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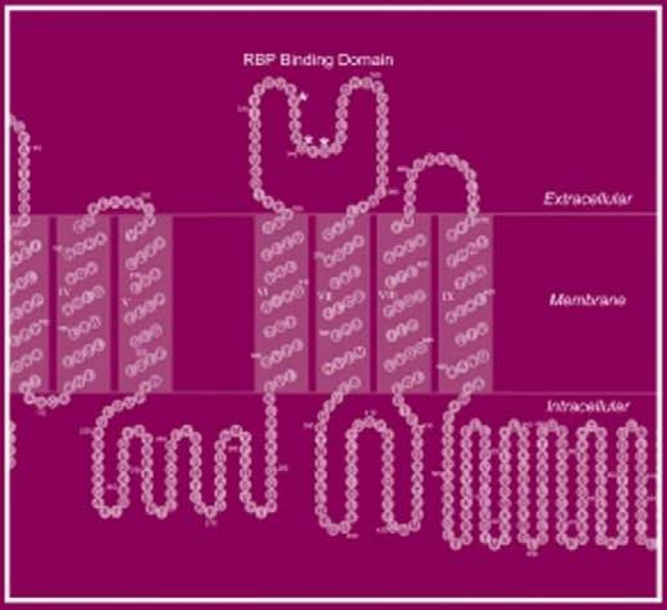
Kwang W. Jeon



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CHAPTER ONE

The Membrane Receptor for Plasma Retinol-Binding Protein, A New Type of Cell-Surface Receptor

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Abstract

Vitamin A is essential for diverse aspects of life ranging from embryogenesis to the proper functioning of most adult organs. Its derivatives (retinoids) have potent biological activities such as regulating cell growth and differentiation. Plasma retinol-binding protein (RBP) is the specific vitamin A carrier protein in the blood that binds to vitamin A with high affinity and delivers it to target organs. A large amount of evidence has accumulated over the past decades supporting the existence of a cell-surface receptor for RBP that mediates cellular vitamin A uptake. Using an unbiased strategy, this specific cell-surface RBP receptor has been identified as STRA6, a multitransmembrane domain protein with previously unknown function. STRA6 is not homologous to any protein of known function and represents a new type of cell-surface receptor. Consistent with the diverse functions of vitamin A, STRA6 is widely expressed in embryonic development and in adult organ systems. Mutations in human STRA6 are associated with severe pathological phenