based on electron microscopic observations, caveolae and caveolin-containing membrane domains of the plasma membrane have been described to have various curvatures and shapes (Ω, elongated flask, curved membrane invaginations with wide opening and narrow-neck). This morphology strongly suggests that caveolae can pinch off from the plasma membrane and could be involved in endocytosis operating in parallel with clathrin-mediated endocytosis. In spite of the morphological evidence, whether caveolae could really pinch off from the plasma membrane has been debated for a long time. A study using green-fluorescent-protein (GFP)-tagged caveolin-1 fusion protein revealed that the exchange of caveolin-1-GFP between the plasma membrane and intracellular pools was surprisingly slow, indicating that plasma membrane caveolae are immobile structures.[1] Uptake of albumin receptor (gp60) localized in caveolae of endothelial cells was inhibited by overexpression of caveolin-1,[35] suggesting that caveolin-1 itself can be responsible for stabilizing caveolae at the plasma membrane. The overexpression of caveolin-1 significantly reduces the internalization of autocrine motility factor receptor as well, indicating that caveolin-1 is rather a negative regulator of caveolae internalization.[36]

In the absence of endocytotic stimulus, caveolae seem to be quite static. Using a combination of total internal reflection fluorescence microscopy, Pelkmans and Zerial showed, however, that many caveolae are not immobile at the plasma membrane.[10] They are either stored in stationary multi-caveolar structures, or undergo continuous cycles of fission and fusion with the plasma membrane during which they do not travel long distances and they keep their structural integrity while cycling between the cytoplasm and the cell surface.[10] Serine/threonine kinases were shown to regulate this short-distance kiss-and-run dynamics of caveolae.[10]

Increasing evidence has shown that although caveolae are not normally involved in endocytosis, interaction of caveolae or caveolin with specific ligands can trigger the rapid internalization of caveolae. Several ligands like folic acid,[37] albumin,[39] autocrine motility factor,[40] alkaline phosphatase,[41] lactosyl ceramide[42] and pathogens like ganglioside-bound cholera toxin,[41] SV40 virus,[2,43] polyoma virus,[44] echovirus1,[45] HIV virus,[46] respiratory syncytial virus[47] and certain FimH-expressing bacteria[48] are known to be internalized by caveolae. Binding of various ligands to caveolin/caveolae, cross-linking of caveolar components and accumulation of receptors in caveolae promote downstream signaling events that result in caveolar internalization.[49-54] Stimulated internalization of caveolae changes the mode of transport from short-range to long-rang cycles.

Based on recent morphological and biochemical data, it has been widely accepted that caveolae take part in endocytosis and caveola-mediated endocytosis functions via a true uptake mechanism parallel to the clathrin-mediated pathway. Being ligand-triggered, caveolar endocytosis provides a highly regulated pathway for uptake of specific substances.

Caveolar Budding and Pinching Off from the Plasma Membrane

Caveolar budding is regulated by kinases and phosphatases. Treating cells with genistein, a tyrosine kinase inhibitor, rapidly blocks the pinching off of caveolae[55] indicating that internalization involves proteins regulated by phosphorylation of tyrosine. In endothelial cells, endocytotic stimuli induce a signaling cascade including G-protein-coupled Src activation.[35,54,56] Caveolin was first described as a substrate of a viral tyrosine kinase (v-Src).[49] It appeared that the cellular homologue of this kinase—c-Src—is able to phosphorylate caveolin-1 on tyrosine 14[53] and can bind to the scaffolding domain of caveolin-1.[50-51] Palmitoylation of caveolin-1 at cysteine 156 contributes to the coupling of Src to caveolin-1.[50,52] Binding of albumin to gp60 (receptor for albumin localized to caveolae) in endothelial cells induces tyrosine phosphorylation of both gp60 and caveolin-1.[54] Tyrosine kinase inhibitors (herbimycid A and genistein) prevented the gp60-activated vesicle formation and albumin endocytosis.[54] These data suggest that caveolin-1 phosphorylation is the initial step in caveolar internalization.

Lee et al[57] showed that the other caveolin isoform, caveolin-2 also undergoes Src-induced phosphorylation on tyrosine 19. Phospho-caveolin-2 (TyrP19) was strictly colocalized with phospho-caveolin-1 (TyrP14) indicating that the simultaneous phosphorylation of caveolin-1 and caveolin-2 might be equally important in the regulation of caveolae pinching off from the plasma membrane. Similarly, phosphorylation of caveolin-2 induced the internalization of caveolae in peritoneal macrophages.[58]

Sowa et al[13] mapped two serine phosphorylation sites in the N-terminal region (23 and 36) of caveolin-2. They showed that phosphorylation of each site contributes to caveolin-1 dependent caveolae formation. Mutation of serine 36 to alanine markedly reduced the number of plasma membrane-attached caveolae. Phosphorylation of serine 23 had marginal influence on the formation of caveolae at the plasma membrane but enhanced the number of uncoated vesicles in the cytoplasm. When both serine 23 and 36 residues were replaced by alanine, surface-attached caveolae were eliminated but the appearance of noncoated vesicles in the cytoplasm was markedly increased. These results suggest that dephosphorylation of serine residues of caveolin-2 might also be important to regulate caveolae pinching off from the plasma membrane.

Phosphatases also seem to play an important role in caveolar internalization (Fig. 1). Vanadate, a tyrosine phosphatase inhibitor stimulates caveolar endocytosis by causing hyperphosphorylation of tyrosine residues of caveolin-1.[59] Treatment with a serine/threonine phosphatase inhibitor (okadaic acid) causes a massive mobilization of caveolae.[41,42,58,59] Okadaic acid is a polyether metabolite, isolated from the black marine sponge Halichondria, which inhibits protein phosphatases, especially PP1 and PP2A.[60] PP1 and PP2A are two major classes of serine/threonine protein phosphatases that dephosphorylate a broad spectrum of substrates.[61] The exact mechanism by which serine/threonine phosphatase (PP1 and PP2A) inhibitors can stimulate caveolar endocytosis is not known. Since PP2A is a specific inhibitor of Src kinase, one possible explanation for okadaic acid stimulation of caveolar internalization is that these sequential inhibitions activate Src kinase which can phosphorylate caveolin-1 and/or -2.[58]

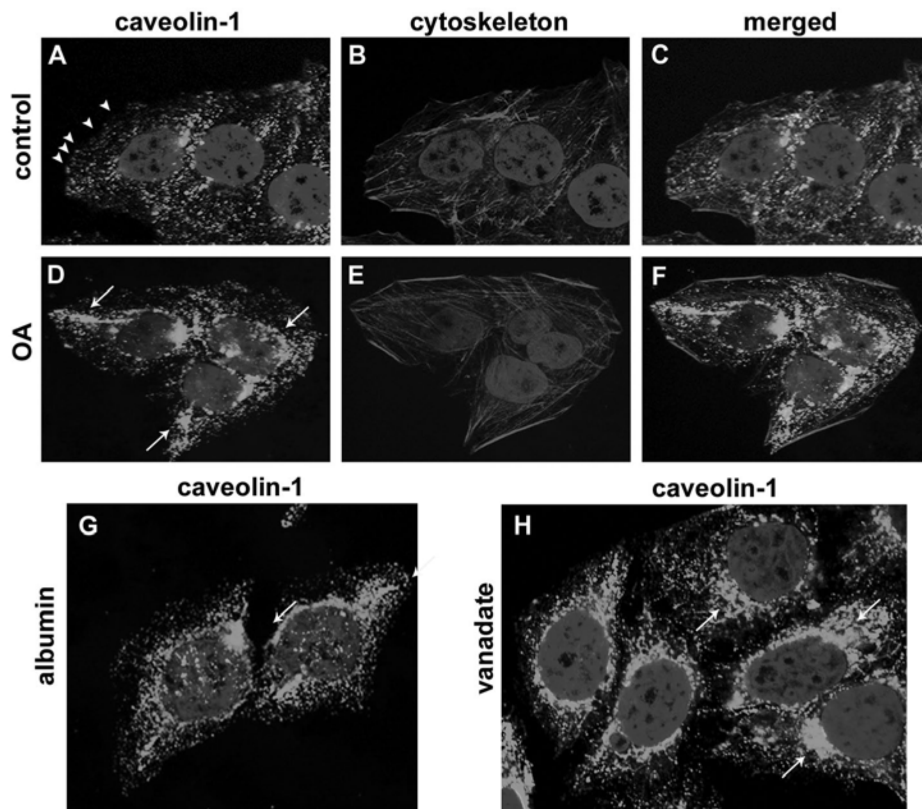

Figure 1. Stimulated internalization of caveolae followed by immunolocalization of caveolin-1 in HepG2 cells. A-C) In control cells caveolin-1 can be detected at the cell surface (white arrowheads) and also in the cytoplasm. D-F) Okadaic acid treatment significantly changes the cytoplasmic distribution of caveolin-1. Large fluorescent aggregates (white arrows) are present in the cytoplasm mainly in the perinuclear region. Okadaic acid treatment causes a discrete change in the cytoskeletal network as well. G-H) Vanadate (a tyrosine phosphatase inhibitor) and albumin treatment induce similar redistribution of caveolin-1, indicating that the internalization of caveolae is regulated by kinases and phosphatases. A color version of this image is available online at www.landesbioscience.com/curie.

The GTP-binding protein dynamin is known to play an important role in the pinching off of clathrin coated vesicles.[62] It was surprising when dynamin was found to be recruited also to caveolar membranes.[63-64] Dynamin, however, is not a permanent component of caveolae. Association of dynamin with caveolae appears to be a transient phenomenon.[2] Predescu et al[65] found a very strong association of intersectin—an important partner of dynamin in clathrin-dependent endocytosis—and dynamin in endothelial cells that were not transfected with a dynamin mutant. It seems likely that in the dynamin assembly, intersectin may serve as a scaffolding protein recruiting dynamin to generate a high local concentration required for collar formation. Subsequent to the hydrolysis of GTP, dynamin triggers the fission of caveolae by constricting its neck.

The cytoskeleton has also an important function in regulating the steady state distribution of caveolae.[29] Whereas the cortical actin cytoskeleton appears to confine

caveolae/caveolin-1 to the cell surface acting as a simple physical barrier to the detachment of caveolae, microtubules serve as tracks for the transport of caveolae to the cytoplasm. Thus, the internalization of caveolae depends on the integrity and/or reorganization of the cytoskeleton,[41] and local disassembly of cortical actin network is essential to initiate the inward transport of caveolae along microtubules. Reorganization of the actin cytoskeleton was found to be crucial for SV40 virus entry.[2] Accumulation of virus in caveolae initiates a signaling cascade that leads to tyrosine phosphorylation and depolymerization of cortical actin cytoskeleton. Actin monomers are recruited to the virus-loaded caveolae and actin patches are formed. After internalization of caveolae, the cortical cytoskeleton returns to its normal pattern. Okadaic acid can also cause reorganization of the actin cytoskeleton, leading to caveolar internalization.[1]

Detailed total internal reflection microscopy and computational analysis of caveolar structures at the cell surface[10] showed that in the absence of endocytotic stimulus, caveolae were in equilibrium between individual and aggregated multicaveolar states. The individual caveolae are dynamic, undergoing short-range cycles of fusion and internalization (kiss-and-run) just below the cell surface. The multi-caveolar complexes are rather static and connected to the cell surface. Two serine/threonine kinases were identified (KIAA0999 and MAP3K2) to specifically regulate the kiss-and-run dynamics, while Src and a putative phosphatidyl inositol 4-phosphate kinase were found to be responsible for the regulation of the aggregation of caveolae at the cell surface.[10]

Summary: The caveolar pinching off from the plasma membrane is regulated by kinases and phosphatases. Phosphorylation of caveolin(s), recruitment and activation of dynamin at the caveolar neck and reorganization of the cytoskeleton are essential for caveolar fission from the plasma membrane. Stimulated internalization of caveolae (travelling of caveolar structures inside of the cytoplasm) involves a switch from short-range to long-range cycles. This switch is possibly established by a signaling cascade coordinating the actin turnover, the microtubules and the transport along these filaments.[29]

INTRACELLULAR ROUTE OF CAVEOLAE

Caveolae were shown to be equipped with several components of the fusion machinery. NSF, SNAP, WAMP were described to concentrate in caveolae and possibly associate with caveolin-1.[66-68] Less extensively documented are, however, the trafficking and the intracellular fate of caveolae. Since internalized caveolae co-opt the same mechanism used in trafficking by other vesicles,[67-69] it is obvious that they can dock to and fuse with cytoplasmic organelles. The basic question is whether the internalized caveolae can fuse with endosomes and follow the classical endocytotic degradative pathway, or the caveolar endocytosis involves other cellular compartments, bypassing the classical endocytotic organelles.

Stimulated caveolar internalization is always accompanied with the appearance of grape-like multicaveolar complexes (Fig. 2). Studying the entry of SV40 viruses taken up by caveolae, the virus particles were detected in these multicaveolar complexes of neutral pH, distinct from classical endocytotic compartments.[43] These multicaveolar complexes never fused with lysosomes; thus, viruses could escape lysosomal degradation. Since these structures were labeled with caveolin-1, they were named caveosomes.[43,70] Until now, only a few electron microscopic pictures have been published about the morphology of these organelles.[43,71]

According to recent studies, caveosomes are multicaveolar structures of heterogeneous morphology that are supposed to be early endosome-equivalent intermediate organelles

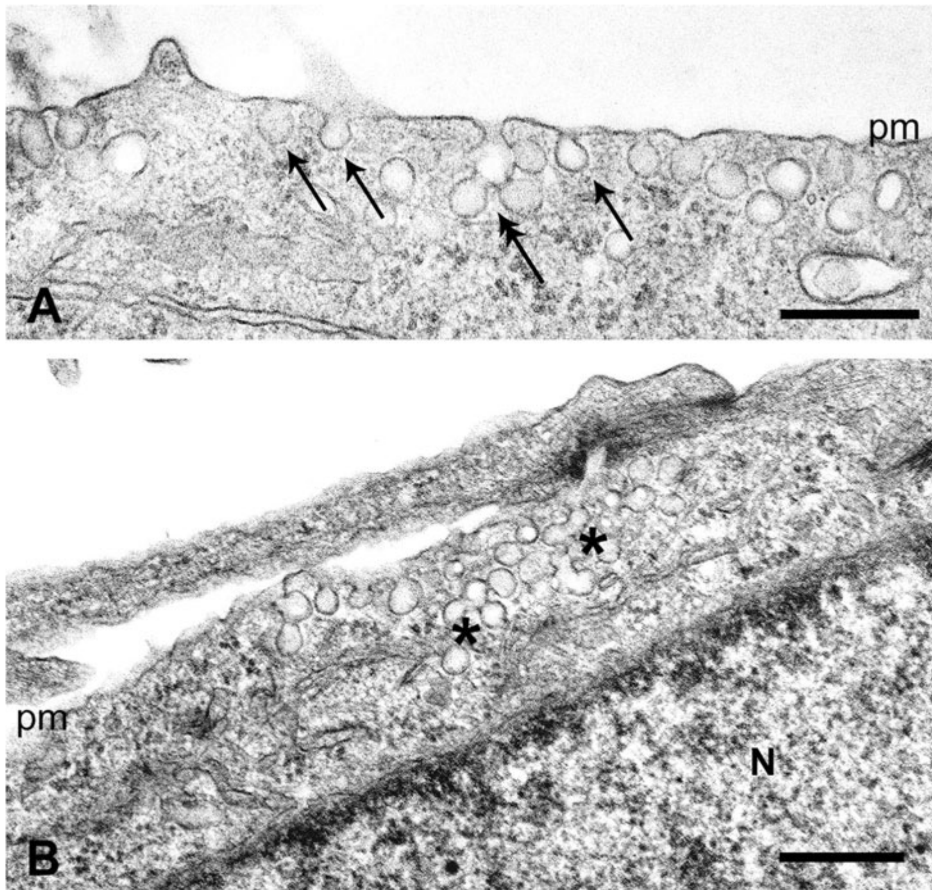

Figure 2. Phosphatase inhibitor (okadaic acid; OA) treatment causes the appearance of large caveolar cluster. In control HepG2 cells (A), single caveolae (arrows) and small caveolar clusters (double arrow) are present at the plasma membrane. When caveolar internalization was stimulated by okadaic acid (B), grape-like multicaveolar clusters (stars) appeared close to the cell surface. N: nucleus; pm: plasma membrane. Bars: 200 nm. B) Adapted with permission from Kiss AL et al. J Cell Mol Med 2009; 13:1228-37.[91]

in caveolar endocytosis. Because of their grape-like appearance they can not be easily distinguished from caveolar-clusters present at the plasma membrane. When studying caveolar internalization in HepG2 cells, we found that many of these caveosome-like, multicaveolar complexes were connected with the cell surface by a narrow tubular plasma membrane invagination (Fig. 3A,B), but some of them seemed to be independent structures in the cytoplasm. When Ruthenium red (Ru red)—an electron-dense dye—was used as an extracellular tracer, many of these structures were Ru red positive (Fig. 3C,D) indicating that they were still connected with the cell surface. These results support the idea that a significant portion of these multicaveolar complexes described as caveosomes are not independent structures.

The later fate of caveosomes is not entirely known. Viruses were described to be sorted from caveosomes into vesicular and tubular structures that travel along microtubules

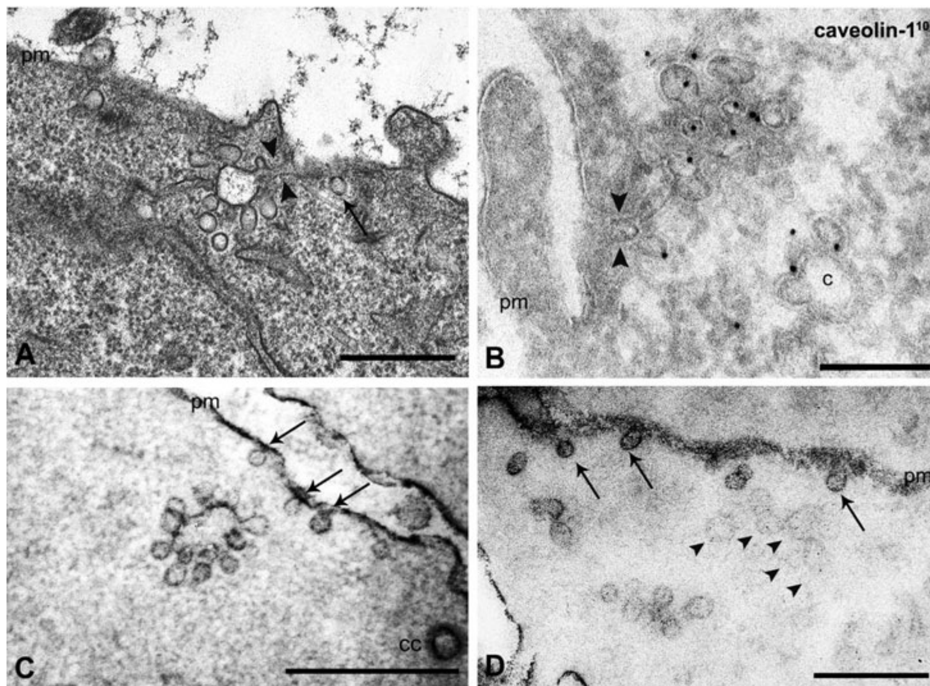

Figure 3. Caveolar clusters are connected to the plasma membrane. A,B) Many of the caveolar clusters are connected to the cell surface through narrow tubular plasma membrane invagination. Arrowheads in (A) and (B): A) transmission EM picture; B) immunolocalization of caveolin-1 on ultrathin frozen section. C,D) The morphology of these caveolar clusters strongly suggest that they are independent structures in the cytoplasm. When Ruthenium red—a specific cellular surface marker—was used, many of these clusters were strongly labeled, indicating that they are still connected to the plasma membrane. Some of these clusters contain only a few Ru red crystals, arrowheads on (D), suggesting that the tubular membrane invagination by which they are connected to the cell surface is very narrow. (Arrows: single caveolae) c: caveosome; cc: clathrin coated vesicle; pm: plasma membrane. Bars: 200 nm. A,B) Adapted with permission from Kiss AL et al. J Cell Mol Med 2009; 13:1228-37.[91.] C,D) Adapted with permission from Kiss AL et al. Pathol Oncol Res 2009; 15(3):479-86.[92]

to the smooth endoplasmic reticulum.[43,45] There are data, however, showing that ligands internalization by caveolae can be directed to the classical endocytotic organelles. Cholera toxin entering cells by caveolar-mediated endocytosis passes through early endosomes and accumulates in the Golgi complex.[72] It was also shown that under normal conditions caveolae carrying SV40 virus particles can transiently interact with early endosomes.[73-74] The existence of alternative caveolar trafficking routes, involving caveosomes and early endosomes, raises the questions of whether the downstream caveosomes interact with the classical endocytotic compartments or ligands taken up by caveolae are transported by organelles bypassing the degradative pathway. The subcellular distribution of caveolin could provide insights into the endocytotic pathways. Caveolin-1 in many cells is evident at the cell surface and within the Golgi complex and only partial colocalization can be detected with endosomal markers such as EEA1, a marker of the early sorting endosome[74] or CD63, a late endosomal marker.[75] When caveolar endocytosis was induced by albumin, the number of CD63 and caveolin-1 double labeled multivesicular bodies or late endosomes

significantly increased.[75] Studying the long-term internalization of albumin in HepG2 cells, albumin was found to accumulate in large, caveolin-1 positive caveosome-like caveolae clusters (Fig. 4). The number of caveolin-1 and CD63 containing multivesicular bodies significantly increased, indicating that caveolae-mediated endocytosis of albumin resulted in an increased caveolar trafficking along the classical endosomal degradative pathway.[75]

Analysing the involvement of several small GTPases in the trafficking of caveolae, Pelkmans et al[73] found that caveolae can move to early endosomes in a Rab5-dependent manner. Although caveolae dock to and fuse with endosomal membrane, their membrane domains do not mix with each other. After some time, caveolin-1 subdomains can pinch off again from early endosomes as membrane vesicles. If caveolae transiently fuse with early endosomes, one can suppose that they should be able to pick up some fluid from that compartment and potentially carry it to caveosomes, suggesting that the traffic is bidirectional. Experiments using a fluid phase tracer Lucifer yellow (LY) showed that after a long time incubation, small but significant amounts of LY accumulated in caveosomes.[73] Human polyoma virus (JCV) are known to enter the cell by clathrin-dependent endocytosis and are transported immediately to early endosomes. They can also be sorted to a caveolin-1 positive endosomal compartment. This transport is dependent on Rab5-GTPase.[76] These experimental data clearly show that there is a communication between caveolae-mediated endocytosis and classical endocytotic pathways (caveosomes and endosomes) and

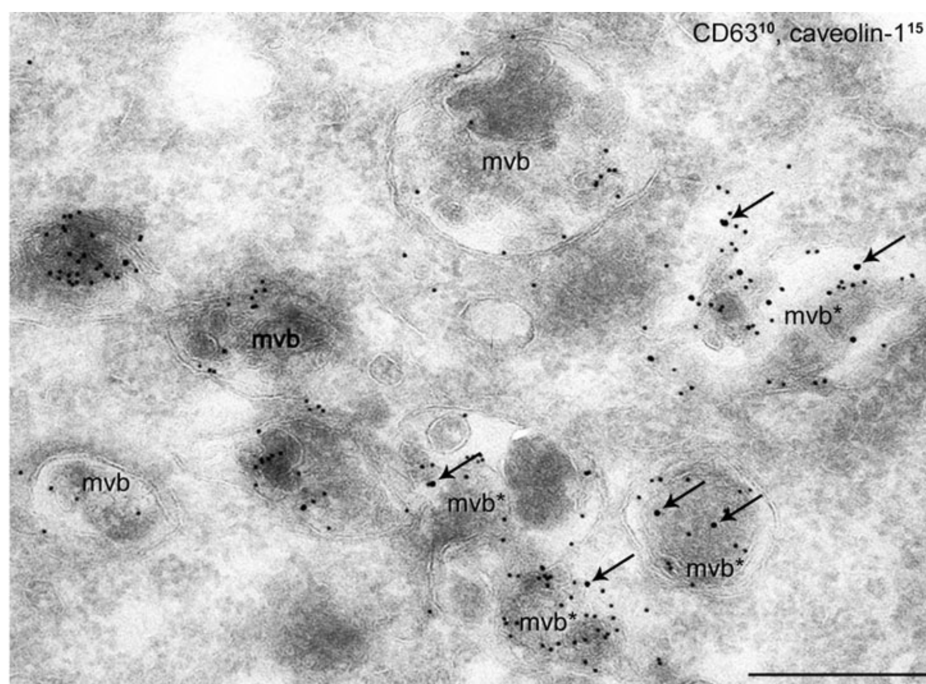

Figure 4. Caveolin-1 and CD63 double labeling in ultrathin frozen section. Caveolin-1 (arrows) is present in CD63 positive late endosomes indicating that albumin-stimulated internalization drives caveolin-1 to a degradative pathway through CD63 positive late endosomes. mvb: CD63 labeled multivesicular bodies; mvb*: caveolin-1 and CD63 double labeled multivesicular bodies. Bars: 200 nm. Adapted with permission from Kiss AL et al. J Cell Mol Med 2009; 13:1228-37.[91]

strongly suggest that the communication is bidirectional. Rab5-GTPase should prolong the caveolar interaction with endosomes.

The morphological entities by which this communication can occur are not known. Caveolar vesicles continously docking on and fusing with caveosomes and early endosomes[18] are good candidates for this function. During their kiss-and-run interaction with endocytotic compartments the caveolar coat remains intact. Caveolin-1 containing subdomains pinching off from the early endosomes and/or caveosomes as vesicles can function as mediators between the two pathways. It can not be excluded, however, that caveolar clusters can pinch off "en mass" from the plasma membrane and fuse with endosomes. Alternatively, caveolae can move to and from the transitional ER to cis-Golgi complex, resulting in the entrapment of stable caveolar domains and an enlarged Golgi complex.[18] This membrane traffic is regulated by Arf1, another small GTPase.

REGULATION OF CAVEOLAR TRAFFICKING

Kinases and phosphatases have a regulatory role in the fusion of caveolar carriers with endosomal compartments and/or caveosomes. Sorting, distribution and transfer of the cargo to its final destination strongly depend on kinases. Using high-throughput RNA interference and automated image analysis Pelkmans et al[77] identified six kinases that regulate coat stability, caveolar clustering, kiss-and-run dynamics and long-distance cycling of caveolae. With this genome-wide screen they could show the specific role of kinases at different stages of the caveolar assembly and transport. A large group of these kinases was found to function in various signaling systems indicating that endocytotic transport and signal transduction are tightly coupled.

Not only phosphorylation but also dephosphorylation of membrane-associated receptors or proteins can be critical to determine the sorting. The regulatory role of phosphatases, however, is less known. There are data indicating that PP2A—a serine/threonine phosphatase—plays an important role in endosomal sorting and movement of endocytotic compartments along the microtubules.[78-80] Via dephosphorylation of proteins present in the endosomal membrane, PP2A can regulate the maturation of endosomes and the fusion of endosomes with lysosomes.[78,81,82] When PP2A is inhibited, the classical endocytotic sorting is blocked and the cargo can stop in one of the intermediate compartments. The small T-antigen of SV40 virus is known to bind and inhibit PP2A,[83] by which it can interfere with the maturation of the virus and caveolin containing endosomes (caveosomes) and endosome/lysosome fusion. This might explain why the SV40 virus is retained in caveosomes resulting in an escape from lysosomal degradation. Caveolin-1 itself is known to interact with and inhibit PP2A[84] which can result in an accumulation of caveolae in caveosomes. It seems likely that the interaction of the cargo with caveolar components, caveolin itself or any of the regulatory kinases and/or phosphatases can be an important determinant for the final destination of the cargo. The interaction of the cargo with the components of the machinery provides a specific, "custom-made" pathway for the entrance of different particles and molecules.

It has been suggested that cholesterol itself plays an important role in intracellular transport.[33] Normally, cholesterol is found in the plasma membrane and early endosomes and is sorted away from late endosomes and lysosomes.[85] Recent studies have revealed that cholesterol is not a passive component of endosomal membranes, but is rather directly involved in the sorting and transport of endocytotic vesicles.[86]

CONCLUSION

Although caveolae are quite static lipid domains of the plasma membrane, under specific conditions (eg. binding of specific ligands to their receptors), they can pinch off from the plasma membrane. Nowadays it is generally accepted that caveolae-mediated endocytosis functions as a true uptake mechanism in parallel to the clathrin-mediated pathway. Being ligand-triggered, caveolar endocytosis provides a more selective and highly regulated pathway for uptake of specific substances. Caveolar endocytosis is regulated by kinases and phosphatases. Tyrosine phosphorylation of caveolin-1 (and possibly caveolin-2) can initiate budding and internalization of caveolae. The GTP-binding protein dynamin, that is temporally associated with caveolae triggers fission of caveolae by constriction of its neck subsequent to the hydrolysis of GTP. The internalization of caveolae depends on the integrity and/or reorganization of the cytoskeleton: local disassembly of cortical actin network is essential to initiate the inward transport of caveolae.[41] Once caveolae pinch off from the plasma membrane, they may associate with motor molecules that propel them along the microtubules.[29] Whereas the cortical actin cytoskeleton appears to confine caveolae/caveolin-1 to the cell surface acting as simple physical barriers to the detachment of caveolae, microtubules serve as tracks for the transport of caveolae to the cytoplasm.

After stimulated internalization, grape-like multicaveolar complexes called caveosomes appear in the cytoplasm.[2,43,70] It is still debated whether these caveolar clusters are independent entities since many of them are connected to the cell surface by very narrow tubular plasma membrane invaginations. Viruses accumulate in these multicaveolar complexes of neutral pH, distinct from classical endocytotic compartments.[43] They never fuse with lysosomes thus viruses can escape lysosomal degradation. Other ligands using caveolae to enter the cells, however, are present in early endosomes and follow the classical endocytotic route. Long-term incubation with albumin also results in the appearance of caveolin-1 in late endosomes/multivesicular bodies indicating that caveolae or caveosomes communicate with the classical endocytotic compartments. These data strongly suggest that although the first step of the uptake is different, the intermediate organelles (caveosomes/early endosomes) can communicate with each other. Thus, ligands internalized by caveolae can be directed to the classical endocytotic pathway.

The route followed by different ligands is regulated by small GTPases (Rab molecules), kinases and phosphatases. Figure 5 summarizes the possible routes for caveolae after internalization. It seems likely that the interaction of the cargo with caveolar components, caveolin itself or any of the regulatory kinases and phosphatases can have a determining influence on the final destination of the cargo. The interaction of the cargo with the machinery components can result in a specified, "custom-made" pathway for the entrance of different particles and molecules.

Since numerous signaling molecules have been identified to accumulate in caveolae, they can also be called "signaling organelles".[87] Large groups of kinases identified in endocytosis are members of various signaling pathways supporting the idea that endocytotic transport and signal transduction are tightly coupled and that caveolar internalization should play an important role in the regulation of signal transduction as well.

It has to be pointed out that there are caveolae that are immobile and never pinch off from the plasma membrane. Smooth muscle cells have large numbers of caveolae at their plasma membrane which enlargea the surface area by 80%. These caveolae, however, are never taking part in endocytosis, perhaps because their caveolar protein lacks tyrosin

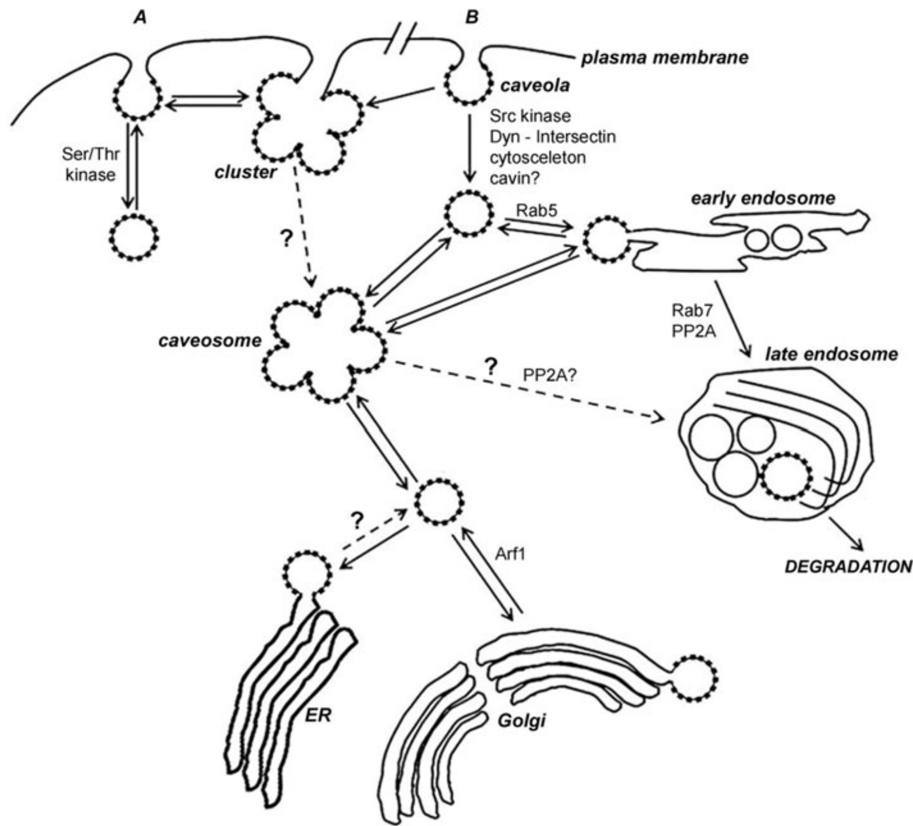

Figure 5. Intracellular trafficking of caveolae. A) The kinase-regulated short-range cycles of caveolae: Individual caveolae continuously internalize and fuse with the plasma membrane (kiss-and-run) or are stored in small multicaveolar clusters. B) Stimulated internalization of caveolae. Phosphorylation of caveolin(s), association of dynamin with the neck of caveolae, reorganization of the cytoskeleton result in pinching off of caveolae from the plasma membrane. Pinched off single caveolae may fuse with pre-existing caveosomes, but they can also fuse with early endosomes in a Rab5-dependent manner. Stimulated caveolae internalization results in the formation of large caveolar clusters. While many of these clusters remain connected to the plasma membrane, some of them may detach ("en mass") from the plasma membrane and form caveosomes. The caveosome/endosome pathways seem to be bidirectional. Further sorting from early to late endosome depends on Rab7 and PP2A. The small GTPase Arf1 regulates caveolar trafficking between caveosomes and the transitional ER or cis-Golgi complex.

residues that could be phosphorylated.[88] Therefore, one of their most important roles seems to be the regulation of calcium movements in smooth muscle cells.[89-90]

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CHAPTER 3

CAVEOLIN-1: Role in Cell Signaling

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Abstract: Caveolins (Cavs) are integrated plasma membrane proteins that are complex signaling regulators with numerous partners and whose activity is highly dependent on cellular context. Cavs are both positive and negative regulators of cell signaling in and/or out of caveolae, invaginated lipid raft domains whose formation is caveolin expression dependent. Caveolins and rafts have been implicated in membrane compartmentalization; proteins and lipids accumulate in these membrane microdomains where they transmit fast, amplified and specific signaling cascades. The concept of plasma membrane organization within functional rafts is still in exploration and sometimes questioned. In this chapter, we discuss the opposing functions of caveolin in cell signaling regulation focusing on the role of caveolin both as a promoter and inhibitor of different signaling pathways and on the impact of membrane domain localization on caveolin functionality in cell proliferation, survival, apoptosis and migration.

INTRODUCTION: COMPLEXITY OF CAVEOLIN MEMBRANE DOMAINS

Whereas caveolae were identified in the 1950s by electron microscopy as plasma membrane invaginations, caveolin-1 (Cav1) was initially identified as a 22kDa phosphoprotein substrate of Src kinase and subsequently localized to caveolae.[1,2] Cav1 was also observed in the Golgi apparatus, the plasma membrane and vesicles suggesting a role as a trans Golgi network transporter.[3] Cav1 was then quickly characterized as a raft-enriched protein responsible for signaling regulation.[4] Cav1α is a 178 amino

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acid protein (24 kDa) whereas Cav1β is encoded from methionine 32 and contains only 148 amino acid (21 kDa). Cav1α is the best characterized in terms of function. Cav2 and Cav3 are encoded by different conserved genes and display a high degree of similarity with Cav1.[5] Cav1 and Cav2 are coexpressed in many tissues (adipocytes, endothelial cells and fibroblasts) whereas Cav3 is enriched primarily in differentiated muscle cells.[6,7] The role of Cav2 in signaling regulation is poorly characterized while Cav3 appears to function similarly to Cav1.[7-9] This chapter will therefore focus on the role of Cav1 in cellular signaling.

Structure of Cav1

Cav1 is a hairpin membrane protein with N and C-terminal cytoplasmic tails separated by a hydrophobic segment (amino acids 102-134) that does not cross the membrane.[10,11] Cav1 displays two main functional domains: the tyrosine 14 phosphorylation site and the oligomerization domain that also contains the scaffolding domain. Cav1α (but not Cav1β lacking the first 32 amino acids) is phosphorylated on tyrosine 14 (Y14) by Src kinase as well as Fyn, Yes and c-Abl.[12-14] This phosphorylation can occur chronically or punctually in response to growth factor treatment or integrin activation, with various consequences as detailed in the other sections.[15,16] Cav1 is also phosphorylated on serine 80 which has been proposed to regulate Cav1 and cholesterol trafficking.[17,18] Cav1 oligomerizes through amino acids 1-101; oligomers can include Cav2 and interact with the actin cytoskeleton through filamin A.[19-21] This region contains the scaffolding domain (juxtamembrane 82-101 amino acids) which is responsible for Cav1 interaction with Src family proteins, G proteins, phospholipases, protein kinase A, protein kinase C, adenylate cyclase, nitric oxide synthases, tyrosine kinase receptors and Ras family GTPases.[22-25] Moreover, this domain and the C-terminal 135-178 amino acids are responsible for Cav1 localization at the plasma membrane.[11] The scaffolding domain allows direct binding to cholesterol which participates in raft organization and cholesterol transport.[26-29] Finally, Cav1 is palmitoylated on cysteines 133, 143 and 156 which is required for cholesterol binding and transport to caveolae and for interaction with Src leading to Cav1 Y14 phosphorylation.[30,31] Here, we detail the functions of the Cav1 scaffolding domain and tyrosine 14 as critical regulators of cell signaling.

Cav1-Associated Membrane Domains: Rafts, Caveolae and Cav1 Scaffolds

Lipid rafts are plasma membrane domains enriched in cholesterol and sphingolipids where multiple signaling and responses are regulated. Rafts were initially characterized biochemically as detergent resistant membranes (DRMs), however they have since been shown to be very dynamic and transient structures. Cav-associated structures represent subdomains of lipid rafts and include caveolae but also Cav1 scaffolds, flat oligomerized Cav1-associated domains that do not form caveolar invaginations at the cell surface (Fig. 1).

Noncaveolar rafts are planar 1-1000 nanometer ordered structures enriched in glycosylphosphatidylinositol (GPI)-anchored proteins. They are difficult to visualize because of their transient properties and flat shape. Tools have been developed to study rafts, such as fluorescent B subunit of Cholera toxin, which binds the ganglioside GM1, GFP-tagged GPI-proteins and drugs such as filipin and nystatin that sequester cholesterol, or β-methylcyclodextrin that extracts cholesterol. Recently new dyes such as Laurdan

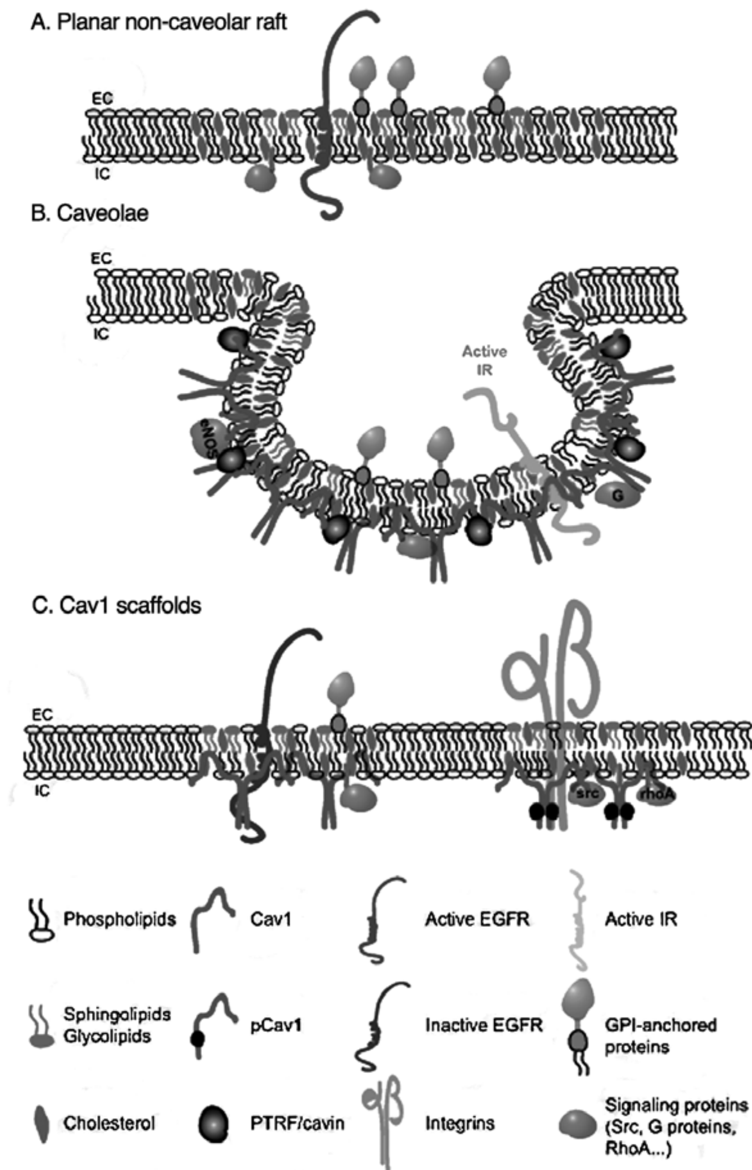

Figure 1. Cav1-associated membrane domains: Rafts, caveolae and Cav1 scaffolds. A) Planar Rafts: Dynamics and transient cholesterol-enriched domains where receptors and signaling proteins (including GPI-anchored proteins) accumulate and signal. EGFR has been isolated in this fraction after activation and planar rafts are thought to be the site of EGFR signaling. B) Caveolae: Stable invaginated raft domains enriched for Cav1 and Cav1-associated PTRF/cavin. IR, NOS or Gαq are thought to signal in caveolae, whereas Gαs is thought to be inhibited. C. Cav1 scaffolds: Poorly characterized noncaveolar Cav1 oligomers thought to be negative regulatory domains that inhibit EGFR signaling. pCav1 is proposed here to form a similar domain at or near focal adhesion sites where Src and RhoA are regulated according to a loop of activation/deactivation, promoting pCav1-dependent cell polarity and migration. A color version of this image is available online at www.landesbioscience.com/curie.

have been characterized to distinguish ordered versus disordered membrane regions.[32] Phosphatidyl inositol-4, 5-Phosphate (PtdIns(4,5)P_2) is enriched in DRM fractions and might define another raft subdomain. PtdIns(4,5)P_2 is a key signal messenger: its cleavage by phospholipase C produces inositol triphosphate leading to calcium release and its phosphorylation produces the messenger and docking lipid Phosphatidyl inositol-3,4,5-Phosphate. PtdIns(4,5)P_2 is found in caveolae and noncaveolar DRMs where it recruits different signaling proteins through their PH (Pleckstrin homology) domain.[33,34] PtdIns(4,5)P_2 rafts might be the site of regulation of signaling events, actin cytoskeleton organization and dynamics, as well as membrane ruffling.[35-37]

Caveolae are stable invaginated raft subdomains containing 100-200 Cavs resulting from multi-step assembly.[38,39] Cav1 oligomerizes in the endoplasmic reticulum after synthesis and is transported to the Golgi apparatus where oligomerized Cav1 interacts with cholesterol.[29,40] This large complex is transported to the plasma membrane where PTRF/cavin-1 is recruited and stabilizes caveolae at the plasma membrane.[41] Expression of PTRF/cavin-1 and the other cavins, SRBC/cavin-2 and SDPR/cavin-3, are critical regulators of caveolae formation, morphology and dynamics.[42] The requirement for additional cavins in addition to Cavs for caveolae formation indicates that, in cells expressing reduced cavin levels, Cav1 must necessarily have functions outside of caveolae.

The Cav1 scaffold corresponds to flat, oligomerized Cav domains at the plasma membrane that do not form caveolae.[43] They likely correspond to the minimal ~15 oligomerized Cav1 structure that has been identified in native acrylamide gels and by crosslinking.[44-47] The Cav1 scaffold has been poorly described in comparison to caveolae and planar rafts, probably because electron microscopy is required to distinguish these structures from caveolae. The concept of the Cav1 scaffold came from the fact that (1) Cav1 has been shown to regulate signaling events in cells lacking caveolae such as neurons.[48] (2) The level of Cav1 expression defines the formation of caveolae and the regulation of eNOS activity without any correlation between these two events.[46,49] Finally, (3) Cav1 oligomerization regulates EGFR activity independently of caveolae formation.[50] The Cav1 scaffold therefore defines a domain distinct from caveolae.

Characterization of the localization of receptors and their downstream signaling partners to these membrane domains remains a complex and difficult process that has best been shown using tremendously high speed image acquisition of live cells.[51,52] With respect to Cav1, how and why a receptor is regulated by its localization in planar rafts, caveolae or Cav1 scaffolds in different systems is not clear.

Cav1: Up and Down Regulation in Cancer

Expression of Cav1 appears to be modified in response to various factors, from green tea polyphenols to long bone fracture.[53,54] In cancer cells both increased and decreased Cav1 expression has been described, as well as Cav1 gene mutations. The relationship between tumor aggressiveness and Cav1 expression is an open question and might be cancer specific consistent with different roles for Cav1 as a function of the protein expression profile of a particular cell type. The locus 7q31.1 containing Cav1 gene is a fragile genomic region: it is often deleted in cancers suggesting that Cav1 is a tumor suppressor.[55,56] However, Cav1 expression is strongly correlated with poor prognosis in breast and prostate cancer.[57-59] The sporadic mutation P132L of the

Cav1 gene has been identified in 16% of a breast cancer cohort.[60] Expression of this mutant traps endogenous Cav1 intracellularly suggesting that P132L Cav1 mutant acts as a dominant negative.[61] However, both negative and positive effects on tumor cell migration have been observed with the P132L Cav1 mutant, confirming the complexity of the mechanisms associated to Cav1.[62,63] Altogether, Cav1 expression can be differentially regulated in cancer, showing the complexity associated to this scaffolding protein in disease progression. Subsequent sections detail the ambivalent actions of Cav1 on cellular signaling.

SCAFFOLDING DOMAIN AND TYROSINE KINASE RECEPTOR REGULATION

Cav1 recruits and regulates various signaling proteins through the interaction of its scaffolding domain with the hydrophobic sequence *ψXψXXXXψ* or ψXXXXψ*XX*ψ where ψ is an aromatic residue[23,64] (Table 1). For instance, activity of EGF receptor (EGFR,[65]), NO synthase,[66] G proteins,[4] protein kinase C[23] and Src family proteins are negatively regulated by this interaction, whereas insulin receptor (IR),[24] Eph receptors[67] or Estrogen Receptor[68] are activated. Differential regulatory roles of Cav1 for different receptors and signaling pathways highlights how Cav1 can exhibit divergent roles in cellular signaling leading to complex involvement in disease.

Complexity of Tyrosine Kinase Receptor Regulation by Cav1

Intriguingly, Cav1 can both negatively and positively regulate tyrosine kinase receptors (TKR). For instance, EGFR and the NGF receptor are both negatively regulated, whereas IR and Eph Receptors are positively regulated.[24,48,65,67] EGFR and IR display different structures and signaling patterns which may explain why Cav1 regulates them differently. EGFR is classically represented as a single transmembrane monomer which dimerizes upon EGF treatment, whereas IR is composed of an extracellular α subunit linked to the transmembrane β subunit forming dimers stabilized by disulfide bonds. Phosphorylated tyrosines of activated EGFR recruit SH2 domain proteins such as Grb2, Shc, SHP-1 or phospholipase C, whereas activated IR phosphorylates Insulin Receptor Substrates (IRS) such as IRS-1, GAB-1, Shc, APS, p60Dok and c-Cbl. Altogether, EGFR and IR represent TKRs differently regulated by Cav1. Indeed, Cav1 has been shown to interact with EGFR to prevent its activation and EGF-induced cell migration, whereas Cav1 interaction with IR promotes its activation playing a key role in glucose metabolism and diabetes.[24,65,69,70] This may be due in part to differential Cav1 involvement in EGFR and IR endocytosis. In addition to its regular clathrin-dependent endocytosis, EGFR can also be internalized by a Cav1-dependent process leading to perinuclear trafficking and/or degradation.[71,72] In contrast, Cav1-deficient mice exhibit insulin resistance due to accelerated degradation of IR suggesting that Cav1 stabilizes IR and limits its turnover and degradation,[70,73] consistent with the negative regulatory role of Cav1 in raft-dependent endocytosis.[74,75] However, EGFR and IR have been both shown to interact directly with Cav1 and to signal in a DRM dependent way and EGF and insulin can induce Cav1 phosphorylation.[65,99,213-215] EGFR and IR therefore share similar and opposing particularities relative to Cav1 regulation. Why Cav1 negatively regulates EGFR and positively IR remains an open and intriguing question. Indeed, as it

Table 1. Non-exclusive lists of Cav1 interacting partners

Regulated Factor	Binding Region of Cav1	Role of Cav1	References
Caveolae Formation			
Cholesterol	Scaffolding domain and cysteines 133, 143 and 156	Transport to plasma membrane and downregulation of raft endocytosis	26,27,29,75
PTRF/Cavin-1	n.d.	Caveolae formation	41,184
SDPR/Cavin-2	Recruited to caveolae through PTRF	Caveolae formation	185
SRBC/Cavin-3	n.d.	Caveolae formation and functions	186
MURC/Cavin-4	n.d.	Caveolae formation	187
Receptor Tyrosine Kinase			
EGF receptor	Scaffolding domain	Prevent activation	50,65,69,77,88
		Promote activation	84-87
		No effect	82,83
TGFβ receptor I	Scaffolding domain	Prevent activation	188
NGF receptors (p75 and TrkA)	Scaffolding domain	Prevent activation in caveolae	48,141
Insulin receptor	Scaffolding domain	Promote activation	24,70
EphB1 receptor	Scaffolding domain	Promote activation by stabilization at membrane level	67
Gprotein Coupled Receptors			
Estrogen receptor alpha	1-182 region	Promote activativation	68,189
Angiotensin II receptor (AT1-R)	Scaffolding domain	Cav1 stabilizes AR expression, AR not localized in caveolae	190,191
Glutamate receptor mGluR1	Scaffolding domain	Internalization	192
G Proteins			
G proteins (alpha and trimeric)	Scaffolding domain	Prevent activation	4,193,194
Galpha s		Prevent activation	12,195
Galpha i and alpha o		Prevent activation	194
G alpha q		Prolong signal	155
Protein Kinases			
Protein kinase A	Scaffolding domain	Prevent activation	196,197
Protein kinase C	Scaffolding domain	Promote activation	23,198,199
		Inhibition	200
Phospholipases			
PLC	Scaffolding domain	Promote activation	77
PLD	Scaffolding domain	Promote activation	201,202
		Prevent activation	203

continued on next page

Table 1. Continued

Regulated Factor	Binding Region of Cav1	Role of Cav1	References
Other Signaling Proteins			
ERK1/2	Scaffolding domain	Prevent activation	89,90
Akt	n.d.	Maintain activation	91
NO synthase	Scaffolding domain	Prevent activation	25,66
		Promote activation	152-154
Adenylate cyclase	Scaffolding domain	Attenuate signal	195,204
Ras GTPase (H, N and K)	Scaffolding domain	Inhibition	12,23,193,205,206
Src	Phosphorylated Cav1 required	Down regulation	
Csk	N-terminal	Promote activation	177,207
TRAF2	N-terminal	n.d.	177
IRS-1	n.d.	Promote signaling	208
Grb2		Promote signaling	209

has been shown for EGFR,[50] differential expression of caveolae versus Cav1 scaffolds, not to mention Cav1 phosphorylation, may contribute to the differential regulation of TKR activity by Cav1.

Regulation of EGF Receptor Signaling by Cav1

As mentioned above, Cav1 directly binds EGFR to negatively regulate EGF signaling and EGF-induced cell migration.[65,69] However, where inactive and activated EGFR are located in the plasma membrane and how caveolae, noncaveolar rafts and Cav1 scaffolds regulate EGFR signaling, remains discussed. Inactive EGFR is found in raft fractions and in Cav1 immunoprecipitates whereas activated EGFR is lost in enriched Cav1 raft fractions, suggesting that inactivated EGFR is clustered in caveolae and leaves this membrane domain upon activation.[22,76,77] However, electronic microscopy revealed that EGFR is not present in caveolae in the absence of EGF.[71,78] Moreover, activated EGFR has been detected in DRMs and in Cav1 immunoprecitates and PLCγ accumulates in Cav1 enriched raft fractions after EGF treatment suggesting that EGFR signaling occurs in raft microdomains.[65,77] It is then possible that EGFR accumulates in different raft subtypes as a function of its activation state (Fig. 1). Indeed, EGFR is colocalized at the nanoscale level with the ganglioside GM1 independently of cholesterol and addition of EGF provokes the coalescence of different raft domains which might represent the site of EGFR signaling.[79] The role of Cav1 in raft-dependent EGFR regulation remains unclear and might involve regulation by Cav1 scaffolds. Cav1 scaffolds can regulate raft internalization independently of caveolae formation[80] and a similar phenomenon may participate to EGFR regulation. Moreover, inactive EGFR has been found to associate with oligomeric Cav1 domains preventing its activation independently of caveolae.[50] This localization might be under the control of the glycan-binding protein galectin-3 which prevents EGFR accumulation in Cav1 scaffolds. Finally, the ratio between the amount of presented EGF and the level of EGFR might also define plasma membrane compartmentalization of activated receptor.[81] EGFR might be internalized through a clathrin-dependent pathway at low concentrations of EGF and through a Cav1-dependent

pathway at high concentrations.[71] It is possible that a threshold defined by the ratio between EGFR and EGF determines the plasma membrane domain localization of EGFR and how it signals. Altogether, different membrane microdomains might regulate EGFR signaling as a function of cellular context. Characterization of the expression and role of Cav1 scaffold domains might be key to resolving these controversies.

Divergent roles of Cav1 have also been observed. Cav1 loss has been shown not to affect EGFR signaling.[82,83] Cav1 might also promote EGFR signaling. Cav1 overexpression promotes EGFR activation and Cav1 knockdown by siRNA decreases EGF signaling leading to tumor cell migration and proliferation.[84,85] EGFR activation by mechanical stretch or by angiotensin II was shown to be dependent on Cav1 and probably on Cav1 phosphorylation since a nonphosphorylatable Cav1 mutant prevented EGFR activation.[86,87]

To reconcile observations of positive and negative roles of Cav1 on EGFR activation, it can be postulated that Cav1 regulation of EGFR function could be signaling pathway dependent. It is clear that the EGF-induced Grb2-Sos-Ras pathway leading to ERK1/2 activation is negatively regulated by Cav1,[88] but activation of the PI3 Kinase pathway may require expression of Cav1.[85] Independently of its role on EGFR, Cav1 interacts with ERK1/2 through its scaffolding domain which prevents activation whereas Cav1 interaction with Akt maintains its activation and increases cell survival.[89-91] Focal adhesion kinase (FAK) may also be required for EGF signaling, suggesting crosstalk between EGFR and focal adhesions leading to cell migration and proliferation.[85] Indeed, EGFR is known to promote cell migration via different mechanisms including the regulation of FAK phosphorylation and integrin endocytosis probably by EGFR interaction with focal adhesion proteins and/or Src.[92,93] In these pathways, Cav1 might intervene at different levels: Cav1 could be required for integrin/FAK interaction with EGFR, for integrin endocytosis and for pCav1 dependent regulation of FA dynamics.[59,94-96]

EGF induces context-dependent Cav1 phosphorylation with various consequences. Activated EGFR is known to stimulate Src activity which is responsible for Cav1 phosphorylation.[10,97] Whereas Cav1 phosphorylation was initially proposed to be insulin specific and not EGF- or PDGF-dependent in adipocytes, EGF induced Src-dependent phosphorylation of Cav1 was demonstrated in 2000.[98,99] At this point, both results have been observed (Table 2). EGF-induced Cav1 phosphorylation might be cell specific and probably associated with specific expression levels of different partners such as the ganglioside GM3.[100] Various consequences of EGF-induced Cav1 phosphorylation have been described. EGF can induce tyrosine 14 specific caveolae formation at the site of cell-cell contacts which might be associated with caveolae fusion, endocytosis and targeting to endosomes.[101-103] This

Table 2. Possible Cav1 phosphorylation state

Factor	Phosphorylation or Not	References
Insulin	Phosphorylation	98,99
EGF	Phosphorylation	85,98,103
	No phosphorylation	72,99,210,211
PDGF	No phosphorylation	99
EphrinB2	Phosphorylation	67
Shear stress	Phosphorylation	16
	Dephosphorylation	212

provokes E-cadherin endocytosis and loss of cell-cell contacts after short EGF treatment, as well as Cav1 and E-cadherin down-regulation after chronic EGF treatment.[104,105] This might also promote cell migration through ERK1/2, FAK and PI3 Kinase.[85]

Initially, Cav1 was described as a negative regulator of EGFR signaling. However questions remain open concerning the generality and the consequences of this mechanism. Among the numerous shadows over the Cav1/EGFR couple, the veracity of these complex mechanisms remains to be demonstrated during development, homeostasis and cancer development.

CAV1 REGULATION OF CELL PROLIFERATION, SURVIVAL AND DIFFERENTIATION

Cav1 regulates cell fate during developmental and pathological process at various levels: Cav1 is involved in cell proliferation, survival, senescence, differentiation, adhesion, migration and invasion. Different pathways are regulated by Cav1 in these processes, mainly MAP kinase, PI3 Kinase and the noncanonical Wnt pathways, leading to regulation of transcription. Once again, the literature reveals that Cav1 can act both positively and negatively on these pathways.

Cav1 is an Antiproliferative Factor that Down-Regulates Cyclin D1 Expression

Reintroducing Cav1 in human breast cancer cells decreased growth rate demonstrating for the first time an antiproliferative action of Cav1.[106] The fact that Cav1 deleted mice display mammary epithelial and vascular hyperplasia and that Cav1$^{-/-}$ mouse embryonic fibroblasts (MEF) are hyperproliferative strongly strengthens an antiproliferative role of Cav1.[61,107] Mechanistically Cav1 induces scaffold domain dependent-cell cycle arrest in G0/G1 by up-regulating p53, increasing p21 and decreasing cyclin D1 expression.[8] Cyclin D1 is induced by several pro-proliferative signaling pathways such as ERK1/2, PI3K or β-catenin-TCF/LEF.[108-110] Cav1 can prevent proliferation by down-regulating one or more of these pathways.

Cav1 Prevents MAP Kinase and PI3 Kinase Signaling Pathways

The INK4α locus is considered to be a major tumor suppressor locus: its inactivation is observed in many tumors and provokes cell cycle deregulation due to overactivity of p16^{INK4a} and p19^{AR}, the two distinct products of the locus. The proliferative effect of INK4α loss is potentiated by Cav1 depletion: in INK4α depleted mice, Cav1 loss is associated with increased ERK1/2 activation, increased cyclin D1 expression and decreased p21.[111] Moreover Cav1$^{-/-}$ mice revealed cardiac hypertrophy associated with ERK1/2 overactivity.[112,113] Cav1 might negatively regulate ERK1/2 through direct interaction of its scaffolding domain.[89,90] The PI3K/Akt pathway can also be involved in Cav1 negative regulation of cell growth, in parallel, upstream or independently of the ERK1/2 pathway.[85,114,115] For instance in murine ES cells, the lectin galectin-1 was shown to increase cell growth according to the pathway Src>Cav1>PI3K>mTor.[116] Moreover, depletion of Cav1 is associated with high levels of Rac and Akt activity and increased Cyclin D1 levels, leading to ERK1/2 independent cell proliferation.[117] Altogether, Cav1 negatively regulates

ERK1/2 and/or Akt signaling leading to decreased cyclin D1 expression thereby preventing cell proliferation.

Cav1 Down-Regulates the β-Catenin-TCF/LEF Signaling Pathway

Cav1 also negatively regulates the noncanonical Wnt pathway. β-catenin is part of the cell-cell junction complex and a key component of the Wnt pathway (reviewed in ref. 118). In the cytoplasm, phosphorylated β-catenin is associated with GSK3β in a complex containing APC, axin and CKIα which is continuously degraded. When Wnt is presented to cells, cytoplasmic nonphosphorylated β-catenin associates with the transcription factor TCF/LEF which is then activated and translocated to the nucleus. Cav1 interacts with β-catenin at cell-cell contacts and is able to recruit β-catenin to caveolae fractions preventing its degradation but also its transcriptional activity.[119] In addition, the 1-60 amino acid region of Cav1 regulates TCF/LEF transcription activity leading to decreased cyclin D1 expression and cell proliferation.[120] Cav1 sequestration of β-catenin down-regulates the expression of survivin decreasing cell proliferation and survival: this process might involve accumulation of an E-cadherin/β-catenin/caveolin complex at cell-cell junctions.[121,122]

Down-regulation of these two pathways by Cav1 suggests that Cav1 can suppress tumor growth through regulation of cyclin D1 expression. However, a few examples have shown that Cav1 can also increase cell proliferation such as in tumor cells where Cav1 overexpression is associated with ERK1/2 and Akt overactivity.[114,123] How Cav1 regulates these signaling pathways remains unclear: whether Cav1 acts as an individual protein, an oligomerized scaffold or through caveolae may contribute to defining the role of Cav1.

Cell Survival and Drug Resistance to Cell Death

Cav1 plays a complex role in cell apoptosis and anoïkis (apoptosis triggered by cell detachment from the matrix): Cav1 has been observed as a pro-apoptotic or pro-survival factor in various models. Again, the basis for the contradictory roles of Cav1 stays unexplored.

Cav1 as Pro-Apoptotic Factor

Whereas strong evidence shows that Cav1 prevents proliferation, the literature is not as abundant concerning the negative role of Cav1 on survival. As mentioned above, Cav1 prevents survivin expression by decreasing β-catenin/TCF-LEF transcription activity in various epithelial, fibroblastic and tumor cells.[121,122,124] Moreover, Cav1 sensitizes epithelial and fibroblastic cells to apoptosis through PI3 kinase.[125] However, the role of Cav1 as a pro-apoptotic factor has been poorly illustrated compared to its role in cell survival.

Cav1 as a Pro-Survival Factor

Cav1 has been mainly described as a pro-survival and antidrug agent in cancer therapy. Cav1 regulation of cell signaling is very complex and might be tissue and pathology specific. For instance, Cav1 expression increases hepatoma cell survival by promoting survivin-mediated survival pathway.[126] In heart, Cav1 promotes cell survival by promoting the MAP Kinase p38 activity in association with Akt phosphorylation and expression of Bcl-2.[127] Cav1 is also required for Src-mediated survival of intestinal epithelial cell anoïkis in a mechanism which may involve PI3K and ERK1/2 pathway regulation.[216]

These data suggest that the Cav1 regulated pathway may be tissue specific. In tumors, Cav1 expression correlates with increased cell survival and drug-resistance to cell death according to different mechanisms. In breast cancer cells, Cav1 increases cell proliferation and prevents anoïkis in association with upregulation of IGF-I receptor expression and signaling.[114,123] The Cav1 scaffolding domain might interact with and inhibit the PP1 and PP2A phosphatases promoting Akt and ERK1/2 activity.[128] Other pathways have been proposed to be regulated by Cav1. For instance, Cav1 overexpression provokes TRAIL receptor, DR4, internalization, preventing cell death of human hepatocarcinoma cells.[129]

It has been shown that Cav1 might promote cell survival in a pCav1 dependent mechanism[130] and Cav1 expression, phosphorylation and localization might be critical to define its pro- and antisurvival roles. Altogether, Cav1 is often considered as a pro-survival factor with a predominant role in ERK1/2 and Akt pathway regulation. However, the different mechanisms that have been highlighted suggest that a general scheme might be inappropriate whereas the cell context might define the Cav1 signaling pathway.

Regulation of Cell Phenotype and Differentiation

Cav1 has been proposed to participate in various differentiation processes such as adipocyte or oligodendrocyte differentiation,[131-134] or tissue formation such as angiogenesis and vessel or bone formation.[135-137] Cav1 was implicated in neuronal differentiation despite some controversies. Cav1 has been detected in various neuronal tissues and neurons such as dorsal root ganglion neurons or hippocampal neurons.[138-140] Cav1 might be a negative regulator of neuritic processing and neuronal differentiation. Primary neurite outgrowth and branching are decreased in Cav1 overexpressing cells probably due to inhibition of NGF receptors p75 and TrkA.[48,141,142] Indeed, TrkA interaction with the Cav1 scaffolding domain, as well as raft disruption, prevent receptor activation.[48,143] Cav1 overexpression might also prevent FGF-induced ERK1/2 activation and p21-activated kinase 1 (PAK1)-dependent neurite outgrowth.[144] However, these studies were mainly performed in PC12 cells in which Cav1 expression is controversial. Cav1 is not detected in native PC12 cells in some studies[48,143] whereas other studies described Cav1 expression in PC12 cells and TrkA localization and signaling in caveolae.[138,145] Cav1 expression has also been reported to change in immortalized hypothalamic neurons in function of the differentiation stage.[146] In this case, it would be crucial to understand how Cav1 expression is regulated and if it is involved in nervous system formation.

Cav1 has also been involved in epithelial-mesenchymal transition (EMT). The role of Cav1 is again controversial since both negative and positive regulation have been described. During EGF-induced EMT of breast cancer cells, Cav1 expression is decreased and might be associated with loss of E-cadherin expression through regulation of the β-catenin-TCF/LEF pathway.[104,105] However Cav1 up-regulation has been also observed in a FAK expression-dependent mechanism in different carcinoma models.[147] Moreover, Cav1 overexpression with its partner ID-1 (Inhibitor of Differentiation-1) might be enough to induce EMT in prostate cancer cells.[148] However, the signaling pathways regulated by Cav1 have been poorly explored: it is possible that the role of Cav1 is tissue specific as might be the case for cell proliferation and survival.

In conclusion, Cav1 regulates various signaling pathways that participate in cell fate. It is not possible to define a general model since positive and negative regulation of transduction cascades and associated proliferation, survival or differentiation can

be found in the literature. How Cav1 regulates these pathways is not very well defined. It is difficult to know if Cav1 acts as a plasma membrane domain regulator through caveolae or Cav1 scaffolds, or if its phosphorylation state is involved.

CAV1 REGULATION OF SIGNALING PATHWAYS IN CELL MIGRATION

Cell migration is caused by a complex mechanotransduction process that leads to cell movement and reorganization of the underlying extracellular matrix. Cav1 is strongly involved in processes associated with cell motility.

Regulation of Cell Migration

Individual and collective cell migration in 2 dimensions are complex oriented signaling processes that lead to adhesion of cells to the extracellular matrix or to its neighbors and provokes cytoskeleton reorganization. Individual migration requires high actin dynamics as well as accurate, strong and polarized adhesion organization to allow the cell to anchor and exert traction. Cells strongly adhere to the substrate through the formation of focal adhesions, a platform of signaling proteins such as FAK, Src, paxillin and Crk and scaffolding proteins, such as talin, vinculin and α-actinin, linking matrix bound integrins to the underlying actin cytoskeleton. These structures exhibit strong turnover during cell migration: the mechanisms of formation and dissociation of focal adhesions are regulated by phosphorylation of its components, in particular FAK and Src, and by activation of RhoGTPases (reviewed in ref. 149,150). Cav1 involvement in cell migration has mainly been studied in endothelial and fibroblastic cells, which respectively highlight that Cav1 is required for cell polarity and focal adhesion organization. This involves multiple transduction pathways, such as MAP Kinases, RhoGTPases or G proteins and a key role of Cav1 phosphorylation.

Cav1 Participates to Polarity of Endothelial Migrating Cells

Cav1 expression is required for endothelial cell polarization and subsequent directional migration.[151] During migration, Cav1 staining and caveolae formation increase in the trailing edge of the motile cell where local calcium waves, NO and Gαq protein also accumulate.[152-154] Cav1 might participate to Ca^{2+} release through activation of Gαq protein leading to phospholipase C recruitment to caveolae where it produces diacylglycerol and inositol triphosphate (IP3).[77,155] Altogether, these observations suggest that Cav1 might contribute to compartmentalize cell signaling events leading to oriented cell migration. Indeed, Cav1 interaction with eNOS through its scaffolding domain allows local recruitment of the inactive enzyme, which is released and activated upon local calcium transients and interaction with calmodulin.[156,157] Local NO production then leads to endothelial cell migration, proliferation and increased vascular permeability. In 3 dimensions, Cav1 is localized to the leading edge of endothelial cells transmigrating through a filter pore.[154] This process is dependent on tyrosine 14 whereas accumulation at the rear of 2D migrating cells appears to require amino acids 46-66.[158] This suggests that Cav1 and its phosphorylation, might regulate distinct signaling pathways in 2D and 3D.

Phosphorylated Cav1 Regulates Focal Adhesion Signaling and Structure

Cav1 interacts with β1-integrin to allow cells to adhere and migrate.[159,160] The urokinase receptor uPAR is found in the Cav1-integrin complex and might be involved in this interaction.[160-162] This receptor binds the urokinase uPA to activate its serine proteinase function leading to cleavage of plasminogen and production of plasmin, both involved in cell migration and invasion.[163] uPAR is a cofactor in cell adhesion and migration that forms a complex with Cav1 and engages β1-integrin triggering outside-in signaling resulting in cytoskeleton reorganization and cell migration.[160] Cav1 binding to integrins recruits and activates Src to promote FAK phosphorylation and ras/ERK signaling. These results suggest a direct regulation of FA organization by Cav1 leading to cell migration.

pCav1 is a crucial regulator of FA dynamics and cell migration. Overexpression of a nonphosphorylatable Cav1 mutant (Cav1Y14F) results in loss of cell motility.[62,96,104] pCav1 has been observed in focal adhesions but, as mentioned above, this particular localization has been questioned and is apparently a consequence of crossreactivity of the anti-pCav1 antibody with phosphopaxillin.[164] Cav1 and a Cav1 phosphomimetic mutant (Cav1Y14D) are not observed in focal adhesions, but Cav1 is found in tumor cell protrusions in a Y14 dependent process suggesting that Cav1 might locally regulate focal adhesion signaling and dynamics and thereby cell migration.[59,96] Indeed, pCav1 promotes FAK stabilization in focal adhesions destabilizing these structures to promote their turnover and subsequent cell migration. Moreover, Cav1 phosphorylation is required for high level organization of plasma membrane in focal adhesions, whereas this organization is lost in cells in suspension which present a high level of endocytosed caveolae and rafts.[94,165] pCav1-mediated plasma membrane organization might differentially localize signaling partners or differentially regulate their activity in the plasma membrane, allowing outside-in signal leading to cell migration.[166] Altogether, these observations suggest that pCav1 might indirectly regulate focal adhesion turnover through organization of signaling proteins in particular plasma membrane domains. In this case, the role of Cav1 oligomerization and Y14 phosphorylation as well as compartmentalization of focal adhesion partners remains to be explored.

Thus, Cav1 is a crucial regulator of migration by promoting cell polarization and regulating focal adhesion dynamics. Cav1 and pCav1 might act by interacting with focal adhesion and calcium signaling partners and organizing them into plasma membrane domains. However, the relationship between Cav1 plasma membrane domains and pCav1 function, localization and interactions remains poorly defined.

Central Role of Cav1 in Outside-In and Inside-Out Integrin Signaling: The Cav1-Src-Rho Loop

Adhesion to ECM and migration is dependent on Src and RhoGTPases (RhoA, Rac and Cdc42) activity (reviewed in ref. 167). RhoGTPases are activated when bound to GTP thanks to Guanine nucleotide Exchange Factors (GEFs). RhoGTPases hydrolyze their GTP thanks to the GTPase Activating Proteins (GAPs).[168] Adhesion to the fibronectin matrix triggers RhoA-dependent Src and FAK recruitment and phosphorylation in focal adhesions.[169] In return, engagement of integrins triggers Src-dependent p190GAP phosphorylation which when activated inhibits RhoA enabling Rac1 activation that promotes lamellipodia formation, cell spreading and nascent focal adhesions.[170-174] Activated Rac1 has been proposed to interact with Cav1 at focal adhesion sites leading

to down-regulation of Rac1 by degradation.[175] The spatiotemporal relationship of Cav1 phosphorylation and recruitment to focal adhesions during cell spreading and migration remains unclear. However, as mentioned above, Cav1 is observed in lamellipodial protrusions and pCav1 appears to be critical for focal adhesion turnover, cell polarity and migration.[59,94,96,151,176] Cav1 might be phosphorylated by integrin-activated Src leading to downregulation of the kinase due to pCav1 activation of Csk.[16,177] Through inactivation of Src-p190RhoGAP activity, pCav1 might then promote RhoA activity leading to focal adhesion maturation and dissassembly.[176] Indeed, activated RhoA leads to Src activation forming an activation/deactivation loop resulting in focal turnover, cell polarity and directional migration.[59,96] Altogether, Cav1 might define a regulatory membrane domain at the periphery of migrating cells that dynamically regulates focal adhesion organization and turnover. Indeed, Cav1 has been shown to regulate lipid ordered domain organization at the level of focal adhesions.[165] How Cav1/pCav1 regulates raft organization and signaling cascades at focal adhesion sites remains an intriguing and important question.

Cav1 in Mechanotransduction

Cav1 participates to the transmission of mechanical stimuli, such as mechanical stress, involved in cell reorganization and migration, through signaling regulation which modulates cell adhesion and cytoskeleton organization.[178,179] Endothelial cells undergoing shear stress display increased Cav1 expression associated with ERK1/2 activation and caveolae formation.[181,217] Caveolae formation might occur at the luminal surface of endothelial cells resulting in local signaling via eNOS and G proteins.[178-181] Moreover, endothelial shear stress might trigger transient Cav1 phosphorylation and association with β1-integrin leading to Csk recruitment to focal adhesions promoting myosin light chain (MLC) phosphorylation.[16,182] This suggests that pCav1 interacts with and activates Csk, which then activates RhoA, Rho-kinase and MLC phosphorylation leading to cytoskeleton reorganization and regulation of cell tension.[59,177,183] Moreover, raft disruption prevents this pathway suggesting that pCav1 function is also dependent on raft membrane domain organization.[182]

Altogether, Cav1 and its phosphorylated form are critical regulators of cell-matrix adhesion and migration through the recruitment of signaling proteins and the formation of signaling loops that allow focal adhesion remodeling and cell-cell tension. It is still not clear if pCav1 activity requires formation of a plasma membrane domain similar to the Cav1 scaffold.

CONCLUSION: Cav1 OR THE YIN AND THE YANG OF SIGNALING REGULATION

Cav1 signaling regulation is an ambiguous process since both negative and positive regulation of signaling events can be found in the literature. Initially, Cav1 was thought to be a tumor suppressor protein. In fact, it is difficult to predict at this point if over expression or lack of expression of Cav1 is a good or bad prognostic in cancer progression since Cav1 can be an antiproliferative factor, but also an anti-apoptotic factor and a pro-migratory protein. Cav1 might act differentially according to the partners expressed, the triggering signal and the cellular context, such as the stage of differentiation during development or the stage of disease progression in cancer and other pathologies. Finally, the role of

plasma membrane domains in Cav1 regulation needs to be clarified. The respective roles of caveolae and Cav1 scaffolds are difficult to assess and frequently assimilated. However, they likely function differently and interact with different partners. In addition, where pCav1 is localized and whether it defines another plasma membrane domain remains a challenging question.

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CHAPTER 4

REGULATION OF eNOS IN CAVEOLAE

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Abstract: Caveolae are a specialized subset of lipid domains that are prevalent on the plasma membrane of endothelial cells. They compartmentalize signal transduction molecules which regulate multiple endothelial functions including the production of nitric oxide (NO) by the caveolae resident enzyme endothelial NO synthase (eNOS). eNOS is one of the three isoforms of the NOS enzyme which generates NO upon the conversion of L-arginine to L-citrulline and it is regulated by multiple mechanisms. Caveolin negatively impact eNOS activity through direct interaction with the enzyme. Circulating factors known to modify cardiovascular disease risk also influence the activity of the enzyme. In particular, high density lipoprotein cholesterol (HDL) maintains the lipid environment in caveolae, thereby promoting the retention and function of eNOS in the domain and it also causes direct activation of eNOS via scavenger receptor class B, Type I (SR-BI)-induced kinase signaling. Estrogen binding to estrogen receptors (ER) in caveolae also activates eNOS and this occurs through G protein coupling and kinase activation. Discrete domains within SR-BI and ER mediating signal initiation in caveolae have been identified. Counteracting the promodulatory actions of HDL and estrogen, C-reactive protein (CRP) antagonizes eNOS through FcγRIIB, which is the sole inhibitory receptor for IgG. Through their actions on eNOS, estrogen and CRP also regulate endothelial cell growth and migration. Thus, signaling events in caveolae invoked by known circulating cardiovascular disease risk factors have major impact on eNOS and endothelial cell phenotypes of importance to cardiovascular health and disease.

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INTRODUCTION

The endothelium is critically involved in neovascularization and in the regulation of the structure and function of established blood vessels. Endothelial cells generate signaling molecules such as nitric oxide (NO), prostacyclin and endothelin, which serve diverse autocrine and paracrine functions, and they form a monolayer which modulates local hemostasis and thrombolysis and provides a nonpermeable barrier protecting the underlying vascular smooth muscle from circulating growth-promoting factors.[1-4] As the guardian of the vascular wall, the endothelium is the “first-responder” to multiple physical, biochemical and cellular events occurring in the vessel lumen.

Over the past two decades, our knowledge of endothelial cell responses to external stimuli has been enhanced by the identification and study of signaling molecules and their interactions in caveolae, which are a specialized subset of lipid rafts that are prevalent on the endothelial cell plasma membrane. Caveolae are enriched in cholesterol, glycosphingolipids, sphingomyelin and lipid-anchored membrane proteins. This chapter will highlight recent advances in our understanding of endothelial cell signaling events in caveolae, with a focus on mechanisms regulating the activity of the resident enzyme endothelial NO synthase (eNOS). Emphasis is placed on processes which provide mechanistic coupling between circulating factors known to modify cardiovascular disease risk and their abilities to directly govern endothelial function. After reviewing the mechanisms underlying eNOS localization in caveolae, the regulation of the enzyme through interaction with caveolin, the major structural protein in caveolae, will be discussed. Next, the promodulatory actions of high density lipoprotein cholesterol (HDL) and estrogen on eNOS will be outlined. Representing the more recent consideration of disease-related eNOS antagonism, the inhibitory actions of C-reactive protein (CRP) will also be reviewed. Finally, key questions guiding the future direction of research in this field will be highlighted.

eNOS LOCALIZATION IN ENDOTHELIAL CELL CAVEOLAE

eNOS is one of the three isoforms of NOS which generates NO upon the conversion of L-arginine to L-citrulline.[1,2,5-9] eNOS is activated by increases in intracellular calcium and by changes in phosphorylation mediated by upstream kinases. In particular, with many agonists, tyrosine kinases activate PI3 kinase/Akt kinase to stimulate eNOS enzymatic activity by causing the phosphorylation of Ser-1177.[10,11] In certain contexts, there are also alterations in Thr-497 phosphorylation, which attenuates enzyme activity.[12] The NO produced prevents thrombosis, adhesion molecule expression and apoptosis and it promotes endothelial cell growth and migration and vasodilation while blunting vascular smooth muscle cell growth and migration. Diminished NO production or bioavailabilty has been implicated in the pathogenesis of systemic and pulmonary hypertension and in multiple other vascular disorders including atherosclerosis.[13]

Since eNOS activity is regulated by diverse extracellular stimuli and the NO produced is a labile, cytotoxic molecule with paracrine functions,[5,6] the intracellular site of NO synthesis has a major influence on the biological impact of the enzyme. eNOS is primarily associated with caveolae[14,15] and optimal targeting of the enzyme to caveolae requires its N-terminal myristoylation and palmitoylation. The glycine in position 2 is myristoylated and the cysteines at positions 15 and 26 are palmitoylated[15] (Fig. 1A). In addition, the status

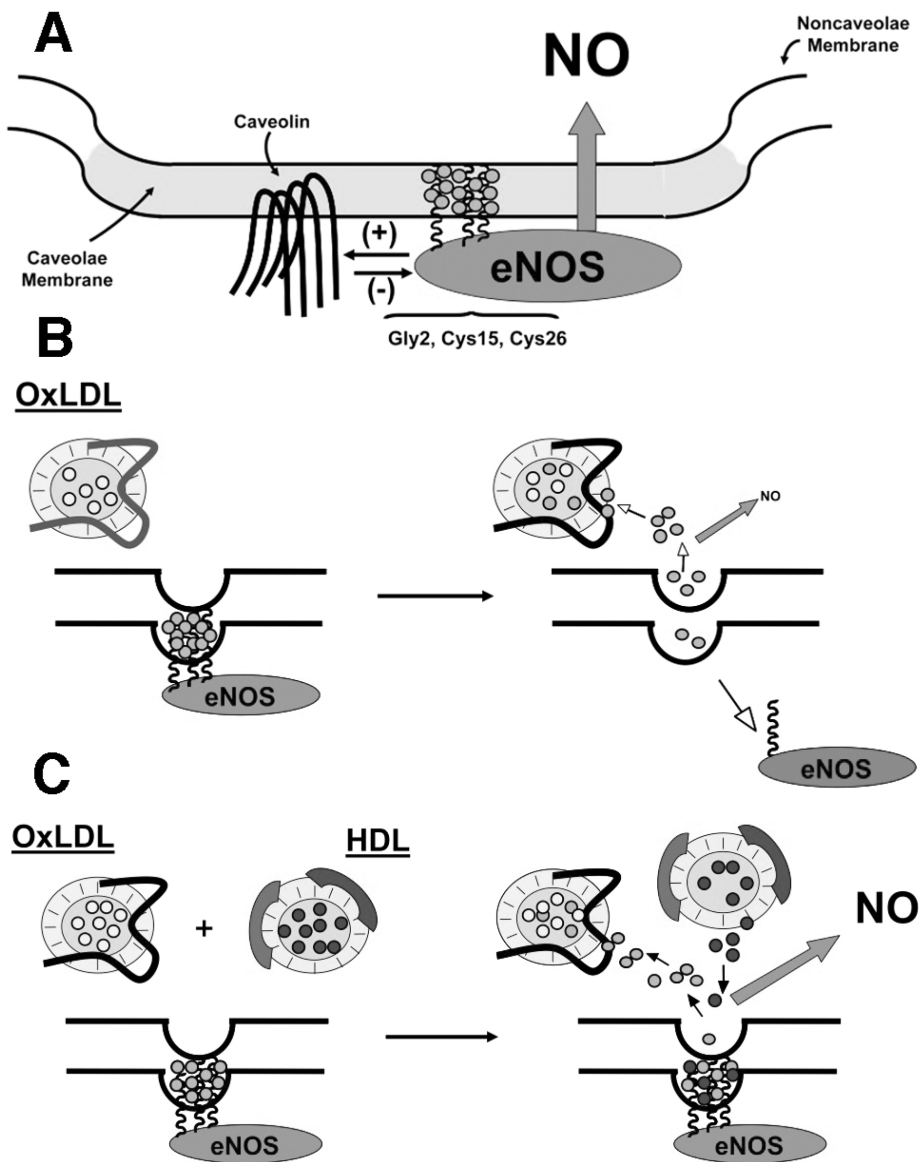

Figure 1. Endothelial nitric oxide synthase (eNOS) localization and interaction with caveolins in cholesterol-rich caveolae. A) eNOS is localized in plasma membrane caveolae enriched in cholesterol- (orange circles) through the myristoylation and palmitoylation of the protein (shown as black wavy lines). Gly2 of eNOS is myristolyated and Cys15 and Cys26 are palmitoylated. Caveolins bind to eNOS through their scaffolding domain to negatively regulate eNOS enzyme activity, which results in vascular dysfunction in a variety of pathologic conditions. B) By disrupting the cholesterol-rich lipid environment in caveolae, oxidized LDL (OxLDL) causes the displacement of eNOS from caveolae, thereby decreasing the capacity for enzyme activation (depicted by change from green to red). C) Counteracting caveolae cholesterol depletion by oxLDL, high density lipoprotein cholesterol (HDL) provides cholesterol esters (blue circles) to maintain caveolae total cholesterol content and thereby retain eNOS in the domain to optimize NO production. A color version of this image is available online at www.landesbioscience.com/curie.

of cholesterol, which is a key component of the unique lipid environment in caveolae is critical to normal eNOS function.[16] Oxidized LDL (oxLDL) causes depletion of caveolae cholesterol in cultured endothelium via the scavenger receptor CD36, leading to eNOS redistribution away from the plasma membrane and a diminished capacity to activate the enzyme[16,17] (Fig. 1B). Paralleling the findings in cell culture, the administration of the eNOS agonist acetylcholine (Ach) to wild-type mice with eNOS localized normally in caveolae results in a fall in blood pressure, whereas Ach does not alter blood pressure in hypercholesterolemic apoE null mice in which eNOS is not present in caveolae. In contrast, normal eNOS localization in caveolae and normal Ach-induced blood pressure responses occur in apoE/CD36 double knockout mice.[18] Thus, pathologic lipoprotein and cholesterol status disrupts eNOS subcellular localization to caveolae and thereby attenuates the function of the enzyme. This mechanism may be operative in the early stages of hypercholesterolemia-induced vascular disease, when there is impairment of eNOS responses to receptor-dependent stimuli.[1,2,19]

Counteracting caveolae cholesterol depletion by oxLDL via CD36, HDL maintains caveolae cholesterol content, retains eNOS in the domain and thereby preserves the capacity for eNOS activation. This process is not related to the inhibition of cholesterol removal from caveolae by oxLDL; instead, it is due to the provision of cholesterol esters by HDL[17] (Fig. 1C). Moreover, scavenger receptor class B Type I (SR-BI), the high affinity receptor for HDL, is in endothelial caveolae and it mediates the ability of HDL to reverse the impact of oxLDL on eNOS localization and function.[17] Thus, in the presence of oxLDL, the HDL/SR-BI tandem preserves the lipid environment within caveolae, thereby maintaining normal eNOS localization and function and possibly explaining a portion of the antiatherogenic properties of HDL.

CAVEOLIN AND THE REGULATION OF eNOS

Caveolins are the major coat proteins of caveolae and they directly interact with other intracellular proteins through amino acids 82-101 that comprise the putative scaffolding domain[20-22] (Fig. 1A). Studies in cultured endothelial cells demonstrate that eNOS has the capacity to directly interact with caveolin-1 or caveolin-3 and that this interaction results in the inhibition of NO production.[23-25] In vivo experiments further support the role of caveolin-1 as a negative regulator of eNOS, with basal and stimulated eNOS activation and eNOS-dependent relaxation enhanced in blood vessels from caveolin-1 knockout mice.[26,27] Recent work using a membrane-permeable form of the caveolin-1 scaffolding domain in mice demonstrates that the peptide is a potent inhibitor of eNOS in intact blood vessels.[20] In these studies, exposure of the blood vessels to the peptide blocked Ach-induced relaxation, but it had no effect on relaxation responses to the direct NO donor sodium nitroprusside. The importance of eNOS regulation by caveolins has been further demonstrated in a variety of disease models. Genetic ablation of caveolin-1 in mice on apoE null background inhibits the progression of atherosclerosis through promotion of NO production, reduction of LDL infiltration into the artery wall and decreased leukocyte-endothelial cell adhesion. Conversely, the overexpression of caveolin-1 in endothelium enhances atherosclerotic lesion expansion.[28,29] In addition, the blockade of HMG CoA reductase with statin-based drugs reduces caveolin levels and promotes eNOS activation in apoE null mice, yielding improvements in NO-dependent heart rate and blood pressure.[30] Furthermore, in a rat

model of cirrhosis, caveolin-1 is overexpressed and there is enhanced interaction of caveolin-1 with eNOS resulting in the suppression of basal and stimulated production of NO, which may increase portal pressure.[31] In another model of cholestasis in rats, there is an upregulation of sinusoidal caveolin-1 and a decrease in eNOS activity.[32] Elevated expression of caveolin-1 has also been found in patients with hepatocellular carcinoma and hepatitis C-related cirrhosis.[33] As such, the upregulation of caveolin-1 may contribute to endothelial dysfunction in the diseased liver.[33-35] Thus, caveolin is an important negative regulator of eNOS that influences vascular function in a variety of pathologic conditions.

HDL ACTIVATION OF eNOS

In addition to modifying the subcellular localization of eNOS when the lipid environment in caveolae is overtly altered, HDL is a potent agonist of eNOS.[36,37] HDL stimulates the enzyme through SR-BI and the molecular mechanism by which SR-BI mediates intracellular signaling to eNOS has been elucidated (Fig. 2). In

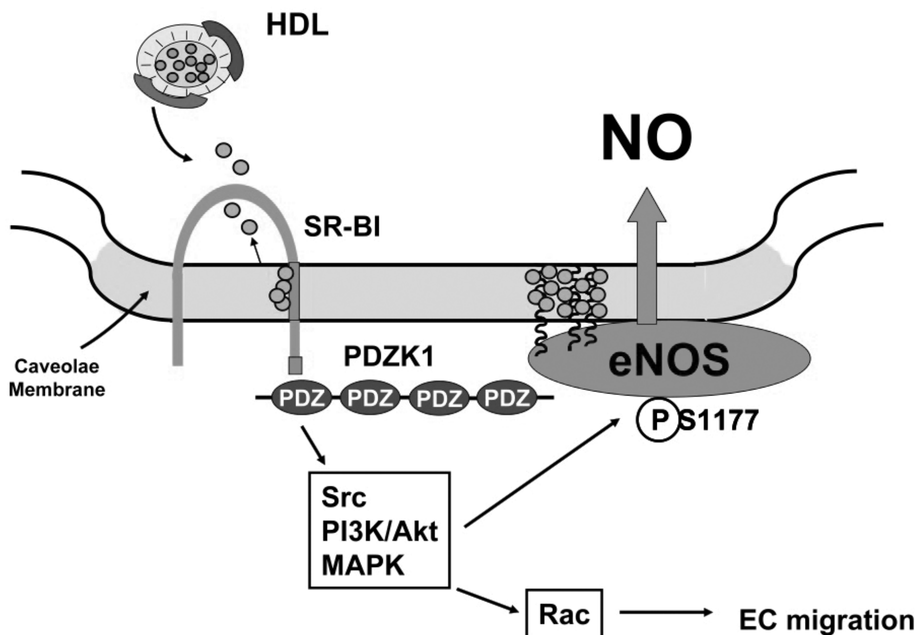

Figure 2. High-density lipoprotein cholesterol (HDL) mediates endothelial cell signaling via SR-BI in caveolae. HDL binding to caveolae-associated SR-BI induces cholesterol efflux (shown as orange circles) and initiates signal transduction by processes that require the adaptor protein PDZK1, which contains four PDZ domains and binds to the C-terminus of SR-BI. The C-terminal transmembrane domain of SR-BI, which binds membrane cholesterol, is also required for HDL/SR-BI-initiated signaling. HDL-SR-BI-PDZK1 activates Src family kinases, MAPK, PI3K and Akt kinase, which phosphorylates eNOS at Ser-1177, to activate the enzyme and stimulate NO production. In an eNOS-independent manner, HDL-induced kinase activation also leads to increased Rac activity and lamellipodia formation and ultimately to enhanced endothelial cell migration. A color version of this image is available online at www.landesbioscience.com/curie.

cultured endothelial cells, short-term exposure to HDL or methyl-β-cyclodextrin causes eNOS stimulation of similar magnitude and the activation by both agents is mediated by SR-BI.[38] Studies using a variety of chimeric mutants of SR-BI showed that the C-terminal transmembrane domain is indispensable for HDL signaling. In addition, studies employing a photoactivated derivative of cholesterol revealed that membrane cholesterol binds directly to the C-terminal transmembrane domain. These findings indicate that signal initiation by HDL requires cholesterol flux and that SR-BI may serve as a cholesterol sensor on the plasma membrane. Studies comparing the functions of SR-BI and the splice variant SR-BII, which differ only in their C-terminal cytoplasmic domains, have further indicated that the C-terminus of SR-BI is required for signal initiation. The C-terminus interacts with the PDZ domain-containing protein PDZK1.[39,40] PDZK1 is expressed in endothelium and it is required for HDL-induced activation of eNOS and endothelial cell migration both in culture and in mouse models.[41] HDL binding to SR-BI-PDZK1 causes the activation of Src, which leads to parallel activation of PI3 kinase/Akt and MAP kinases and their resultant independent modulation of eNOS via its phosphorylation at the critical Ser-1177 and unknown mechanisms, respectively.[42] Importantly, functional coupling of SR-BI to eNOS is demonstrable in isolated endothelial cell caveolae, revealing that all of the molecular machinery required for HDL-induced eNOS simulation is associated with caveolae.[36] Through eNOS-independent mechanisms SR-BI/PDZK1 activation of kinases also stimulates endothelial cell migration by activating the small GTPase Rac.[38]

ESTROGEN MODULATION OF eNOS

There is considerable evidence that the hormone estrogen has potent direct actions on vascular cells including endothelium.[43,44] These processes may underlie the lower susceptibility to vascular disease in premenopausal females compared with males and the cardiovascular benefits of estrogen replacement therapy when provided under certain circumstances.[45] Many of the vascular actions of estrogen are mediated by increases in bioavailable NO. Whereas a portion of this effect relates to the upregulation of eNOS expression, an important contribution is derived from the nonnuclear activation of eNOS by estrogen.[46,47]The basis for the nonnuclear effects of estradiol (E_2) on eNOS function and the role of estrogen receptors (ER), which classically serve as transcription factors, has been elucidated (Fig. 3). Studies in cultured cells indicate that a subpopulation of ERα is localized to endothelial cell caveolae where they are coupled to eNOS in a functional signaling module.[48,49] A variety of mechanisms have been proposed to underlie ERα localization to caveolae. Palmitoylation of ERα occurs at Cys-447 of the receptor and the palmitoylation enhances membrane localization and the capacity for signal initiation by ERα.[50,51] In addition, within the ERα ligand binding domain there is a caveolin-1 interaction site which promotes targeting to caveolae.[52] E_2 binding to the membrane ERα induces intracellular signal transduction through G protein coupling of the receptor, which entails direct protein-protein interaction between Gαi and amino acids 251-260 of the receptor.[53,54] The downstream signaling occurs via liberated Gβγ and it involves activation of Src, MAPK, PI3 kinase and Akt, which leads to phosphorylation of eNOS at the critical Ser-1177.[55-58] The importance of nonnuclear, caveolae-initiated E_2-ERα signaling was clearly demonstrated in recent work in which an E_2-dendrimer conjugate

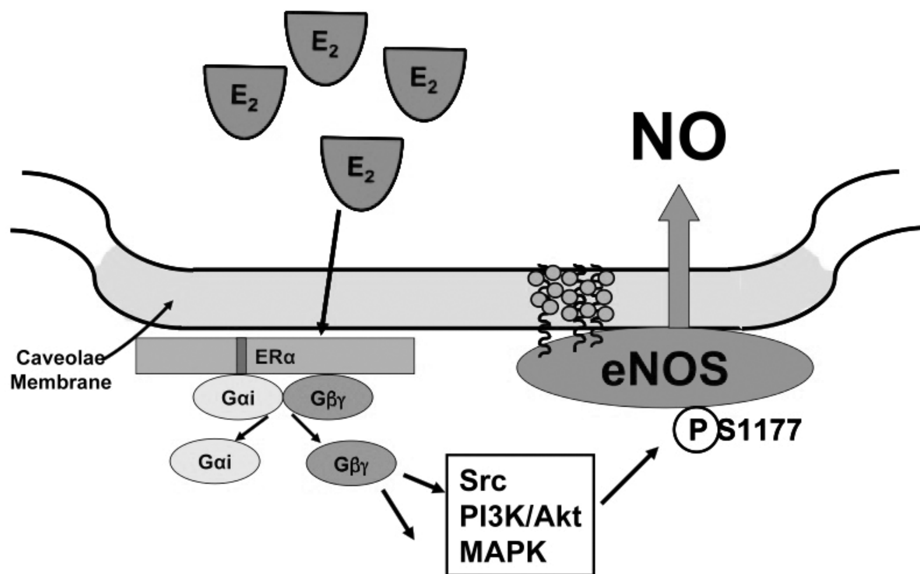

Figure 3. Estrogen stimulates eNOS through an ERα-G protein complex in caveolae. Caveolae-associated ERα directly bind, to Gαi via amino acids 251-260 of the receptor (shown as red rectangle). Estradiol (E_2) binding to ERα triggers the dissociation of the Gαi-βγ complex and liberated Gβγ activates Src family kinases, MAPK and PI3 kinase/Akt kinase, resulting in Ser-1177 phosphorylation and the activation of eNOS. A color version of this image is available online at www.landesbioscience.com/curie.

(EDC) that is excluded from the nucleus stimulated eNOS, endothelial cell proliferation and endothelial cell migration via ERα to a degree comparable to that observed with E_2. Studies with EDC further showed that eNOS activation underlies the endothelial cell growth and migratory responses to estrogen.[46] In mice, E_2 and EDC equally promoted carotid artery reendothelialization in an ERα- and G protein-dependent manner and both agents attenuated the development of neointimal hyperplasia following endothelial denudation in hypercholesterolemic mice.[46] Thus, the effects of estrogen on eNOS which are central to cardiovascular physiology are mediated by nonnuclear ER functioning in caveolae.

CRP MODULATION OF eNOS

C-reactive protein (CRP) is an acute phase reactant that is positively correlated with cardiovascular disease risk and endothelial dysfunction.[59-66] The brief treatment of cultured endothelial cells with recombinant human CRP fully attenuates eNOS activation by HDL, E_2, vascular endothelial growth factor and insulin.[67] More recent studies have demonstrated the inhibitory effects of CRP on eNOS in mouse models.[68,69] In transgenic mice expressing elevated serum levels of CRP, carotid artery reendothelialization after perivascular electric injury is blunted, mimicking the effect of NOS antagonism on reendothelialization in wild-type mice.[68] Furthermore, CRP transgenic mice are hypertensive due to supersensitivity to angiotensin II caused by

a reduction in vascular angiotensin receptor subtype 2 expression that results from a decrease in bioavailable NO.[69] The mechanisms underlying CRP antagonism of eNOS have been elucidated (Fig. 4). Fc receptors for IgG, which bind immune complexes and mediate their effects in immune response cells, display high affinity for CRP.[70-75] The inhibitory Fc receptor FcγRIIB is expressed in endothelial cells in culture and in endothelium in vivo.[67] A requirement for FcγRIIB in CRP inhibition of eNOS has been demonstrated both in cultured endothelial cells using blocking antibody and in mouse models of Ach-induced increases in carotid artery vascular conductance. Furthermore, reconstitution experiments in cell lines overexpressing sham, wild-type FcγRIIB or a mutant FcγRIIB revealed that the ITIM (immunoreceptor tyrosine-based inhibitory motif) in the cytoplasmic domain of the receptor is required for CRP actions on eNOS.[76] CRP binding to FcγRIIB induces the activation of the phosphatase SHIP1 (src homology 2 domain-containing inositol 5′-phosphatase 1), which attenuates signaling downstream of PI3 kinase and thereby diminishes eNOS phosphorylation at Ser-1177.[76] In addition to attenuating the activation of eNOS by various agonists, CRP binding to FcγRIIB blunts eNOS gene transcription and both processes likely

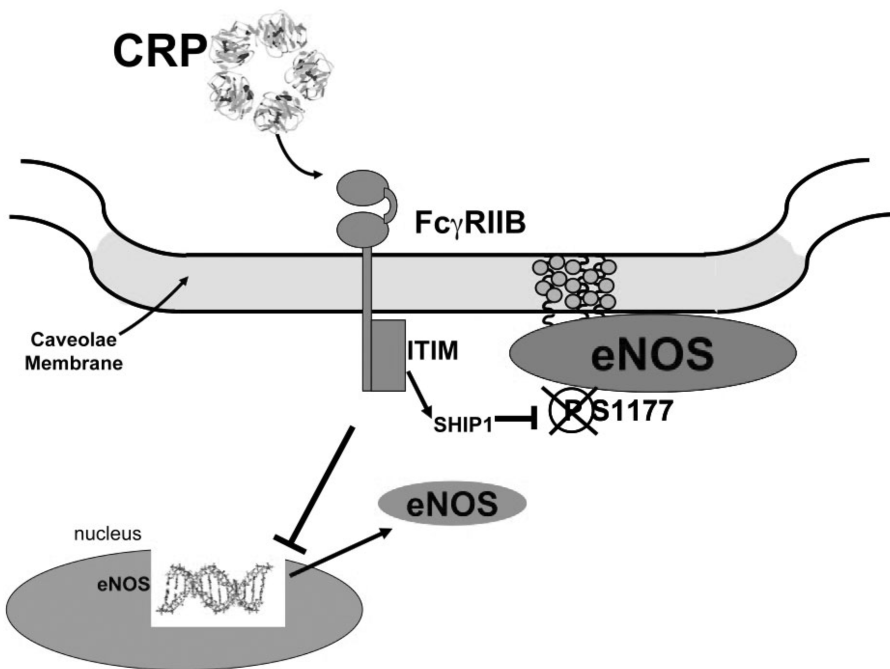

Figure 4. C-reactive protein inhibits eNOS via FcγRIIB in endothelium. CRP binding to the IgG receptor FcγRIIB induces the activation of the phosphatase src homology 2 domain-containing inositol 5′-phosphatase 1 (SHIP1) by processes that require the immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain of the receptor. SHIP1 activation antagonizes eNOS by attenuating the phosphorylation of the enzyme at Ser-1177. CRP also decreases eNOS protein expression by blunting eNOS gene transcription. Although FcγRIIB localization to caveolae has been observed in immune response cells, comparable localization in endothelial cells is yet to be directly demonstrated. A color version of this image is available online at www.landesbioscience.com/curie.

contribute to the attenuation of endothelial cell migration caused by CRP.[69] In B-cells, FcγRIIB is localized to lipid rafts.[77] Similar caveolae-raft localization of FcγRIIB in endothelial cells is yet to be demonstrated.

CONCLUSION

It is now evident that multiple processes converge in caveolae to regulate eNOS. The localization of the enzyme in caveolae is critical to normal NO production induced by various agonists. The interaction of eNOS with caveolins negatively regulates the activity of the enzyme and the modulation of the interaction and subsequent changes in bioavailable NO are important factors in a variety of vascular disease states. HDL and SR-BI play important roles in maintaining normal eNOS localization and activity and estrogen binding to ER in caveolae also stimulates eNOS. In contrast, the cardiovascular disease risk factor CRP antagonizes eNOS through FcγRIIB. A variety of important endothelial cell functions are modified by these processes and consequently these signaling events in caveolae have profound implications on cardiovascular health and disease.

There are multiple questions remaining to be answered in this area of research. The mechanisms by which HDL-induced cholesterol flux initiates signaling are currently unknown. The specific roles of the C-terminal transmembrane and cytoplasmic domains of SR-BI in signaling also warrant further study. Although FcγRIIB localization to caveolae/rafts has been demonstrated in immune response cells, comparable localization and the mechanisms regulating such targeting in endothelial cells are yet to be revealed. In addition, there is limited understanding of the endothelial cell genes that are regulated by the signaling that occurs in response to HDL, estrogen and CRP actions in caveolae. Importantly, other signaling modules in caveolae governing endothelial cell phenotype likely exist and need to be identified. Furthermore, the developmental and disease-related implications of these processes await additional study in intact animal models. Moreover, the contribution of genetic variation in caveolae resident molecules to differences in vascular disease risk in humans is yet to be fully interrogated. With continuing elucidation of the molecular components and signaling processes occurring in endothelial cell caveolae, it is expected that valuable new lessons will be learned from the guardian cell of the vascular wall to ultimately yield new strategies to prevent and treat vascular disease.

ACKNOWLEDGEMENTS

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SECTION II

CAVEOLAE AND CAVEOLINS IN HUMAN DISEASES

CHAPTER 5

RECENT DEVELOPMENTS IN THE INTERACTIONS BETWEEN CAVEOLIN AND PATHOGENS

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Abstract: The role of caveolin and caveolae in the pathogenesis of infection has only recently been appreciated. In this chapter, we have highlighted some important new data on the role of caveolin in infections due to bacteria, viruses and fungi but with particular emphasis on the protozoan parasites *Leishmania* spp., *Trypanosoma cruzi* and *Toxoplasma gondii*. This is a continuing area of research and the final chapter has not been written on this topic.

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INTRODUCTION

The first steps in the initiation of an infection are the attachment and entry of a pathogen into a host cell. It has long been assumed that an understanding of these initial events may result in new methods for control and treatment of infections. A role for caveolae and caveolin proteins in these processes has only recently been investigated. In this chapter, it is not our intent to review all of microbiology and describe how each and every micro-organism interacts with caveolae and caveolin proteins, but rather to focus our attention on some recent important new developments. Pathogens enter mammalian cells to escape the immune system of the host and/or as part of their requirement to maintain a replicative cycle. In the past several years, this topic has been reviewed by others.[1-5] These articles highlighted the role of caveolae and caveolin proteins, such as caveolin-1 (Cav-1) and lipid rafts in the entry of diverse pathogens into the host cell. As discussed in detail in other chapters in this book, Cav-1 is a critical structural protein in the formation of the flask shaped caveolae lining the plasma membrane. In addition, the role of caveolins in the pathogenesis of infection may be related to an effect on components of the immune system such as lymphocytes and macrophages.[6,7]

VIRUSES

Caveolae are enriched in cholesterol and glycolipids, such as the glycosphingolipid GM1, glycosylphosphatidylinositol-anchored proteins and caveolin. Caveolae–mediated viral entry into human cell lines has been described and viruses that enter cells via caveolae apparently act as signaling ligands, triggering signal transduction events and actin rearrangement in the host cell, resulting in pathogen uptake (Table 1).[8,9]

The mechanisms by which a virus gains entry via caveolae are still not completely understood. The SV40 virus may bind to the MHC Class I complex, which recruits Cav-1 to the site of viral attachment from preformed caveolae and the associated lipid rafts are then formed around the virus. Alternatively, the SV40 virus bound to MHC may be associated with preformed caveolae. Regardless of the precise mechanism, SV40 containing caveolae pinch off from the plasma membrane and are transported to the endoplasmic reticulum via a caveosome.[8] Polyoma virus, Echovirus, Respiratory Syncitial Virus (RSV) and the filoviruses (Ebola and Marburg viruses) are additional examples of viruses that are associated with Cav-1. The effects of lipid raft disrupting agents on Ebola infection indicate that membrane lipid rafts are important in the entry of filoviruses.[1]

The HIV-1 receptors are associated with lipid rafts in T-cells and the disruption of the integrity of these lipid rafts likely inhibits HIV infection. The blood brain barrier (BBB) integrity is maintained by tight junctions,[10,11] and an intact BBB is crucial for preventing the trafficking of HIV into the brain. Early in the course of HIV infection, virus crosses the BBB via HIV-1 infected monocytes in macrophages and microglial cells in the brain. During the subsequent inflammatory response, leukocytes enter into the central nervous system through breaches in the BBB. Weiss et al [12] demonstrated that HIV-1-Tat protein is a powerful pro-inflammatory agent that causes transendothelial cell migration of monocytes. Recently, Zhong et al[13] demonstrated that Tat-mediated activation of Ras signaling is regulated by Cav-1 in brain endothelial cells and that inhibition of Cav-1 and Ras signaling attenuates Tat-induced disruption of the tight junction proteins.

Table 1. Summary of papillomavirus entry pathways

HPV Type	Pathway Identified	Methods Used	Ref.
BPV1	clathrin mediated entry, shuttling from endosomes to caveosomes	biochemical inhibitors, colocalization studies, caveolinl shRNA, dominant negative Cav-l, 293 cells, pseudovirions	27
BPV1/HPV16	clathrin mediated entry	biochemical inhibitors, colocalization studies, C127 cells, virions and VLPs	19
HPV16	clathrin/caveolae independent, lipid raft independent, dynamin independent, tetraspanins involved	siRNA KO of clathrin, cavl, dynamin and tetraspanins, biochemical inhibitors, caveolin –/– cells, dominant negative inhibitors 293TT and HELA cells, pseudovirions	23
HPV16	clathrin mediated entry, shuttling from endosomes to caveosomes	biochemical inhibitors, colocalization studies, caveolinl shRNA, HaCaT cells, pseudovirions	26
HPV16/HPV31	clathrin mediated entry (HPV16), caveolar uptake (HPV31)	biochemical inhibitors, dominant negative inhibitors, HaCaT cells, pseudovirions	22
HPV16/HPV31	clathrin mediated entry	biochemical inhibitors, 293TT and COS7 cells, pseudovirions	20
HPV31	Caveolar mediated uptake, Rab5 mediated shuttling to endosome	biochemical inhibitors, colocalization studies, dominant negative Rab5, HaCaT cells, pseudovirions	28
HPV16/31 and 58	clathrin mediated entry (HPV16 and 58) caveolar uptake (HPV31)	biochemical inhibitors colocalization studies, COS7 cells, VLPs and pseudovirions	18
HPV33	noncaveolar uptake	biochemical inhibitors, COS7 and HELA cells, pseudovirions	21

Papillomaviruses (PVs) infect the mucosal and cutaneous stratified squamous epithelia. These infections are associated with both benign and malignant neoplasias and the human papillomaviruses (HPVs) cause virtually all cases of cervical cancer.[14] The 8 kb, circular viral genome is encapsulated by a complex of L1 (major) and L2 (minor) structural proteins.[15] Upon binding to heparan sulfate proteoglycans, the PV capsid undergoes a series of conformational changes resulting in the N-terminus of L2 becoming sensitive to cleavage by furin.[16] A recent study suggested that these conformational changes may occur on the extracellular matrix prior to transfer to the cell.[17] Earlier work employed chemical inhibitors and/or microscopic localization to suggest that HPV16, HPV33, HPV58 and Bovine PV1 (BPV1) use a clathrin-dependent pathway, whereas similar studies suggest the use of a caveolae-dependent pathway for HPV31.[18-22] However, more recent work suggests that HPV31 may also use a clathrin-dependent pathway.[20] Other authors have suggested that PVs can enter cells independent of either pathway.[23]

The lack of in vitro culture methods for the production of infectious virus has limited the study of PV entry. Most studies have used viral-like particles (VLPs) produced in insect cells or pseudovirions in which the L1/L2 capsid carries a reporter gene. It is possible that the use of these laboratory-made particles and nontarget cell lines could explain the observed differences between entry mechanisms. For example, it has been demonstrated that HPV16 enters dendritic cells and Langerhans cells via distinct pathways.[24]

The observations that a simian virus, JC virus (a polyoma virus), enters cells via clathrin-mediated endocytosis before being shuttled to caveolae-derived vesicles[25] led to the identification of a similar pathway for HPV16 and BPV1.[26,27] Based on recent studies, HPV16 virions first colocalize with markers of early endosomes; then, beginning at around 20 minutes post-entry, increasing amounts of the virons colocalize with Cav-1. Four hours post-entry the HPV16 virions are present in the endoplasmic reticulum. These observations strongly suggest that HPV16 and BPV1 enter the host cell via a clathrin-dependent pathway, after which they get shuttled from endosomes to caveolae. Conversely, HPV31 enters cells through the caveolar pathway followed by Rab5-dependent shuttling towards endosomes.[28] Since Rab5 controls the transport from endosomes to caveosomes and vice-versa, it is tempting to speculate that both HPV16 and HPV31 could shuttle back and forth between the caveolar and endosomal pathways following entry. This hypothesis is supported by the ultrastructural observation that both HPV16 and HPV31 end up in similar looking vesicles.[18] However, studies in which cells have been co-infected with HPV16 and HPV31 have not shown colocalization of both viral types.[23] It is possible that the differences in entry half-time (4 hours for HPV16 vs 14 hours for HPV31) could explain these observations. Thus, it appears that HPV31 has evolved to (predominantly) enter cells through a uniquely different pathway from the other tested PV types. This is surprising since HPV16 and HPV31 are evolutionary more closely related to each other than HPV16 is to HPV58 or BPV1 (HPV16 and HPV31 share approximately 83% amino acid similarity (PAM250 matrix) across the L1 structural protein). One hypothesis is that these two highly related viruses evolved to use different entry mechanisms to avoid competition. However, the much higher prevalence of HPV16 may suggest otherwise. One key observation is that it appears that the HPV16-E5 protein up-regulates Cav-1 at the plasma-membrane of cervical cells. Taken together, these findings suggest that caveolin proteins might play a role in PV entry, but it will be necessary to sort out discrepant results and intertype differences in order to adequately assess their role.

BACTERIA

There is limited information regarding the role of caveolins in bacterial infection. However, early studies suggested that caveolae may be an important alternative pathway for endocytosis of bacteria.[1,2,4,5] Chemical agents have been used to study the function of caveolae including nystatin, filipin and methyl-β-cyclodextrin (MβCD). These agents disrupt the cholesterol enriched lipid rafts. Many pathogens require lipid rafts for entry and colocalize with markers of caveolae to invade host cells. However, only a small number of bacterial pathogens have actually been shown to require caveolin expression for host cell entry.

Escherichia coli is an important cause of human infection including those of the urinary and gastrointestinal tracts. Studies involving *E. coli* invasion into mast and bladder epithelial cells have revealed that caveolae-dependent endocytosis is a mechanism for bacteria to invade both phagocytic and nonphagocytic cells. Importantly, intracellular bacteria can colocalize with Cav-1 and compounds that cause disruption of caveolae by removing membrane cholesterol inhibited bacterial invasion. *E. coli* uptake and invasion is dependent on the organization of lipid rafts and Cav-1 expression.[29] Furthermore, *Campylobacter jejuni,* is an important cause of diarrhea world-wide whose invasion of intestinal epithelial cells is dependent on Cav-1 expression.[30]

In an intraperitoneal model of sepsis using lipopolysaccharide injection, Cav-1 null mice were observed to be resistant to lung injury and their mortality was reduced due to a reduction in inflammation.[31] However, in a Cav-1 null mouse model of *Salmonella typhimirium*, an important cause of human diarrhea and systemic illness, Medina et al[32] found that higher levels of proinflammatory cytokines, chemokines and nitric oxide accompanied increased bacterial burden in the spleen. Surprisingly, no differences in *S. typhimurium* invasion of macrophages between Cav-1 null or wild type macrophages were observed.

Infections with *Pseudomonas aeruginosa* are observed most commonly in hospitalized immunocompromised individuals, resulting in urinary tract infections, pneumonia and sepsis. This is especially true of those individuals on mechanical ventilation and those suffering from severe burns. Also, over 80% of individuals with the genetic disorder Cystic fibrosis (CF) have pulmonary infection with *P. aeruginosa*.[33] Individuals with CF have a defect in the transmembrane conductance regulator (CFTR).[34,35] In normal individuals, infection with this bacteria stimulates the formation of lipid rafts that contain the CFTR thus allowing the organism to invade the respiratory epithelium and initiate inflammatory and apoptotic processes leading to shedding of bacteria-containing epithelial cells.[35-37] However, in CF patients these responses are absent and *P. aeruginosa* becomes established as a chronic infection.[38] Mechanistically, knockdown of either Cav-1, which also eliminates Cav-2, or Cav-2 alone reduces the uptake of *P. aeruginosa* into rat bronchial epithelial cells.[39] Importantly, Bajmoczi et al[40] demonstrated that following entry into host cells, *P. aeruginosa* colocalizes with Cav-1 and CFTR. More recently, increased mortality, bacterial burden and inflammation was demonstrated in Cav-1 KO compared with wild type mice, which correlated with a decreased ability of Cav-1-deficient neutrophils to phagocytose *P. aeruginosa*. Additionally, the colonization of *P. aeruginosa* was more efficient in Cav-1 KO mice. Taken together, these preclinical observations strongly suggest that Cav-1 contributes to innate immunity to *P. aeruginosa* infection, which may have clinical implications for human CF patients.[41]

PROTOZOAN PARASITES

Leishmania

Depending on the species, infection with members of the genus *Leishmania* may result in cutaneous, mucocutaneous, or visceral leishmaniasis. These diseases are found wide-spread in tropical and sub-tropical areas of the world and are major causes of morbidity and mortality. Additionally, leishmaniasis is an opportunistic infection in patients with HIV/AIDS.[42]

Leishmania spp. have a life cycle consisting of two stages, the promastigote and the amastigote. The extracellular promastigote develops in the gut of the sand fly vector until it becomes a fully virulent metacyclic promastigote. Metacyclogenesis is a process during which surface molecules associated with virulence, such as lipophosphoglycan (LPG) and MSP (called GP63), are modulated in their expression and/or posttranslational modifications.[43-45] Attainment of full promastigote virulence coincides with the feeding cycle of the insect vector.[43-45] During a blood meal, the sand fly inoculates the parasite in the skin whereupon it is phagocytosed first by neutrophils and then by macrophages, the ultimate host cell.[46] Inside the macrophage, parasites transform from promastigotes to amastigotes over two to five days. Thereafter, amastigotes are the only form found in the mammalian host. Amastigote replication leads to the release of amastigotes from infected macrophages; amastigotes in turn are taken up by non-infected macrophages, thus spreading the infection.[47,48]

Leishmania enter macrophage phagosomes that ultimately fuse with lysosomes. The survival of *Leishmania* spp. in this hostile intracellular environment has been primarily attributed to the capacity of amastigotes to withstand the phagolysosomal compartment and to the ability of the parasite to down-modulate macrophage activation.[49-54] Accumulating evidence demonstrates that different external stimuli induce distinctive types of macrophage activation with divergent pro and/or anti-inflammatory profiles.[55-57] *L. infantum chagasi* is a cause of visceral leishmaniasis. Infection of BALB/c mouse macrophages with *L.i chagasi* promastigotes initiates a pattern of gene expression that is neither classically activated nor alternatively activated, but which demonstrates a novel type of macrophage activation characterized by an anti-inflammatory profile and an increase in the caveolae-related molecules dynamin-2, Cav-1 and Cav-3.[58] This increase in caveolae components upon *L. i. chagasi* infection suggests the presence of a feedback mechanism that may be triggered by the depletion of surface caveolae upon parasite uptake.

Employing confocal microscopy, virulent *L. i. chagasi* promastigotes were found to colocalize with the caveolae markers GM1 and Cav-1 both during entry and up to 24 hours after murine macrophage infection (Fig. 1). Entry of promastigotes via Cav-1 correlated with a delay in lysosome fusion of approximately 24 to 48 hours; a time coinciding with their promastigote-to-amastigote conversion.[48,59] Colocalization of promastigotes with Cav-1 also correlated with increased parasite survival. In contrast, serum opsonization of attenuated (avirulent) *L. i. chagasi* promastigotes precluded parasites to enter macrophages through lipid rafts/caveolae. Unlike virulent *L. i. chagasi,* avirulent parasites entered compartments that fused early with lysosomes usually within the initial three hours of infection and failed to survive in macrophages.

Because caveolae are enriched in cholesterol, transient treatment of macrophages with the cholesterol chelating agent MβCD was used to investigate the role of lipid rafts/caveolae in Leishmania infection. MβCD treatment does not affect macrophage

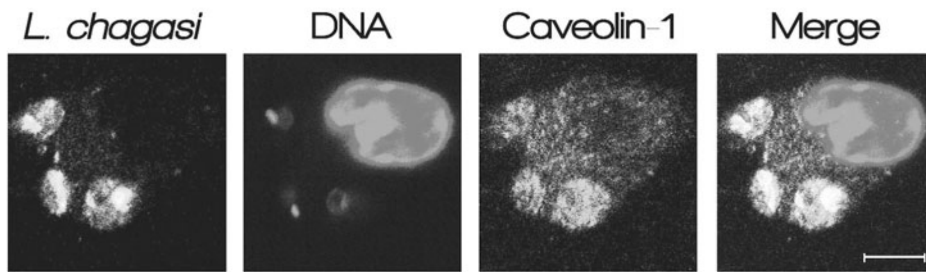

Figure 1. Intracellular *L. i. chagasi* colocalizes with caveolin-1. Bone marrow macrophages were infected with carboxy-fluorescein diacetate succinimidyl (CFSE) labeled *L. i. chagasi* promastigotes (green). Macrophage and parasite DNA was stained with TO-PRO-3 (blue). Confocal microscopy was used to assess colocalization of markers at serial time points. Caveolin-1 (red) clustered at the entry site of *L. i. chagasi* promastigotes and remained associated with parasites for 24 to 48 hours after infection. Shown is a picture taken at 24 hours of infection. Scale bar: 5 μm. A color version of this image is available online at www.landesbioscience.com/curie. Reproduced with permission from Adesse et al. Cell Cycle 2010; 9:1639-1649.[81]

viability if the cell membrane cholesterol is extracted while preserving the intracellular cholesterol pools.[59-61] Under these conditions, pretreatment with MβCD significantly impaired parasite entry and inhibited replication for up to 72 hours after phagocytosis, even though surface cholesterol was restored by 4 hours after treatment. Furthermore, macrophage pretreatment with MβCD accelerated the rate of lysosome fusion and led to rapid intracellular killing. Side by side comparisons showed that transient disruption of lipid rafts/caveolae caused virulent metacyclic *L. i. chagasi* to enter macrophages through a phagocytic pathway, leading to early lysosome fusion and intracellular death, resembling the entry of attenuated parasites.[59]

Surface LPG and MSP on metacyclic promastigotes are able to bind and inactivate the serum protein C3b to C3bi, facilitating parasite uptake through the macrophage receptor CR3, which localizes in caveolae.[62-65] A comparison of the receptors used by avirulent log phase versus virulent metacyclic *L. i. chagasi* in human macrophages demonstrated that metacyclic parasites preferentially enter through CR3 but not the mannose receptor and that the metacyclic parasites colocalize with Cav-1 during the initial hour of infection. In contrast, log-phase parasites ligated both the mannose receptor and CR3 and failed to colocalize with Cav-1. The ability of metacyclic, but not log phase promastigotes to associate with CR3 and Cav-1 also correlated with a slower kinetics of lysosome fusion and increased parasite survival.[66]

The amastigote form of the parasite expresses low levels of MSP and no LPG; the latter of which inhibits lysosome fusion with promastigotes. Amastigotes can survive in the phagolysosomes and it has been suggested that the unique nutritional requirements of the amastigote can only be met in this degradative compartment.[67-69] As such, amastigotes might not benefit from entering macrophages through a cholesterol-rich/caveolae uptake mechanism that delays lysosome fusion. Indeed, side by side experiments with metacyclic promastigotes and hamster-derived amastigotes of *L. i. chagasi* supported this hypothesis. Transient depletion of cholesterol from BALB/c mouse macrophages did not affect the entry of amastigotes or their kinetics of lysosome fusion (submitted manuscript, Rodriguez, Gaur, Allen and Wilson), suggesting that the entry and survival of amastigotes is independent of cholesterol-rich microdomains including caveolae. Taken together, these results suggest that virulent, but not attenuated or log phase promastigotes,

are able to exploit a caveolae-mediated pathway to facilitate their entry and intracellular survival by a mechanism that includes delayed lysosome fusion until their conversion into amastigotes and the establishment of infection.

Trypanosoma cruzi

Trypanosoma cruzi causes Chagas' disease. It is an important cause of acute myocarditis, chronic cardiomyopathy and gastrointestinal disorders in endemic areas.[70] Chagas' disease is found in endemic areas of Mexico, Central and South America. In recent years, there has been an increased recognition of Chagas' disease among immigrants from endemic areas into North America and Europe. Chagas' disease is also an opportunistic infection in patients with HIV/AIDS.[71-73]

The parasite has a complex life cycle. During a blood meal from an infected mammalian host, the insect vector ingests blood-form trypomastigotes, which undergo transformations and after 3 to 4 weeks, infective, nondividing metacyclic trypomastigotes are present in the hindgut of the vector and are deposited with the feces of the vector during subsequent blood meals. Transmission to the new host occurs when the parasite-laden feces contaminate oral or nasal mucous membranes, the conjunctivas, or other vulnerable surfaces. Other modes of transmission include blood transfusion, organ donation, congenital, breast milk, ingestion of contaminated food or drink and laboratory accident.

When the trypomastigotes enter a host cell they transform into amastigotes where they multiply by binary fission and again transform to trypomastigotes. Trypomastigotes are released as the host cell ruptures and disseminate through the lymphatics and the bloodstream to find new cells to invade. The precise mechanism(s) by which the parasite enters the host cell is not entirely understood, however several receptors have been implicated in this process. Although any nucleated mammalian cell can be parasitized, those of the cardiovascular system, including cardiac myocytes, cardiac fibroblasts, endothelial cells and vascular smooth muscle cells, as well as cells of the reticuloendothelial, nervous and muscle systems and adipose tissue, appear to be favored.

T. cruzi infection is characterized by an intense inflammatory reaction accompanied by an upregulation of cytokines and chemokines.[73,74] Pathological examination of the cardiovascular system in both human samples and experimental acute chagasic myocarditis reveals inflammation, myonecrosis, vasculitis and numerous parasite pseudocysts. In chronic chagasic cardiomyopathy, inflammation, fibrosis, myocytolysis and vasculitis persist but there are few parasites in the infected tissues. In many chronically infected individuals there is a dilated cardiomyopathy. In order to understand the mechanisms involved in progression of disease and the development of the resulting cardiomyopathy, many groups have focused their research either on the in vitro infection of cardiac cells with *T. cruzi* or through the use of murine models of infection.

Caveolin is a negative regulator of extracellular signal-regulated kinases (ERK) and cyclin D1.[75] Thus, a reduction in the expression of Cav-1 and Cav-3 generally results in the upregulation of ERK activity and an induction of *cyclin D1* expression, which contribute to cardiac myocyte hypertrophy and ultimately cardiomyopathy. Interestingly, Cav-1 and Cav-3 null mice as well as the Cav-1/Cav-3 double null mice display a cardiomyopathic phenotype associated with cardiac myocyte hypertrophy and interstitial fibrosis.[76-78] Thus, it was of great interest that during the acute phase of *T. cruzi* infection a reduction in the expression of Cav-1, Cav-2 and Cav-3 was observed. This was accompanied by activation

of ERK, activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and increased cyclin D1 expression.[79,80] The change in Cav-1 expression in infected mice was the result in part of infection of the cardiac fibroblasts since cardiac myocytes do not express Cav-1. At 60 days post-infection, which is considered the sub-acute/chronic phase, there was a reduction in Cav-3 expression which normalized by day 180 post-infection,[81] though the increase in the expression of ERK persisted. Thus, it would appear that the initial reduction in Cav-3 expression may trigger an increase in ERK, leading to cardiac myocyte hypertrophy.

Chagas' disease is also a vasculopathy and in carotid arteries obtained from *T. cruzi* infected mice there was a reduction in the expression of Cav-1 and Cav-3 and activation of ERK, cyclin D1 and endothelin-1(ET-1).[82] These findings may in part explain the vasoconstriction observed as a result of this infection. *T. cruzi* infection also results in a reduction of Cav-1 and Cav-2 expression and an increase in activated ERK, cyclin D1 and ET-1.[82,83] In addition, infection of cardiac myocytes results in a reduction of Cav-3 expression and a concomitant increase in activated ERK (Fig. 2).[81] Taken together *T. cruzi* infection results in cardiomyopathy and a vasculopathy in which caveolin, ERK, cyclin D1 and ET-1 contribute to cardiovascular remodeling and the pathogenesis of chagasic heart disease.

Employing the highly virulent Tulahuen strain of *T. cruzi* infection, Medina et al[84] infected both Cav-1 wild type and Cav-1 null mice and while the resulting mortality was 100% in both genetic backgrounds, death was slightly delayed in the wild type mice. The parasitemia in the Cav-1 null mice was significantly reduced compared with wild type mice. In both groups there were numerous pseudocysts, myonecrosis and marked inflammation. Interestingly, infection of cultured cardiac fibroblasts obtained from Cav-1 null and wild type mice revealed no differences in infectivity. Determination of serum levels of several inflammatory mediators revealed a significant reduction in IFN-γ, TNF-α and components of the nitric oxide pathway in infected Cav-1 null mice, while infection of wild type mice resulted in an increase in these inflammatory mediators. The defective production of chemokines and cytokines observed in vivo is, in part, attributed to Cav-1 null macrophages. These results suggest that Cav-1 may play an important role in the normal development of immune responses. Recently, Barrias et al demonstrated that infection of mouse peritoneal macrophages with the Y strain of *T. cruzi* was impaired when cells were treated with MβCD.[85] The contributions of caveolin in Chagas' disease are not entirely understood and investigators are continuing to explore these interactions.

Toxoplasma gondii

Toxoplasma gondii is a ubiquitous Apicomplexan obligate intracellular protozoan parasite of mammals and birds. It has long been recognized as being an important cause of congenital infection with chorioretinitis and central nervous system manifestations and has also emerged as an opportunistic pathogen in immune compromised hosts where it primarily causes encephalitis.[86-88] Although overwhelming disseminated toxoplasmosis has been reported, the predilection of this parasite for the central nervous system causing necrotizing encephalitis and the eye causing chorioretinitis constitutes its major threat to patients. The development of these diseases is a consequence of the transition of the resting or latent bradyzoite stage to the active rapidly replicating form, the tachyzoite stage.[86-87,89-93] It is likely that in chronic toxoplasmosis tissue cysts (bradyzoites) regularly transform to tachyzoites and that these active forms are removed or sequestered by the immune system, while some invade host cells differentiating to new tissue cysts.[86,89,90,93]

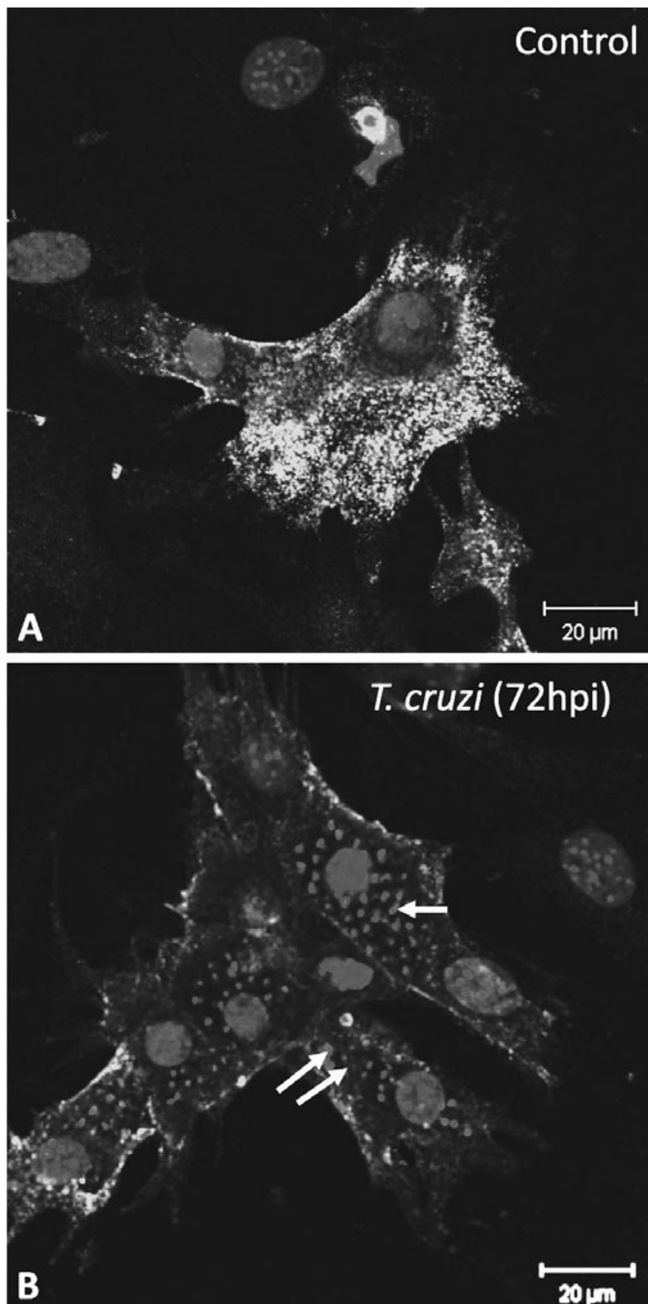

Figure 2. Caveolin-3 (Cav-3) expression is diminished after *Trypanosoma cruzi* infection: Cardiac myocytes were isolated from mouse embryos and infected with trypomastigotes. Confocal microscopy showed that uninfected cultures displayed abundant Cav-3 immunoreactivity (A), including peripheral staining. Cav-3 signal was reduced among highly parasitized myocytes (B) and was present predominantly at the cell periphery. DNA staining by DAPI permitted visualization of host cell nucleus and the amastigote kinetoplastid DNA. Bars = 20 mm. Reproduced with permission from Adesse et al. Cell Cycle 2010; 9:1639-1649.[81]

T. gondii replicates within a parasitophorous vacuole, isolated from host vesicular traffic.[94] *T. gondii* are capable of actively invading host cells[95,96] and these invasion processes require parasite motility, orientation toward the host cell and sequential discharge of three secretory organelles termed micronemes, rhoptries and dense granules.[97,98] Host cell cholesterol is required for entry and intracellular replication of this and other pathogens.[99-102] Central roles for cholesterol are suggested at the attachment, penetration and intracellular multiplication stages. Depletion of membrane cholesterol leads to a loss of invaginated caveolae.[103] The predominant sterol in *T. gondii* membranes is cholesterol and it has been unequivocally demonstrated that this parasite is auxotrophic for this major lipid.[101] *T. gondii* actively intercepts low-density lipoprotein (LDL)-derived cholesterol that has transited through host lysosomes by a yet undefined mechanism. Cholesterol trafficking from mammalian lysosomes to intravacuolar *T. gondii* requires functional host Niemann–Pick type cholesterol proteins (NPC), which are known to mediate cholesterol egress across the endosomal (mainly NPC1) and lysosomal (mainly NPC2) membranes,[104] and trafficking is independent of pathways involving the host Golgi and endoplasmic reticulum.[101] The uncoupling between LDL uptake and cholesterol biosynthesis occurring during *T. gondii* infection[101] leads to the assumption that these pathways are dramatically perturbed in infected cells.

Caveolae/caveolins have been proposed to function in a number of cholesterol-trafficking steps. These steps include selective cholesterol uptake from HDL via the scavenger receptor SR-B1 located in caveolae (cholesterol influx) and the delivery of newly synthesized cholesterol from the endoplasmic reticulum membrane caveolae, where it is delivered to HDL (cholesterol efflux).[105] However, studies have shown that high levels of host caveolae vesicles are not needed for trafficking of LDL-derived lysosomal cholesterol to Toxoplasma in mammalian cells.[101] It has been suggested that a probable scenario during *T. gondii* infection is that following LDL receptor-mediated endocytosis, cholesterol is liberated from LDL cholesteryl ester in early hydrolytic compartments containing the enzyme acid lipase. The cholesterol then effluxes from the NPC-containing late endosome/lysosome before trafficking to the parasitophorous vacuole. The post-endolysosomal movement of cholesterol to the parasitophorous vacuole is blocked by inhibitors of vesicular transport but does not require vesicle fusion or host endolysosome fusion with the parasitophorous vacuole. Transit to the parasitophorous vacuole is direct, rather than across host organelles, e.g., endoplasmic reticulum, Golgi[101,106] or through the host plasma membranes.[107] However, the parasitophorous vacuole is accessible to sterol acceptors in the medium by an unknown mechanism. Neither the host sterol carriers SCP-2 nor caveolins are involved and the process is independent of vimentin intermediate filaments. Host cholesterol is delivered to the parasitophorous vacuole via lipid extractor-or transporter-like proteins on the parasitophorous vacuole membrane, then trafficked within the vacuolar space, perhaps in association with the tubulo-vesicular network (TVN) secreted by *T. gondii* before internalization into the parasite interior via parasite plasma membrane proteins and storage as cholesteryl esters in lipid bodies.

Rhoptries are elongated club-shaped organelles containing a densely packed granular material in their basal bulbous portion and are related to secretory lysosomes or exosomes.[108] *T. gondii* rhoptries are formed via the endocytic pathway[109] and contribute to the formation of the *Toxoplasma* and *Plasmodium* parasitophorous vacuole membranes (PVMs) by releasing their contents from the anterior end of the parasite during invasion.[98,110,111] *Toxoplasma* rhoptries also contain lipids, including large amounts of cholesterol and phosphatidylcholine.[112,113] In these organelles, the cholesterol/phospholipid molar ratio (1.5/1) is too high for lipid bilayer stability, suggesting that some rhoptry cholesterol

molecules may be organized in a crystalline array inside the organelle. Although it is plausible that rhoptry cholesterol is incorporated into the PVM during invasion and that rhoptry discharge can effectively compensate for the absence of caveolae in host cells, this possibility requires testing.

The biogenesis of the PVM surrounding *Toxoplasma* is only partially understood. Capacitance measurements in patch-clamped host cells indicate that at least 80% of the membrane in the nascent vacuolar membrane is host cell derived.[114] This is consistent with observations showing that fluorescent tracers inserted into the host plasma membrane before infection were incorporated into the nascent PVM of *Toxoplasma*[110,115,116] as well as into the *Plasmodium* PVM.[117] Additionally, multiple lines of evidence suggest that rhoptry contents contribute directly to formation of the vacuolar membrane.[109,110,118-120] These apparently conflicting observations can be resolved by postulating a two-step process of invasion. Initial discharge of the rhoptry contents directly into the host cytoplasm leads to coalescence of multivesicular structures, which then fuse with the nascent vacuole that is derived primarily from the host cell plasma membrane.[121] Whether and how cholesterol contributes to this unusual process of cell invasion by *T. gondii* is not clear. Studies have evaluated the ability of cholesterol-depleted parasites to invade normal cells, of cholesterol-depleted cells to be invaded by untreated parasites, as well as the ability of normal parasites to invade caveolin-minus cells. The results demonstrate that *T. gondii* is dependent upon host plasma membrane cholesterol to trigger organelle discharge. Neither rhoptry-derived cholesterol nor caveolae microdomains in the host cell plasma membrane are required to complete invasion. These results identify a heretofore unexpected mechanism by which cholesterol regulates microbial entry into mammalian cells.

Lisanti and colleagues[122,123] discovered that purified caveolae microdomains contained an abundance of signaling molecules, such as Src-like tyrosine kinases and heterotrimeric G proteins and proposed that Cav-1 and caveolae may serve as docking points for numerous cell surface receptors, which, when activated by ligand binding, are recruited to caveolae.[122,123] It has been demonstrated that GTPase activity of G protein-subunits could be suppressed by a peptide derived from the NH2 terminus of Cav-1 termed the caveolin scaffolding domain, demonstrating the interdependence of these proteins for functional activity.[124] Finally, studies have shown that interaction of the *T. gondii*-derived molecule cyclophillin-18 (C-18) with a chemokine receptor (CCR5) on dendritic cells is critical for IL-12 (a pro-inflammatory mediator) induction in vivo and the subsequent control of parasite growth.[125] Since CCR5 is associated with G-protein-subunits, it is interesting to consider the possible participation of caveolin in the signaling pathways and transcriptional events downstream of CCR5 triggered by *T. gondii.*

FUNGI

Although significant efforts have led to an appreciation that sterol-rich membrane domains significantly impact the function of fungal membranes,[126] there is limited information regarding the role of lipid rafts and caveolae in the pathogenesis of fungal disease in mammalian hosts. The most in depth analysis of a fungal interaction with cellular membrane rafts comes from a study on *Paracoccidioides brasiliensis,* a dimorphic fungus endemic in the environment in Central and South America.[125] This fungus causes a spectrum of human disease ranging from mild localized infection to

disseminated deep lesions, characterized by granulomatous inflammation. *P. brasiliensis* is frequently acquired via inhalation. Using epithelial alveolar cells, Maza et al[127] demonstrated that host cell lipid rafts are critical for engaging *P. brasiliensis* cells. They demonstrated that ganglioside GM1 appeared to be recruited to the point of adhesion of *P. brasiliensis* to this host cell. Furthermore, pretreatment of the alveolar cells with either MβCD to deplete host cell cholesterol, or nystatin, to bind host cholesterol, significantly reduced fungal adhesion.

Pneumocystis species are frequent pulmonary pathogens in hosts with compromised cellular immune responses, especially individuals with AIDS and the fungus can intimately engage Type I alveolar epithelial cells.[128] Although *Pneumocystis* associates with cells, the fungus is an extracellular pathogen. Nevertheless, the fungus interdigitates with the alveolar cells and activates caveolae of the host cells, resulting in the close proximity of plasmalamellar vesicles in the areas of contact. Presumably, this allows the fungus to parasitize the host cells for nutrients.

Beta-glucans are major components of many fungal cell walls including that of *Candida albicans*, which is the most prevalent cause of systemic mycoses. Beta-glucans from *C. albicans* can interact with very long fatty acid-containing lactosylceramide lipid rafts on the plasma membrane of human neutrophils, inducing migration of the neutrophil toward the fungus[129,130] and, presumably, enhancing phagocytosis of the yeast.

Histoplasma capsulatum, a dimorphic fungus endemic to the Mississippi and Ohio River Valleys of the USA and in regions within Central and South America, causes pulmonary and disseminated infections, especially in individuals with compromised immunity. Calcium binding protein (CBP) is a well known virulence factor for the fungus that recently was shown to have structural homology with mammalian saposin B.[131] Hence, it has been proposed that CBP may interact with host glycolipids, including those present in caveolae. This finding underscores the probable broad and yet undiscovered import of caveolae in interactions with pathogenic fungi. Future work will continue to elucidate the functional importance of caveolae interactions with fungi during adhesion, internalization and internal processing.

CONCLUSION

A growing list of pathogens, including viruses, bacteria and their associated toxins, fungi and even prions, can interact with caveolae membrane domains.[2] The intracellular trafficking of these agents via caveolae differs dramatically from the usual route of ligands internalized by clathrin-mediated endocytosis. The use of caveolae for cellular entry allows the pathogen to avoid classical endosome-lysosome trafficking and, consequently, avoid degradative compartments within the cell.

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CHAPTER 6

CAVEOLIN-1 AND BREAST CANCER:
A New Clinical Perspective

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Abstract: The current chapter focuses on the role of Caveolin-1 (Cav-1) in cellular growth with an emphasis on its implication in breast cancer initiation, progression, clinical prognosis and as a potential therapeutic target. The role of Cav-1 as a tumor suppressor in breast cancer has emerged in the past few years, with dual functions on both cancer epithelium as well as the cancer stroma. Its multiple functions as a regulator of estrogen signaling and kinase activity and its newly found role as an important factor controlling the dynamic relationship between cancer epithelia and stroma position Cav-1 as a new therapeutic target for the treatment of breast cancer.

INTRODUCTION

The discovery of Caveolin-1 (Cav-1) as part of a structural network of caveolar domains associated with the plasma membrane was a major finding that led to the unraveling of several interesting roles for this protein in cancer. Domain mapping studies have quickly identified an important region spanning amino acids 82-101 of the Cav-1 protein, named the caveolin scaffolding domain (CSD).[1,2] This region was shown to be sufficient and necessary for the binding of several proteins involved in cell proliferation such as Src family members, protein kinase C, the epidermal growth factor receptor, H-ras and Neu.[3-5] The discovery of caveolin-interacting domains within cellular kinases involved in cancer growth initiated several studies that further confirmed the role of Cav-1 in cancer.

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CAVEOLIN-1, A POTENT SUPPRESSOR OF GROWTH IN BREAST CANCER CELLS

Interestingly, Cav-1 expression is most abundant in differentiated cells such as epithelial and endothelial cells, fibroblasts and adipocytes.This finding suggests an antiproliferative role of Cav-1. In fact, a significant decrease of Cav-1 protein and mRNA levels in NIH 3T3 fibroblasts transformed with v-abl and H-ras (G12V), two potent oncogenes, confirmed this hypothesis.[6] Although insightful, the decrease in Cav-1 levels associated with transformation was not sufficient to confirm its direct role in oncogenesis. Further experiments in which Cav-1 levels were re-introduced in oncogene-transformed NIH 3T3 cells successfully restored anchorage-dependent growth through inhibition of the Ras/MAPK pathway.[7] Interestingly, these studies also confirmed a primordial role for Cav-1 in the formation of caveolae structures, which reappeared following Cav-1 expression in v-abl and H-ras (G12V)-transformed cell lines.[7] Additional studies also demonstrated that cellular transformation could be induced using a Cav-1 antisense approach through the activation of the p42/44 MAP kinase cascade.[8]

The mapping of *Cav1* to a tumor suppressor locus (7q31) that is often deleted in several types of cancers further motivated the research interest on this protein.[9] Interestingly, primary cultures of mammary epithelial cells isolated from Cav-1 knockout (Cav-1 KO) mice demonstrated an unusual growth pattern when cultured in a three-dimensional matrigel culture system in vitro. Indeed, when allowed to grow in matrigel, WT mammary epithelial cells adopted a spheroid-like structure with a hollow lumen surrounded by a single layer of cells.[10] In contrast, Cav-1 KO mammary epithelial cells formed larger and irregular acini that underwent complete luminal filling.[10] Cav-1 KO acini displayed increased phospho-ERK1/2 and grew independently of epidermal growth factor (EGF), contrary to their wild-type equivalent.[10] When further examined, Cav-1 KO acini exhibited defects in cell matrix adhesion caused by increased matrix metalloproteinases (MMP2 and 9), resulting in decreased levels of collagen IV.[10] When grown on glass coverslips, these Cav-1-depleted acini underwent an epithelial to mesenchymal transition (EMT) characterized by a spindle-shaped appearance, resulting in the mislocalization of E-cadherin and β-catenin, two hallmarks of EMT.[10] Mechanistically, a lack of Cav-1 expression in mammary acini caused the constitutive activation of phospho-smad-2, a direct mediator of TGF-β signaling, while total smad-2 levels remained unchanged.[10]

As expected, several human breast cancer cell lines also display decreased Cav-1 expression levels compared to benign mammary epithelial cells.[11,12] In fact, low Cav-1 levels in MCF-7 and T47D human breast cancer cell lines make these cells especially suitable to study Cav-1-dependent cell growth.[11,13] Indeed, Cav-1 re-expression in these cells was sufficient to decrease their proliferation and even prevented them from forming colonies in soft agar, highlighting the important role of Cav-1 in breast cancer.[7,11,14] Further analysis of these cells suggested that Cav-1 overexpression was sufficient to decrease their transformation potential by 45%. Concomitantly, a decrease in matrix metalloprotease secretion was also observed, suggesting that Cav-1 can directly modulate invasion through this pathway.[11] In order to further analyze the role of Cav-1 in tumor growth in vivo, a human breast cancer cell line overexpressing Cav-1 was injected subcutaneously in nude mice and allowed to grow for one month. As expected, the resulting tumors from MCF-7 cells overexpressing Cav-1 were much smaller than those from control MCF-7 cells, suggesting that Cav-1 expression inhibits the growth of breast cancer cells in vivo.[15] When further analyzed, the MCF-7/Cav-1 tumors had

significantly less proliferating but more apoptotic cells, both events possibly contributing to decreased overall tumor size.[15] To further investigate the role of Cav-1 in breast cancer cell migration, a metastatic mammary adenocarcinoma cell line (MTLn3) was used. As predicted, adenovirus-mediated overexpression of Cav-1 in this cell line caused a decrease in cell migration, anchorage-independent growth and epidermal growth factor-stimulated lamellipod extension, reverting these cells to a nonmotile phenotype.[13] These results suggest that Cav-1 can also prevent the growth and invasion of cells that have already acquired metastatic properties.

INSIGHTS FROM THE CAVEOLIN-1 KNOCKOUT MOUSE MODEL

Most xenografts studies have attributed a growth-inhibiting role for Cav-1 in already transformed or invasive cancer cells. Although insightful, there was a need to explore how Cav-1 contributes to mammary gland development in a whole mouse model. For this purpose, mice with a Cav-1 deletion were generated. The mammary glands of virgin Cav-1 KO mice demonstrated intraductal hyperplasia with areas of fibrosis, suggesting a direct role of Cav-1 in mammary hyperplasia in the developing mammary gland.[16] Although these mice do not spontaneously develop mammary tumors, a lack of Cav-1 expression accelerates the development of mammary lesions in cancer-prone models. As such, when bred with MMTV-PyMT (mouse mammary tumor virus-polyoma middle T antigen) mice, a transgenic mouse model of breast cancer, the resulting PyMT/Cav-1 null mice showed accelerated onset of mammary tumors as well as increased lung metastases.[17] Consistent with its previously mentioned role in cellular invasion, Cav-1 re-expression in Met-1 cells, a highly metastatic cell line derived from PyMT mice, significantly decreased metastases formation.[18] These results suggest that Cav-1 deletion in combination with other cancer-promoting changes greatly accelerates tumor growth and metastases formation in vivo.

CAVEOLIN-1 MUTATIONS FOUND IN BREAST CANCER PATIENTS

Independent reports have described the presence of a Cav-1 mutation consisting of a proline to leucine substitution at position 132 (P132L) in primary breast tumors.[16,19,20] To further understand the implication of such a Cav-1 mutation on cell growth, the effect of Cav-1 (P132L) on cellular signaling pathways was examined. Interestingly, the presence of this modified Cav-1 form induced cellular transformation and promoted invasion of NIH-3T3 cells. Mechanistically, Cav-1 (P132L) is believed to cause the mislocalization and intracellular retention of wild-type Cav-1, therefore acting as a dominant negative mutant.[19] When overexpressed in mammary tumor cells, Cav-1 (P132L) caused a significant increase in cell migration, invasion and metastases formation, suggesting that Cav-1 mutations are unfavorable.[18] This is potentially clinically relevant as patients harboring Cav-1 mutations have an 82% chance of cancer recurrence.[21] The presence of a Cav-1 (P132L) mutation could be detected by immunohistochemistry as a punctate expression pattern in breast cancer patients.[20] Interestingly, only estrogen receptor (ER)-positive patients with well-differentiated breast cancer harbored this mutation. Since Cav-1 mutations associate with cancer recurrence, and almost half of ER-positive patients develop tamoxifen resistance, there is a possibility that a P132L mutation in breast

tumors could predict a poor response to tamoxifen. As such, early immuno-detection of Cav-1 mutations in these patients could become a routine detection method that could help better stratify patients and assign them to more effective therapies.

CAVEOLIN-1, A REGULATOR OF ESTROGEN-DEPENDENT SIGNALING AND PROLIFERATION

Estrogen has been linked to breast cancer for some time now. In fact, extended exposure to estrogen caused by early menarche and late menopause or even hormonal therapy correlate with increased risk of breast cancer incidence.[22] Estrogen binds the estrogen receptor alpha (ERα), a member of the steroid receptor family, which mediates the proliferative functions of estrogen. While only 10-15% of normal breast epithelial cells express this receptor, ERα becomes up-regulated in breast cancer cells, making 70% of all invasive breast cancers ER-positive.[23] Importantly, breast tumor cells expressing ERα have been shown to divide actively. Mechanistically, ERα can control the growth of breast cancer cells by regulating the expression of genes involved in proliferation.[24]

Since an important proportion of breast tumors segregate with ER-positivity, a large amount of research has focused on this important pathway. In fact, the most effective standard therapy to cure ER-positive breast cancers is currently tamoxifen, which successfully prevents estrogen-dependent growth. Unfortunately, almost half of patients under this therapy eventually become resistant and their cancer can quickly recur. As such, understanding the molecular mechanisms controlling the activation of ERα is crucial to find new alternatives and/or combination therapies that could prevent tumor growth and recurrence.

Recently, Cav-1 has been found to be an important regulator of ERα levels and response to estrogen treatment both in vitro and in vivo.[12,25] Although Cav-1 was demonstrated to be involved in cellular transformation and tumor growth, its implication in estrogen signaling remained completely uncharacterized. A retrovirus-mediated poly-A gene trapping approach was utilized to inactivate numerous genes involved in the transformation of the mammary epithelial cell line MCF10A. Two clones (ST1 and ST3) were further analyzed and they demonstrated a 50% decrease in Cav-1 mRNA and protein levels. Interestingly, these clones were able to grow in an anchorage-independent fashion when compared to MCF10A control cells.[26] It was later shown that ERα was up-regulated in these Cav-1 haplo-insufficient mammary epithelial cells, while ERβ remained unchanged. As expected, treatment of these Cav-1 haplo-insufficient cells with 17β-estradiol, the predominant sex hormone in females, caused an increase in anchorage-independent growth. More interestingly, while control MCF10A cells were unable to form tumors in vivo, a pooled population of ST1 and ST3 clones resulted in tumors of at least 0.5 cm^3 when injected in ovariectomized nude mice treated with 17β-estradiol. These results directly suggested that Cav-1 serves as a negative regulator of estrogen-stimulated proliferation in mammary cells through the regulation of ERα levels.

Thus, there was a need to examine how Cav-1 modulates the response of the mammary gland to estrogen in vivo. As previously mentioned, Cav-1 KO virgin mice develop mammary hyperplasia with wall thickening which consists of up to three to four layers of epithelial cells. Interestingly, Cav-1 KO mammary glands also demonstrated an increase in the levels of luminal ERα. However, it still remained unclear if the hyperplasia observed in mammary glands of Cav-1 KO mice was a result of increased

sensitivity to estrogen. To directly examine such a possibility, a bilateral ovariectomy was performed in order to decrease the levels of endogenous ovarian hormones in these mice. Remarkably, the ovariectomy completely reversed the hyperplasia observed in the mammary ducts of Cav-1 KO mice.[25] Since the ovariectomy procedure consists of the depletion of all ovarian-produced hormones including estrogen and progesterone, it remained unknown which of the two specifically caused the mammary gland to proliferate. As shown in Figure 1, chronic treatment of ovariectomized Cav-1 KO mice with 17β-estradiol for 60 days resulted in mammary ductal lesions resembling those found in human DCIS (ductal carcinoma in situ), while the wild-type mammary glands lacked such structures upon hormonal stimulation.[25] Furthermore, morphological analysis revealed that these mammary lesions lacked smooth muscle actin expression, suggesting a break in the surrounding structural myoepithelial layer, a sign of microinvasion and potential spreading to the surrounding stroma.[25] In addition, the stroma surrounding the Cav-1 KO mammary lesions showed exaggerated amounts of vascularization, which is usually associated with a worse clinical outcome in patients.[27] These lesions also expressed more estrogen-regulated proteins such as the progesterone receptor (PR).[25] As such, elevated PR expression levels confirmed that ER was hyperactivated in Cav-1 deficient mammary glands. Mechanistically, Cav-1 KO mammary lesions showed increased PCNA (proliferating cell nuclear antigen), phosphorylated retinoblastoma protein (pRB) and phospho-ERK1/2, all reflective of increased cell cycle activity.[25] Whether progesterone treatment could further modulates estrogen's response in the mammary gland lacking Cav-1 still remains to be determined.

Cav-1 could regulate the proliferative actions of estrogen through a decrease in its co-activators such as CAPER, a transcriptional activator of ERα and JUN/AP-1.[28] In fact, in the absence of Cav-1, CAPER is upregulated both at the mRNA and protein levels, and the protein localizes to the nucleus.[25] Also, published reports suggest a potential role for Cav-1 in the development of tamoxifen resistance. In fact, mice that lack Cav-1 expression have elevated levels of B23 (nucleophosmin), a nucleolar marker predictive of tamoxifen resistance.[25,29] However, direct experimental evidences linking Cav-1 expression to tamoxifen response and clinical outcome are still needed.

CAVEOLIN-1, A NOVEL STROMAL GATE KEEPER

The failure to successfully treat breast cancer is currently believed to be caused by recurrence, resistance to chemotherapy and spreading to other distant organs. These processes are incompletely understood and are currently a challenge in effectively eradicating this disease. The microenvironment surrounding the tumor (cancer stroma) has recently attracted a lot of attention due to its newly found dynamic contribution to tumor pathogenesis. In fact, several studies were conducted that elegantly showed a direct growth-promoting role of stromal cells isolated from primary tumors.[30] More specifically, the growth of epithelial cells could be enhanced by the addition of cancer-associated fibroblasts (CAFs) while normal fibroblasts could not achieve such an effect.[31] Several reports have even suggested that CAFs are rate-limiting for tumor progression.[32,33] Mechanistically, fibroblasts associated with tumors are highly proliferative and share several characteristics with those found in scar tissues, such as keloids.[34-36] Since CAFs change the way tumors grow, a myriad of studies have been focused on understanding the mechanisms controlling their growth.

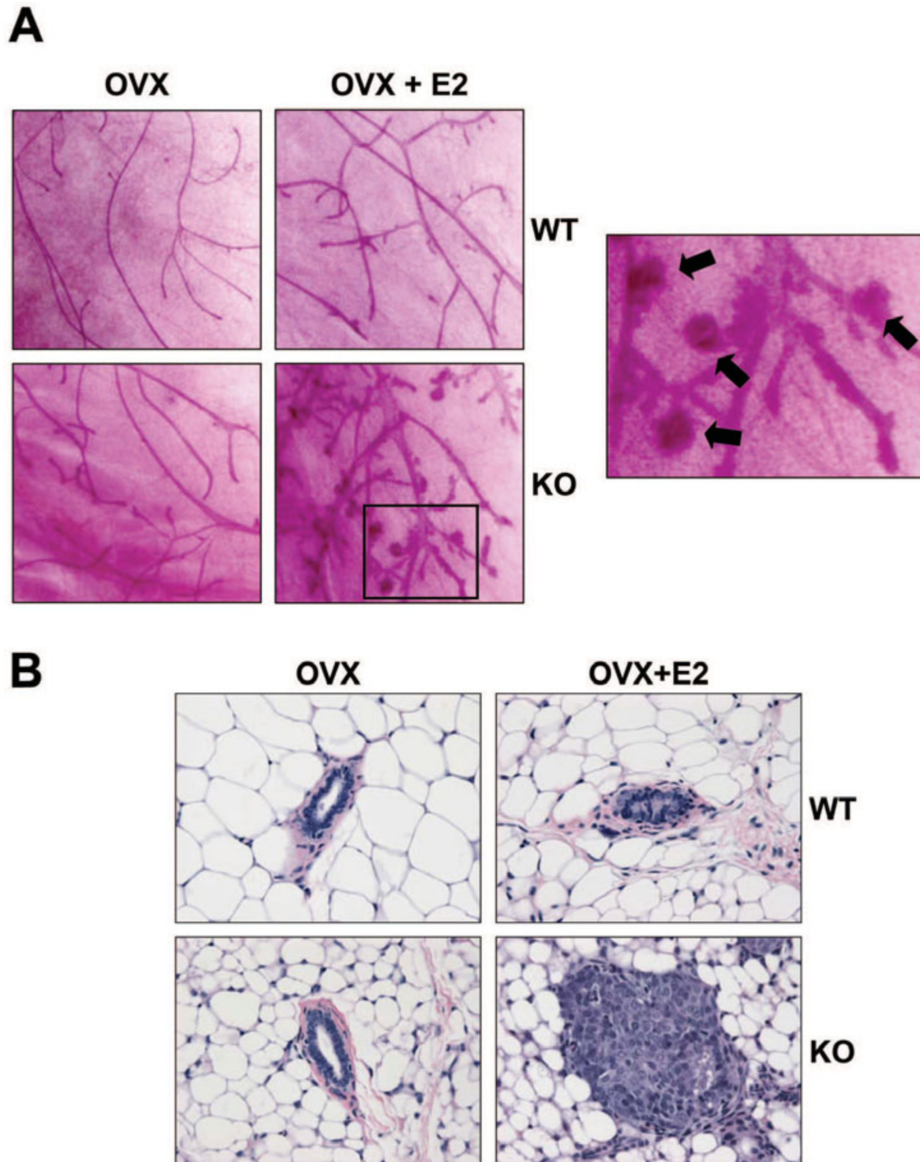

Figure 1. Cav-1 KO Mice Develop Mammary Lesions that Morphologically Resemble Human DCIS. (A) Representative images of Carmine dye-stained mammary whole mounts derived from wild type (WT) and Cav-1 knockout (KO) mice treated with 17-b-estradiol (E2; 250 μg/day for 60 days). A higher magnification of Cav-1 KO lesions is shown in the boxed area (solid arrows). Although WT mice show some minor response to E2, Cav-1 KO mice are markedly hyper-responsive to this hormone. (B) Mammary glands of WT and Cav-1 KO mice subjected to the above treatment were surgically excised, formalin-fixed, paraffin-embedded and subjected to histochemical analysis. Interestingly, mammary glands of E2-treated Cav-1 KO mice display foci that resemble human ductal carcinoma in situ (DCIS), with complete luminal filling, as represented by H&E stain. OVX indicates ovariectomized. Figure modified with permission from Mercier et al. Am J Pathol 2009; 174:1172-1190.[25]

Until recently, the role of Cav-1 in cancer was strictly focused on its epithelium-dependent functions, while completely disregarding its effect on the tumor-associated stroma. However, Cav-1 has recently emerged as a novel stromal regulator of cancer growth and has even been named a "stromal gate keeper".[37,38] More specifically, the analysis of freshly isolated CAFs from invasive breast tumors revealed a notable decrease in Cav-1 protein levels, an induction of BrdU incorporation as well as increased levels of pRB, all consistent with increased cell cycle activity. Since Cav-1 has previously been linked to growth suppression, its re-expression in CAFs was suspected to inhibit their growth. In order to prove such an effect, CAFs were treated with a cell permeable Cav-1 peptide which resulted in growth inhibition through a RB-dependent pathway.[39] Most interestingly, CAFs isolated from patients with low stromal Cav-1 expression revealed a unique RB-regulated gene signature that was associated with cancer recurrence and tamoxifen resistance.[39] These findings were the first to attribute a potential prognostic value for Cav-1 levels within the cancer stroma. In order to further confirm this interesting finding in tumor specimens, a large patient cohort was used to assess stromal Cav-1 expression levels by immunohistochemical analysis and to determine its association with clinical prognosis. As expected, a low stromal Cav-1 expression was predictive of poor clinical outcome as reflected by early tumor recurrence, lymph node metastases and tamoxifen resistance.[40] The prognostic value of stromal Cav-1 was independent of pre-existing epithelial markers such as ER, PR, HER2 and retain a prognostic value even in triple negative breast tumors.[40,41] Furthermore, a low stromal Cav-1 expression in patients with pre-invasive lesions such as DCIS, effectively predicted progression to invasive breast cancer, a powerful tool that could be used to better stratify breast cancer patients early on.[42] We believe that these effects could be linked in part to the antiproliferative effects of Cav-1 on CAFs, through regulation of the RB pathway.

THE AUTOPHAGIC TUMOR STROMA MODEL AND CAVEOLIN-1

Although a general contribution of the tumor stroma to cancer growth is well-established, the exact cellular processes involved remain mostly undefined. As a result, we have recently proposed a new model that can accurately explain the dynamics between the stroma and the epithelial tumor compartment. This new notion can be summarized as "the autophagic tumor stroma model of cancer". Autophagy or macroautophagy, is a cellular process described 50 years ago that allows cells to control their cellular metabolism by turning on a survival mode.[43] For example, when cells are starved and lack sufficient nutrients, a redistribution of internally-stored energy is made by the release of nutrients from non-essential subcellular structures. More specifically, starved cells can sequester cellular components within a vacuole that can fuse with lysosomes to eventually digest its contents and export the nutrients back to the intracellular pool to be used again.[44,45] While this cellular process seems complex and auto-destructive, it represents a fundamental pathway that is constantly utilized at in normal growth and allows degradation of aged organelles. Autophagy has been implicated in several types of pathology such as myopathies, neurodegenerative diseases as well as cancer.[43]

As described above, a lack of stromal Cav-1 has recently emerged as a strong independent predictor of prognosis in both breast and prostate cancers.[40-42,46] The

generation of a coculture system that closely mimics the “epithelial-stromal dynamic” was necessary to understand how the stroma regulates epithelial tumor growth. Thus, we exposed human breast cancer cells (MCF-7) to normal stromal fibroblasts (HTERT-BJ1) for 3-5 days and noted several interesting observations. These cocultures revealed that cancer cells can dynamically induce oxidative stress in adjacent fibroblasts as reflected by an increased production of reactive oxygen species (ROS).[47] The increased oxidative stress in CAFs causes the induction of autophagy through the production of hypoxia inducible fator-1 (HIF1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). More specifically, autophagy in CAFs is mediated by the digestion of their mitochondria, which then obligates these cells to undergo aerobic glycolysis. This type of metabolic pathway produces L-lactate and pyruvate, which can be secreted and used by adjacent cancer epithelial cells as an extra source of fuel. This event makes stromal cancer cells catabolic, by breaking down complex molecules into smaller components to be used as energy. Epithelial cancer cells thus become the recipients of stromal catabolites, as they incorporate the external source of L-lactate and pyruvate into their TCA cycle to produce new ATP molecules and energy. This energy transfer can be seen as a “battery-operated tumor growth” as depicted in Figure 2. We also observed that oxidative stress in CAFs is more likely to induce an anti-oxidative response in adjacent cancer epithelial cells, making them resistant to apoptosis-induced cell death.[38,47] In addition to these observations, production of ROS in fibroblasts induced local DNA damage response in adjacent cancer cells as reflected by double strand DNA breaks as well as aneuploidy, which result in an inappropriate number of chromosomes.[38,47] These results suggest that stromal fibroblasts might cause the acquisition of epithelial mutations and therapy resistance. In summary, the tumor-associated stroma promotes epithelial cancer cell growth through transfer of nutrients as well as acquisition of genomic instabilities and resistance to apoptosis (Fig. 3). When further examined, this relationship appears symbiotic, as epithelial cancer cells are also capable of affecting and reprogramming their neighboring fibroblasts by decreasing their Cav-1 protein levels. This model has also been renamed the reverse Warburg effect.

These interesting studies have permitted the elucidation of how Cav-1 levels become downregulated in the cancer stroma. Since hypoxia is a well-described autophagy inducer, cocultures of cancer epithelial and fibroblast cells were exposed to this stimulus. While fibroblasts exposed to normoxia had normal levels of Cav-1, those exposed to hypoxia, either alone or in cocultures, had decreased Cav-1 protein levels. Treatment of these hypoxic fibroblasts with chloroquine, an autophagy inhibitor, prevented the plunge of Cav-1 levels. Treatment with N-acetyl-cysteine (NAC), an anti-oxidant, also prevented the decrease in Cav-1 levels. These results confirmed that a decrease in stromal Cav-1 levels is caused by the presence of autophagy and oxidative stress.[47] As a proof of principle, direct knockdown of Cav-1 expression in stromal fibroblasts cocultured with cancer epithelial cells caused autophagy in fibroblasts as well as resistance of adjacent epithelial cancer cells to apoptosis. In summary, Cav-1 stromal levels might become a novel indicator of autophagy, oxidative stress and even hypoxia in tumors from breast cancer patients.[38]

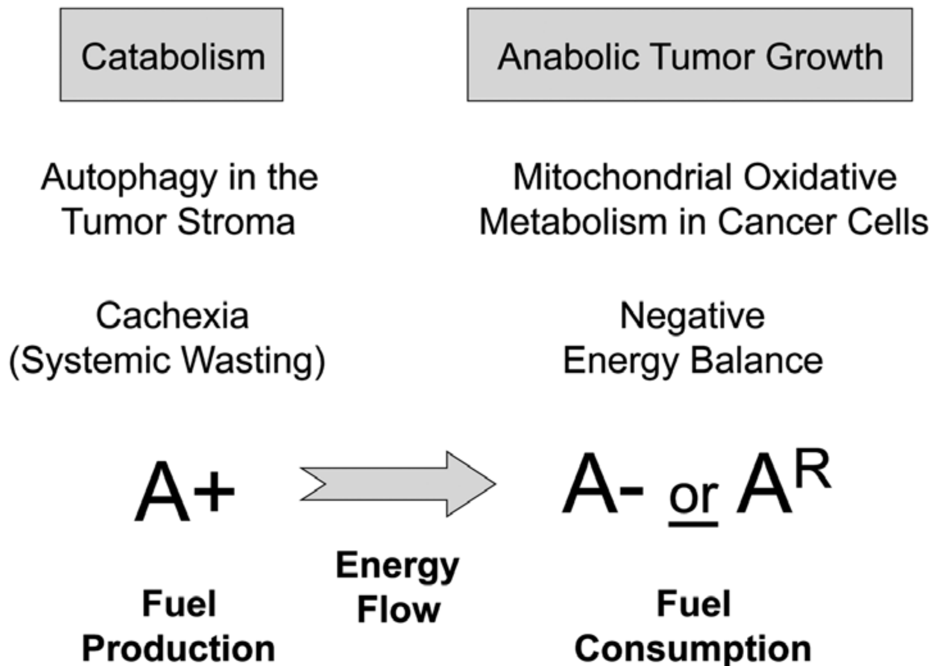

Figure 2. Diagram representing the energy transfer between the tumor stroma and the epithelial tumor. CAFs embedded in the tumor stroma undergo autophagy that results in the production of nutrients that become a new energy supply for adjacent cancer epithelial cells. This energy transfer is unilateral, as the stroma becomes catabolic, while the epithelial tumor cells become anabolic by accepting the stromal catabolites. This fuel production and consumption may be viewed as a battery-operated system where A+ signifies the presence of autophagy resulting in energy production while A– represents an energy consumption through oxidative mitochondrial metabolism and the absence of autophagy. A^R indicates the development of genetic resistance to autophagy in cancer cells. The arrow indicates the direction of fuel transfer from the cancer stroma to the epithelial cancer cells. Figure modified with permission from Martinez-Outschoorn et al. Cell Cycle 2010; 9(21):4297-4306.[42]

CONCLUSION

In summary, Caveolin-1 has emerged as a regulator of both epithelial- and stromal-dependent tumor growth. However, more studies that further characterize the effects of stromal Cav-1 on tumor growth with a focus on new stromal autophagic therapies, such as rapamycin, are needed to improve the clinical outcome and survival of breast cancer patients. More interesting functions of this protein are still being discovered and more studies will be needed to decipher these novel Cav-1 regulated cellular pathways.

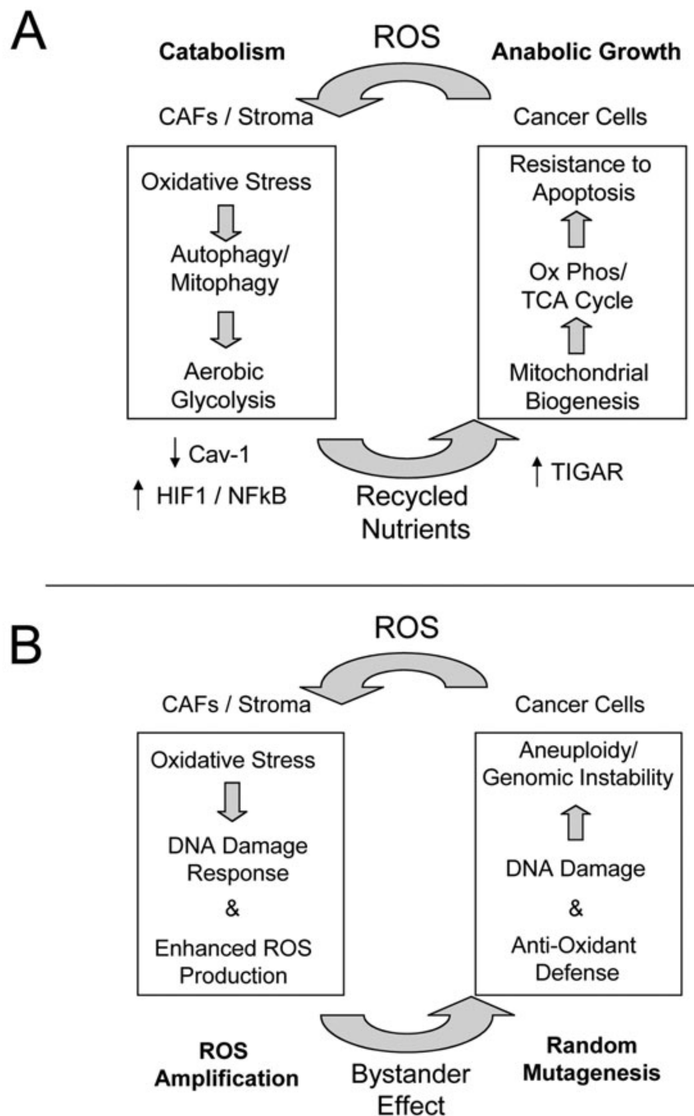

Figure 3. Summary diagram which recapitulates the symbiosis relationship between the tumor stroma and the cancer epithelia. A) Epithelial cancer cells provoke oxidative stress through the production of reactive oxygen species (ROS) in adjacent stromal cancer-associated fibroblasts. Oxidative stress stimulates the induction of stromal autophagy, through the degradation of mitochondria (mitophagy) as well as degradation of Cav-1 protein levels. Autophagic processes in the stroma result in the generation of a nutrient rich pool (catabolism) to be transferred and utilized through oxidative mitochondrial metabolism in adjacent cancer epithelial cells (anabolism). This transfer of nutrient protects cancer cells from apoptosis-induced cell death. B) Local random mutagenesis response secondary to stromal ROS production due to increased oxidative stress in CAFs. Stromal ROS production causes aneuploidy and genomic instability in cancer cells contributing to a symbiotic co-evolution of the cancer stroma and cancer cells, which results in an autophagic tumor stroma model of cancer metabolism. CAFs, cancer-associated fibroblasts, ROS, reactive oxygen species. Figure modified with permission from Martinez-Outschoorn et al. Cell Cycle 2010; 9(21):4297-4306.[42]

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CHAPTER 7

CAVEOLIN-1 AND PROSTATE CANCER PROGRESSION

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Abstract: Caveolin-1 was identified in the 1990s as a marker of aggressive prostate cancer. The caveolin-1 protein localizes to vesicular structures called caveolae and has been shown to bind and regulate many signaling proteins involved in oncogenesis. Caveolin-1 also has lipid binding properties and mediates aspects of cholesterol and fatty acid metabolism and can elicit biological responses in a paracrine manner when secreted. Caveolin-1 is also present in the serum of prostate cancer patients and circulating levels correlate with extent of disease. Current evidence indicates that increased expression of caveolin-1 in prostate adenocarcinoma cells and commensurate downregulation of the protein in prostate stroma, mediate progression to the castration-resistant phase of prostate cancer through diverse pathways. This chapter summarizes the current state of our understanding of the cellular and physiologic mechanisms in which caveolin-1 participates in the evolution of prostate cancer cell phenotypes.

INTRODUCTION

Prostate cancer (PCa) is an androgen-sensitive malignancy that affects middle-aged or older men. PCa is the most common noncutaneous male cancer and a leading cause of cancer death in Western countries. At this writing, PCa claims about 28,000 lives per year in the US. Organ-confined prostate adenocarcinoma is essentially curable in the majority of cases by surgery or radiotherapy. However, there is no effective treatment for nonlocalized disease, which in the developed world generally emerges unpredictably

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after a course of androgen suppression within several years after primary therapy begins. Once primary treatment has failed, there is no effective therapeutic strategy. Because hormone ablation is standard-of-care for nonlocalized disease, recurrence following therapy is characterized as the hormone-insensitive or "castrate-resistant" phase. Clinical progression leads to death within 5 years in most cases, even with aggressive therapeutic intervention. Limited advances in alternative chemotherapeutic modalities have been made in the past decade, however prolongation of survival in the context of the few reported successes against castration resistance has been extremely modest.

In the developed world, PCa is also greatly over-treated. About 70% of patients who receive therapy harbor cancer that would not be clinically threatening during their lifetimes. However, with current technology it is not possible to distinguish indolent cancers from those likely to progress. Many advances in this field are needed to identify new therapeutic strategies and targets that will improve overall survival, as well as quality of life for patients diagnosed with PCa. New biomarkers are also necessary to inform treatment decisions, especially as they relate to novel therapeutic approaches.

Caveolin-1 (Cav-1) is a 21-24 kDa multi-functional signaling protein and lipid transporter that has the distinction of being both a circulating PCa biomarker and a mediator of PCa progression. Cav-1 is the major structural protein within caveolae, small membranous organelles that reside in the cytoplasm or appear as invaginations of the plasma membrane. Cav-1 acts as a scaffold within these structures to organize numerous molecular complexes, thereby regulating a variety of cellular events. Alterations in expression of Cav-1 have been described in a number of malignancies. Increasing evidence points to a dichotomous role played by Cav-1 in cancer, with two prominent cases represented by breast and prostate cancer, in which Cav-1 seems to reduce and promote tumor growth, respectively. Therefore, the Cav-1 protein can be thought of as both a tumor suppressor and a tumor promoter, albeit in different contexts. In PCa, Cav-1 expression correlates positively with aggressive and metastatic potential and serum Cav-1 levels are elevated in patients with PCa but not those with benign prostatic hyperplasia. Several studies have also shown that Cav-1 is capable of actively promoting the metastatic and castrate-resistant phenotypes, suggesting it is not an innocent bystander during disease progression. Because of the diverse mechanistic roles played by Cav-1, its localization within plasma membrane microdomains where many oncogenic proteins also reside and because it circulates in the bloodstream at increased levels in advanced disease, the protein is an attractive focus for therapeutic intervention and biomarker development. The goal of this chapter will be to provide an overview of the current state of knowledge of cell signaling and metabolism in PCa as these processes relate to our understanding of the diverse functional roles of Cav-1.

THE ROLE OF ANDROGEN IN PROSTATE CANCER

Huggins and Hodges reported in 1941 that PCa is dependent on testicular androgens.[1] This critical insight has formed the basis for most of the progress in understanding mechanisms of PCa progression, as well as the development of therapeutic strategies up to the present day.[2-4] Androgens are sterolic hormones that work by binding with high affinity to the androgen receptor (AR), a member of the class I subgroup of the nuclear receptor superfamily of transcription factors.[5] The primary androgenic ligands for the AR are testosterone and its metabolite, 5α-dihydrotestosterone (DHT). DHT is the principal

bioactive androgen in the prostate. Following ligand binding, generally in the cytoplasm, the AR translocates to the nucleus where it binds as a homodimer to chromosomal regions containing short palindromic androgen response elements (AREs) within androgen responsive genes. Nuclear and cytoplasmic AR interacts with many positive and negative regulatory proteins (collectively termed "coregulators") to recruit RNA polymerase II and its associated cofactors to regulate gene expression.[6-9] Like other steroid hormone receptors, such as estrogen, progesterone and mineralocorticoid receptors, AR is highly networked to diverse biochemical mechanisms and is therefore functionally robust. In prostatic cells in vivo, the AR is a key mediator of cell cycle transit, cell differentiation, cell survival and apoptotic mechanisms, secretory processes and metabolism.[5]

Despite decades of study by many laboratories, the precise role of the AR in PCa is still not well understood. Androgen suppression inhibits PCa progression temporarily in humans and can cause regression of experimental prostatic adenocarcinoma in animal models. Studies on somatic AR mutations in human PCa, combined with in vitro and in vivo modeling approaches indicate that, in most instances, the AR appears to mediate pro-survival and pro-growth pathways in prostate tumor cells.[5] This conclusion is consistent with retention of AR expression by cancer cells even in hormonally suppressed conditions and frequent genomic amplification of the AR,[10-16] indicating a likely condition of selective pressure at the AR coding locus for higher expression levels during progression to hormone-refractory disease. However, clinical experience with androgen suppression and androgen replacement therapy also suggests that AR may inhibit prostate tumor progression in a minority of cases, or in certain phases of the disease. Data from animal models, cell line studies and analyses of human tissues are consistent with the concept that the AR takes on different and sometimes opposing roles, depending on the nature of the genetic and epigenetic alterations underlying tumor formation and progression.[17-19] For example, AR expressed by the prostatic stroma appears to play different physiologic roles and to be functionally distinct from AR expressed in epithelial cells.[20] The differential signaling capacities of the AR and the mechanisms underlying them, are presently an active area of research.

AR is expressed in the majority of castrate-resistant prostate cancers and a wide variety of functional somatic mutations in the AR have been identified in human prostate tumors, suggesting that the AR continues to play a significant role under conditions of hormonal suppression, which is still the gold standard for treatment of nonlocalized disease. The manner in which the normally ligand-dependent AR continues to function under conditions of limiting androgen is poorly understood. Several possible strategies for maintenance of AR activity in the hormone-repressed state have been verified experimentally, including AR gene amplification;[10-14] somatic mutations that result in hypersensitivity to androgen or alterations in sensitivity to other steroid hormones, such as progesterone;[21,22] hormone-independent mechanisms of activation through growth factor receptor-mediated mechanisms;[23-28] and intratumoral synthesis of androgen in the castrate condition.[29-31] Some or all of these mechanisms likely operate in patients, possibly even in different phases of disease in individual patients.

CAVEOLIN-1 AND THE ANDROGEN RECEPTOR

In the absence of androgen, the AR is present largely in the cytoplasm, where it is sequestered from the transcriptional machinery. In normal males, the AR is largely present in the nucleus in hormone-sensitive tissues. The AR can form complexes with a wide variety of proteins, including classical signal transduction proteins that reside primarily

in the plasma membrane and in cytoplasmic membranes. These interactions, some of which have been shown to produce unambiguous physiologic effects, include signaling proteins in the epidermal growth factor receptor (EGFR) and insulin/insulin-like growth factor (IGF) pathways.[28,32-34] Lu et al demonstrated that in the presence of androgen, AR and Cav-1 form a complex identifiable in caveolin-enriched, low-density membrane fractions in sucrose gradients.[35] Caveolin-enriched membrane fractions isolated with non-ionic detergents in combination with sucrose gradient centrifugation are believed to represent the residue of cholesterol-rich, "lipid raft" membranes that exist in the in vivo state. Lipid raft membrane microdomains, where the metaphor of a "raft" refers to a less fluid condition than other components of the membrane, have been implicated in many signal transduction processes.[36] Association between Cav-1 and AR in caveolin-enriched fractions was found to be largely androgen-dependent.[35]

Lu et al identified the AR N-terminal and the N-terminal of Cav-1 as subdomains within the two proteins that mediate their physical association. Consistent with these observations, enforced expression of Cav-1 was found to potentiate androgen-dependent signaling, while down-regulation of Cav-1 inhibited androgen signaling. These findings suggest that Cav-1 has the potential to hypersensitize the AR to low androgen, thereby providing a Cav-1-dependent mechanism for the AR to continue to function under conditions of hormonal suppression.

Because the AR-Cav-1 interaction occurs preferentially in lipid raft membranes, these studies also suggest the possibility that Cav-1 intervenes in the androgen-AR axis in a "nongenomic" manner; that is, independently of the AR's involvement in the transcriptional machinery. Several classical steroid hormone receptors, including the AR, have been found to signal nongenomically, i.e., where hormone-dependent signals are processed by the receptors in the absence of their binding DNA.[37,38] Although this is a controversial and very active area of research, a conserved palmitoylation (S-acylation) site has been identified on ERα and ERβ, progesterone receptors A and B and the AR that appears to mediate these receptors' associations with cell membranes and Cav-1, as well as some of the nongenomic signaling effects that have been shown to arise from these associations.[39] The defined AR interaction domain on Cav-1 (residues 1-60) does not overlap with the Cav-1 scaffolding domain (residues 82-101),[35] which has been shown to bind and regulate a wide range of signaling proteins, particularly kinases.[40,41] These distinct domains provide a potential mechanism for Cav-1/AR interaction to facilitate cross-talk with an extensive signaling network without translocation of AR to nuclei. For example, in the LNCaP human PCa cell line, increased androgen promotes an interaction within minutes between AR and Src, a Cav-1 binding protein, which mediates cell proliferation and differentiation signals.[42-44] The manner in which Cav-1 might modify these types of membrane-proximal, hormone-dependent interactions in tumor cells is still not well understood. Cav-1 may act in the role of an AR cochaperone, similarly to other proteins capable of modifying or potentiating androgenic signals, such as Bag-1L, FKBP52 and hsp27.[45-47] Such cochaperone proteins are likely to cooperate to amplify hormonal signals. Hsp27, which forms a complex with AR in the presence of androgen and like Cav-1 can potentiate AR activation, has been shown to facilitate trafficking of sex steroid receptors to the plasma membrane,[48] where Cav-1 in PCa cells resides in increased amounts.[49] Cav-1, by virtue of its ability to associate with many signaling proteins in the sequestered environment of caveolae, may in theory coordinate a large array of these cooperative events.[50] Nongenomic hormonal signals evoked by steroid receptors have also been shown to cooperate and amplify genomic signals mediated by transcriptional activation of the same receptors.[35,37,51] Therefore, Cav-1 localized to

membrane-bound caveolae may still exert profound, yet indirect effects on the classical AR gene activation machinery.

Chromosomal loss at the *PTEN* (phosphatase and tensin homolog) tumor suppressor locus is common in PCa.[52] The PTEN protein is a phosphatidtyl-3,4,5-trisphosphate 3-phosphatase that negatively regulates phosphatidylinositol-3,4,5-trisphosphate, a lipid intermediate in the phosphoinositide 3′-kinase (PI3K)/Akt pathway.[53] The well-described physiologic consequence of inactivating *PTEN* mutations is PI3K/Akt pathway signaling, a potent tumor cell growth and anti-apoptotic mechanism that plays an important role in metastatic disease in many cancers. The extent to which PI3K/Akt activation affects signaling through the AR is still poorly understood. AR was shown by Cinar et al to interact with the oncogenic serine-threonine kinase Akt1 within low-density membrane fractions similar or identical to those described in the above studies of Cav-1/AR interaction.[54] These findings suggest the possibility that convergences in the AR and Akt1 signaling mechanisms may under some circumstances involve Cav-1. However, the AR and Akt1 membrane interaction described by Cinar et al was Cav-1-independent, indicating that Cav-1 is not obligatory for cross-talk between these critical signaling pathways. Significantly, however, in PCa cells Cav-1 was identified as a negative regulator of the serine-threonine phosphatases PP1 and PP2A, which normally dephosphorylate Akt and thereby attenuate signaling through the PI3K/Akt pathway.[55] This regulatory role, which also affects other oncogenic kinases, such as PDK1, has the effect of potentiating Akt signaling and thereby increasing resistance of PCa cells to pharmacologic challenge. Thus, inhibition of tumor-suppressing phosphatases is one mechanism whereby overexpression of Cav-1 in PCa cells can promote tumor cell survival. Under conditions where PP1 and PP2A activity was suppressed by Cav-1, AR localization to nuclei was enhanced in a hormone-independent manner, suggesting that Cav-1 may mediate cooperative signaling between Akt and AR signaling mechanisms at the transcriptional level. Cav-1 was also shown to coprecipitate with PTEN,[56] pointing to the possibility that Cav-1-dependent effects on the PI3K/Akt pathway may arise at multiple levels.

EVIDENCE OF CAV-1 INVOLVEMENT IN PROSTATE CANCER

The first suggestion that Cav-1 might be an important protein in PCa came from an unbiased gene expression screen of isogenic mouse primary vs metastatic PCa cell lines.[57] In this seminal report by the Thompson laboratory, the *cav-1* gene was identified as upregulated in the metastatic lines using differential display-PCR. The same study described elevated expression of Cav-1 protein in human PCa in comparison to normal epithelial cells. Increased frequency of Cav-1 positivity was demonstrated in tissue from lymph node metastases in comparison to primary cancer, suggesting that overexpression of Cav-1 is a relevant feature of castrate-resistant disease. This and subsequent studies have shown that normal prostate epithelia are minimally reactive with anticaveolin-1 antibodies, while endothelial cells and smooth muscle cells of the prostate stroma express the protein at substantial levels. In contrast, Cav-1 expression trends quantitatively higher in prostate adenocarcinoma cells than in normal epithelia. A series of studies, including from our group, have confirmed that elevated expression of the protein is a marker of poor prognosis in localized human PCa and correlates positively with Gleason grade (the standard PCa grading system) and other indicators of aggressive disease. One study reported that the frequency of Cav-1-positive primary prostate tumors increased from 38% in the hormonally naive patient group to 73%

in the hormone refractory group.[58] Poor prognostic features that have been reported to correlate with increased Cav-1 expression include: lymph node metastasis, positive surgical margins, extraprostatic extension, seminal vesicle involvement, tumor angiogenesis and biochemical recurrence following surgery.[49,58-62] Additional reports have demonstrated that co-expression of Cav-1 and the oncoprotein c-Myc is a significant prognostic indicator of time to disease progression following surgery[63] and that Cav-1 expression is increased in PCa in African-American in comparison to Caucasian-Americans.[64] African-American men are at higher risk for aggressive PCa.

Our group demonstrated that levels of fatty acid synthase (FASN), the enzyme responsible for most long-chain fatty acid synthesis in tumor cells and Cav-1 are coordinately upregulated in human prostate tumors and physically interact.[61] In this study, levels of FASN and Cav-1 discriminated between localized and metastatic cancers, with the two proteins occupying similar subcellular locations in a tumor subset, suggesting a functional relationship that could play a role in PCa metabolism. FASN is the sole intracellular producer of palmitate in tumor cells and Cav-1 is a palmitoylated protein. Another study from our laboratory that characterized palmitoylated (S-acylated) proteins in prostate tumor cells on a proteome scale identified Cav-1 as a central node of a novel signaling network localized to lipid raft membranes[50] (Fig. 1). These findings imply that Cav-1 may play an important role in lipid-dependent metabolic pathways relevant to oncogenesis.

Inactivation of the endogenous *cav1* gene in the mouse was able to attenuate prostate tumor progression in the TRAMP model of autochthonous PCa, which is driven by the potent T-antigen (Tag) oncogene under the control of a prostate-specific promoter (probasin).[65] Immunohistochemical analysis of the level of Tag expression and localization to nuclei with the temporal appearance of elevated Cav-1 expression in TRAMP/*cav1* ($^{+/+}$) animals indicated that the ectopic expression of Tag is not the basis of Cav-1 upregulation seen in TRAMP animals (consistent with the human data), but rather that Cav-1 overexpression is a feature of activation of endogenous processes as a result of the carcinogenic process, consistent with findings using human tissues. Although this is an artificial system with limited mechanistic relevance to human PCa, it does demonstrate the capability of the intact *cav1* gene to promote tumor progression from the native environment of the prostate in vivo. These studies also revealed that loss of only one *cav1* allele was sufficient to greatly retard prostate tumor progression in the TRAMP model, suggesting that partial loss of Cav-1 expression is sufficient to inhibit PCa progression. More recently, we found that genetic ablation of Cav-1 in TRAMP mice causes a dramatic reduction of FASN levels,[60] suggesting that Cav-1 might play a role in regulating over-expression of this enzyme, which is common in PCa and other malignancies.

The consequences of *cav1* genetic loss using this mouse PCa model are in contrast to studies in the murine mammary gland, most of which suggest that Cav-1 exerts a tumor-suppressor rather than a tumor-promoting role.[66,67] This marked disparity between two hormone-sensitive, secretory organs that are frequently compared and considered similar in many ways, is intriguing and provides striking evidence for a context-dependent physiologic role for the Cav-1 protein.

Experimental evidence supporting the involvement of Cav-1 in castrate-resistant PCa was first presented by the Thompson group using an approach where Cav-1 expression was manipulated in mouse PCa cell lines.[68] Suppression of Cav-1 expression converted androgen-insensitive cells to an androgen-sensitive phenotype and selection for androgen resistance in vivo correlated with increased Cav-1 levels. These findings provided compelling early evidence that increased Cav-1 expression is one component of the means by which PCa becomes resistant to androgen ablation therapy.

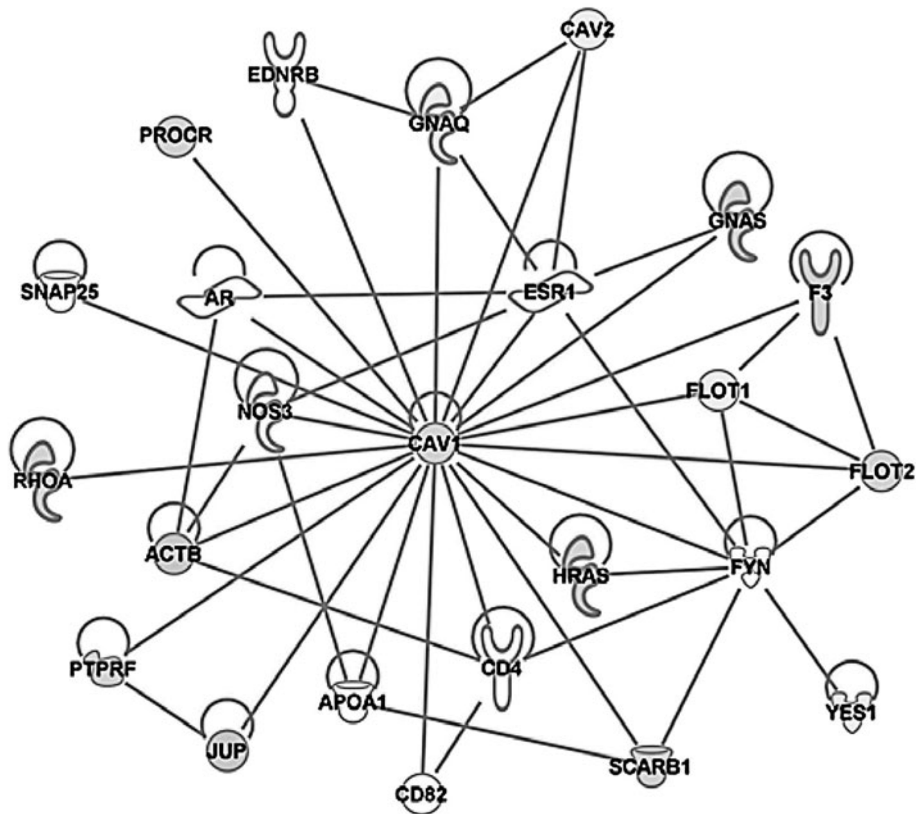

Figure 1. Caveolin-1 is the central node of a presumptive signaling network comprising multiple proteins that may be modified by dynamic and reversible S-acylation (palmitoylation). Gray nodes indicate S-acylated proteins that were identified by mass spectrometry in DU145 human prostate cancer cells. Originally published in Yang W et al. Mol Cell Proteomics 2010; 9:54-70;[50] © the American Society for Biochemistry and Molecular Biology.

Further studies revealed that testosterone stimulates *cav1* transcription in prostate cancer cells and that increased prostate tumor cell viability and clonal growth in culture arising from increases in testosterone concentration in the medium are mediated by Cav-1.[69] Because the AR continues to function in the hormone-repressed state, these findings provide direct evidence that AR signaling can modulate mechanisms controlling Cav-1 gene and protein expression and that the AR is likely to promote increased Cav-1 levels during disease progression. Cav-1 was also shown to promote metastasis in experimental models.[58,65,69] The pro-survival function of Cav-1 is not restricted to the androgen-AR axis because Cav-1 has been shown to suppress apoptotic signals from sources other than androgen withdrawal from hormone-sensitive cells, such as apoptosis induced by enforced *c-myc* expression.[70] Low levels of Cav-1 expression are also sufficient to confer a pro-survival advantage to prostate tumor cells. Experiments in animals using anti-Cav-1 antibodies and antisense approaches have demonstrated that Cav-1 is a potential therapeutic target.[68]

CELL-ASSOCIATED CAVEOLIN

Cav-1 is the primary protein constituent of the caveolar form of lipid raft microdomain and is responsible for the invaginated architecture of caveolae, recognizable as flask-shaped structures in electron micrographs. Homozygous ablation of the *cav1* and *cav3* genes in mice is sufficient to abolish caveolae formation in all organs.[71] Caveolae cannot be studied in the absence of caveolin, providing an essential challenge in determining whether a given effect of caveolar loss or gain is attributable to the membrane microdomain or to the caveolin protein itself. Consequently, the role of caveolae in PCa is still poorly understood.

"Flat" lipid rafts that do not contain caveolin proteins and consequently do not form membrane invaginations, have been described.[36] They are biochemically similar to caveolae and contain relatively high levels of glycosphingolipids and cholesterol. Normal prostate tissue has high cholesterol content, about the same concentration as the liver. Schaffner and colleagues provided the first evidence that lowering cholesterol levels alters prostate tissue homeostasis.[72] These investigators showed that prostate regression was evoked in animals by oral administration of hypocholesterolemic agents, such as the polyene macrolide candicidin.[72,73] Candicidin and structurally similar compounds bind to cholesterol and inhibit its absorption through the intestine. A similar cholesterol-targeting strategy was used in an experimental PCa study by Solomon and coworkers in which an FDA-approved drug (ezetimibe), which blocks the intestinal cholesterol transporter NPC1L1, was used to lower circulating cholesterol and the effect on growth of human PCa xenografts determined.[74] Cholesterol was also raised in another arm of the study using an isocaloric diet manipulation. Ezetimibe was found to inhibit growth of human LNCaP xenografts in mice and an unanticipated, dramatic antitumor angiogenesis effect of the drug was observed. Conversely, the high cholesterol diet, which increased circulating cholesterol, promoted tumor angiogenesis and tumor xenograft growth. These findings are significant because the LNCaP model is Cav-1-negative; consequently, the cholesterol-dependent effects on tumor growth cannot be attributed to Cav-1.

An important role for cholesterol in prostate tumor growth has recently received support from experimental and clinical observations. Older observations reported a correlation between the presence of cancer and increases in tissue cholesterol content along with evidence that irregularities in lipid metabolism underlie the basis for cholesterol accumulation in the prostate.[73,75] Several prospective observational series have now shown that long-term cholesterol-lowering therapy using HMG-CoA reductase inhibitors (aka "statins") reduces the risk of aggressive prostate cancer, suggesting that cholesterol localization in prostate tumor cells can promote either cell proliferation or other aggressive behaviors.[76-79] The prostate normally synthesizes large amounts of cholesterol, comparable to the liver. Androgens stimulate lipogenesis in human PCa cells by promoting transcription of genes, such as those encoding FASN and HMG-CoA reductase (the rate limiting step in cholesterol biosynthesis). Several unbiased studies have shown that androgens regulate lipogenesis in several cell types at the genome level.[80-82] Studies in the Cav-1-negative LNCaP cell line have demonstrated a role for membrane cholesterol in signal transduction mechanisms relevant to castrate-resistant PCa. The pathways shown to be affected by targeting membrane cholesterol in various ways include interleukin-6 to STAT-3, AR and Akt signaling mechanisms.[54,83-86] One explanation for cholesterol-dependent effects on signal transduction involves perturbations of signaling through multi-protein complexes that reside within lipid raft microdomains.[54,84,86] Another explanation involves the role of cholesterol as a metabolic precursor of androgens and the ability of tumor cells to

synthesize sufficient levels of androgen to promote tumor cell growth.[29] The relative contribution of lipid raft-dependent mechanisms, in comparison to effects on anabolic metabolism arising from perturbations in cholesterol homeostasis, remain to be assessed.

Studies in Cav-1-negative LNCaP cells are informative because they have been used to demonstrate the importance of cholesterol-dependent mechanisms employing lipid rafts, but show that Cav-1 expression is not required to elicit tumor growth or tumor cell survival effects. Whether lipid raft pathways present in Cav-1-negative cells might be enhanced by upregulation of Cav-1 is unknown. The physiologic distinction between caveolar and flat rafts is unclear.[36] Along with Cav-1 (or Cav-3 in the case of skeletal and cardiac muscle), caveolae formation requires a collaborating protein, polymerase I and transcript release factor (PTRF), also called cavin-1.[87-89] PTRF is a soluble, cytosolic protein that is recruited to the membrane to generate caveolae. Cav-1 remains associated with the plasma membrane in cells in which PTRF is silenced, despite the loss of caveolae, but lateral mobility and lysosomal degradation of Cav-1 are increased. At this writing, a single study has reported that PTRF expression is decreased in human PCa tissue.[90] If this finding is confirmed and expanded, the implications are that PCa cells in vivo may over-express Cav-1 in the absence of typical caveolar architecture. The biological implications of such a scenario are unknown. The stepwise, hierarchical assembly of Cav-1/PTRF complexes within the ER, Golgi structures and the plasma membrane suggests that caveolar membranes, distinct from flat rafts, perform specialized roles.[91]

Cav-1 regulates lipoprotein uptake and thereby affects lipoprotein and triglyceride metabolism.[92,93] Cell associated functions that involve the Cav-1 scaffolding domain, such as kinase regulation, are also unlikely to be replicated by flat lipid rafts unless the Cav-1 activity can be provided by another signaling protein. Cav-1 overexpression may feed forward onto lipid raft signaling more generally because the protein is a regulator of intracellular cholesterol level. Because Cav-1 is a mediator of LDL uptake, increases in Cav-1 in adenocarcinoma cells may promote cholesterol accumulation in cancer nodules. Increases in cholesterol within or near the cancerous tissue may then alter signal transduction mechanisms through more generic lipid raft mechanisms, or by stimulating intratumoral synthesis of androgen. One anticipated consequence of cholesterol accumulation in normal or malignant prostate tissue would be an increase in tissue inflammation, a potential effector of both benign urologic disease as well as cancer.

SECRETED CAVEOLIN

The studies described above point to an important role for increases in Cav-1 expression in the progression of PCa to castrate-resistant disease. Although over-expression of Cav-1 is common in PCa and tracks with aggressive disease, there is still considerable heterogeneity in the immunostaining patterns seen in human tissues. Cav-1-positivity, as measured by conventional immunohistochemistry, ranges from 40-60% cells in metastatic tumors.[49,57] These findings of tumor heterogeneity with respect to patterns of expression of the Cav-1 protein suggest the possibility that Cav-1 might be exported into the extracellular space and play a role in the tumor microenvironment as a component of paracrine or endocrine signaling mechanisms.

Cav-1 is in fact secreted by mouse and human prostate tumor cells in culture and secretion can be stimulated by androgen in Cav-1-expressing cells expressing AR.[58] Cav-1 is also detectable in human serum, can be measured using enzyme linked absorbent

assay (ELISA) and in its circulating form has been shown to be informative clinically as a biomarker capable of predicting disease recurrence.[58,94,95] Biochemical fractionation suggests that in its soluble form the protein associates with some kind of lipoprotein particle.[96] Experiments with conditioned media indicate that Cav-1-expressing PCa cells secrete a bioactive component that can promote cell survival and clonal growth and which can be inhibited with anti-Cav-1 antibodies,[58] indicating that Cav-1 is a critical component of this paracrine mechanism. Expression of Cav-1 in Cav-1 deficient prostate tumor cells was shown to promote tumor growth in vivo and Cav-1-secreting cells were demonstrated to be targetable in vivo using anti-Cav-1 antibodies, which elicited a therapeutic effect in model systems.[58] Using a xenograft LNCaP model, Bartz et al showed that subcutaneous tumors seeded with Cav-1-deficient cells became Cav-1-positive when Cav-1 expressing tumors were grown on the contralateral side.[96] These findings suggest that Cav-1 circulating in the blood can be incorporated into tissue sites distant from the point of secretion and that circulating Cav-1 can exert autocrine, paracrine and/or endocrine effects within the tumor microenvironment and at other locations.

Di Vizio et al recently reported the identification of a novel type of bioactive vesicular particle, within the size range of 1-10 μm, secreted by prostate tumor cells in response to epidermal growth factor (EGF) and/or silencing of the actin nucleating protein, DRF3.[97] DRF3 is encoded by a gene (*DIAPH3*) that undergoes chromosomal deletion at high frequency in aggressive prostate cancer.[97] These microvesicles, termed "oncosomes," contain Cav-1, indicating that the circulating form of Cav-1 may be transported in a wide range of particle classes and sizes. Oncosome secretion elicited by EGF receptor activation coincides with dramatic changes in cell shape, cytoskeleton structure and localization of the phosphorylated form of Cav-1 (Fig. 2). Preliminary characterization by mass spectrometry of other protein cargo of oncosomes produced by LNCaP cells suggests that this large class of particle could serve as a mechanism for the widespread dissemination of cell-free signaling complexes with bioactive capability.[97] The role of Cav-1 in these signaling events is unknown, but antibodies against Cav-1 have been shown to inhibit bioactivity detectable in prostate cancer cell secretions.[58] When considered in combination with published data that Cav-1 levels in serum are potentially clinically informative, particularly when combined with other disease parameters such as preoperative PSA, these experimental findings suggest that examining the contents of Cav-1-enriched particulate fractions in serum using mass spectrometry-based proteomics methods may provide a source of new biomarkers with clinical relevance.

STROMAL CAVEOLIN

Stromal cells react to the presence of epithelial tumors by initiating a complex cascade of events that are still poorly understood. Adenocarcinomas promote a "desmoplastic" reaction in which cancer-associated fibroblasts take on the characteristics of myofibroblasts more characteristic of wound healing. Phenotypic transformations in the stromal population may conceivably arise from changes in the gene expression program of the native stromal cells, recruitment of stem cell populations, or epithelial-to-mesenchymal or endothelial-to-mesenchymal transitions. It is now well established that these "cancer-associated fibroblasts" (CAF) are active participants in tumorigenesis via mechanisms that involve remodeling of the extracellular matrix, secretion of bioactive proteins of several kinds (particularly TGFβ1) and induction of angiogenesis.[98,99] CAF

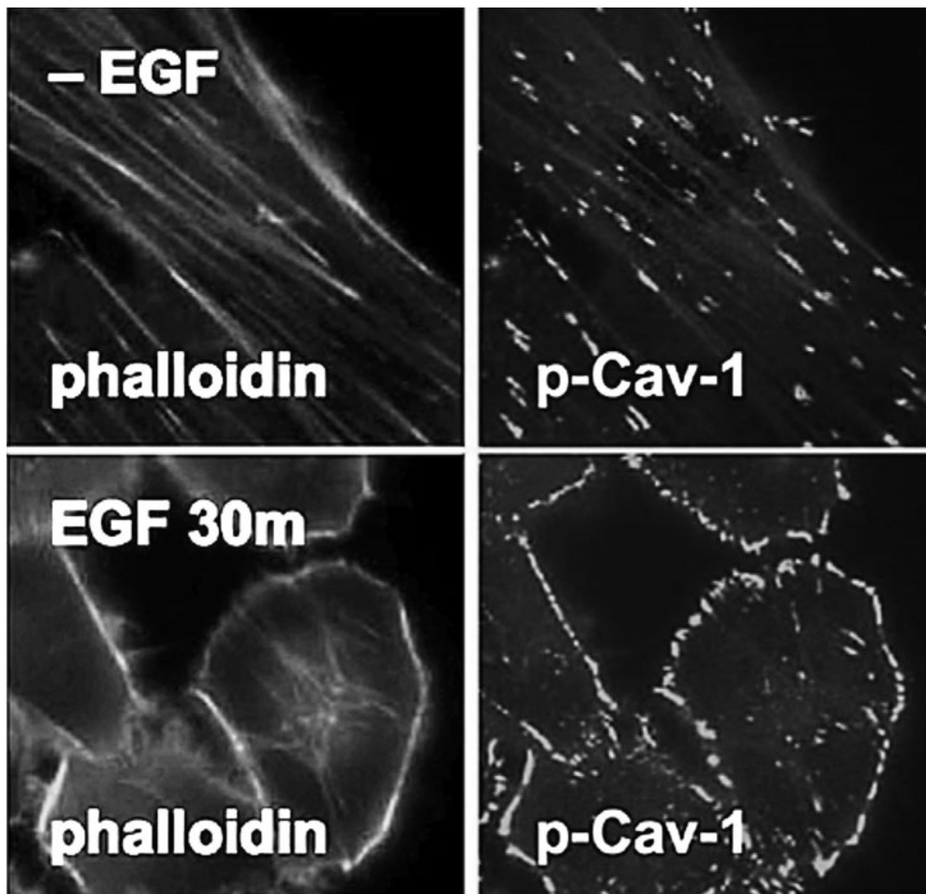

Figure 2. EGF induces rapid changes in cell shape, reordering of the cytoskeleton and redistribution of phosphorylated caveolin-1 (Tyr14) to the plasma membrane in DU145 human prostate cancer cells.

communicate with a variety of cells in the tumor microenvironment, including endothelial cells, immune cells, inflammatory cells, bone marrow-derived stem cells, adipocytes and carcinoma cells, through paracrine signaling mechanisms that can promote tumor expansion and matrix invasion. Recent studies of CAF in human tumors strongly suggest a direct role of the stroma in progression to advanced disease.[100-102]

In contrast to the upregulation of Cav-1 seen in aggressive PCa cells, studies of NIH3T3 fibroblasts transformed with several oncogenes demonstrated that Cav-1 levels were down-regulated and caveolae formation was ablated, by these manipulations.[103-105] Consistent with these experimental data, CAF isolated from breast cancer patients showed down-regulation of Cav-1.[100,106] Tumor implantation into the mammary fat pad of Cav-1($^{-/-}$) mice resulted in a significant enhancement of tumor growth, suggesting that Cav-1-negative stroma is tumor promoting. RNA profiling of human breast CAF indicated that Cav-1 downregulation corresponded with a gene signature resembling that seen with functional repression of the RB1 tumor suppressor gene. Additional data suggest that loss of stromal Cav-1 can be used clinically as a novel biomarker of poor

clinical outcome in breast cancer patients, with Cav-1 loss predictive of recurrence-free survival and progression to invasive disease or metastases.[107] Similar to breast cancer, limited studies in PCa have shown that loss of stromal Cav-1 is positively correlated with increased Gleason score and progression to metastatic disease.[108] Additional unpublished data from our group indicate that silencing of Cav-1 in prostate stromal cells promotes PCa tumor cell migration and predicts disease-free survival. Collectively, these and other reports of the stroma reaction to adjacent tumor, the so-called "reactive stroma," are consistent with the hypothesis that carcinoma growth, survival and progression are mediated in some cases by Cav-1 downregulation in the stromal compartment.[98,99,109]

CONCLUSION

Cav-1 is a multi-functional protein that promotes PCa progression when expressed at elevated levels by adenocarcinoma cells and when downregulated in prostate stromal cells. The specific mechanisms where by Cav-1 takes on oncogenic cellular activities are not well understood, but its role as a binding partner of many signal transduction proteins and as a mediator of cholesterol and fatty acid metabolism, are likely to be critical aspects of its oncogenic function. The evidence we have described indicates that increased Cav-1 expression promotes phenotypic transformation from an androgen-sensitive phenotype to an androgen-insensitive phenotype. Although the biochemical data described in this chapter strongly support a mediating role for Cav-1 in signaling through the androgen-AR axis, it is nevertheless remarkable that a single protein that resides primarily at extranuclear membrane sites and primarily at the plasma membrane when expressed at high levels, is capable of conferring androgen-insensitivity to PCa cells. Because Cav-1 is found in both cell-associated and secreted forms, it is important to consider how the protein may operate in several distinct tissue compartments during disease progression. Future studies will employ new technologies and approaches to dissect these diverse functions. Because of its role in a wide variety of pathways and processes, studies of Cav-1 and its signaling partners are likely to provide new avenues toward the treatment of castrate-resistant PCa.

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CHAPTER 8

CAVEOLINS AND CAVEOLAE, ROLES IN INSULIN SIGNALLING AND DIABETES

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Abstract: Much data in the scientific literature demonstrate a fundamental involvement of caveolae in insulin action, although particular aspects remain matters of debate. The insulin receptor and part of the downstream signalling mediators are localized in or recruited to caveolae. Moreover, as part of the signalling, insulin receptors are rapidly endocytosed by caveolae in response to the hormone. The insulin regulated glucose transporter GLUT4 appears to localize to caveolae after insulin-stimulated translocation to the plasma membrane, while the endocytosis of GLUT4 may involve a clathrin-mediated process. Insulin resistance due to dysfunction of insulin signalling in target tissues is a primary cornerstone of Type 2 diabetes. Lack of caveolae makes animals and human beings insulin resistant, but there is presently no evidence that caveolae play a role in the pathogenesis of insulin resistance in obesity and Type 2 diabetes.

INTRODUCTION

Insulin is perhaps the most important anabolic hormone and has, as such, a primary role in directing nutrients to synthetic processes and storage in connection with a meal. However, between meals and during fasting, a falling insulin concentration mobilizes stored energy reserves. Most cell types express receptors for insulin, but the highest concentrations of the receptor are found in the major metabolic target cells of the hormone: muscle, adipose tissue and liver. Liver in particular also has a quantitatively important function to dispose of circulating insulin after receptor-mediated uptake. Insulin further has important effects in pancreatic β-cells that produce the hormone and in the brain to regulate feeding and satiety.

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This chapter examines the localization of the various components of the insulin signalling network and metabolic processes to caveolae and then examines the involvement of caveolae in the pathogenesis of insulin resistance in Type 2 diabetes and obesity.

INSULIN SIGNALLING IN CAVEOLAE

Localization of Insulin Receptors in Caveolae

Insulin regulates its target cells via an intracellular signalling network which involves the ligand-activated insulin receptor (IR). IR is a tetrameric protein consisting of two α and two β subunits that span the plasma membrane. In the plasma membrane, IR is mainly localized in caveolae with, at least in adipocytes, very little receptors found in the membrane outside caveolae. Electron microscopy, immunofluorescence confocal microscopy, plasma membrane fractionation and functional studies have established that IR is localized to caveolae in adipocytes (Fig. 1).[1-7] Earlier electron microscopic analysis of metal-labelled insulin also indicated, with hindsight, the presence of IR in caveolae.[8,9] However, IR was first considered not to be localized in caveolae,[10-12] a conclusion eventually demonstrated to be wrong. Corley-Mastick et al[13] reported that IR catalyzed the phosphorylation of caveolin-1, but that it was not present in caveolae isolated as a detergent resistant fraction of cells. It was later demonstrated that IR is solubilised from caveolae by detergent treatment.[1] Souto et al[12] failed to detect IR or other signalling proteins after immunoprecipitation of caveolin from the microsomal fraction of adipocyte homogenates. However, although not commented on by the authors, after density-gradient centrifugation, the small amount of IR present cofractionated closely with caveolae. After insulin stimulation, an increased intracellular level of IR was recovered in an uncharacterized fraction that almost certainly represents endocytosed IR.[12] Finally, using electron microscopy, Carpentier et al[10] had reported that IR was localized in clathrin coated pits of 3T3-L1 adipocytes, but have recently confirmed the localization of IR in caveolae.[6]

Adipocyte plasma membrane have a very high density of caveolae, with caveolae constituting one-third of the membrane area.[14] A quantitative analysis of caveolae reveals that there are fewer insulin receptors than caveolae in an adipocyte.[14] A similar relationship has been reported for specific gangliosides such as GD3 or GM1 that are concentrated in caveolae, but on average there is no more than one molecule of GD3 or GM1 per caveola.[15] However, caveolae that are immunolabelled for IR are often labelled multiple times,[1,3,8] indicating that there are several receptors per caveola in a subset of adipocyte caveolae. Likewise, it is likely that GD3 and other glycosphingolipids present in equimolar or lower levels in caveolae are concentrated in subsets of caveolae. Indeed, subclasses of caveolae with different functions have been isolated from adipocytes and characterized.[5,16]

Furthermore, caveolae in muscle,[17] rat liver,[18] hepatocytes,[19] endothelial cells,[20,21] and pancreatic β-cells[22] have been described to harbour, at least a fraction of, the plasma membrane IR. In liver of rats fed a cholesterol rich diet, caveolae contained more IR and insulin activation of IR was enhanced compared to rats fed a chow diet.[18] Most interestingly, expression of caveolin-3 in livers of diabetic mouse models improved glucose metabolism and enhanced insulin sensitivity.[23] In particular, insulin stimulation to increased glycogen synthesis was improved. It is not clear why the authors chose caveolin-3, but presumably caveolin-3 can act as a caveolin-1 substitute in hepatocytes. There is no evidence of a regulated translocation of IR in or out of caveolae in the plasma membrane,[24] as reported for some hormone receptors.[25]

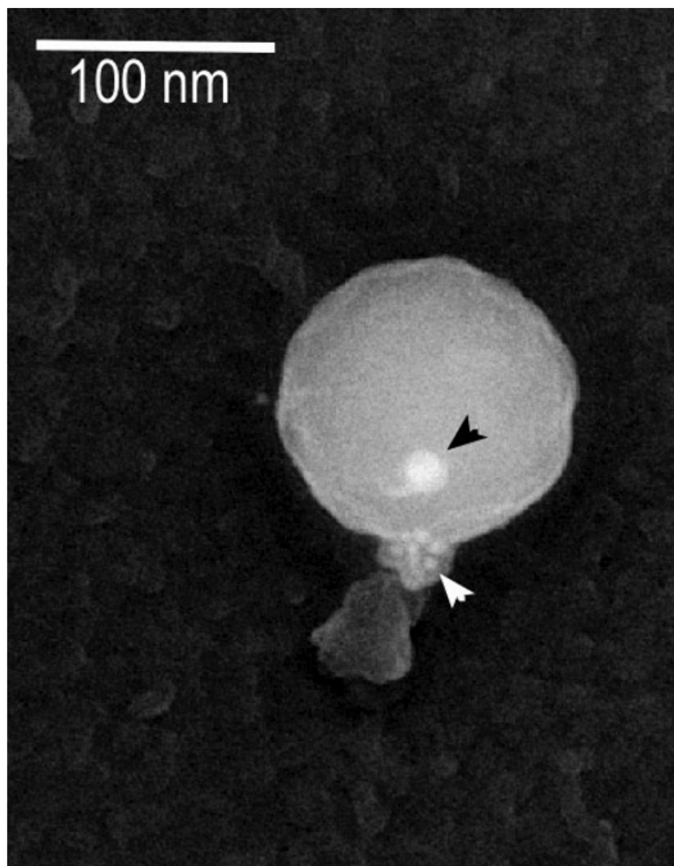

Figure 1. Electron micrograph of the inner surface of the plasma membrane from a human adipocyte. A single caveola attached to the plasma membrane is seen by immunogold labelling of the insulin receptor (large gold particle, black arrowhead) and caveolin-1 (small gold particles, white arrowhead). Reprinted with permission from Karlsson M et al. Eur J Biochem 2004; 271:2471-2479.[3] ©2004, John Wiley and Sons.

However, in an hepatoma cell line lacking caveolae and caveolin, IR was reported to translocate into detergent-resistant lipid rafts in response to insulin treatment,[26] the relevance of which remains to be understood. In pancreatic β-cells, signalling by IR of both A and B types was impaired by cholesterol depletion or by expression of a dominant-negative caveolin-1 mutant,[22] indicating that also in these cells IR is a caveolar protein.

Endocytosis of Insulin Receptor by Caveolae

In parallel with insulin activation, IR is endocytosed in a caveolae-mediated process that appears to be triggered by the autophosphorylated IR catalyzing the phosphorylation of caveolin-1 at tyrosine-14.[24] The endocytosed receptor passes through a caveosome-like endosomal compartment before it is dephosphorylated and returned to the plasma membrane.[24] Under steady-state conditions, only a few percent of IR is in the intracellular compartment in adipocytes.[24,27] Also, human adipocytes do not degrade insulin to any

appreciable extent,[27] implicating that endocytosis and fusion with lysosomes is only of minor importance in adipocytes. This is in sharp contrast with observations made in the liver and hepatocytes where IR endocytosis is much slower and a major part of insulin is routed to lysosomes for degradation and removal from the circulation. In the latter case, the endocytosis of IR may be mediated by clathrin coated pits (see below).

Caveolae-mediated endocytosis of IR is important for signalling as it is critically required for a rapid (1-2 min) negative feedback that enhances dephosphorylation and deactivation of the autophosphorylated and activated IR.[27,28] Blockade of caveolae-mediated endocytosis of IR and the endocytosis-dependent feedback increased signalling strength several fold in human adipocytes.[27] This process, which directly involves caveolae, constitutes an interesting novel target for the treatment of insulin resistance in Type 2 diabetes.

Insulin Receptor Signalling in Caveolae

In contrast to many other receptors and signalling proteins found in caveolae, IR signalling is not inhibited by caveolin. On the contrary, the effect of caveolar localization of the IR could be to augment signalling. This notion has direct support in experiments where overexpression of caveolin-1 or -3 in HEK293T cells enhanced insulin-stimulated phosphorylation of the insulin receptor substrate-1 (IRS1) without affecting IR autophosphorylation.[29] Accordingly, caveolae-depletion in rat adipocytes inhibited insulin-stimulated phosphorylation of IRS1 without affecting IR autophosphorylation.[4] Likewise, after overexpression of caveolin-1 in Cos-7 cells, insulin-mediated phosphorylation of the transcription factor Elk-1 was enhanced, while the phosphorylation of ERK2 was blocked.[30] When transfected in Cos-7 cells, IR harboring mutations in the caveolin-binding motif was expressed at substantially lower levels as compared to the wild-type receptor.[30] This is in agreement with a requirement for caveolin-1/caveolae to maintain cellular levels of IR (see below). The interaction between caveolae/caveolin-1 and IR and mechanisms for localisation of IR to caveolae, have been reviewed in details elsewhere.[31] Interestingly, the ganglioside GM3, which is normally present both inside and outside of caveolae in adipocytes,[15] was shown to interfere with the IR–caveolin-1 interaction in 3T3-L1 adipocytes and mice lacking GM3 exhibited enhanced insulin sensitivity.[7,32]

Insulin Receptor Downstream Signalling in Caveolae

Signalling specificity in the context of shared signalling intermediates involves clustering and compartmentalization, with caveolae as a prime candidate to achieve a specificity of insulin signalling that can be further augmented by targeted endocytosis of the activated receptor by caveolae.[24] When activated by insulin binding, autophosphorylation of IR activates the protein kinase domains of the receptor toward downstream signalling proteins. In particular, IRS1 binds to the autophosphorylated IR and is immediately phosphorylated at specific tyrosine residues. Thus, phosphorylated IRS1 acts as a hub that can interact with different downstream signalling proteins containing phosphotyrosine-binding SH2-domains. Interestingly, in human adipocytes, but not in murine adipocytes, IRS1 colocalized with IR in caveolae under basal, non-insulin-stimulated conditions.[3,33] Insulin therefore induces a two-fold increase in plasma membrane-associated IRS1 in human adipocytes but more than a ten-fold increase in rat adipocytes.[33] The caveolar localization of IRS1 in human adipocytes appears specific to the human IRS1

protein as human-IRS1 binds to caveolae in both human and rat adipocytes in the absence of insulin, while rat-IRS1 does not bind to caveolae in adipocytes from either species.[33] In rat liver, both IRS1 and the phosphoinositide 3-kinase (PI3kinase) were enriched in caveolae after insulin treatment.[19] The significance of the localization of IRS1 in caveolae is demonstrated by the different effects of cholesterol and caveolae depletion in human compared to rat adipocytes (see below).[3,4] Also, the finding that caveolin-1 knockout reversibly increased the degradation of IRS1,[34] similarly to findings for IR (see below),[35-37] supports a direct interaction between IRS1 and caveolae/caveolin-1.[34,38]

The tyrosine-phosphorylated IRS1 protein binds and activates PI3kinase, through the SH2-domain of PI3kinase and generates phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) from the precursor phosphatidylinositol-(4,5)-bisphosphate (PIP2) in the plasma membrane. PIP3 in turn, binds and concentrates 3-phosphoinositide-dependent kinase-1 (PDK1) and its substrate, protein kinase B/Akt (PKB), through their pleckstrin-homology (PH) domains. PKB can then be efficiently phosphorylated at threonine 308 by PDK1 and at serine 473 by mTORC2 to activate the protein kinase. PIP2 has been shown to localize in plasma membrane caveolae in A431 cells[39] and recently, with high resolution microscopy, to be concentrated in caveolae in human fibroblasts.[40] It is probable that insulin induces the production of PIP3 in caveolae. Indeed, in response to insulin, PI3kinase was found to colocalize with IR in caveolae of rat livers.[19] Moreover, in response to insulin, PKB and PDK1 were reported to localize to caveolae in cultured muscle cells,[41] likely as a result of the increased concentration of PIP3 in the caveolae membrane. Expression of caveolin-3 in the liver of mice improved insulin sensitivity and strongly enhanced insulin-induced phosphorylation of IRS1 and PKB.[23] There is also some evidences from the human A431 cell line suggesting that the phospho-tyrosine specific protein phosphatase PTP1B, which is a major phosphatase acting on IR and IRS1, is localized in caveolae.[42]

CAVEOLAE DEPLETION AND INSULIN SIGNALLING

Caveolae Depletion In Vitro

A dependence of insulin signalling on intact caveolae was suggested by experiments involving depletion of cholesterol and hence caveolae, in cells or, more specifically, in the plasma membrane. Partial depletion of plasma membrane cholesterol with methyl-β-cyclodextrin destroys caveolae structure in primary cultures of human,[3] rat[4] and 3T3-L1[43] adipocytes. In rat adipocytes, partial extraction of cholesterol interfered with IR-mediated phosphorylation of IRS1, without affecting insulin binding or IR autophosphorylation.[4] In human adipocytes with a caveolar colocalisation of IRS1 and IR, in contrast, cholesterol depletion affected neither activation of IR nor phosphorylation of IRS1, but blocked downstream signalling and phosphorylation of protein kinase B.[3] Also, depletion of cholesterol in pancreatic β-cells impaired insulin signalling.[22] Similarly, inhibition of cholesterol biosynthesis in 3T3-L1 preadipocytes disrupted caveolae and reversibly impaired insulin signalling to phosphorylation of PKB and inhibited insulin stimulation of glucose uptake, without changes in IR protein levels.[44] Although cholesterol depletion is not a very specific treatment as it also affects noncaveolar rafts and can affect clathrin-coated pits, it dramatically affects caveolae[4,14] and indicates that, while not necessarily required for IR activation, caveolae have a critical role in insulin signalling. In a different approach to examine the role of caveolae in IR signalling, caveolin-1 was knocked down in 3T3-L1

adipocytes, which increased the degradation of IR,[37] indicating a requirement for caveolin/caveolae to stabilize IR in these cells (see below). In pancreatic β-cells, expression of a dominant-negative caveolin-1 mutant impaired insulin signalling.[22] In conditionally immortalized skeletal muscle cells that lack caveolin-3, insulin activation of PI3kinase and PKB was strongly inhibited.[45]

Caveolae Depletion In Vivo

Caveolin-1$^{-/-}$ mice lack caveolae in all tissues except muscles that express caveolin-3. On a high fat diet, they develop postprandial hyperinsulinemia demonstrating that the animals are insulin resistant (reviewed in ref. 46). With age, the adipose tissues and adipocytes of these animals exhibit increased derangement and atrophy (reviewed in ref. 47). Not surprisingly, the caveolin-1 null mice have drastically reduced levels of the circulating adipokines leptin and adiponectin.[48] One form of human congenital generalized lipodystrophy (CGL3) is the result of a mutation in the caveolin-1 gene, which introduces a stop codon at the beginning of the transcript.[49] Similarly to caveolin-1$^{-/-}$ knockout mice, these patients' cells lack caveolae. The main feature of the lipodystrophy is a nearly complete lack of adipose tissue. Patients are severely insulin resistant, but this may not necessarily depend only on the lack of caveolin-1 and caveolae, but on the consequential lack of adipose tissue. Lack of caveolae robs the adipocytes of their primary protection against the detergent effects of fatty acids. Fatty acids are normally produced and disposed of in adipocytes and uptake from and release to the circulation involves transport of huge amounts of fatty acids over the plasma membrane of these cells.[14] Fatty acids are effective detergents that rapidly dissolve the adipocyte plasma membrane if allowed to accumulate.[50] Fatty acids are therefore normally channelled through the detergent resistant caveolae.[51-53] Indeed so potentially harmful are fatty acids that, upon uptake from the circulation, they are converted into harmless triacylglycerol in a specific subset of caveolae dedicated to this task.[5,16,52] The lack of caveolae and the consequential failure to cope with fatty acids is likely an important aspect of the lipoatrophy in caveolin-1 deficient patients and animals. Such conclusion finds further experimental support in recent developments in the biogenesis of caveolae that was found to require not only caveolin, but also the major protein constituent of caveolae polymerase I transcript release factor (PTRF)/cavin-1.[54,55] PTRF/cavin-1$^{-/-}$ mice also lack caveolae and present with dyslipidemia and insulin resistance. The mice are reportedly lean but the adipose tissue has not been fully examined.[56] Similarly to caveolin-1$^{-/-}$ mice (see below), the levels of IR were reduced in muscle and adipose tissue of PTRF/cavin-1 null mice.[56] Recent discoveries of patients with null mutations in the gene encoding PTRF/cavin-1 report congenital generalized lipodystrophy (CGL4) with heterogeneous additional pathological features and insulin resistance, hyperinsulinemia and hypertriglyceridemia.[57-59]

Through a protocol of normal or over feeding of newborn mice which followed over- or under-nourishment during suckling, a small set of genes was identified that correlate with adipose expansion and is independent of genes and transcription factors associated with adipogenesis.[60] Among the proteins that are highly correlated with adipose expansion (i.e., fat accumulation), are caveolin-1, caveolin-2 and PTRF/cavin-1. These findings strongly underscore the importance of caveolae for adipocyte fatty acid/triacylglycerol metabolism. In line of these findings, the expression of caveolin-1 was strongly upregulated in human visceral and subcutaneous adipose tissue in obese subjects

compared to lean subjects.[61] There were, however, no difference between obese subjects with or without Type 2 diabetes.[61]

Muscle dysfunctions are found in caveolin-3$^{-/-}$ mice and in patients harbouring mutations of the caveolin-3 gene; both mice and men lack or have reduced levels of caveolae in muscle tissues because of the specific absence of caveolin-3 in muscle. The caveolin-3 knockout mice eventually develop increased adipocity, glucose intolerance and insulin resistance, presumably as a result of impaired insulin signalling and hence impaired GLUT4 catalyzed uptake of glucose (see below),[35,62] although the amount of GLUT4 protein increased markedly in the skeletal muscle.[35] Striking findings were insulin resistance in the liver and strongly reduced glucose uptake by the adipose tissue. In addition, the levels of the adipokines leptin and adiponectin were increased and decreased, respectively.[35] Evidently, caveolin-3 is important for whole body energy homeostasis in mice. Defects in insulin signalling and the insulin resistance observed in caveolin-3$^{-/-}$ mice were reversed by genetic transfer of caveolin-3.[62] However, in humans with caveolin-3 mutations, no major defects in insulin signalling or glucose homeostasis have been reported.[63]

Stabilization of the Insulin Receptor in Caveolae

An interesting aspect of caveolin-1 and caveolin-3 deficiency is a reduction of the amount of IR in adipocytes[36,37] and skeletal muscle,[35] respectively. This decrease appears to be due to enhanced degradation of IR. Reduced amounts of IR were also found after caveolin-1 knockdown in 3T3-L1 adipocytes.[37] In addition, when transfected in Cos-7 cells, IR carrying mutations in the caveolin-binding motif was expressed at substantially lower levels compared to the wild-type receptor.[30] Likewise, a few patients with syndrome of extreme insulin resistance have been found to harbour mutations in the caveolin-binding motif of IR, which result in accelerated degradation of the receptor.[64-69] Interestingly, in cells lacking caveolae because of PTRF/cavin-1-knockout, the levels of IR are reduced in both adipose and muscle tissues.[56] This suggests a protective role of caveolin/caveolae to segregate IR from degradation by proteasomes or lysosomes. In adipocytes with functional caveolae, the IR is rapidly internalized (half-life of 1-2 min) in response to insulin and then apparently quickly recycled back to the plasma membrane. It is well known that in other cell types, primarily hepatocytes, insulin is internalized at a much slower rate (half-life of 10 min in Fao hepatoma cells[70]). This process may be mediated by clathrin and coupled to insulin degradation in the lysosomes, which is a major function of insulin endocytosis in hepatocytes. This is compatible with the findings that IR is degraded in both caveolin-1 and -3 knockout mice that lack caveolae, especially since the increased degradation in skeletal muscle is initiated by insulin.[35]

GLUT4 AND INSULIN-REGULATED GLUCOSE TRANSPORT IN CAVEOLAE

The insulin-regulated glucose transporter protein-4 (GLUT4) isoform is specifically expressed in metabolic target tissues of insulin: adipose tissue, and skeletal and heart muscles. These tissues also express the GLUT1 isoform but this transporter is not significantly affected by insulin. Liver and pancreatic β-cells express GLUT2 which is

also not regulated by insulin. Under basal conditions, in the absence of insulin, GLUT4 is largely sequestered intracellularly. Insulin enhances glucose uptake into the cells by inducing the translocation of GLUT4 to the plasma membrane,[71,72] where the transporter after integration into the membrane will catalyze glucose transport down its concentration gradient. This gradient is maintained by a rapid metabolism of cellular glucose via its phosphorylation by hexokinase. GLUT4 is constitutively endocytosed and this process may be slowed down by insulin, at least in adipocytes.[73] In the plasma membrane, GLUT4 has been repeatedly found in caveolae using a variety of techniques. However, this has been challenged by findings that fail to confirm the association of GLUT4 with caveolae or caveolin. Overall, it appears that plasma membrane GLUT4 clusters in caveolae, under both basal and insulin-stimulated states, but that endocytosis of GLUT4, particularly during insulin stimulation, may involve a clathrin-mediated process.

Localization of GLUT4 in Caveolae

Localization of GLUT4 in plasma membrane caveolae, especially after insulin treatment, has been indicated by the insolubility of GLUT4 during detergent extraction of whole 3T3-L1 adipocytes[74] or purified plasma membranes from primary adipocytes.[75] GLUT4 was also recovered in caveolae after purification of caveolae from adipocyte plasma membranes without detergent treatment.[5] Immunogold electron microscopic examination of plasma membranes from 3T3-L1 adipocytes has shown that a large percentage, but not all, of GLUT4 is localized in caveolae, in both control and insulin-stimulated cells.[76,77] Immunogold electron microscopy of L6 myotubes also revealed GLUT4 in noncoated invaginations resembling caveolae, although labelling for caveolin was not determined.[73] In rat hearts, immunoprecipitation of caveolin-3 coprecipitated GLUT4.[78,79] In a different approach, knockdown of a fatty acid hydroxylase in 3T3-L1 adipocytes increased lipid mobility in rafts and inhibited insulin-stimulated glucose uptake by routing GLUT4 to lysosomal degradation.[80] Cholesterol sequestration from the plasma membrane with β-cyclodextrin, which destroys caveolae, inhibited GLUT4 translocation and insulin-stimulated glucose uptake in conditionally immortalized mouse skeletal muscle cells.[45] Insulin-stimulated glucose uptake was similarly inhibited in immortalized skeletal muscle cells from caveolin-3$^{-/-}$ mice.[45] Knockdown of caveolin-1 in 3T3-L1 adipocytes dramatically increased GLUT4 degradation and, consequently, decreased its cellular levels.[37] Likewise, in the muscle and adipose tissue of PTRF/cavin-1$^{-/-}$ mice lacking caveolae, the amount of GLUT4 was reduced.[56] It is interesting to note that several reports have described decreased amounts of GLUT4 and increased degradation of GLUT4 in adipocytes after depletion of caveolae, which also appears to be the case with IR (see above). Note, however, that in muscles of caveolin-3$^{-/-}$ mice, the amount of GLUT4 is increased,[35] indicating that GLUT4 localization in caveolae may be quantitatively more important in adipocytes. It should also be noted that in skeletal muscle, much of the glucose uptake takes place in the T-tubules[81] that may be formed from caveolae,[82-84] but caveolin-3 is mostly associated with sarcolemmal caveolae in mature skeletal muscle fibers.[82,85]

It has long been known that in response to insulin, GLUT4 association with the plasma membrane is followed, with a lag time of several minutes, by the productive insertion of GLUT4 in the membrane to allow glucose uptake.[86-88] Interestingly, GLUT4 appearance in the caveolae fraction of rat adipocytes, but not GLUT4 association with the plasma membrane, in response to insulin, coincided with and paralleled the increase in glucose uptake by the cells.[75] Moreover, knockdown of caveolin-1 in 3T3-L1 adipocytes did not

interfere with GLUT4 translocation to the plasma membrane, but rather repealed the lag time,[89] suggesting that the lag is caused by the slow insertion or transfer of GLUT4 to caveolae. Using TIRF microscopy, GLUT4 has been found mainly as immobile clusters in the plasma membrane, with or without insulin treatment.[90] In response to insulin, GLUT4-containing vesicles fuse with the plasma membrane and most GLUT4 is immediately dispersed but rapidly clusters again in the plasma membrane.[90] Insulin increases both the density of GLUT4 clusters and the amount of GLUT4 in each cluster.[90] The rapid clustering of GLUT4 in the plasma membrane is compatible with a caveolar localization and the presence of large numbers of caveolae and with immunogold electron microscopic localization of GLUT4 in caveolae.[76] However, this observation is less easily reconciled with a clathrin-coated pit localization of GLUT4 and the small number of clathrin-coated pits found at any one time in the plasma membrane of adipocytes.[8]

GLUT4 has also been found, by electron microscopy, in clathrin-coated pits only[91,92] or neither in clathrin-coated pits nor caveolae.[93,94] Such failures to detect GLUT4 in caveolae by electron microscopy can be explained with the methodological considerations discussed in references 76 and 77. A reported failure to detect GLUT4 in the detergent-resistant fraction of the adipocyte plasma membrane is likely explained by the use of far too much detergent in relation to the amount of cell membranes.[95] It is important to realize that the detergent resistance of caveolae is not absolute but relative, as caveolae and rafts require higher temperature, more detergent and longer time for solubilisation compared with the surrounding plasma membrane. When caveolin-1-GFP and GLUT4-mCherry chimera were transiently cotransfected into primary rat adipocytes and examined by TIRF microscopy, a low extent of colocalization was interpreted as evidence against localization of GLUT4 in caveolae.[90] This may, however, not necessarily be correct as caveolin-1-GFP incorporates into existing stable caveolae at a very low rate.[96] Failure to detect colocalisation with GLUT4 may therefore reflect a very low fractional incorporation of caveolin-1-GFP in caveolae. A problem when studying primary rat adipocytes is that insulin-stimulated glucose uptake is severely impaired after overnight culture of the cells,[97] which is required to express fluorescent-tagged proteins. In addition, after more than 24h in culture, GLUT4 has been reported to relocalize in the cells.[98] A further inherent complication when examining plasma membranes by TIRF microscopy is that caveolae membrane may be 50-100 nm away from the flat cell membrane.[14] Because the fluorescence intensity will fall off exponentially with the distance from the cell-glass interface this will markedly affect fluorescence intensity in caveolae compared with the flat portions of the membrane.

Endocytosis of GLUT4

GLUT4 is constitutively endocytosed from the plasma membrane in a process that appears to involve both caveolae- and clathrin-mediated endocytosis, to varying extents in muscle and adipose cells, as reviewed in reference 73. Early findings have indicated that GLUT4 is endocytosed from caveolae in rat adipocytes,[75,89,95,99] but not necessarily in a caveolae-mediated endocytosis. Notably, in contrast to endocytosed IR,[24] intracellular GLUT4-containing vesicles do not contain caveolin.[100,101] A considerable body of evidences implicate both caveolae and clathrin in the endocytosis of GLUT4 from the plasma membrane in rat adipocytes,[90,102] 3T3-L1 adipocytes[99,103] and myoblasts.[104] Blot and McGraw[99] reported that the majority of GLUT4 was internalized by caveolae and a minority by clathrin-coated pits and that insulin blocked caveolae-dependent endocytosis. Antonescu et al[104] found evidences for both clathrin-dependent and clathrin-independent mechanisms

for GLUT4 internalization in myoblasts. Although, clathrin knockdown completely inhibited clathrin-mediated endocytosis, GLUT4 endocytosis was only reduced by 50%. Neither process was affected by insulin in these cells. By confocal immunofluorescence microscopy, only a minor fraction of GLUT4 was localised with clathrin in the plasma membrane of L6 myotubes.[73] TIRF microscopy has demonstrated that, directly after exocytosis, GLUT4 is dispersed but then clusters in the plasma membrane[90,98,103] and later colocalizes with clathrin in the plasma membrane.[90,103] Stenkula et al[90] found that the preclustered GLUT4 in the plasma membrane recruits clathrin for endocytosis.

As already discussed, caveolae are relatively static structures in the plasma membrane[96,105,106] that are rapidly endocytosed in response to specific stimuli such as loading of cargo,[107] or in response to insulin[24] or other ligands.[108] GLUT4 itself could serve as such a signal, but the constitutive endocytosis of GLUT4 appears to be retarded in response to insulin, at least in adipocytes,[73] i.e., in response to GLUT4 accumulation. Clathrin-coated pits, on the other hand, form as they are required for a slower internalization of cargo and at any one time very few clathrin-coated pits exist in the plasma membrane of primary adipocytes.[8,9,14] The density of clusters of GLUT4 in the plasma membrane, following maximal insulin stimulation,[90] corresponds to about 10% of the density of caveolae in the plasma membrane,[14] which by far outstrips the density of clathrin-coated pits.[8,9,14] This argues against GLUT4 clusters representing clathrin-coated pits, but is easily reconciled with GLUT4 clustering in a sub-population of caveolae before recruitment of clathrin and endocytosis of GLUT4. The localization of GLUT4 in the membrane has obviously not been unequivocally settled and one reason for this could be that GLUT4 is internalized by both caveolae and clathrin mediated processes, or even from caveolae through a clathrin mediated process.

It is apparent that so far scrutiny has not been directed at carefully testing whether GLUT4 is located in caveolae or not, but rather at showing that it is located in caveolae or not. Taken together, however, the available findings suggest that under basal conditions, a small fraction of GLUT4 in the plasma membrane is localized in caveolae and is constitutively endocytosed. Upon insulin-stimulated exocytosis, GLUT4 is dispersed in the membrane, but a majority of GLUT4 clusters in caveolae for glucose uptake and thus, clustered GLUT4 can be endocytosed in a clathrin-dependent process. The untangling of these events will be important but demanding.

CAVEOLAE IN INSULIN RESISTANCE AND TYPE 2 DIABETES

Mechanisms of Insulin Resistance in Type 2 Diabetes

Insulin resistance and Type 2 diabetes are closely linked to obesity, although not everybody who has Type 2 diabetes is obese. Insulin resistance appears to originate in an expanding adipose tissue and it is not just obesity per se that causes the insulin resistance.[109] Whether a normal adaptation to an expanding adipose tissue/obesity or a pathological phenomenon, insulin resistance is manifested as a functional change in insulin signal transduction. In human adipocytes and skeletal muscle obtained from patients with Type 2 diabetes, the phosphorylation of IRS1 by IR appears to be the first malfunctioning step.[97,110] In human adipocytes and in contrast to murine models, this is, at least in part, due to the attenuation of a positive feedback loop from mTOR-raptor to phosphorylation of IRS1 at serine 307 (human sequence).[111-113] This in turn is coupled to the down-regulated mTOR-raptor activity,[113] which can result from impaired mitochondrial

function, hypoxia, inflammation, or ER-stress. These conditions have all been associated with the development of insulin resistance in different systems.[113]

Caveolae in Insulin Resistance and Type 2 Diabetes

We have seen how caveolae play a pivotal role in organising insulin signalling at the plasma membrane and intracellularly after caveolae-mediated endocytosis of IR. The previous discussion has clearly shown how dysfunctional caveolae can result in insulin resistance, both in numerous experimental systems as well as in human beings. An outstanding question that remains to be answered is how caveolae function relates to the pathogenesis of insulin resistance in Type 2 diabetes.

Aging male but not female mice of the JYD strain have been shown to develop insulin resistance in the absence of obesity with dramatically reduced levels of caveolin-1 in adipose and muscle tissues.[114] Both insulin sensitivity and caveolin-1 expression were normalized after orchidectomy.[114] In an attempt to address the question in humans, we determined the amount of caveolin-1 in plasma membranes as well as cholesterol and caveolin-1 in isolated caveolae from adipocytes obtained from a group of insulin resistant patients with Type 2 diabetes and compared with weight and age-matched nondiabetic subjects. Neither the amounts of cholesterol nor the amounts of caveolin-1 differed between the two groups (M. Karlsson and P. Strålfors, unpublished), suggesting that caveolae are usually not structurally impaired in adipocytes from Type 2 diabetic humans. On the contrary, Catalan et al[61] reported that caveolin-1 expression in adipose tissue was increased in obesity, but not further affected in obese subjects with Type 2 diabetes. As discussed above, the increased adipocyte expression of caveolin-1 in obesity may be a metabolic response to the increased demands of obesity and the increased transport of fatty acids over the plasma membrane.

Although evidence is lacking for a direct effect of caveolae disruption or malfunctioning in insulin resistance of Type 2 diabetics, it may well be a critical factor or a contributing factor in some cases, as Type 2 diabetes and insulin resistance are pleiotropic conditions with a wide range of possible primary and secondary causes. A case in question is the endothelium lining the blood vessels of the major target tissues of insulin. The endothelial cells can have an impact on the tissue responses to insulin and energy homeostasis. Endothelial cells regulate blood flow through tissues and between tissues and secrete a variety of signalling factors that participate in the regulation of inflammation, immunity and haemostasis. In humans, Type 2 diabetes is associated with endothelial dysfunction[115] and an increased risk of cardiovascular disease development (see Dr. Frank's chapter). Endothelial dysfunction may furthermore be part of the underlying insulin resistance. First, insulin directs blood to and controls the blood flow through muscle and adipose tissue.[116] This regulation involves the control of eNOS activity by caveolae and thus regulates local vasodilation and blood flow (see Dr. Shaul's chapter). Second, as a physical barrier to hormones and nutrients, endothelial cells also provide transport from the blood to the interstitial space and peripheral cells. Equally important is the transport from the interstitial space to the circulation of cytokines and adipokines such as leptin and adiponectin. Small molecules may pass between the cells, while others are transported via receptor-mediated or receptor-independent fashions utilizing transcytosis or endocytosis/exocytosis. Endothelial cells in the adipose and muscle tissues are enriched in caveolae and caveolae-related transcellular channels that participate in signalling and in the transport across the endothelium. Endothelial cells were shown to transport insulin through the cells in a caveolae-mediated process,[20,117] which apparently involves binding to the insulin receptor in caveolae[20,21] as well as signalling through the

receptor.[118] Any defects in insulin transport from the blood vessels into the underlying muscle or adipose cells, or impairment of insulin control of the blood flow can contribute to insulin resistance. However, knockout of IR in mouse endothelial cells has no apparent effect on whole body insulin sensitivity, suggesting that IR is not needed or not rate-limiting for insulin transport through the endothelium, as least not in mice.[119] It is not yet clear to what extent this pathway may be responsible for the insulin resistance in Type 2 diabetes, or whether it is a consequence of, or part of the etiology of the disease.

CONCLUSION

There is very strong experimental support for the presence and activation of IR in caveolar domains of the plasma membrane, and IRS1 is certainly a caveolae protein under basal conditions in human adipocytes. Moreover, as part of the signalling, IR is rapidly endocytosed by caveolae in response to insulin stimulation. Although much evidences support a caveolar localization of GLUT4 in the plasma membrane, this remains to be unequivocally demonstrated. Interference with caveolae structure or function, in vitro or in vivo, in experimental animals or in human beings, is associated with defects in insulin signalling and insulin resistance. These data clearly demonstrate a potential role for caveolae dysfunction in the pathogenesis of human diseases that are characterized by insulin resistance. To date, however, there is no strong evidence linking caveolae or caveolar dysfunction to insulin resistance in human obesity or Type 2 diabetes, the major conditions associated with insulin resistance.

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CHAPTER 9

ATHEROSCLEROSIS, CAVEOLAE AND CAVEOLIN-1

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Abstract: Atherosclerosis is a disease of the blood vessel characterized by the development of an arterial occlusion containing lipid and cellular deposits. Caveolae are 50-100 nm cell surface plasma membrane invaginations that are believed to play an important role in the regulation of cellular signaling and transport of molecules among others. These organelles are enriched in sphingolipids and cholesterol and are characterized by the presence of the protein caveolin-1. Caveolin-1 and caveolae are present in most of the cells involved in the development of atherosclerosis. The current literature suggests a rather complex role for caveolin-1 in this disease, with evidence of either pro- or anti-atherogenic functions depending on the cell type examined. In the present chapter, the various roles of caveolae and caveolin-1 in the development of atherosclerosis are examined.

INTRODUCTION

The Development of Atherosclerosis: Current Understanding

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in industrialized nations. Atherosclerosis is the primary cause of CVD and is mainly characterized by the formation of plaques that develop in the arterial wall. This wall consists of three distinct cellular layers: the intima, the media and the adventitia. The intima is the innermost monolayer of the artery formed by endothelial cells (ECs) and

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internal elastic lamina. The media consists of smooth muscle cells (SMCs) embedded in an extracellular matrix (ECM). The adventitia is the outer layer of the arterial wall and is made up predominantly of fibroblasts and ECM.[1]

Plaque formation is a complex multistep process that is initiated by the accumulation of lipoproteins in the arterial intima and followed by the infiltration of monocytes at lesion sites. Lipoprotein infiltration mainly involves low-density lipoprotein (LDL), which acts as a molecular suitcase for the transport and delivery of lipids to peripheral tissues. Thus, increased plasma LDL levels have been linked to increased risk of CVD.[2] Importantly, the entrapment of LDL particles and their subsequent modification (e.g., oxidation or aggregation) in the sub-endothelial space of arteries[3,4] have been demonstrated to play a major role in the initiation of atherosclerosis.[5] As a result, the transfer of LDL from the blood stream to the sub-endothelial space may be the defining initial step for the atherosclerotic process.

The presence of modified LDL particles in the sub-endothelial space induces early inflammation via the activation of ECs. This inflammatory process is initiated by the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intracellular cell adhesion molecule-1 (ICAM-1), P-Selectin and E-Selectin.[6] Selectins play a key role in the primary interaction between monocytes and the endothelium, namely tethering and rolling of monocytes at the surface of activated endothelial cells.[7] Subsequently, monocytes differentiate into macrophages that can take-up large amounts of modified LDL and eventually become foam cells, which are enriched in cholesteryl esters (CE). The presence of T-lymphocytes, foam cells and macrophages in the intima further contribute to the inflammatory response via the secretion of chemokine and cytokine molecules, such as Monocyte Chemoattractant Protein-1 (MCP-1) and Tumor Necrosis Factor-α (TNF-α).[8]

In the early stages of this process, foam cell formation occurs via the uptake and subsequent accumulation of modified LDL in macrophages.[9] Ingested lipoprotein particles are degraded into cholesterol, amino acids and fatty acids in the lysosomes. Excess cholesterol is stored in lipid droplets as cholesteryl esters (CE).[10] These cholesterol-loaded macrophages transform into foam cells since the expression of receptors responsible for lipoprotein uptake (i.e., scavenger receptors) is not regulated by cellular cholesterol levels.[11] The scavenger receptors CD36 and Scavenger Receptor class A (SR-A) are receptors that bind modified LDL. Contrary to the LDL-receptor, mRNA levels of these receptors are not regulated by cellular cholesterol levels. Consequently, mice deficient in either of these receptors exhibit reduced atherosclerotic lesions.[12,13] This phenotype is likely due to impaired modified LDL uptake by macrophages and, consequently, reduced fatty streak formation.

During all stages of lesion progression, macrophages may undergo apoptosis[14,15] and with prolonged cholesterol loading, macrophages show characteristics of necrosis.[16] In vitro experiments have shown that free cholesterol (FC)-loading or oxidized LDL (oxLDL) treatment of macrophages leads to necrosis that is characterized by disruption of the plasma membrane and swelling of cellular organelles.[16-18] Other possible causes for macrophage death in atherosclerotic lesions include growth factor deprivation[19] and the exposure to factors such as inflammatory cytokines and nitric oxide.[20] These observations highlight the importance of the macrophage phagocytotic properties that would allow an efficient clearance of apoptotic cells. The removal of the resulting apoptotic cells by phagocytosis is carried out by infiltrating macrophages in a process known as efferocytosis.[21] This process is decreased in the more advanced stages of atherosclerosis and, as a result, increased plaque necrosis and inflammation are observed.[9,22] Besides apoptotic macrophages, lesions in the sub-endothelial space at this stage are also composed of proliferating SMCs and an ECM composed of lipid-rich cellular and necrotic debris.[1,23] The secretion of cytokines and

growth factors by macrophages and T-cells further promote the migration and proliferation of SMCs. In turn, these stimulated SMCs produce ECM proteins that can facilitate plaque rupture.[1] All of these events are believed to promote the development of an atheroma and later plaque rupture can eventually lead to blood clot and acute arterial occlusion causing a myocardial infarction or stroke depending on the location.[1,3,4,24]

A good understanding of the molecular mechanisms associated with the development of atherosclerosis has been obtained in mouse models. Mice are normally very resistant to atherosclerosis. However, under specific genetic and dietary conditions, they can develop hypercholesterolemia and extensive atherosclerotic lesions with characteristics that are similar to those observed in humans. In mice, a targeted disruption of the apolipoprotein E gene (*apoe*) is characterized by increased very low-density lipoproteins (VLDL) and LDL associated cholesterol levels in the blood stream. In addition, feeding *apoe*$^{-/-}$ mice with a western-type diet (i.e., enriched in cholesterol) leads to a further increase in plasma cholesterol levels that can reach 1500-2000 mg/dl (~ten times normal values) and therefore accelerates the appearance of lesions in the aorta.[25,26]

Caveolin-1 (Cav-1) is expressed in all of the cell types involved in the development of atherosclerosis (i.e., endothelial cells, macrophages and smooth muscle cells). Because of its role in the regulation of cellular cholesterol homeostasis and in numerous signaling pathways, it has been proposed to play an important role in atherosclerosis together with caveolae. The objective of this chapter is therefore to provide a better understanding of the role of Cav-1 and caveolae in the complex process of atherosclerosis development at the cellular and molecular levels. The study of complex diseases such as atherosclerosis is challenging because of its multi-factorial origin, most notably environmental and genetic. Based on data generated by various laboratories, including ours, we believe that the study of Cav-1 will allow the development of novel scientific approaches to study atherosclerosis by examining the different steps associated with the development of this disease. In this chapter, we present a caveolae-based approach to dissect the various steps, in particular, intimal LDL accumulation, endothelial, macrophage and SMC function. These cell types are directly involved in the development of atherosclerosis at different stages during disease progression. More specifically, we underline the multifaceted and sometimes opposing roles of Cav-1 in ECs, macrophages and SMCs. Finally, we present a working model for Cav-1 function in atherogenesis.

Caveolae: Discovery and Biochemical Properties

"Caveolae", a term coined by Yamada,[27] are small, 50-100 nm, flask-shaped plasma membrane invaginations, first identified by Palade in 1953 and described as "little caves" due to their appearance by electron microscopy.[28] This type of vesicular structure is a subtype of lipid rafts, which are plasma membrane microdomains enriched in sphingolipids and cholesterol.[29] The particular lipid composition of caveolae/lipid rafts is responsible for the insolubility observed in non-ionic detergents (e.g., Triton X-100) at 4º C and a light buoyant density after sucrose gradients ultracentrifugation.[30] These properties have been instrumental for the purification and biochemical characterization of these structures.[31-35] They are involved in the regulation of signal transduction events, endocytosis, transcytosis, membrane trafficking and the regulation of cholesterol homeostasis.[33,36] They are highly sensitive to cholesterol depletion as treatment of cells with cholesterol-binding agents (e.g., cyclodextrin) flatten these structures.[37,38] Caveolae are characterized by the presence of the protein Cav-1. Caveolae and Cav-1 are abundant in terminally differentiated cells,

including fibroblasts, epithelial cells, adipocytes and ECs.[39,40] Cav-1-deficient mice lack caveolae in all of the cell types normally expressing Cav-1. These findings indicate that Cav-1 is required for the formation of caveolae.[41-43]

Caveolae and Caveolins Structure and Function

The molecular makeup of caveolae has remained mysterious for four decades after their initial morphological description. The discovery of Cav-1 as a major structural protein component of caveolae has since provided new insights into the multifaceted function of caveolae and caveolins.[38,44] Cav-1 was first identified through a screening of tyrosine-phosphorylated proteins in Rous sarcoma v-Src positive cells. This protein was detected in caveolae by immuno-electron microscopy and protein sequencing identified it as the previously characterized VIP21 protein (Vesicular integral-membrane protein of 21 kDa).[45,46] Two additional caveolin protein isoforms have also been identified by sequence identity. Together, they form the caveolin protein family, which consists of three proteins (Caveolin-1, Caveolin-2, Caveolin-3) that are well-conserved from *C. elegans* to mammals.[38]

Cav-1 has an unusual topology (Fig. 1) with the middle portion of the protein (~33 amino acids) embedded into the cytoplasmic leaflet of the lipid bilayer and its amino and carboxy termini in the cytosol, thus forming a hairpin-like structure.[44] Cav-1 and Cav-3 homo-oligomerize, while Cav-1 and Cav-2 form hetero-oligomers, via the caveolin oligomerization domain (COD, residues 61-101).[47] After synthesis in the endoplasmic reticulum (ER), Cav-1 forms high molecular oligomeric complexes with either itself or Cav-2. In skeletal muscle and cardiac myocytes, Cav-3 is the main structural component of caveolae.[48] Cav-1 oligomers organize themselves within the membrane to form a higher order umbrella-like structure (Fig. 1). As the complex traffics through the Golgi network, a higher order complex of well over 1000 subunits (Cav-1 oligomers) eventually leads to the formation of caveolae at the plasma membrane in association with cholesterol and sphingolipids. Cav-1 protein levels are highly dependent on cellular cholesterol levels.[49-52] In addition, this protein has a high affinity for cholesterol.[53-56] The initial Cav-1 oligomers allow to anchor various receptors and signaling molecules.[57,58] Besides, this structure promotes the invagination and bending of the membrane through the caveolin-induced asymmetrical conformation.

Domain mapping and deletional analysis have identified a Cav-1 scaffolding domain (CSD, residues 82-101), which allows Cav-1 to mediate protein-protein interactions and modulate signal transduction pathways. Several cytoplasmic and transmembrane proteins and downstream signaling molecules have been shown to preferentially localize to caveolae and interact with Cav-1. These molecules include Src-family tyrosine kinases, p42/44 MAPK and endothelial nitric oxide synthase (eNOS). Cav-1 can hold these signal transducing molecules in an inactive state until they are activated by the appropriate stimulus.[57,59] These properties allow caveolae and caveolins to regulate signal transduction and act as platforms for compartmentalization, engaging signaling molecules in a manner similar to lipid rafts. This function has been proposed in the "caveolin signaling hypothesis".[60]

ABSENCE OF CAVEOLIN-1 DECREASES ATHEROSCLEROSIS DEVELOPMENT

The first direct indication suggesting that Cav-1 plays a role in atherosclerosis has come from findings obtained in our laboratory. We have shown a major reduction of

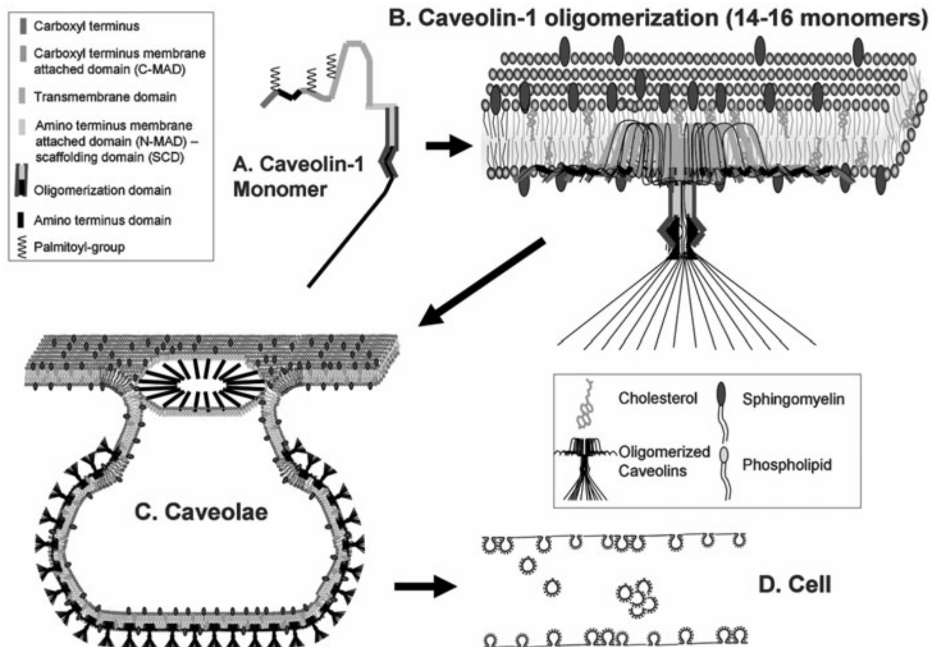

Figure 1. Caveolae Organization. A) Representation of Cav-1 hairpin-like structure with its domains, including the scaffolding domain (CSD) which allows Cav-1 to bind and regulate kinases and other downstream signaling pathways. B) Representation of Cav-1 oligomer formation (14-16 monomers) with an umbrella-like structure embedded within the plasma membrane enriched in cholesterol and sphingomyelin. C) Higher ordered complex of well over 1000 subunits eventually forms caveolae within the lipid bilayer of the plasma membrane. D) Examples of caveolae structures observed in differentiated cells. Grape-like structures plasma membrane attached caveolae are shown.

atherosclerosis in caveolin-1-deficient (*cav-1*$^{-/-}$) mice in the *apoe*$^{-/-}$ genetic background. These double knock-out mice displayed reduced aortic lesions by up to 70% compared to *apoe*$^{-/-}$ mice alone despite remarkably elevated levels of circulating plasma cholesterol.[61,62]

However, Cav-1 is expressed in all the cell types involved in the development of an atheroma. Nevertheless, its expression levels and function are different depending on the cell type. In fact, current studies suggest that Cav-1 has both a pro- and anti-atherogenic role that is context-dependent based on the cell type in which it is expressed.[61] The various roles of Cav-1 in atherosclerosis will be discussed in the following sections.

Role of Caveolin-1 in the Regulation of Endothelial Cell Function

Caveolae and the Regulation of LDL Transcytosis

Elevated plasma LDL cholesterol levels have been associated with increased risk for heart disease development. As a consequence, all mouse models used for atherosclerosis studies exhibit abnormal lipoprotein profiles. Transcytosis and retention of LDL are believed to be the initiating events that lead to downstream processes such as activation

of ECs and subsequent monocyte recruitment. The transcytosis process is defined as the transfer across ECs of a molecule (e.g., LDL) from the lumen to the subendothelial side of a blood vessel. Its occurrence may be related to the presence of Cav-1 in ECs, since it was suggested that caveolae could mediate LDL transcytosis[63] (Fig. 2). Other molecules that are known to transcytose across ECs are albumin[64] and transferrin.[65] Interestingly, ECs that lack Cav-1 display impaired transcytosis of albumin.[66,67]

Transcytosis is the first function that has been ascribed to caveolae[68] and fifteen years later it was shown that the majority of LDL transcytosis occurs via caveolae.[69] Endothelial caveolae are thought to play a role in transcytosis via receptor-mediated transfer of LDL across ECs[61,68] or fluid phase transfer of LDL across ECs. A third pathway by which LDL could cross the endothelial barrier might be via a paracellular transport, which could occur between two ECs (Fig. 2). However, the latter pathway is unlikely to occur since LDL particles may be too large (20-30 nm) to fit between the tightly apposed ECs. In fact, Simionescu et al have shown that the transfer of molecules via the paracellular pathway is limited to those in the 3-6 nm range.[70] Moreover, Vasile et al[69] have shown that LDL particles are endocytosed in small amounts in ECs by receptor-dependent and receptor-independent processes. In addition, caveolae have also been shown to be responsible for the transcytosis of LDL and HDL across ECs of the blood brain barrier.[71,72] Finally, in a recent study, we have shown that Cav-1-deficient mice present defects in the aortic uptake of LDL particles, both in vivo and in vitro.[63] In direct support of these findings, we have confirmed that downregulation of the Cav-1 protein in human umbilical vein endothelial cells leads to an over 50% reduction in LDL uptake (S Pavlides and PG Frank unpublished data). The latter studies demonstrate a critical role for caveolae-mediated transcytosis of LDL particles from the vascular lumen to the sub-endothelial space. Furthermore, they also indicate an important pro-atherogenic function for Cav-1 and caveolae in ECs. Therefore, the requirement for high cholesterol

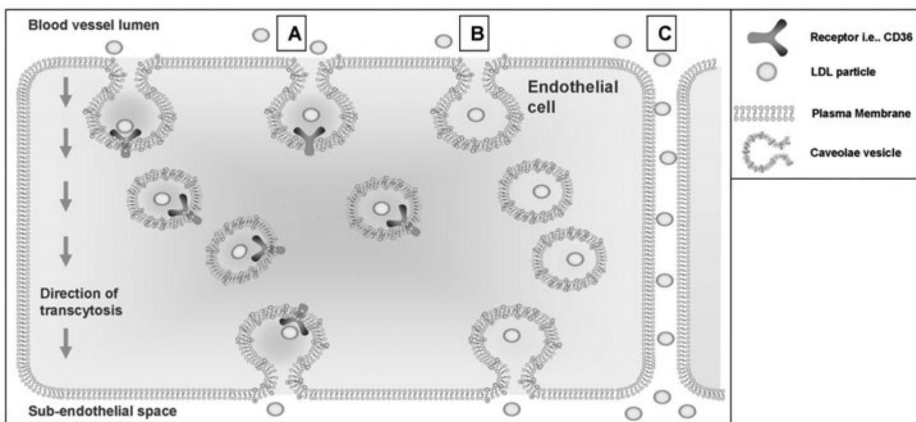

Figure 2. Transcellular model of LDL transfer across endothelial cells. LDL is transferred from the lumen to the sub-endothelial space via three possible transcellular pathways: A) Receptor-mediated transcytosis: LDL binds specific receptors that are found in caveolae (such as CD36). Caveolae vesicles endocytose and transfer LDL across the cell, where caveolae fuse with plasma membrane on the basal side to release LDL within the intima. B) Fluid phase-mediated transcytosis: LDL particles engage in caveolae vesicles in a nonspecific manner and are transferred across the cell to the intima. C) Paracellular pathway: LDL particles are transported between the space of two apposed cells in a caveolae-independent manner.

circulating levels in animal models susceptible to atherosclerosis is not sufficient but transcytosis of LDL across ECs is a prerequisite for the initiation of atherosclerotic lesion progression. Blockage of this process could prevent atherosclerotic lesion development. Further research into the factors regulating this step may lead to the development of novel drugs for the treatment of vascular diseases.

Caveolin-1: Role in the Regulation of eNOS Function and Inflammation

Endothelial Cav-1 is implicated in vascular inflammation, which is a critical element in the development of atherosclerosis. In that regard, the endothelial nitric oxide synthase (eNOS) has been demonstrated to play an important role in inflammation. eNOS is an enzyme produced by endothelial cells and it is palmitoylated and myristoylated.[73-75] These posttranslational modifications are a common feature of many signaling proteins that are targeted to caveolae.[33] Moreover, eNOS interacts with the Cav-1 scaffolding domain and is tonically inhibited by Cav-1 in vascular ECs.[42,76,77] eNOS is a complex dimeric enzyme, which activity is highly regulated. Intracellular calcium concentration rises upon agonist (e.g., acetylcholine) stimulation of ECs. This reaction leads to the binding of calmodulin to intracellular calcium and the newly-formed complex effectively displace Cav-1 from eNOS and associates with the latter. Dissociation of eNOS from caveolin allows the production of nitric oxide (NO).[78] Dysregulated eNOS activity, due to the lack of one of the major cofactors can have adverse effects by inducing superoxide production[79] (see Chapter 3 for additional details relating to the role of Cav-1 in the regulation of eNOS function).

Activation of eNOS is associated with protective effects on lesion formation via decreased expression of adhesion molecules, such as VCAM-1.[80,81] VCAM-1 is a protein that belongs to the immunoglobulin superfamily, which also includes integrins and selectins.[82] Under basal, unstimulated physiological conditions, VCAM-1 is not expressed. However, under specific pro-inflammatory conditions, such as in the presence of cytokines like Tumor Necrosis Factor (TNF)-α or Interleukin (IL)-1β, ECs are activated and quickly synthesize VCAM-1.[83] Previous studies have demonstrated the important role of VCAM-1 in the development of atherosclerosis.[84,85] Our studies using *cav-1*$^{-/-}$*apoe*$^{-/-}$ and *apoe*$^{-/-}$ mice have shown that the absence of Cav-1 in endothelial cells could lead to a reduction in VCAM-1 production.[62] In addition, Fernandez-Hernando et al[86,127] have demonstrated the direct role of Cav-1 in the regulation of adhesion molecule expression. In this study, Fernandez-Hernando et al have shown that the re-expression of Cav-1 in endothelial cells of *cav-1*$^{-/-}$*apoe*$^{-/-}$ is sufficient to reverse the effect observed on VCAM-1 expression. In addition, these authors have also shown that the expression of other markers of inflammation (ICAM-1, E-selectin and P-selectin) is reduced in *cav-1*$^{-/-}$*apoe*$^{-/-}$ mice.[86,127] Taken together, these data suggest that endothelial Cav-1 plays an essential role in the regulation of endothelial cell activation.

Summary

The current literature strongly suggests that endothelial Cav-1 and caveolae plays critical roles in the development of atherosclerosis. Moreover, contrary to its role in macrophages and smooth muscle cells (See the following two sections), a clear pro-atherogenic role has been demonstrated for endothelial Cav-1.[62,86] It may regulate lipoprotein and cholesterol accumulation in the intima.[63] In addition, we and others have demonstrated direct and indirect effects for Cav-1 in the regulation of endothelial-mediated

inflammation.[62,86] Finally, Cav-1 may also play an important role in the regulation of endothelial cell replacement in injured blood vessels. In that case, the presence of Cav-1 may limit cellular replacement in injured blood vessels, thereby promoting lipoprotein accumulation in the intima and eventually atheroma growth.[87]

Role of Caveolin-1 in the Regulation of Macrophage Function

Macrophage Apoptosis during Atherosclerotic Development

Macrophage apoptosis occurs at all stages of atherosclerotic lesion development after foam cell formation.[14,15] Cav-1 has been implicated in the regulation of apoptosis in a number of cell types such as endothelial cells[88] and smooth muscle cells.[89] Extensive analysis shows that Cav-1 expression sensitizes certain types of cells to chemicals that induce apoptosis. For example, NIH 3T3 cells that overexpress Cav-1 are more sensitive to apoptosis mediated by the protein kinase inhibitor staurosporine. Conversely, NIH 3T3 cells that have been depleted of Cav-1 become resistant to apoptosis induced by staurosporine.[90] Similar results have been obtained with the bladder epithelial cell line T24.[90] Studies have shown that regulators of apoptosis, such as the TNF-α receptor[91] and caspase-3[88] localize to caveolae and their function may depend on the presence of caveolae. These data suggest that the localization of apoptotic regulators within caveolae is critical for apoptosis.

Several studies have now examined the role of molecules regulating macrophage apoptosis in the development of atherosclerosis. In vivo studies using bone marrow transplantation of cells lacking the pro-apoptotic gene *Bax* have revealed that decreased macrophage apoptosis leads to increased early lesion size and cellularity.[92] Similar results were obtained in bone marrow transplantation experiments using cells lacking the pro-apoptotic (or tumor-suppressor) gene *p53*.[93] Both of the above studies have used mouse models susceptible to atherosclerosis such as $apoe^{-/-}$ and $ldlr^{-/-}$ mice. However, these bone marrow-derived macrophages lacking the pro-apoptotic genes (*bax, p53*) have been shown to display increased cellular proliferation, which may further contribute to the increased lesion size.[92] On the other hand, mice that lack the anti-apoptotic factor, AIM (*a*poptosis *i*nhibitor expressed by *m*acrophages) have been shown to develop smaller early atherosclerotic lesions compared to their $ldlr^{-/-}$ control group.[94] The above examples are indicative of an inverse relationship between apoptosis and early atherosclerotic lesion development. Increased apoptosis leads to decreased cellularity and therefore, reduced lesion size. Conversely, decreased apoptosis leads to increased cellularity and therefore, increased lesion size.

In mouse peritoneal macrophages (MPMs), Cav-1 expression is up regulated during simvastatin-induced apoptosis of macrophages where Cav-1 colocalizes with phosphatidylserine (PS).[49] In their study, Gargalovic and Dory have suggested that Cav-1 may be involved in the externalization of PS during early apoptosis and that increased expression of Cav-1 in MPM may serve as an early marker for apoptosis.[51] Recently, we have shown that the absence of Cav-1 in MPMs is associated with increased accumulation of CE and decreased free cholesterol (FC).[95] CE is a neutral lipid that is stored in lipid droplets within the cytoplasm. It is the accumulation of CE that leads to the formation of foam cells.[96] This event is concomitant with reduced FC synthesis and increased acyl coenzyme A: cholesterol acyltransferase (ACAT) activity.[95] Increased FC levels in the ER are toxic for macrophages and may lead to the activation of the unfolded protein response (UPR) and eventually to apoptosis.[17,48,97] Our findings[95] are in agreement with the first line of defense against cholesterol toxicity, which, in macrophages, is the esterification

of FC into CE by the enzyme ACAT.[98] Therefore, efficient conversion of FC into CE is considered a survival mechanism.[17,48,99] These results suggest that *cav-1*$^{-/-}$ macrophages may be more resistant to the toxic effects of FC and less susceptible to apoptosis than wild-type macrophages. Thus, if cell death (apoptosis) is inefficient and cholesteryl ester is stored more efficiently in macrophages lacking Cav-1, cellularity may be amplified in association with enhanced foam cell formation. This hypothesis is in agreement with our preliminary studies in which we observe an increased in early atherosclerotic lesion development in wild-type mice transplanted with bone marrow obtained from caveolin-1-deficient mice (S. Paulides and P.G. Frank, unpublished data).

Phagocytosis: Role in Lesion Development and Progression

The process of efferocytosis by macrophages infiltrating the atherosclerotic lesion is crucial for the containment of the atheroma. Many groups have suggested that apoptosis is linked to phagocytosis[100] and others have shown that at early stages of lesion development, foam cell accumulation and apoptosis are regulated by the levels of phagocytosis. In early lesion development, the process of phagocytosis is considered favorable because it helps prevent further expansion of the atheroma by decreasing cellularity and lessening the inflammatory cascade. Macrophage apoptosis is associated with diminished lesion cellularity and decreased lesion progression in early lesions, in which phagocytic clearance of apoptotic macrophages seems to be efficient.[92-94] Moreover, even if the initial response is the engulfment of foam cell apoptotic bodies by neighboring macrophages, phagocytes may become engorged with apoptotic foam cell "remnants," including abundant lipids. Eventually, the capacity of macrophages to carry out this process can be exceeded. However, in later more complex lesions, this balance is disrupted and apoptotic cell clearance is usually defective and this defect leads to advanced plaque formation.[22,101]

Data suggests that Cav-1 is involved in the modulation of macrophage inflammatory responses (e.g., to oxLDL) and in the clearance of apoptotic cells at lesion sites.[102,103] Electron microscopy studies have shown that Cav-1 is linked to the process of phagocytosis and cannibalism (an act of engulfing live cells)[104] among malignant tumor cells, through images of caveolae-like structures (caveolae-caveolae fusion) at the site of cell contact between the phagocyte and the tumor cell. Additional studies have shown that Cav-1 is present in endolysosomes of phagocytes, indicating that caveolae may contribute to the formation of the "cannibalistic vacuole".[104] Our laboratory has shown that *cav-1*$^{-/-}$ MPM have decreased phagocytic clearance ability of apoptotic thymocytes and fluorescein-labeled *E.coli* K-BioParticles.[102] To further expand on these results, Li et al,[102] have also shown that phagocytosis is impaired in wild-type MPM treated with methyl-β-cyclodextrin, which disrupts caveolae by depleting cholesterol from the plasma membrane. If Cav-1-deficient cells have reduced phagocytic capabilities, it may follow that Cav-1 is important for the proper clearance of apoptotic foam cells by macrophages in the arterial intima. In that case, macrophage Cav-1 may play an anti-atherogenic role since it allows macrophages to cleanup apoptotic foam cells and cellular debris at lesion sites.

Macrophage and Inflammation

Macrophages have also been shown to play a critical role in the regulation of vascular inflammation during atherogenesis.[1] Interestingly, Cav-1 has previously been shown to play a role in the regulation of this process.[105] Overexpression of Cav-1 in macrophages

leads to reduced secretion of TNF-α and IL-6, whereas downregulation of Cav-1 leads to increased TNF-α and IL-6 secretion.[106] In that case, signaling via the NFκB/Akt pathway is also increased. Therefore, Cav-1 is believed to play an anti-inflammatory role and prevent activation via the NFκB/Akt pathway.[106] In addition, the pro-apoptotic role of Cav-1 in macrophages may prevent a prolonged inflammatory response and may reduce the recruitment of T-cells and monocytes into atherosclerotic plaques. Taken together, these data suggest that the absence of Cav-1 in macrophage may lead to events (apoptosis-inflammation) that synergistically contribute to accelerated plaque progression in early lesions.

Summary

In general, most of the studies have provided evidence for a role of Cav-1 in the regulation of macrophage function. We propose that Cav-1 plays a role in the regulation of cellular cholesterol homeostasis, apoptosis and inflammation. In each of the function examined, we and others have shown that macrophage Cav-1 has antiatherogenic properties.

Role of Caveolin-1 in the Regulation of Vascular Smooth Muscle Cell Migration and Proliferation

Vascular tunica media is mainly composed of SMCs that can contract or relax and, as a consequence, allow the modification of blood vessel shape and blood pressure. Like the aforementioned macrophages and endothelial cells, SMCs play a critical role in atherosclerosis development. Early studies have shown that diet-induced atherosclerosis alters vascular smooth muscle morphology and/or function in rabbit,[107] swine,[108] nonhuman primate[109,110] and humans.[111] In particular, an increase in SMC proliferation leads to increased arterial wall thickness and intracellular lipid accumulation. In parallel, various research groups have realized that SMCs can present either a contractile or a synthetic phenotype.[112-115] The latter cellular state involves the acquisition of proliferative, migrating and secreting machineries, which play key roles during atherosclerosis development.[116-118]

Caveolae structures have been detected in association with the plasma membrane of brain vascular SMCs by freeze fracture ultrastructure techniques.[119] Similar to striated muscle cells, vascular SMCs express Cav-1, -2 and -3;[120-123] however, in contrast to striated muscle cells, caveolin-3 has been detected to a lesser extent in vascular SMCs.[122-124] Interestingly, while genetic deletion of Cav-3 in mice prevents caveolae formation in striated muscle cells,[125,126] Cav-1 genetic ablation is sufficient to considerably diminish the number of caveolae in vascular SMCs.[43,127-129] Moreover, while Cav-1 is expressed in all vascular SMCs, caveolin-3 expression appears to be restricted to arterial rather than venous SMCs.[130]

Regulation of SMC migration

The main role of SMCs is to aid in the distribution of blood through vascular smooth muscle contraction and relaxation. This vascular smooth muscle function is in part controlled by NO produced by eNOS in ECs,[131] although studies have also demonstrated the existence of NOS activity in SMC.[132,133] As mentioned earlier, Cav-1 regulates NO production in ECs. Therefore, a great amount of evidence in this field has shown that Cav-1 indirectly regulates SMC function. Consequently, it has been shown that aortic rings from Cav-1-deficient mice fail to contract properly when

exposed to phenylephrine and the NO-mediated relaxation effects of acetylcholine significantly increases compared to the effect observed in aortic rings obtained from wild-type mice.[42,43] Supporting the increased production of NO in *cav-1*$^{-/-}$ aortic rings, treatment with the NOS inhibitor L-NAME, causes a significantly greater contraction of aortic rings obtained from *cav-1*$^{-/-}$ mice than of those obtained from wild-type mice.[42] More recently, endothelial re-expression of Cav-1 in *cav-1*$^{-/-}$ mice has been shown to rescue low flow-mediated dilation[134] and restored SMC contractility.[135] Taken together, these data suggest that signaling mediated by NO in SMCs is regulated by endothelial Cav-1. Interestingly, several studies have now confirmed the link between defective NO-signaling and atherosclerosis. These observations have been made in human subject as well as in animal models.[136] The current literature suggests a critical role for Cav-1 in this pathway. Interestingly, endothelial Cav-1 expression has been shown to be upregulated in hypercholesterolemic subjects.[137] This fact is sufficient to explain by itself the abnormal NO-mediated vasorelaxation observed in these patients. These findings have more recently been confirmed in mice overexpressing Cav-1 in endothelial cells only.[86]

NOS activity and presence of the nNOS isoform have been demonstrated in SMC.[130,132,133] In addition, Cav-3 is expressed in SMCs[120-123] and nNOS has also been shown to interact with the scaffolding domain of Cav-3.[138] Taken together, these observations point towards the idea that Cav-3 may also regulate NO production in SMCs and may therefore play an important role in the development of CVD. However, this hypothesis has not been yet addressed. In general, the current literature suggests that Cav-1 and/or Cav-3 may play a key role in the vasorelaxation of SMCs via their ability to regulate NO production. Since NO can regulate SMC function,[139] Cav-1 and Cav-3 may therefore, indirectly regulate the phenotype of SMCs. This process may be relevant for the pathogenesis of atherosclerosis.

Before proliferating, SMCs migrate into the vascular intima to form part of the occlusive mass found in atheromas.[140] SMC migration is controlled by a set of molecules including PDGF, angiotensin II, TGFβ and FGF[141] that activate tyrosine kinases.[142] Cav-1 expression has been implicated in the regulation of a number of signaling pathways that regulate SMC migration. This is possibly due to the fact that Cav-1 negatively regulates and stabilizes key players (i.e., kinases) implicated in various signaling cascades.[143-147] Moreover, Cav-1 may also modulate vascular protease activity and SMC migration.[148] Taken together, these data are consistent with those obtained in our laboratory where we have shown that aortic SMCs from *cav-1*$^{-/-}$ mice have an increased migratory potential compared to aortic SMCs obtained from wild-type mice.[149]

Role of Caveolin-1 in the Regulation of SMC Proliferation

It is well accepted that SMCs have the capacity to acquire proliferative properties (i.e., synthetic phenotype) at the initial stages of atherosclerosis.[109,150] Since Cav-1 has been shown to regulate various signaling pathways involved in the control of cellular proliferation, we can expect Cav-1 to play an important role in the regulation of vascular SMC proliferation. In agreement with this idea, several in vitro studies have demonstrated an antiproliferative function for Cav-1 in vascular SMC.[120,127] For example, Schwencke et al (2005) have demonstrated that in the absence of Cav-1, primary SMCs display increased proliferative properties.[127] Recently, vascular SMCs have been found to proliferate in response to static pressure correlating with Cav-1 downregulation and the activation of ERK1/2.[151] ERK1/2 has

been shown to be negatively regulated by Cav-1 in various studies.[146,149,152] Besides the ERK pathway, Cav-1 may regulate other signaling pathways involved in the regulation of SMC proliferation. These pathways include integrin/focal adhesion kinase[153] and tissue factor.[154]

Correlative studies have also shown that Cav-1 expression is reduced in human vascular SMC from atherosclerotic lesions[127,155] and in neointimal hyperplasia.[89] Neointima formation is a process characterized by SMC proliferation and extracellular matrix deposition in the vascular intimal layer. To evaluate the role of Cav-1 in the pathogenesis of neointimal lesions, we have used *cav-1*$^{-/-}$ mice as a model system. The right common carotid artery of wild-type and *cav-1*$^{-/-}$ mice was ligated just proximal to its bifurcation. The changes in vessel wall geometry in response to flow reduction in *cav-1*$^{-/-}$ and wild-type mice were determined by measuring the luminal, intimal and medial areas of carotid arteries after vessel ligation. Our results demonstrate that Cav-1-deficiency is associated with increased neotimal formation with the concomitant activation of the p42/44 MAP kinase cascade and upregulation of cyclin D1.[156] In support of these findings, Schwencke et al (2005) have shown that proliferation of SMCs from *cav-1*$^{-/-}$ mice is inhibited when re-expressing Cav-1.[127] Under specific conditions (i.e., cyclic strain), Cav-1 may also be involved in the activation of pro-proliferative signals.[157] However, this line of research will require further investigations.

Recent studies have shown that Cav-1 may influence vascular protease activity and potentially stabilize atherosclerotic lesions. Rodriguez-Feo et al[148] have demonstrated that low levels of SMC Cav-1 promotes plaque instability with increased lipid core size, macrophage infiltration and increased secretion of IL-6, IL-8 and matrix metalloprotease-9 activity.[148] This study implies that the absence of Cav-1 in SMCs could be directly related to impaired inflammatory responses that contribute to the formation of an atherosclerotic lesion.

Summary

In summary, these findings suggest that Cav-1 and caveolae in SMCs play an important role in the regulation of SMCs phenotype. They suggest that Cav-1 may play both antiproliferative and antimigratory roles. Therefore, we propose that SMC Cav-1 may have an anti-atherogenic role during the development of atherosclerosis.

CONCLUSION

A role for Cav-1 during atherogenesis has first been demonstrated in our laboratory. We have shown that double knockout mice *cav-1*$^{-/-}$*apoe*$^{-/-}$develop significantly less atherosclerotic lesions than the control *apoe*$^{-/-}$ mice. It is proposed that endothelial Cav-1 promotes atherogenesis through its role in the transcytosis of LDL across ECs from the blood stream into the subendothelial intima. In addition, endothelial Cav-1 appears to play an important role in the regulation of vascular inflammation. By contrast, macrophage Cav-1 may have an anti-atherogenic role. Supporting this hypothesis, we have recently shown that Cav-1 regulates intracellular cholesterol homeostasis and accumulation of CE in macrophages, but decreased FC[95] indicating a possible survival function.[16,17,99] In addition to the latter finding, we have also shown that macrophages lacking Cav-1 present impaired phagocytosis properties.[102] These findings indicate that macrophage Cav-1 can contribute to reduced atherosclerotic lesion cellularity. Another anti-atherogenic role of Cav-1 may be linked to its ability to reduce the production of cytokines by macrophages submitted to a pro-inflammatory stimulus.[106] Furthermore, Cav-1 expression in SMCs is

hypothesized to be anti-atherogenic by inhibiting migration and proliferation of this cell type during atherosclerosis progression. Finally, these findings suggest rather complex and sometimes opposing roles for Cav-1 supporting a cell-context dependent paradigm for all three types of cells that play a central role during atherosclerosis development.[61] A better understanding of the role of Cav-1 in vivo is required to better define the various functions of Cav-1. It is expected that these studies will provide us with a better rational for the treatment of patients with CAD.

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CHAPTER 10

CAVEOLINS AND HEART DISEASES

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Abstract: Caveolins serve as a platform in plasma membrane associated caveolae to orchestrate various signaling molecules to effectively communicate extracellular signals into the interior of cell. All three types of caveolin, Cav-1, Cav-2 and Cav-3 are expressed throughout the cardiovascular system especially by the major cell types involved including endothelial cells, cardiac myocytes, smooth muscle cells and fibroblasts. The functional significance of caveolins in the cardiovascular system is evidenced by the fact that caveolin loss leads to the development of severe cardiac pathology. Caveolin gene mutations are associated with altered expression of caveolin protein and inherited arrhythmias. Altered levels of caveolins and related downstream signaling molecules in cardiomyopathies validate the integral participation of caveolin in normal cardiac physiology. This chapter will provide an overview of the role caveolins play in cardiovascular disease. Furthering our understanding of the role for caveolins in cardiovascular pathophysiology has the potential to lead to the manipulation of caveolins as novel therapeutic targets.

INTRODUCTION: CAVEOLINS IN THE CARDIOVASCULAR SYSTEM

Originally identified in the 1950s by electron microscopy investigating cellular ultrastructure, caveolae appear as morphologically identifiable ampullate pits or vesicles at the plasma membrane.[1] Caveolae, 50-100 nm invaginations in the plasma membrane, have been identified to some degree in most differentiated cell types and are especially abundant in adipocytes, muscle and endothelial cells.[2] Caveolae greatly increase the surface area of the cell and are involved in macromolecular transport, maintenance of plasma membrane integrity, vesicular trafficking, signal transduction

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and mechanotransduction.[3-6] Mammalian caveolae contain three integral structural proteins, termed caveolin (Cav) 1, 2 and 3 (Cav-1, Cav-2 and Cav-3). Caveolins, acting as scaffolding proteins, are able to concentrate lipids (cholesterol and glycosphingolipids),[7] signaling proteins (heterotrimeric G-proteins, H-Ras, nitric oxide synthase, epidermal growth factor receptor, protein kinases and Src-like kinases),[8] and structural proteins (dystroglycan, M-phosphofructokinase and dysferlin)[9-11] in caveolae.

Cav-1 exists as two isoforms via alternate translational initiation from methionines 1 and 32, resulting in Cav-1α (residues 1–178) and Cav-1β (residues 32–178). Cav-1 is a 22–24-kDa integral membrane protein consisting of 178 amino acid residues. The central region of Cav-1 contains a string of 33 hydrophobic amino acids that function as a membrane anchor, allowing Cav-1 to assume a hairpin configuration with both N- and C-terminal domains facing the cytoplasm. Residues 61–101 of Cav-1 function to direct the self-oligomerization of Cav-1 and as a plasma membrane bound scaffold for recruiting specific caveolin-interacting protein components of caveolae. Cav-1 expression in heart is not restricted to endothelial cells and fibroblasts but also is found in myocytes.[12-14] Knocking out Cav-1 in mice produces a cardiac phenotype similar to hypertrophic cardiomyopathy in humans.[15] Reexpression of Cav-1 in endothelium rescues the vascular, cardiac and pulmonary defects in global Cav-1 knockout (KO) mice.[16]

Cav-2 has three known isoforms (α, β, γ) with a tissue distribution that largely overlaps with Cav-1. Cav-2 does not assemble into large oligomers or drive caveolae formation by itself. Cav-2 protein is stabilized when in heteroligomeric complexes with Cav-1. In the absence of Cav-1, Cav-2 is detected as a mixture of monomers or dimers retained in the golgi complex. Several laboratories detected caveolin-2 mRNA or protein in cardiac preparations,[17] endothelial cells,[18] fibroblasts,[19] and cardiomyocytes.[20] Cav-2 forms hetero-oligomeric complex with Cav-3 suggesting Cav-2 can influence caveolae biogenesis in striated muscle cells, including cardiomyocytes.[20] Parallel increases in Cav-2 and Cav-3 were seen during cardiac development in cardiomyocytes.[20] A distinct immunocytochemical staining pattern for Cav-2 and Cav-3 in cardiomyocytes also suggests that Cav-2 may have additional unrecognized functions in cardiomyocytes.[20] Phosphorylation of Cav-2 on serines 23 and 36 modulates Cav-1 dependent caveolae formation in LNCaP cells.[21] Phosphorylation of Cav-2 on tyrosine 19 or 27 produces different cellular localization patterns, phosphorylation at tyrosine19 produces a docking site for Src homology domain-2 containing proteins during signal transduction.[22] Cav-2 KO mice do not show vascular system dysfunction or lipid disorders (atherosclerosis); however, mice lacking Cav-2 show severe pulmonary dysfunction without disruption of caveolar invaginations.[23]

Cav-3 is necessary for the formation of caveolae in cardiac, skeletal and some smooth muscle.[24,25] Cav-3 is exclusively present in cardiomyoctes throughout development but tightly regulated.[26] Histochemical and cytochemical analyses further demonstrate that Cav-3 is most abundantly expressed from postnatal day 1 to death.[27,28] Loss of Cav-3 results in muscle degeneration, cardiomyopathy and increases in adipose tissue deposition.[29,30] Increased Cav-3 levels can be paradoxically associated with muscle dystrophy, cardiomyopathy, protection from ischemia/reperfusion and inhibition of cardiac hypertrophy.[31-35] These differences are likely due to global vs cardiac myocyte specific expression of Cav-3. All three caveolin knockouts show cardiovascular phenotypes, which point to the fact that caveolins play a key role in the regulation and maintenance of signaling events involved in the cardiovascular system.

ATHEROSCLEROSIS

Cell type specific surveys of caveolin proteins demonstrate abundant expression in endothelial cells,[36] smooth muscle cells,[37] fibroblasts[38] and myocytes[39] of the cardiovascular system. It is therefore not surprising that alterations in caveolin abundance are associated with vascular disease. The number of caveolae was found to be significantly lower in endothelial cells from atherosclerotic lesions,[40] thereby fueling the suggestion that alterations in caveolae structure on the plasmalemma may underlie the development of endothelial dysfunction in atherosclerosis. Studies using KO mice revealed that the lack of caveolin protein could lead to various pathological conditions related to the vascular system.[41] Elimination of Cav-1 by deleting exon 3 of the Cav-1 molecule impaired nitric oxide (NO) signaling in the cardiovascular system resulting in an aberration in endothelium dependent relaxation, contractility and maintenance of myogenic tone.[41] Aortic rings from exon 1 and 2 Cav-1 targeted knockouts showed abnormal vasoconstriction and vasorelaxation responses to various stimuli.[42] Zhao et al[43] generated Cav-1 KO mice that showed marked chronic increases in pulmonary artery pressure resulting in right ventricular hypertrophy. Knockout of different exons of Cav-1 all result in similar cardiovascular phenotypes. Scaffolding domains within the caveolin binding domain allow direct interaction with endothelial nitric oxide synthase (eNOS). Studies have supported the conclusion that in basal conditions, eNOS becomes hyperactivated in the absence of Cav-1.[44] Razani et al[42] observed that eNOS activity was upregulated in Cav-1 null animals and this activity could be blunted by specific NOS inhibition. Similarly, downregulation of endogenous Cav-3 in cardiomyocytes by transgenic overexpression of a Cav-3 mutant induced an increase in cardiac eNOS activity.[45] In general, Cav-1 KO mice have a reduction in systemic arterial blood pressure corresponding with high NO production and impaired left ventricular contractility. The hypothesis that caveolin is a key negative modulator of eNOS activity gains strength from these independent approaches. A number of cardiovascular risk factors have been demonstrated to alter the organization of caveolar domains. Increased levels of LDL-cholesterol result in increased Cav-1 which in turn reduces NO.[46] Oxidized-LDL also inhibits vascular NO production; however, unlike LDL-cholesterol it disrupts the caveolae structure by specifically removing cholesterol from caveolae.[46] Pharmacological therapies in cardiovascular disease management utilizing statins have recently been demonstrated to augment eNOS activity by down regulating Cav-1.[47] A recent study using Cav-1/apolipoprotein E double-knockout mice showed that the lack of Cav-1 in apolipoprotein E knockout mice significantly reduces the development of atherosclerosis.[48] Developing strategies that target the eNOS-Cav system may lead to novel therapies for vascular dysfunction. Caveolin allosterically inhibits the basal activation of molecules like eNOS; however, agonist stimulation can also activate eNOS signal transmission[49] via enhanced receptor affinity or receptor-effector coupling suggesting the existence of a caveolae/eNOS paradox.[50] Clearly, caveolins have multiple functions in the vascular system and more research is required to elucidate the molecular mechanisms involving caveolins in atherosclerosis.

ARRHYTHMIAS

In cardiac cells, caveolae contain ion gated channels (primay voltage-gated sodium (Na) channel (Nav1.5),[51] voltage-dependent calcium (Ca) channels (Cav1.2),[52] voltage-driven

potassium (K) channels (Kv1.5),[53] ATPases (Na/K and Ca)[54] and Na/Ca exchangers).[55] Recent studies have linked mutations in Cav-3 with human ventricular arrhythmias and sudden cardiac death.[56] The Cav-3 mutations associated with Long QT syndrome Type 9 (LQT-9) interfere with the association between Cav-3 and the Nav1.5 ion channel, resulting in an increase in the late Na current.[57] Long QT-3 is also associated with genetic variants in the gene encoding Cav-3 resulting in an increase in the late Na current.[58] In sudden infant death syndrome, a Cav-3 mutation (V14L, T78M and L79R) functionally disrupts normal sodium channel function.[59] In other cardiomyopathies, myocardial infarction and hypertrophy models, the arrhythmias seen may be secondary effects due to alterations in morphological caveolae and Cav-3 protein expression on the cell surface. It is clear that changes in caveolae and caveolin proteins can lead to dysregulation and dysfunction of ion channels in caveolar microdomains thus potentially altering the risk of arrhythmias in cardiovascular diseases.

CARDIAC HYPERTROPHY AND HEART FAILURE

Cardiac hypertrophy can be defined as an increase in cardiomyocyte size that can be beneficial and adaptive (physiological) or a maladaptive (pathophysiological) response to hemodynamic stress resulting from pressure or volume overload. Cardiac hypertrophy is a critical determinant involved in cardiac remodeling and the progression of heart failure. Cardiac growth during hypertrophy results primarily from an increase in the size of the individual cells rather than an increase in the number of cells. When the ventricle is subjected to hemodynamic stress, the ventricular myocytes experience morphological and biochemical changes that mark the initiation of hypertrophy.[60-64]

Cav-1 and -3 have been shown to be involved in cardiac hypertrophy. Cav-1 KO results in cardiac hypertrophy and induces contractile dysfunction.[65] Hearts from Cav-1 KO mice show a progressive cardiac hypertrophy characterized by increased cardiomyocyte size and interstitial fibrosis. Impairment of heart function in Cav-1 KO mice is characterized by a dilated cardiomyopathy with an enlarged left ventricular diameter, wall thinning, decreased systolic function and decreased contractility.[66,67] Additionally, Cav-1 KO mice have markedly increased pulmonary artery pressures and hypertrophied right ventricles that may be due to pulmonary fibrosis.[41,43] Thus, it seems likely that knockdown of Cav-1 affects ventricular function by a direct action on cardiac muscle cells and indirect effects secondary to pulmonary changes. Moreover, Cav-1 has been shown to prevent the development of monocrotaline-induced right ventricular hypertrophy.[68] Although gross histological and functional changes within the heart are a hallmark of Cav-1 depletion, Cav-1 KO mice exhibit hyperactivation of the p42/44 mitogen-activated protein kinase (MAPK) cascade in isolated cardiac fibroblasts[66] and nitric oxide synthase in endothelial cells. It has been hypothesized that hyperactivation of the p42/44 MAPK in cardiac fibroblasts results in an elevated secretion of growth factors and a concomitant hypertrophic response of neighboring cardiomyocytes.[66]

It is known that caveolae contain numerous signaling molecules involved in cardiac hypertrophy including but not limited to alpha adrenergic receptors, Gq proteins, phospholipase C, epidermal growth factor receptors, Ras, MAPKs, Src kinases, natriuretic peptide receptors and Cav-3.[69,70] Woodman et al[30] have shown that knocking out the gene for Cav-3 results in hyperactivation of the Ras/extracellular signal-regulated kinases (ERK 1/2) signaling pathway, cardiac hypertrophy and

reduced cardiac function. The authors concluded that loss of Cav-3 was sufficient to induce a molecular program of cardiac hypertrophy. Catecholamine-induced cardiac hypertrophy in H9c2 cardiomyoblasts is marked by retarded translocation of Cav-3 to caveolae thus altering the spatial distribution of Cav-3.[71] Cav-1/Cav-3 double KO mice completely lack morphologically identifiable caveolae and develop a severe cardiomyopathic phenotype with left ventricular hypertrophy and dilation.[72] Cav-3 KO mice develop cardiomyopathy characterized by hypertrophy, ventricular dilation and reduced contractility.[30] This hypertrophic phenotype also could be produced by expressing a mutant Cav-3 peptide.[45] Histological analysis of Cav-3 KO myocardium, shows increased cellular infiltration with accompanying interstitial/perivascular fibrosis.[30] These results raise the intriguing hypothesis that expression of Cav-3 may be involved in the regulation of cardiac hypertrophy.

Koga et al[31] have tested this hypothesis in vitro and demonstrated that the overexpression of Cav-3 in neonatal cardiac myocytes using an adenovirus vector attenuated phenylephrine and endothelin induced ERK1/2 activation and blocked myocyte hypertrophy. Voluntary exercise training in rats induces physiological cardiac hypertrophy and increases Cav-3 expression within the heart[73] suggesting expression of Cav-3 may be involved in physiological as well as pathological cardiac hypertrophy. In this regard, increased Cav-3 expression by mild exercise training was shown to inhibit apoptosis and protect the heart against hypertrophy.[74]

There is some evidence for changes in the expression of Cav-3 in models of heart failure and patients with cardiomyopathy. Mutations in Cav-3 are associated with cardiomyopathy in patients.[75] Abdominal aortic banding in rats produces an increase in Cav-3, 28 days after banding.[76] Aortic stenosis in the rat produces an increase in Cav-3 expression at 7 weeks after stenosis and a decrease in Cav-3 expression at 14 weeks as hypertrophy progresses[77] while dogs with hypertrophic cardiomyopathy secondary to perinephritic hypertension show reduced levels of Cav-3 in the heart.[78] Hyperdynamic myocardium in spontaneous hypertensive rats shows less Cav-3 at 6 months of age[69] along with a hypertropic phenotype and the mRNA level of Cav-3 is depressed in rats with pressure overload.[73] Taken together these results suggest Cav-3 expression increases as a result of hypertrophic stimuli and possibly is reduced in later stages of heart failure suggesting a potential compensatory role for Cav-3 in heart failure. However, there are conflicting data for this hypothesis. Global overexpression of Cav-3 in mice can produce severe cardiac tissue degeneration, fibrosis and a reduction in cardiac function.[35] Cav-3 protein is increased in explanted hearts from patients with idiopathic dilated cardiomyopathy.[79] Additionally, Cav-3 protein and sarcolemmal caveolae are increased in dogs with pacing induced heart failure.[80]

Recent work has suggested a role for caveolae in the alterations in adrenergic signaling involved in heart failure. Head et al[81] have shown that Cav-3 interacts with β2-adrenergic receptors in t-tubules of adult cardiac myocytes. Nikolaev et al[82] have implicated lipid rafts in β-adrenergic receptor redistribution in heart failure. The authors could produce a redistribution of β2-adrenergic receptors out of t-tubules in cardiac myocytes similar to heart failure by using methyl-β-cyclodextrin (MBCD), a compound used to disrupt caveolae and lipid rafts, suggesting that alterations in lipid rafts and potentially caveolae were a critical element involved in adrenergic receptor redistribution in heart failure. Future work regarding the role of caveolins in cardiac hypertrophy and heart failure has significant potential to advance our understanding of pathophysiological mechanisms and lead to novel therapeutic targets and strategies for heart failure patients.

MYOCARDIAL ISCHEMIA/REPERFUSION INJURY

Myocardial infarction (MI) is a disorder in which cardiac myocytes undergo necrosis as a consequence of interrupted coronary blood flow. Myocardial infarction commonly is due to an occlusion of an atherosclerotic coronary artery. In 1986, Murray and colleagues[83] discovered that multiple, brief episodes of ischemia, applied before a sustained ischemic insult, did not contribute to ischemic injury, but rather induced an increased tolerance against ischemic damage. Termed ischemic preconditioning (IPC), this intervention has proven to be the most robust and potent application to confer protection against myocardial ischemia/reperfusion (I/R) injury. Preconditioning is mediated via a molecular signaling cascade that has become known as the reperfusion injury salvage kinase (RISK) pathway.[84] PI3K-Akt and ERK1/2 are identified as potential signaling components of the RISK pathway involved in IPC-induced protection. Both prosurvival kinases are activated to confer protection before index ischemia and also at the time of reperfusion.[85,86] Short bouts of ischemia after an index ischemia and prior to reperfusion can also initiate cardiac protection,[87] e.g., improve postischemic dysfunction or reduce infarct size, which has been called postconditioning. Preconditioning and postconditioning that can be induced by sublethal ischemia, several pharmaceuticals and volatile anesthetics have been well documented in the heart and are related to activation of similar kinase cascades.[88,89] Of note, numerous members of the RISK pathway have been shown to be associated with caveolae and caveolins including G-protein coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), Src kinases, G-proteins, H-Ras, nitric oxide synthases, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K) and MEK/ERK kinases (Fig. 1).[70]

Evidence that caveolins may be involved in protecting the myocardium includes the finding that infusion of the CSD peptide of caveolin-1 into ischemic/reperfused hearts results in recovery of cardiac function.[90] Ischemia/reperfusion injury activates p42/44 and p38 MAPK, redistributes Cav-3 and down-regulates expression of Cav-1,[91] thereby limiting the negative impact of Cav-1 on eNOS. The latter effect suggests that one mechanism by which preconditioning produces cardiac protection is via increased generation of NO during lethal ischemia.[92] Ischemic preconditioning may modulate the microenvironment of caveolae and caveolin-associated protein interactions so as to enrich for proteins that promote cardiac protection. This idea is consistent with findings indicating that eNOS and the glucose transporter GLUT-4 translocate to caveolae after preconditioning.[93]

Another line of evidence for involvement of caveolae and caveolins in cardiac protection against myocardial ischemia/reperfusion injury comes from myocyte specific studies on ischemia- and opioid-induced preconditioning.[39] Myocytes treated with methyl-β-cyclodextrin (MBCD) to deplete membrane cholesterol and disrupt caveolae, fail to display IPC and opioid mediated cardiac protection. However, myocytes treated with MBCD preloaded with cholesterol show normal Cav-3 expression in caveolae and maintain the protection against I/R-induced cell death. Similarly, isoflurane induced cardiac protection was abolished in myocytes with disrupted caveolae by treating with MBCD and colchicine.[32] Cav-3 and Cav-1 KO mice are resistant to the cardioprotective effects of isoflurane to myocardial I/R injury.[13,32] Hearts exposed to isoflurane (and other protective stimuli including IPC)[33] show an increased formation of caveolae (Fig. 2) and increased Src phosphorylation and recruitment of C-terminal Src kinase, a negative regulator of Src.[13] Cav-1 knockout mice, resistant to isoflurane-induced cardiac protection, show impaired Src phosphorylation and recruitment of C-terminal Src kinase.

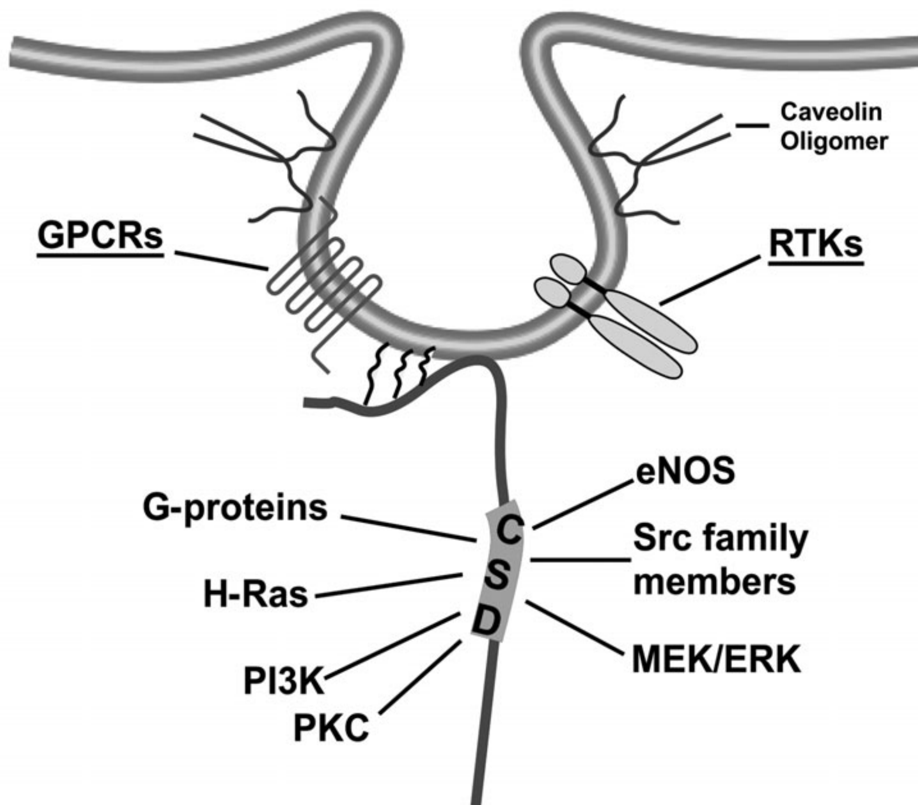

Figure 1. Schematic depicting caveolae, caveolae–resident proteins and proteins interacting with the caveolin scaffolding domain (CSD) involved in preconditioning. RTK, receptor tyrosine kinase; GPCR, G-protein coupled receptor; eNOS, endothelial nitric oxide synthase; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase.

Validation of a role for Cav-3 in cardiac myocyte protection from I/R injury was confirmed by experiments using adenovirus mediated overexpression of Cav-3 in vitro and myocyte-specific overexpression of Cav-3 in vivo.[33] Transgenic mice with cardiac myocyte-specific overexpression of Cav-3 have increased tolerance to myocardial I/R injury. Hearts from Cav-3 overexpressing mice have improved functional recovery after myocardial ischemia and reperfusion and increased basal Akt and GSK3β phosphorylation suggesting augmentation of the RISK signaling pathway in Cav-3 overexpressing mice. The cardiac protection afforded by cardiac myocyte-specific overexpression of Cav-3 could be abolished by PI3-kinase inhibition with wortmannin. Additionally, Cav-3 knockout mice were not able to protected using an ischemic preconditioning protocol. Thus, Cav-3 is both necessary and sufficient to produce cardiac protection from myocardial ischemia/reperfusion injury. These results suggest that caveolins are important mediators of cardiac protection and may become important therapeutic targets for augmenting the preservation of the heart and other organ systems. Future studies are needed to define the proteome of "preconditioned caveolae" in order to identify the proteins and signaling networks that may mediate and modulate cardiac protection.

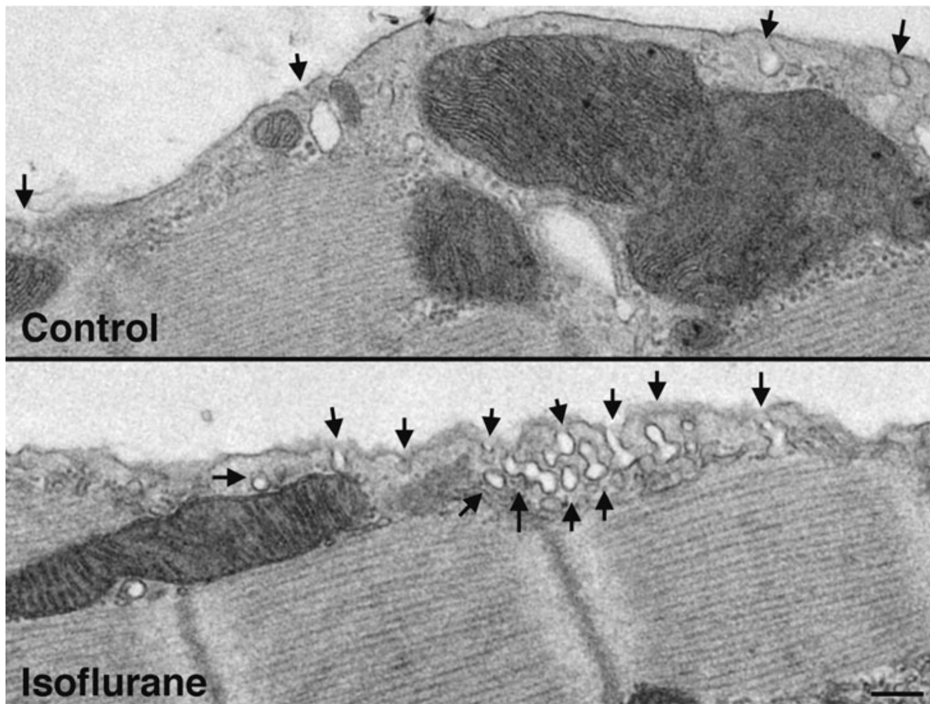

Figure 2. Adult rat cardiac myocytes were exposed to isoflurane or oxygen (control). After isoflurane exposure (30 min, 2.1% isoflurane), cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at room temperature, postfixed in 1% OsO4 in 0.1 M cacodylate buffer (1 h) at room temperature and embedded as monolayers. Representative EM images show that isoflurane increases membrane invaginations that are typical features of caveolae.

CONCLUSION

Cardiovascular diseases are a major source of morbidity, mortality and healthcare resource use worldwide. Cardioprotective strategies geared toward atherosclerosis, arrhythmias, cardiac hypertrophy, heart failure and myocardial ischemia/reperfusion injury are of major interest. The caveolin protein family plays a diverse and critical role in the cardiovascular system. Although some details of the expression pattern of caveolins have been noted in normal and animal models of pathophysiology, understanding of the underlying mechanisms involved in the role for caveolins in cardiovascular disease remains limited and an exciting area for active investigation. Animal studies have shown intriguing evidence for a potential role for the cardioprotective effects of caveolin proteins, providing new insight into mechanisms underlying the orchestral effect of caveolins in coordinating cell-signaling molecules. Further basic science research and eventual randomized clinical trials are needed to define the mechanisms and therapeutic potential of caveolins in patients with cardiovascular disease. Clearly, the role of caveolins in cardiovascular disease is a rapidly growing area and work in this field has significant potential to lead to the design of new therapies and interventions for cardiovascular diseases.

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CHAPTER 11

CAVEOLINS AND LUNG FUNCTION

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Abstract: The primary function of the mammalian lung is to facilitate diffusion of oxygen to venous blood and to ventilate carbon dioxide produced by catabolic reactions within cells. However, it is also responsible for a variety of other important functions, including host defense and production of vasoactive agents to regulate not only systemic blood pressure, but also water, electrolyte and acid-base balance. Caveolin-1 is highly expressed in the majority of cell types in the lung, including epithelial, endothelial, smooth muscle, connective tissue cells, and alveolar macrophages. Deletion of caveolin-1 in these cells results in major functional aberrations, suggesting that caveolin-1 may be crucial to lung homeostasis and development. Furthermore, generation of mutant mice that under-express caveolin-1 results in severe functional distortion with phenotypes covering practically the entire spectrum of known lung diseases, including pulmonary hypertension, fibrosis, increased endothelial permeability, and immune defects. In this Chapter, we outline the current state of knowledge regarding caveolin-1-dependent regulation of pulmonary cell functions and discuss recent research findings on the role of caveolin-1 in various pulmonary disease states, including obstructive and fibrotic pulmonary vascular and inflammatory diseases.

INTRODUCTION

The primary function of the lung is to transfer oxygen from the atmosphere to the blood and carbon dioxide from the blood to the atmosphere, a process known as "gas

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exchange." In order to facilitate efficient gas exchange, with each heartbeat blood is pumped from the right ventricle into the pulmonary artery and through the pulmonary vascular system where the vast surface area of alveolar epithelial cells are in close contact with the extensive blood capillary network.[1] Alveolar epithelial and capillary endothelial cells are in close contact with each other, forming an exquisitely thin barrier[2,3] such that gas transit time between blood and the airspace is minimal. An intricate system of branched tapering bronchi from the trachea to the numerous alveoli conducts air to the distal lung airspaces.[4] Bronchial and vascular smooth muscle cell layers regulate vascular and bronchial diameter, thus optimally matching alveolar airflow, or ventilation, to blood flow, or perfusion.[5] In addition to gas exchange, the lung is constantly producing mucous, which traps inhaled particles, and pulmonary surfactant, which lowers alveolar surface tension, thus preventing alveolar collapse.[6] Due to its position at the interface between the environment and the body's interior, the lung possesses all the necessary tools to mount an innate and adaptive immune response. Finally, the lung synthesizes mediators important to the homeostasis of the organism, including angiotensin II, an important regulator of blood pressure. These diverse functions must be performed by different populations of highly specialized cells. In this Chapter, we present the current state of knowledge on the role of caveolin-1 and caveolin-2 protein expression and function in the various cell types of the lung.[7] We also summarize research findings linking caveolin expression to the pathogenesis of pulmonary disease.

CAVEOLIN IN LUNG DEVELOPMENT

Caveolin family protein members, caveolin-1 and caveolin-2, are co-expressed primarily in endothelial cells and Type I epithelial cells in the adult lung where they function both as structural and signaling proteins due to their localization in specialized plasma membrane domains—caveolae, enriched in cholesterol, sphingolipids, ceramides and other lipids.[8] Importantly, caveolae also contain heterotrimeric G proteins, G-protein coupled receptors, Src family kinases, receptor tyrosine kinases, and other signaling molecules as well as enzymes.[9,10] The presence of these signaling molecules in caveolae places caveolin at the crossroad of multiple signaling pathways. Therefore, it is not surprising that caveolin modulates the activity of various receptors and enzymes or that loss of caveolin-1 expression leads to cellular transformation, implying that caveolin-1 may also function as a tumor suppressor protein.[11]

Expression of caveolins is differentially regulated in various cell types of the developing and adult lung. Lung development begins at embryonic day E9.5 in mice and at around 28 days in humans, when 2 lung buds appear within the endoderm-derived embryonic foregut.[12] At around the same time, primitive hemangioblasts located in the mesoderm surrounding the foregut begin differentiating and give rise to a vascular plexus, which later becomes the peripheral lung vasculature. However, the mechanism by which pulmonary blood vessels develop is not clear.[5,13] Williams and associates showed that mRNA of both caveolin-1α and caveolin-1β isoforms were readily detected at E12.5.[14] At the protein level, expression of caveolin-1α was detected exclusively in endothelial cells of the developing lung (Fig. 1). Postnatally, caveolin-1α was not detected in the lung epithelium until mice were >2 months of age and was observed only in vessels and in Type I epithelial cells.[14] Although the study did not address the expression pattern of caveolin-1β during development, there is some evidence that caveolin-1β is expressed

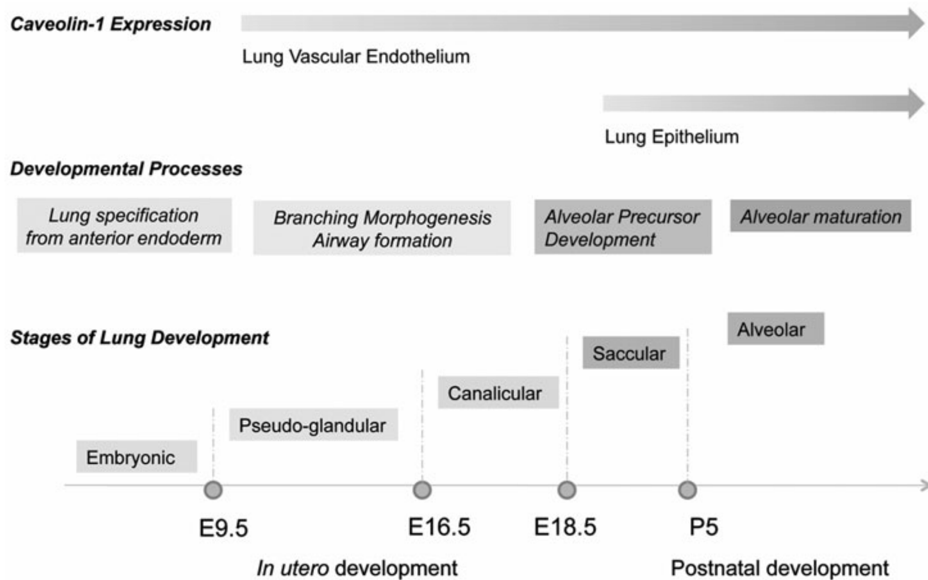

Figure 1. Expression of caveolin-1 in pulmonary epithelium and endothelium during mouse lung development. Caveolin-1 and -2 expression begins at day E10 in pulmonary endothelial cells and E19 in alveolar epithelial cells. The temporal and cell-specific regulation of caveolin expression in the lung may play an important role in the mechanisms which govern branching morphogenesis and alveolar maturation during lung development.

in fetal lung epithelium.[15] Thus, it is tempting to speculate that this finding may relate to the observed delay in lung development in caveolin-1 knockout mice.[16,17] Caveolin-1 null mice lack expression of both caveolin-1 isoforms (α and β) as well as caveolin-2, which is apparently unstable in the absence of caveolin-1.[16] Lung abnormalities in knockout animals are fully realized at 4-5 months of age and are represented by alveolar wall thickening, hypercellularity, and extensive remodeling of pulmonary vessels.[16,18] Interestingly, the caveolin-1 knockout lung phenotype develops at the same time that caveolin-1α expression reaches its highest levels in the alveolar epithelium.

Although studies in global knockout mice provide important information about protein function, they do not discriminate between the various cell types of the lung and thus many questions regarding the role of caveolin-1 in lung development remain unanswered. Still, some insights can be gained from studies of caveolin-1 role in morphogenesis of other organs and tissues.[19,20] The study by Yang and colleagues reports that caveolin-1 deficient mice demonstrate abnormal epithelial differentiation and/or hyperplasia in pancreas, prostate, uterus and breast as well as hypercellularity in lung and the exocrine pancreas.[20] The authors propose that "loss of caveolin-1 function in stromal cells of various organs directly leads to a disorganized stromal compartment that, in turn, indirectly promotes abnormal growth and differentiation of adjacent epithelium."[20] Lung epithelial morphogenesis from specification of ventral foregut endoderm to formation of alveolar septa is driven by epithelial-mesenchymal interactions.[20] Stromal cells such as fibroblasts and endothelial cells present morphogenetic signals to developing epithelium. Interestingly, caveolin-1 expression is detected early in development in the lung endothelium suggesting that it may be important for development of mesoderm

and its proper inductive function. Caveolin-1 knockout lungs display hypercellularity, enlarged airspaces, and nonhomogenous filling of capillaries (Fig. 2). The abnormal architecture of vasculature and alveolar compartment can be attributed to inefficient signaling from mesenchymal cells, which in the absence of caveolin-1 remain in an under-differentiated proliferative state.

Recent research from different groups suggests involvement of caveolin-1 in proliferation and differentiation of cells in the fetal and adult lung. In the lung, caveolin-1 is considered to be a marker of Type I cell differentiation.[21] It was reported that Type II cells cultured in vitro exhibit loss of surfactant protein C (SP-C) expression concomitant with an increase in caveolin-1 expression.[22] Contrary to the long prevailing view that Type II cells do not express caveolin-1, a recent study demonstrated presence of caveolin-1 expression in embryonic E19 rat Type II cells.[23] Moreover, this study reports that caveolin-1 participates in stretch-induced ERK activation and SP-C mRNA expression in isolated Type II cells, suggesting a potential role of caveolin-1 in mechanotransduction during fetal lung development.[23]

There is also a growing interest in the involvement of caveolin-1 in Wnt/β-catenin signaling pathways which regulates development and pathogenesis in vertebrates and invertebrates.[24] In brief, the secreted glycoprotein Wnt binds to Frizzled receptors initiating a cascade which results in displacement of the multifunctional kinase GSK-3β from the APC/Axin/GSK-3β complex.[24] In the absence of Wnt signaling, APC/Axin/GSK-3β ubiquitinates β-catenin and targets it for degradation by the proteasome. Upon

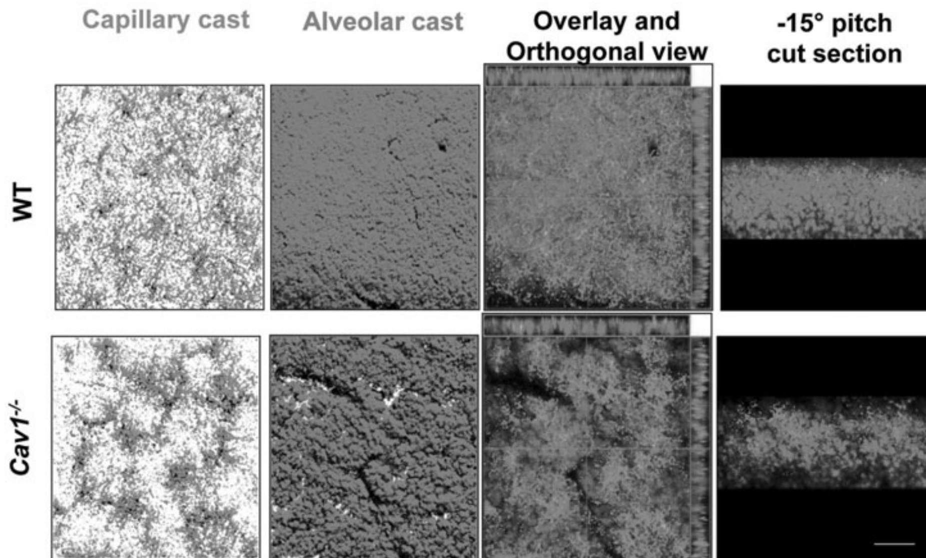

Figure 2. Architectural changes of vascular and alveolar compartments in *Cav1*$^{-/-}$ lungs. Vascular and alveolar casts were generated by chilling lungs after perfusion of fluorescent beads in a solution containing 0.3% agarose via pulmonary cannula and trachea, respectively. 3D rendering of thick lung section confocal z-stack images shows nonhomogeneous filling of capillaries and enlarged airspaces in *Cav1*$^{-/-}$ compared to wild type (WT) lungs. Reproduced with permission from Maniatis NA, Am J Physiol 2008; 294(5):L865-873.[18] © 2008, the American Physiological Society. A color version of this image is available online at www.landesbioscience.com/curie.

Wnt binding, GSK-3β is recruited away from the degradation complex which allows for stabilization of β-catenin protein, nuclear import, recruitment to LEF/TCF DNA-binding factors, and activation of transcription. Wnt target genes include c-myc, Cyclin D, and other genes involved in proliferation and differentiation.[25] Involvement of caveolin-1 in regulating Wnt/β-catenin/Lef-1 signaling was first shown a decade ago.[26] It was found that caveolin-1 efficiently inhibits β-catenin/Lef-1 responsive transcription, possibly by recruiting β-catenin to caveolae. Based on the fact that β-catenin interacts with E-cadherin as a component of intercellular adherens junction adhesion complexes, the authors proposed that recruitment of β-catenin to the membrane reinforces cell-cell adhesion while reducing transcription of Wnt responsive genes.[26] This attractive model places caveolin-1 as a regulator of cancer-associated gene expression as well as intercellular adhesion impairment which is another characteristic feature of malignant transformation. A later study from the same group demonstrated that mammary gland epithelial cells isolated from caveolin-1 knockout mice exhibit hyperactive Wnt/β-catenin signaling, which is likely to be responsible for mammary gland hyperplasia observed in caveolin-1 knockout animals.[26] As mentioned above, the Wnt/β-catenin pathway is known to regulate cell fate decisions and differentiation during development and in the adult organism. The role of β-catenin in differentiation of lung epithelium has been documented.[27] When an activated form of β-catenin was expressed in respiratory epithelial cells of fetal lung, air space enlargement, epithelial cell dysplasia, and appearance of atypical Type II-like cells in the airways were observed postnatally.[27] The role of caveoin-1 in respiratory epithelial differentiation in vivo remains to be tested.

Caveolin-1 is thought to function as a negative regulator of many other signaling cascades involved in proliferation and oncogenesis. For example, caveolin-1 inactivates members of PI3-kinase/Akt-, receptor tyrosine kinase- and MAP kinase-pathways. The general inhibitory action of caveolin-1 on protein kinases is thought to be mediated through a specific sequence, the caveolin scaffolding domain.[28]

CAVEOLIN AND THE ALVEOLAR EPITHELIUM

The alveolar epithelium of higher vertebrates, birds and mammals, which occupies more than 99% of the lung surface area,[29] is formed by Type I and II cells.[21,30] Alveolar Type I (ATI) cells are squamous epithelial cells that comprise roughly 8% of total lung cells but cover 90% of the lung surface area[31] and whose main function is gas exchange. ATI cells contain sparse intracellular organelles, including Golgi apparatus, endoplasmic reticulum, and mitochondria.[32] They also contain abundant cell membrane invaginations and vesicular structures and, according to estimates, 70% of the ATI cell plasmalemmal surface area is contained in such invaginations, which are distributed both on the luminal and interstitial side of the membrane.[32,33] In fact, the density of vesicles per unit volume in ATI cells, at least in some species, matches that of adjacent endothelial cells[34] implying a role of ATI cells in fluid and macromolecular transport processes and host defense.

Recent studies provide evidence for an important role of ATI cells in regulating lung fluid homeostasis. ATI cells express Na^+ channels and Na^+/K^+ ATPase pumps, which control Na^+ transport across the alveolo-capillary membrane.[35,36] As Na^+ moves across cell membranes, water passively follows. ATI cell permeability to water in response to osmotic gradients is the highest of any mammalian cell, indicating that this cell type is a major pathway for water transport between the airspace and the endovascular compartment.[37]

Water permeability seems to be particularly high in plasmalemmal vesicles of ATI cells, presumably due to the presence of water channels, most notably aquaporin-5.[37]

In ultrastructural studies, a large fraction of the aforementioned vesicles in ATI cells were identified as caveolae.[38] Using immunolabeling with anticaveolin-1 antibodies in rat lungs, Newman et al identified caveolin-1 in membrane invaginations of ATI cells as well as free vesicles in the thin cytoplasmic extensions, which lack Golgi.[38] Caveolin-negative invaginations were also found in ATI cells, which, aside from technical limitations leading to incomplete antibody binding, could indicate the presence of a morphologically and functionally heterogeneous population of these membranous structures in these cells. However, electron-dense structures consistent with clathrin-coated pits have not been described in these cells and it is likely that the overwhelming majority of membrane invaginations and plasmalemmal vesicles in ATI cells (as well as endothelial cells) are of the noncoated type, i.e., caveolae.[39,40] A number of other workers reported caveolin-1 expression in ATI cells,[29,41] and thus caveolin-1 seems to be restricted to this cell type in the alveolar epithelium. Interestingly, ATII cells, which are primarily responsible for producing, secreting and recycling surfactant,[6,42] have no caveolae and weak caveolin-1 expression.[22,38,43,44] It has been implied that a certain threshold of caveolin-1 expression may be required for caveolae to be formed.[39] Caveolin-1 expression seems to coincide with the process of differentiation into the ATI phenotype in vitro.[43] In addition, caveolin-1 negatively regulates mechanical stretch-induced differentiation of fetal ATII cells into mature surfactant-producing cells by controlling the Extracellular-Regulated Kinase 1/2 pathway.[23]

ATI cells predominantly express the β-isoform of caveolin-1 lacking the 31-amino acid N-terminal sequence, whereas endothelial cells primarily express the full length α-isoform.[45] Given that the β-isoform does not contain the tyrosine residue at position 14, a substrate of *Src* kinase, it would appear that this important regulatory mechanism of caveolin-1 function may not apply to ATI cells.

Since the alveolar epithelium is a highly absorptive surface for water, it would be attractive to speculate that caveolae function as facilitators of lung water and protein homeostasis under physiological conditions and regulate permeability, thereby contributing to lung edema formation and clearance under pathological conditions. By substantially increasing the plasma membrane surface area, caveolar invaginations could provide space for water channel and Na^+ pump insertion into the membrane as well as removal of proteins from the alveolar space through the process of transcytosis. This notion is supported by the observation that the protein concentration in the alveolar lining fluid is substantially lower than in the plasma and rises during an acute inflammatory insult.[46-48] A number of experimental studies to address the issue of transcytotic protein removal from the airspace have been conducted.[49-52] The results indicate that transcytosis can account for albumin transport through epithelial cells at low concentrations. At high concentrations, such as those encountered during acute lung injury (ALI), soluble protein removal seems to take place overwhelmingly via a passive process, most likely occurring via intercellular junctions.[51] Thus, at present, the role of epithelial caveolae in lung edema clearance, in particular with regard to lung injury, is unclear.

CAVEOLIN AND THE PULMONARY ENDOTHELIUM

As outlined above, the lung's main function is to ensure oxygen saturation of venous blood and carbon dioxide elimination. The relatively unimpeded passage of the entire

cardiac output through the lung is facilitated by a variety of factors, including: (a) an extensive vascular network to accommodate blood flow at rest and during increased flow requirements (during exertion) by mechanisms of vasodilation and vascular recruitment; (b) the production of vasodilators, which help regulate vascular tone and reduce flow resistance; (c) the production of biomolecules, which increase hemofluidity and reduce blood viscosity and propensity for clot development. Nitric oxide (NO) and prostacyclin are the most important of these mediators synthesized primarily by the pulmonary endothelium. In addition to its synthetic function, the pulmonary endothelium forms a semi-permeable membrane, which separates and also connects the interstitium with the intravascular space by controlling fluid, solute and macromolecule exchange across the capillary barrier. Thus, the endothelium not only regulates tissue homeostasis, it also determines lung fluid balance. In pathologic states of increased endothelial permeability, including ALI and the Acute Respiratory Distress Syndrome (ARDS),[53] fluid filtration across the endothelium may exceed the clearance capacity of the lymphatic vessel system resulting in pulmonary edema, impairment of oxygen diffusion, and hypoxemic respiratory failure.[54-56]

Under normal conditions, the endothelium restricts movement of macromolecules and fluid to the perivascular space, allowing passage of only a very small amount of protein, including albumin, the most abundant plasma protein. According to recent evidence, caveolae are important carriers of albumin, thus regulating tissue oncotic pressure and trans-endothelial permeability.[57-60] Binding of albumin to albumin-binding protein gp60 leads to gp60 clustering and association with caveolin-1 on the surface of endothelial cells.[52,61-63] The GTP-binding protein G_i is activated downstream of gp60 and, specifically, the $G\beta\gamma$ subunit of G_i triggers a series of signaling events leading to activation of ^{pp60}*c-Src* tyrosine kinase (*Src*) activation and signaling.[62,64,65] This seems to be a critical step in the endocytic process, as activated *Src* phosphorylates downstream target proteins dynamin-2[64] and caveolin-1,[61,64,66] resulting in dynamin oligomerization into circular structures surrounding the neck of caveolae.[67] Scission ("pinching off") of caveolar invaginations and formation of endocytic vesicles is thought to be accomplished by the constrictive action of activated dynamin-2 rings.[67] This mechanism, which accounts for the overwhelming majority of albumin exchange across the vascular wall, is at the same time coupled to constitutive production of NO by lung endothelial cells by a mechanism involving *Src*, phosphoinositide-3-kinase and Akt activation.[68]

In addition to protein-permeability regulation, caveolar structures are also permeable to water. This was initially ascribed to the presence of aquaporin-1 water channels in caveolae,[69] although this was not confirmed by a subsequent study.[70] More robust evidence has been provided in support of caveolar regulation of inter-endothelial junctional integrity. This action is primarily related to control of endothelial NO synthase (eNOS) function and NO release. In mice lacking caveolin-1, extravascular accumulation of protein is increased, which would appear counter-intuitive, given that these mice also lack caveolae.[71,72] However, it was demonstrated by electron microscopy that microvascular endothelial cells from caveolin-1 knockout lungs also have a higher percentage of "open" inter-endothelial junctions which can be "closed," thus restoring junctional integrity and barrier function, by eNOS inhibition. Thus, these data indicate that excess eNOS-derived NO may be responsible for inter-endothelial junction instability in the absence of caveolin-1.[71,72]

Even though the role of caveolin-1 in pulmonary endothelial homeostasis has been well documented, its involvement in endothelial pathophysiology is considerably less well understood. One important question is whether transcellular protein permeability, the main route of protein influx in the lung under normal conditions, can be accelerated

and contribute to protein-rich lung edema formation during pathologic conditions, the main characteristic of ALI and ARDS. This concept has been addressed using in vitro and in vivo models of neutrophil-and oxidant-induced lung injury. Sun et al[73] and Hu et al[74] showed that caveolin-1 is essential for mediating transcellular albumin shuttling across the capillary endothelium in response to both types of insults, introducing a new paradigm in the mechanistic understanding of endothelial permeability regulation in pathologic conditions such as inflammatory hyperpermeability.[73,74]

ROLE OF CAVEOLIN IN PULMONARY ARTERIAL HYPERTENSION

Pulmonary arterial hypertension, defined as mean pulmonary arterial pressure above 25 mm Hg at rest, is a rare disorder affecting young women more commonly than men. The underlying defect is occlusion of medium- and small-sized arteries in the pulmonary circulation caused by thickening of the endothelial layer, known as the "intima," and the surrounding smooth muscle layer, or "media." The resulting obstruction leads to increased resistance of the pulmonary vascular network to the flow of blood, causing right heart strain and, ultimately, right heart failure and death.[75,76]

Key to disease pathogenesis is considered to be the dysregulated hyper-proliferation of the pulmonary endothelium in response to noxious stimuli, including shear stress, reactive oxygen species, autoimmune processes, and toxins.[77] Consequently, the disease is associated with scleroderma and other connective tissue diseases, blood dyscrasias-including sickle cell disease and thalassemia, congenital cardiac defects-including ventricular septal defect, HIV infection, portal hypertension, and use of anorexigenic drugs.[78] The disease may also be heritable, which is commonly associated with gene mutations in members of the transforming growth factor (TGF) receptor super-family including bone morphogenetic protein receptor-2 and activin receptor-like kinase Type 1.[78] Finally, in some cases, no apparent cause is found.[78] A common feature to the above heterogeneous patient groups is the histological appearance of vaso-occlussive defects, thrombosis, and neo-vascularization, collectively known as "plexiform" lesions. Specifically, the "plexiform" lesion is a glomeruloid structure of proliferating endothelial cells forming neo-vessels.[79] Plexiform lesions are frequently located distal to bifurcation sites of small pulmonary artery branches.[80] The fact that PAH cases share common histological features despite different etiologies supports the notion that the disease constitutes a uniform reaction of the pulmonary endothelium to a variety of insults. This response comprises de-regulated angiogenic proliferation of apoptosis-resistant cells expressing endothelial markers, which replace the damaged normal endothelium.[77,81] These cells are, at least in some cases, monoclonal in origin and express anti-apoptotic factors and endothelial cell growth factor receptors while lacking certain tumor suppressor genes.[79,80] Thus, they share characteristics of neoplastic cells.

Ultrastructurally, endothelial cells observed in lungs from patients with pulmonary hypertension are rich in endoplasmic reticulum, Golgi apparatus, and Weibel-Palade bodies.[82] In advanced plexiform lesions, they contain fibrillar structures and, at least in one report, no caveolae.[83,84] Using microarray analysis of lung tissue samples from patients with PAH, Geraci et al reported reduced caveolin-1 mRNA levels compared to controls.[85] The same group further investigated caveolin-1 expression in human lungs and found that, although caveolin-1 levels in the whole lung homogenate were not different between the PAH and controls groups, caveolin-1 was very weakly expressed in the endothelium of

plexiform lesions and pathological pulmonary arteries.[86] These studies demonstrated that the abnormal endothelium in lungs from PAH patients contains low caveolin-1 levels, although the significance of this finding is only now beginning to emerge.

The role of caveolin-1 in PAH has recently been addressed in a series of experimental studies. Reduction in caveolin-1 expression has been demonstrated in two rat models of PAH, one using the classic PAH inducing agent monocrotaline,[87] and the other using a recently developed method of pharmacologic VEGF receptor-2 antagonism and hypoxia.[86] In the study by Matthew et al rats exposed to monocrotaline showed a reduction in lung caveolin-1 expression as early as 48 hours post-insult. This was associated with activation of the transcription factor STAT3 and DNA synthesis in lung tissue and cultured endothelial cells treated with monocrotaline pyrrole, consistent with the function of caveolin-1 as a negative regulator of cell growth. Moreover, incubation of cultured endothelial cells with monocrotaline pyrrole led to sequestration of eNOS within the cytoplasm and away from the cell membrane, its normal site of action, implying that caveolin-1 down-regulation could lead to reduced NO bioavailability and possibly, reactive oxygen species production by eNOS, thus fueling the development of PAH.[88] The findings of Matthew et al were recapitulated by Jasmin et al who in addition, treated mice with the caveolin-1 scaffold domain peptide which was able to attenuate the development of PAH along with activation of STAT3 and induction of cyclin D1 and D3 expression.[89] Similarly, in the model presented by Achcar et al, caveolin-1 and -2 protein expression was reduced in the vascular lesions of experimental animals.

An important development in the field was the observation that caveolin-1 knockout mice developed PAH and right ventricular hypertrophy.[90] This may be attributable to increased pulmonary vascular resistance arising from an abnormally developed vascular system.[18] Serum nitrate levels in these mice are increased[18,90] but so is oxidative and nitrosative stress,[91,92] possibly the result of eNOS uncoupling. This seems to be a critical issue in the pathophysiology of the caveolin-1 knockout as pharmacologic eNOS blockade in these mice reduced oxidant production and reversed pulmonary hypertension.[92,93] These observations were confirmed by Zhao et al with the aid of eNOS/caveolin-1 double knockout mice in which it was shown that eNOS dis-inhibition in the absence of caveolin-1 was associated with protein kinase G inactivation, which was sufficient to explain the pulmonary vascular phenotype in these mice.[17,94] These studies support the concept that caveolin-1 regulation of eNOS activity is crucial to endothelial homeostasis in the lung. Down-regulation of caveolin-1 and associated eNOS dysfunction could be an important factor in PAH pathogenesis.

ROLE OF CAVEOLIN IN PULMONARY FIBROSIS

Pulmonary fibrosis, the progressive replacement of functional lung parenchyma by scar tissue, may represent the final stage of a variety of disorders, including interstitial lung diseases (idiopathic pulmonary fibrosis-IPF, nonspecific interstitial pneumonitis), collagen/vascular diseases-most notably scleroderma, occupational exposures (silicosis, asbestosis), and less frequently Acute Respiratory Distress Syndrome. Common features in these disorders are the over-production of collagen and extracellular matrix by activated fibroblasts and myofibroblasts, which seems to be driven by TGF-β.[95-99] As pointed out in a detailed review by Del Galdo et al,[100] caveolin-1 seems to be a critical regulator in TGF-β signaling and thus implicated in the pathogenesis of pulmonary fibrosis.

Studies in the caveolin-1 knockout mouse have paved the way for research on the involvement of this protein in fibrotic disorders. Surprisingly, genetic deletion of caveolin-1 in mice was not lethal, although this did result in marked abnormalities in the lung microachitecture. Probably the most prominent finding was the loss of the typical two-layer pattern of the alveolar septa consisting of endothelial and adjacent epithelial cells in favor of a multi-layered, hypercellular appearance resulting in thickened alveolar walls (Fig. 3).[11,101] Areas of increased collagen and reticulin fibers as well as α-smooth muscle actin-expressing cells, most likely myofibroblasts, were also found and these alterations were associated with reduced lung compliance.[16,18,101,102] Moreover, embryonic fibroblasts from these mice proliferated in culture at double the rate of control fibroblasts, indicating that caveolin-1 was required for cell-cycle control in these cells and that, in the absence of caveolin-1, fibroblast hyperproliferation and enhanced collagen production could be expected.[16] TGF-β expression was increased in these mice as well, which resulted in augmented SMAD signaling.[102,103] Interestingly, rescue of caveolin-1 expression selectively in the endothelium partially reduced the levels of TGF-β and collagen I and III, indicating that the fibrosis may be sustained, at least in part, by the endothelium.

On the basis of these data, expression patterns of caveolin-1 were explored in experimental animal models of fibrosis as well as in human cells and lung tissue. Kasper et al described in 1998 the down-regulation of caveolin-1 in ATI cells of mouse lungs rendered

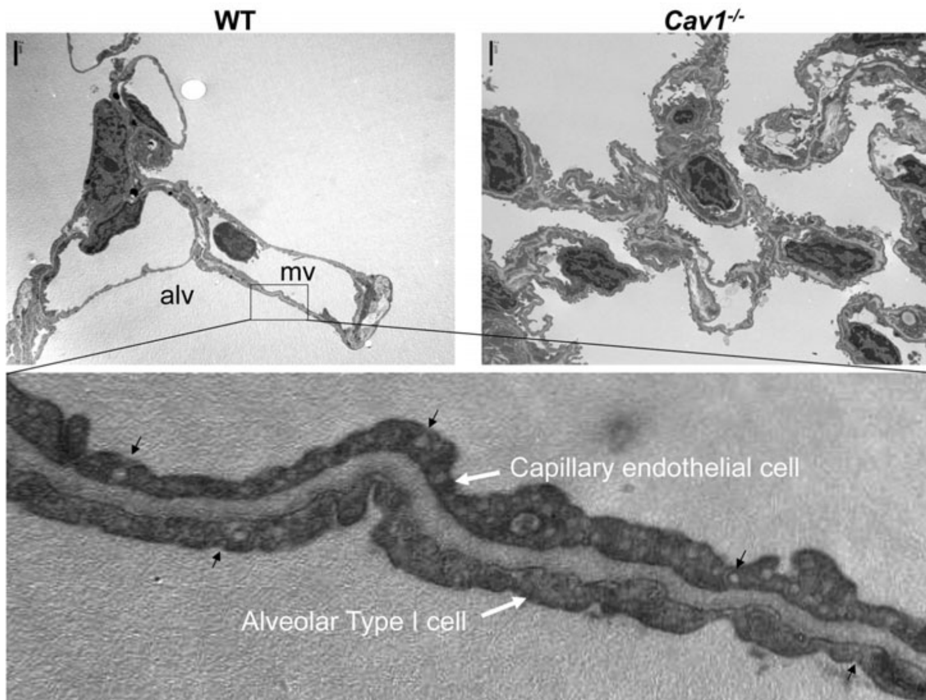

Figure 3. Ultrastructure of alveolar septa in wild type and *Cav1*$^{-/-}$ lungs. Note absence of caveolae, hypercellularity and septal thickening of *Cav1*$^{-/-}$ lung (top right) and abundance of caveolae in thin alveolar, and capillary gas exchange cellular membranes of WT mouse lung (top left and bottom). Images courtesy of Oleg Chaga, PhD., University of Illinois-Chicago.

fibrotic by irradiation.[41] Similar findings were obtained using the bleomycin fibrosis model in mice.[104] Furthermore, caveolin-1 was down-regulated in fibroblasts obtained from patients with IPF and scleroderma-induced lung fibrosis.[105,106] Similarly, caveolin-1 was under-expressed in lung tissues from patients with the above disorders.[104,106,107] In this context, Odajima et al described the loss of caveolin-1 in the metaplastic bronchial epithelium that lines the areas of scarring in fibrotic lungs.[104] These workers argued that loss of caveolin-1 may be associated with abnormal re-epithelialization in lung fibrosis. Interestingly, in at least two studies, treatment of mice exposed to bleomycin with the caveolin-1 scaffold domain peptide was enough to ameliorate fibrosis, suggesting that this part of the protein, which is known to inhibit the activity of various kinases and eNOS, somehow regulates pulmonary responses to pro-fibrotic stimuli.[106,108]

Mechanisms by which caveolin-1 regulates fibroblast function and how this is perturbed in fibrosis include caveolar trafficking of the TGF-β receptor and control of cell signaling processes via the scaffold domain. TGF-β signals by binding and forming a complex with its cell surface receptors, TGF-β RI and II, which possess Ser/Thr kinase activity.[109] Upon receptor activation, the complex is endocytosed via clathrin-, caveolin-1, and early endosome antigen-1 (EEA1)-positive vesicles.[70,109-112] Endocytosis via clathrin-coated pits and EEA-1 positive vesicles can enhance TGF-β signaling by bringing the receptors in close proximity with SMAD effector molecules.[109] Conversely, endocytosis via caveolae seems to facilitate ubiquitinylation and degradation of the receptors, thus turning off signaling. It follows therefore, that in conditions in which caveolin-1 is depleted, TGF-β activity would be dis-inhibited. However, TGF-β may also trigger SMAD-independent pathways, including Mitogen-Activated Kinases (MAPK). In this respect, the p42/44 MAPK pathway, a known activator of collagen synthesis, is upregulated in lung fibroblasts from scleroderma patients or following down-regulation of caveolin-1 with small-interfering RNA.[105] Similarly, the c-Jun terminal kinase pathway (JNK) is also activated in the absence of caveolin-1 and may be suppressed by administering the scaffold peptide.[106,108] These results highlight the importance of caveolin-1 in TGF-β regulation on multiple levels and provide important insights into the mechanisms of TGF-β-induced fibrogenesis. As there is no known therapy for these diseases, caveolin-1-regulated pathways could provide a novel and viable therapeutic approach.

ROLE OF CAVEOLIN IN OBSTRUCTIVE AIRWAY DISEASES

Asthma, one of the most common respiratory diseases, is a condition of episodic cough and dyspnea caused by reversible peripheral airway obstruction. In many instances the disease is allergic in etiology, although other factors including airway irritant substances and emotional or physical stress may trigger asthma attacks. Chronic inflammation of the bronchial mucosa with mucous gland and bronchial smooth muscle hypertrophy and hyperplasia is invariably found in biopsies from asthmatic patients and thus airflow limitations in asthma can be due to airway lumen obliteration by mucous plugs and reduced airway caliber due to bronchoconstriction.[113] In many patients with chronic asthma, airway remodeling, characterized by increased muscle mass and peribronchial fibrosis, has been observed.[113,114] Although the functional consequences and long-term prognosis of this process are still debated, it is becoming increasingly clear that it may lead to a sustained state of airway narrowing. Moreover, contraction of an abnormally thickened airway muscle layer produces greater lumen narrowing and is more effective at occluding peripheral airways than muscle layers

of normal thickness, thus increasing symptom severity during episodic asthma attacks.[113] A host of inflammatory and mitogenic mediators drive bronchial cell responses in asthma, including interleukin-4, -5, -6, -17, and -33, TGF-β, TNF-α, VEGF and others.[115]

Depending on the extracellular environment, airway smooth muscle cells are able to shift between a more differentiated, contractile state, and a synthetic state ("phenotypic plasticity").[116] Cells in the contractile state are elongated and express muscarinic M3 cell-surface receptors, which, in conjunction with the presence of cytoskeletal and contractile-apparatus proteins (α-, γ-smooth-muscle actin, myosin heavy chain, calponin, *h*-caldesmon, SM22, desmin, smoothelin), render them responsive to the length-shortening effects of acetylcholine.[116-118] Transition to this phenotype is triggered by stimuli including TGF-β, laminin and insulin.[119-122] Conversely, in the presence of platelet-derived growth factor (PDGF), fibronectin, and fetal bovine serum, the synthetic phenotype is favored, characterized by a marked capacity to hyperproliferate and synthesize extracellular matrix components.[116,119,123] Caveolae formation seems to be a feature of the differentiated state, and thus it is not surprising that in synthetic cells, caveolin-1, despite being expressed, is mislocalized in a perinuclear distribution away from the membrane.[124]

Given its multiplicity of functions ranging from cell-cycle control to signal transduction regulation, caveolin-1 has been the subject of scrutiny in the field of smooth muscle pathophysiology. Regarding cell signaling control, it appears that caveolin-1 modulates key mechanisms underlying smooth muscle differentiation. This process depends on expression of a specific set of genes under the control of the transcription factor serum response factor (SRF) and is triggered by ligand binding to cell surface receptors, such as the M3 muscarinic receptor, TGF-β, and TNF-α. Communication of surface receptors with the nucleus is facilitated by signaling intermediates, which include the RhoA/Rho kinase pathway, PI3-kinase, and the SMAD system. Although caveolin-1 has no apparent signaling function per se, its contribution in this context lies in coupling and co-ordinating cell signaling networks downstream of cell surface receptors. Importantly, while the RhoA/Rho kinase pathway seems to require caveolin-1 for its activation, SMADs and PI3-kinase are inhibited.[125-128] Thus, caveolin-1 is not only required for smooth muscle cell differentiation, but also for smooth muscle contraction in response to agonists.[116]

With respect to caveolin-1 and cell division, it was shown that incubation of airway smooth muscle cells with PDGF in vitro resulted in accelerated lysosomal degradation of caveolin-1, accompanied by a marked reduction in caveolae and cell proliferation.[123] In this context, siRNA-mediated caveolin-1 gene-silencing activates the p42/44 MAP-kinase pathway and increases cell proliferation.[7] Interestingly, the same group reported later that p42/44 MAP kinases were not found within caveolar structures, indicating that in the cell types examined, the inhibitory effect of caveolin-1 on p42/44 MAP-kinase was indirect.[129] In line with these in vitro findings, modest caveolin-1 down-regulation was demonstrated in an asthma mouse model, predominantly in lung fibroblasts.[130]

From the above presented studies, it is becoming increasingly evident that caveolae are central organelles for the processing of environmental signals which control airway smooth muscle function and differentiation. Accordingly, caveolin-1 and caveolae appear to participate in the pathogenetic mechanisms of bronchial smooth muscle thickening and hypercontractility, which are the anatomic basis of asthma symptoms. To what extent these insights will translate into treatment interventions remains to be determined.

CAVEOLIN CONTROL OF THE INFLAMMATORY RESPONSE

A unique feature of the lung and the gut is that they are in direct contact with the external environment and yet lack the benefits of a protective layer of skin. The lung is the organism's first line of defense against inhaled threats and must possess the armamentarium to perform this task. Tools available to deal with these threats include structures capable of trapping and removing particulate matter such as nasal hair, airway mucous, ciliated airway epithelium, and cough reflexes; antibodies secreted in epithelial lining fluid; and a highly developed cellular and humoral immune system capable of antigen recognition, phagocytosis, and production of antibodies specific to the invading agents. In recent years, it has become apparent that caveolin-1 is not only involved in maintaining homeostasis in healthy tissues but is also involved in disease pathogenesis, with particular respect to infection and inflammation. Work in this field has been motivated by observations that caveolae may serve as entry routes for several micro-organisms, including viruses,[131] bacteria[132] or even immune cells.[133] In addition, caveolin-1 expression may be induced by endotoxin in an NF-κB-dependent manner.[134] Thus, caveolin-1 may be an important factor regulating cellular responses to pathogen invasion and stress in general.

A seminal event in ALI pathogenesis is the breakdown of the pulmonary endothelial and epithelial barrier and the subsequent leakage of plasma fluid in the airspace. As outlined above, an important determinant of microvascular permeability in the lung is eNOS-derived NO which is under the control of caveolin-1. In response to vasoactive agents, including Platelet Activating Factor (PAF) or thrombin, microvascular permeability increases in various vascular beds[62,135] associated with Ca^{2+}-mediated eNOS activation by Ca^{2+}-dependent calmodulin.[136] Caveolin-1 via its scaffold domain[9] may compete with calmodulin for binding to eNOS and thus block eNOS-mediated permeability increases.[135,137,138] This effect could potentially be exploited therapeutically, depending on the availability of nontoxic and effective carrier systems for intracellular delivery of the scaffold domain peptide.

An important function of caveolin-1 in innate immunity lies in the regulation of cellular signaling events which drive the pulmonary response to such insults as invasion of micro-organisms, physical or chemical injury, or systemic inflammatory states. Challenge of caveolin-knockout mice with endotoxin showed that these mice were resistant to LPS-induced mortality, systemic cytokine response, and acute lung injury compared to WT mice.[139,140] The protection of caveolin-1 knockout mice was abrogated upon administration of an eNOS inhibitor, in line with the established finding that caveolin-1 is an endogenous inhibitor of eNOS.

Insight into the significance of caveolin-1 in immune function was gained from experiments involving exposure of knockout mice to live micro-organisms. In the first of these studies, Medina et al infected mice with *Salmonella typhimurium*.[141] These workers noted that caveolin-1 knockout mice exhibited higher mortality rates and bacterial numbers than caveolin-1-expressing mice.[141] Interestingly, levels of several pro- and anti-inflammatory cytokines, as well as blood nitrate, were higher in knockout mice, consistent with an excessive inflammatory response. In later work by the same group, mice were challenged with the parasite *Trypanosoma Cruzi*.[142] A striking feature of this experiment was that levels of major cytokines, including TNF-α, IL-6 and IFN-γ failed to rise in knockout mice to the extent noted in WT mice, despite similar degrees of tissue invasion by the pathogen.[142] In cultured peritoneal macrophages infected by the parasite, reduction in the levels of inflammatory cytokines was documented in cells from knockout mice. Using the cecal ligation and puncture model, which produces peritonitis and sepsis,

Feng et al[143] reported an exuberant production of TNF-α and IL-6 in caveolin-1 knockout mice compared to WT controls. However, this was associated with impaired ability to clear bacteria, increased thymocyte apoptosis, and mortality.

The above studies using different invading pathogens underscore the importance of caveolin-1 in the generation of the innate immune response. This issue has been dealt with by a number of mechanistic studies. A general conclusion to be drawn from these reports, which will subsequently be discussed in more detail, is that caveolin-1 is not only required for proper function of immune cells (primarily neutrophils and macrophages, since lymphocytes do not express caveolin-1), but also for the activation of lung endothelial and possibly epithelial cells in the context of inflammation. However, emerging data also point to a role of caveolin-1 in *limiting* the magnitude of the inflammatory response by interfering with Toll-like receptor-4 (TLR-4) and NF-κB signal transduction pathways. In in vitro assays, Li et al observed decreased phagocytotic ability in cultured primary peritoneal macrophages isolated from caveolin-1 knockout mice compared to WT.[144] This finding was correlated with increased numbers of apoptotic cells found in the thymus of irradiated knockout mice, which resulted from failure of macrophages to clear apoptotic cells.[144] Hu et al focused on the other important cellular component of the innate immune system, the neutrophil. Using in vitro stimulated peripheral blood neutrophils isolated from mice, they observed that the oxidative burst was attenuated in neutrophils lacking caveolin-1, and that caveolin-1 knockout neutrophils also demonstrated reduced migratory capacity and adhesiveness to fibrinogen-coated surfaces.[145] Activation of Rac1 and Rac2 in these neutrophils following challenge with Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) was also attenuated in the absence of caveolin-1.[145] These functional deficits in neutrophils from caveolin-1 knockout mice were associated with an inability to cause microvascular barrier disruption and edema in ex vivo lung preparations perfused with fMLP or PAF-stimulated neutrophils.[145]

By generating mice deficient in both caveolin-1 and eNOS, Mirza et al showed that eNOS derepression due to lack of caveolin-1 expression could result in failure of endotoxin to activate transcription factor NF-κB, thus eliminating the cellular inflammatory response.[140] This effect was mediated by excessive nitration, leading to reduced activity of interleukin-1-receptor-associated-kinase-(IRAK)-4. This work uncovered an important molecular mechanism to explain how fine-tuning of eNOS function by caveolin-1 may regulate the NF-κB pathway activation status. Another mechanism by which caveolin-1 and caveolae may activate the inflammatory response is the endocytosis of the IL-1 receptor, which is a mandatory step in order for the cytokine to activate gene transcription via NF-κB.[146] On a similar note, caveolin-1 seems to be instrumental in the mechanism of up-regulation of inflammatory cytokine MCP-1[147] by environmental toxins as well as for the pro-inflammatory effects of TNF-α on endothelial cells.[148]

Conversely, caveolin-1 may exert inhibitory effects on the inflammatory cascade, as demonstrated by Wang et al in the case of TLR-4.[149] This group demonstrated direct binding of caveolin-1 to TLR-4 in murine macrophages and showed that this binding was inhibitory with respect to the ability of endotoxin to stimulate inflammatory cytokine production via TLR-4 stimulation.[149] They further observed that caveolin-1-mediated recruitment of Heme oxygenase-1 into lipid rafts was able to suppress TLR-4 signaling via carbon monoxide production, indicating that caveolin-1 exerts control on TLR-4 downstream events via multiple pathways.

Summarizing the above studies, it would seem that caveolin-1 and caveolae, besides being a portal of entry for intracellular invasion of micro-organisms, are also central orchestrators

of the innate immune system defensive strategy against these pathogens (Fig. 4). This function may be attributed to the modulation of key signaling pathways including NF-κB, TNF-α, IL-1 and TLR-4, either due to direct binding of their components to caveolin-1 or through other mediators, including NO and CO.

ROLE OF CAVEOLIN IN LUNG TUMORIGENESIS

Lung cancer is the most common cancer-related cause of death in both men and women in the United States. The most common histologic types of lung cancer are small cell lung carcinoma (SCLC) and nonsmall cell lung carcinoma (NSCLC). SCLC is

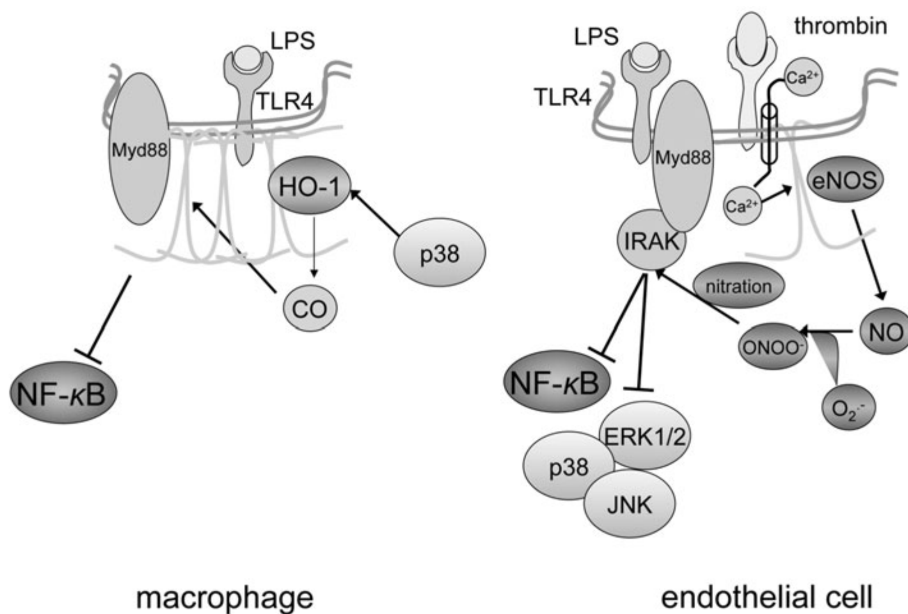

Figure 4. Modulation of Toll-like-receptor-dependent signaling pathways by caveolin-1. Data obtained primarily on macrophages (left panel) favor a suppressive function of caveolin-1 on Toll-like receptor-4 (TLR-4) signaling. This is accomplished on several levels, starting by physical association with the receptor within caveolae, which presumably diminishes binding to adaptor protein MyD88, a required step for propagation of the TLR-4 signal upon docking of lipopolysaccharide (LPS). Activation of heme oxygenase-derived CO production by p38, which is expected to occur following TLR-4 ligation by LPS, is an additional mechanism to turn off the TLR-4 signal (adapted from Wang et al 2009). A seemingly opposing function of caveolin-1 has been advocated in knockdown experiments in endothelial cells (right panel). Excessive free radical (nitric oxide-NO and superoxide-O_2^{-}) production due to lack of caveolin-1-mediated endothelial NO synthase (eNOS) inhibition may lead to nitration of interleukin-1-associated receptor kinase (IRAK)-4, an important intermediate between TLR-4 and its effector molecules nuclear factor-κB (NF-κB) and mitogen-activated kinases (p38, ERK1/2, JNK). Hypothetically, this mechanism could take effect in situations in which eNOS is uncoupled from caveolin-1, as may occur in the presence of vasoactive mediators including thrombin. In this case, dual activation of eNOS due to Ca^{++} influx and dissociation from caveolin-1 could result in production of large amounts of free radicals and tyrosine nitration via the reactive metabolite peroxynitrite ($ONOO^-$). A color version of this image is available online at www.landesbioscience.com/curie.

derived from pulmonary neuroendocrine cells, which are relatively small, grow in diffuse patterns, and express neuroendocrine markers including synaptophysin, chromogranin, CD56, and TTF-1.[150] These tumors are often metastatic at the time of clinical presentation and are responsive to treatment with chemotherapy but invariably relapse, accounting for the very high 5-year mortality rate seen in patients with these tumors. NSCLC are most frequently comprised of, in descending order of frequency, adeno-, squamous-, and large cell-carcinomas.[151] These tumors grow locally and metastasize relatively later in the disease course. When detected at an early stage, they can be cured by surgical resection alone or followed by chemotherapy.[152] At later stages of more extensive local or metastatic spread, the treatment usually includes conventional chemotherapy with or without VEGF or EGF inhibitors, radiation, and symptomatic measures,[151] while surgery is much less commonly performed.[153]

Of importance to the pathogenesis of lung cancer is the effect of carcinogens found in cigarette smoke on the bronchial epithelium.[152] These substances induce premalignant cellular alterations of varying degrees across large areas of bronchial epithelium exposed to cigarette smoke (the 'field cancerization effect'), thus creating the substrate of malignant transformation.[152] Point mutations or chromosomal alterations may result in deletions of tumor suppressor genes and/or activation of proto-oncogenes, leading to malignant transformation and clonal expansion.[152]

Consistent with the first description of caveolin-1 as a substrate of v-*Src*, a tyrosine kinase product of the *v-src* oncogene[10,154-156] which is able to induce avian sarcomas, caveolin-1 has been extensively investigated for its role in cell cycle control and cancer development. Initial reports described caveolin-1 downregulation in cells transformed by various oncogenes, including v-abl and H-ras.[157] Conversely, downregulation of caveolin-1 expression resulted in dis-inhibition of the p42/44 MAP-kinase pathway, loss of anchorage-dependent cell growth, and tumorigenesis in mice.[15] In addition, a number of signaling proteins relevant to tumorigenesis, including the EGF receptor, H-*ras*, and p42/44 MAPK were found to be physically associated with and inhibited by caveolin-1.[15,158] Moreover, caveolin-1 was found to suppress levels of Cyclin D1 which is involved in malignant cell transformation and also to reduce tumor vessel leakiness by regulating eNOS-derived NO levels.[159,160]

On the basis of these and other data, researchers quantified caveolin-1 expression levels in various human cancers. One of the earliest publications was by Racine et al who reported decreased caveolin-1 expression in a variety of human lung cancer cell lines.[161] Using DNA microarrays, Wilkman et al reported reduced mRNA transcripts for caveolin-1 and -2 in human lung adenocarcinomas compared to normal lung tissue.[162] The same group later presented similar findings corroborated by immunohistochemical techniques.[163] In apparent contrast to these data was the report by Sunaga et al who examined cell lines derived from a large number of cancer patients. They showed that caveolin-1 was upregulated in nonsmall cell cancers and down-regulated in small cell cancers.[164] Similarly, Ho et al showed that caveolin-1 was down-regulated in regionally limited adenocarcinomas, whereas it was upregulated in cases presenting with lymph node metastasis. The authors argued that caveolin-1 could be a factor associated with enhanced metastatic potential in cancer cells.[165] These findings were corroborated by Zhang et al who observed enhanced caveolin-1 expression in more advanced lesions.[126] In the same context, Yeh et al studied the properties of a small-cell cancer cell line in vitro. They found that baseline caveolin-1 expression was low and that re-introduction of caveolin-1 into the cells was associated with decelerated growth rate but enhanced metastatic potential.[166]

Of note, a relationship between phosphorylated caveolin-1 and activation of the RhoA pathway was detected in colon cancer cells, which appears to enhance cell motility and the ability of tumor cells to metastasize.[167] Finally, in the same context, Bonuccelli et al showed in breast cancer cells that the P132L caveolin-1 mutation is associated with a differential gene expression profile which favors cancer invasiveness and propensity to extramammary spread.[168]

An interesting twist to the story came in a series of observations in which caveolin-1 expression in cancer cells was monitored following chemotherapy.[169,170] These studies showed that exposure of cultured lung cancer cells to various chemotherapeutics was associated with a strong induction in caveolin-1 expression. Since most studies looking at caveolin-1 expression levels do not report the chemotherapy exposure of donor subjects, the effect of chemotherapy on caveolin-1 expression level cannot be assessed.

In summary, while the majority of in vitro mechanistic data support a role of caveolin-1 as a cell cycle regulator and tumor suppressor, it seems possible that it may also be instrumental in increasing metastatic potential by promoting cell motility. The observation that caveolin-1 may be induced by cytotoxic chemotherapy implies that it could be involved in tumor cell resistance to drugs and emergence of relapse. These findings underscore the biological diversity of human tumors and the multiplicity of caveolin's biological functions.

CONCLUSION

Data from in vitro, animal, and human studies conducted over the past 20 years have revealed, unexpectedly, abnormalities in caveolin-1 expression and caveolin-1-related pathways in a variety of lung diseases and experimental models. However, our understanding of how caveolin-1 is involved in these disease processes is rudimentary, owing in part to the complex phenotype of the global caveolin-1 knockout mouse. Cell-type specific caveolin-1 deletion will further our insights into the many biological roles of this important protein. In addition, search for caveolin-1 mutations and polymorphisms, such as P132L[152] which predicts poor prognosis of breast cancer, may be fruitful in determining the roles of caveolin-1 in lung disease pathogenesis.

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INDEX

A

Adhesion 31, 36, 37, 40-42, 52, 54, 77, 84, 128, 133, 138, 161
Adipocyte 4, 8, 9, 30, 36, 39, 84, 105, 112-122, 130, 145
Akt 35-39, 52, 55-57, 99, 102, 115, 136, 150, 151, 161, 163
Alveolar epithelium 159, 161, 162
Androgen 95-98, 100-103, 106
  receptor 96, 97
Arrhythmia 145, 147, 148, 152
Artery 54, 57, 58, 73, 127, 138, 147, 148, 150, 158, 164, 165
Atherosclerosis 52, 54, 127-131, 133, 134, 136-139, 146, 147, 152
Autophagy 89-92

B

Bacteria 65, 69, 77, 170
Barrier function 163
B-catenin 37-39, 84, 160, 161
Beta-cell 7
Blood brain barrier 66, 132
Blood flow 5, 121, 122, 150, 158, 163
Breast 8, 32, 33, 37, 39, 72, 83-87, 89-91, 96, 105, 106, 159, 173

C

Cancer 8, 15, 32, 33, 37-39, 42, 43, 68, 83-87, 89-92, 95-97, 99-106, 161, 171-173
Cardiac hypertrophy 8, 9, 37, 146, 148, 149, 152
Cardiac myocyte 9, 72-74, 130, 145, 146, 149-152
Cardiac physiology 145
Cardiomyopathy 72, 73, 145, 146, 148, 149
Caveolae 3-10, 14-25, 29-32, 34-36, 38-43, 51-59, 65-72, 75-77, 84, 95, 96, 98, 99, 102, 103, 105, 111-122, 127, 129-136, 138, 145-152, 158, 161-164, 166-171
  -mediated endocytoses 22, 24, 114, 119, 121
Caveolin 4-10, 15-19, 21-25, 29, 38, 43, 51-56, 59, 65-69, 71-76, 83-87, 89, 91, 95-98, 101-105, 111-119, 121, 127, 129-131, 133-137, 145-152, 157-173
  expression 29, 69, 158, 159
  isoform 17
  Caveolin-1 5-9, 15-19, 21-24, 29, 54-56, 66, 71, 83-87, 89, 91, 95-97, 101, 105, 112-119, 121, 127, 129-131, 133-137, 150, 157-173
    knockout mice (Cav-1KO) 150
    scaffold 30-32, 35, 36, 40, 42, 43

Caveolin-2 7-9, 15, 17, 24, 116, 130, 146, 158, 159
Caveolin-3 9, 54, 74, 112, 115-118, 130, 136
Caveosome 5, 7, 10, 19-25, 66-68, 113
Cavin-1 32, 34, 103, 116-118
Cell cycle 37, 71, 74, 87, 89, 91, 92, 97, 172, 173
Cholesterol 3-10, 14-16, 23, 30-32, 34, 35, 51-56, 59, 66, 69-71, 75-77, 95, 98, 102, 103, 106, 112, 113, 115, 118, 121, 127-136, 138, 146, 147, 150, 158
trafficking 7, 30, 75
Clathrin 6, 14, 16-18, 21, 22, 24, 33, 35, 67, 68, 77, 111, 112, 114, 115, 117-120, 162, 167
-coated pit 112, 114, 115, 119, 120, 162, 167
Cystic fibrosis 69

D

Degradation 14, 19, 23, 24, 33, 38, 42, 89, 92, 103, 114-118, 160, 161, 167, 168
Detergent-resistant 14, 30, 112, 113, 116, 119
Differentiation 37, 39, 42, 97, 98, 159-162, 168
Ductal carcinoma in situ (DCIS) 87-89

E

EGF receptor 33-35, 104, 172
Endocytosis 3, 4, 10, 14-22, 24, 33, 34, 36, 37, 68, 69, 75, 77, 111, 113, 114, 117-121, 129, 167, 170
Endosome 5, 10, 14, 19, 21-25, 36, 67, 68, 75, 77, 167
Endothelial cell 4, 5, 8, 15-18, 30, 40, 42, 51, 52, 54-59, 66, 72, 84, 99, 105, 112, 121, 122, 127-129, 131-134, 136, 137, 145-148, 158, 159, 162-165, 170, 171
Endothelial permeability 157, 163, 164
ERK1/2 35-39, 42, 84, 87, 137, 149, 150, 171
Estrogen 8, 33, 34, 51, 52, 56, 57, 59, 83, 85-87, 97

F

Fatty acid 7, 9, 77, 95, 100, 106, 116, 118, 121, 128
Fatty acid synthase 100

G

Ganglioside 16, 30, 35, 36, 77, 112, 114
Glucose transporter 111, 117, 150
GLUT-4 111, 117-120, 122, 150
Glucose uptake 115, 117-120
GPI-linked protein 3, 4, 10
G protein 4, 7, 9, 17, 30, 33, 34, 40, 42, 51, 56, 57, 76, 146, 150, 151, 158

H

Heart disease 73, 131
Heart failure 148, 149, 152, 164
Hepatocyte 112, 114, 117
High-density lipoprotein (HDL) 6, 51-57, 59, 75, 132
HIV 16, 66, 70, 72, 164

I

Insulin receptor (IR) 4, 7, 31, 33, 34, 111-122
substrate-1 (IRS1) 114, 115, 120, 122
Insulin resistance 33, 111, 112, 114, 116, 117, 120-122
Insulin signalling 111, 112, 114-117, 121, 122
Ischemia/reperfusion (I/R) 146, 150-152
Ischemic preconditioning 150, 151
Isoflurane 150, 152

K

Kinase 4, 16-19, 23-25, 29, 30, 33, 34, 36-40, 42, 51, 52, 55-58, 72, 76, 83, 84, 98, 99, 103, 114, 115, 130, 131, 134, 137, 138, 146, 148, 150, 151, 158, 160-165, 167, 168, 170-172

L

Leishmania 65, 70
Lipid 3-6, 9, 10, 14, 15, 24, 29, 30, 32, 42, 51-55, 59, 66, 67, 69-71, 75-77, 95, 96, 98-100, 102, 103, 113, 118, 127-131, 134-136, 138, 146, 149, 158, 170
  raft 3-6, 9, 10, 14, 29, 30, 52, 59, 66, 67, 69-71, 76, 77, 98, 100, 102, 103, 113, 129, 130, 149, 170
Lipodystrophy 116
Lipoprotein 3, 7, 51-55, 75, 103, 104, 128, 129, 131, 133, 134
Liver 55, 102, 111, 112, 114, 115, 117
Low-density lipoprotein (LDL) 7, 53, 54, 75, 103, 128, 129, 131-133, 138, 147
Lung development 158-160
Lung disease 157, 165, 173
Lung function 8, 157
Lung tumorigenesis 171
Lysosome 14, 19, 23, 24, 70-72, 75, 77, 89, 114, 117, 128

M

Macrophage 17, 66, 69-71, 73, 128, 129, 133-136, 138, 157, 169-171
Membrane microdomain 3, 29, 36, 96, 98, 102, 129
Metastasis 100, 101, 172
Methyl-b-cyclodextrin 16, 56, 69, 135, 149, 150
Microenvironment 87, 103-105, 150
Migration 29, 31, 33, 35-37, 40-42, 51, 52, 55-57, 59, 66, 77, 85, 106, 129, 136, 137, 139
Muscle 8, 9, 24, 25, 30, 52, 72, 87, 99, 103, 111, 112, 115-122, 128-130, 133, 134, 136, 145-148, 157, 158, 164, 166-168
Mutation 8, 17, 32, 85, 86, 90, 97, 99, 114, 116, 117, 145, 148, 149, 164, 172, 173
Myocardial ischemia/reperfusion (I/R) injuries 150-152

N

Nitric oxide synthase 6, 30, 53, 130, 133, 146-148, 150, 151

O

Obesity 111, 112, 120-122
Obstructive airway disease 167
Ovariectomy 87
Oxidative stress 90, 92

P

Palmitoylation 15, 17, 52, 53, 56, 98, 101
Papillomavirus 67, 68
Parasite 65, 70-73, 75, 76, 169
Phosphatase 16-18, 20, 23, 58, 99, 115
Phosphatidylinositol-(4,5)-bisphosphate 115
Phosphoinositide 3-kinase (PI3kinase) 36-38, 52, 56-58, 115, 116
Polymerase I transcript release factor (PTRF) 9, 15, 31, 32, 34, 103, 116-118
  PTRF/Cavin-1 34
Postconditioning 150
Prognosis 32, 83, 89, 99, 167, 173
Proliferation 29, 36-40, 57, 83, 84, 86, 98, 102, 129, 134, 136-139, 160, 161, 164, 168
Prostate cancer 8, 32, 39, 43, 89, 95-97, 99, 101, 102, 104, 105
Protein kinase B (PKB) 115, 116
Pulmonary disease 157, 158
Pulmonary endothelium 162-164
Pulmonary fibrosis 148, 165
Pulmonary hypertension 52, 157, 164, 165

R

Raft 3-6, 9, 10, 14, 29-35, 39, 41, 42, 52, 59, 66, 67, 69-71, 76, 77, 98, 100, 102, 103, 113, 115, 118, 119, 129, 130, 149, 170
Reperfusion injury salvage kinase (RISK) 150, 151
  pathway 150
Retinoblastoma protein (RB) 87, 89

S

S-acylation 98, 101
Salmonella 69, 169
Signaling 4-9, 15-17, 19, 23, 24, 29-33, 35-42, 51, 52, 55, 56, 58, 59, 66, 76, 83-86, 95-106, 111-117, 121, 122, 127, 129-131, 133, 136-138, 145-152, 158, 160, 161, 163, 165-172
Signal transduction 3, 4, 6, 7, 23, 24, 51, 55, 56, 66, 97, 98, 102, 103, 106, 120, 129, 130, 145, 146, 168, 170
Smooth muscle cell 8, 24, 25, 52, 72, 99, 128, 129, 133, 134, 136, 145, 147, 158, 168
Src 4, 17, 19, 29-31, 33, 35-38, 40-42, 55-57, 76, 83, 98, 130, 146, 148, 150, 158, 162, 163, 172
  -like kinase 146
Stroma 83, 87, 89-92, 95, 97, 99, 105, 106
Survival 29, 36-39, 70-72, 89, 91, 96, 97, 99, 101, 103, 104, 106, 135, 138

T

Tamoxifen 85-87, 89
Therapy 38, 56, 86, 90, 91, 96, 97, 100, 102, 147, 152, 167
Toxoplasma gondii 65, 73
Transcytosis 3, 4, 7, 121, 129, 131-133, 138, 162
Trypanosoma cruzi 65, 72, 74, 169
Tumor stroma 89, 91, 92
Type 2 diabetes 111, 112, 114, 117, 120-122
Tyrosine phosphorylation 17, 19, 24

V

Vascular disease 54, 56, 59, 133, 147, 165
Virus 5, 16, 19-24, 65, 66, 68, 77, 85, 169

X

Xenograft 85, 102, 104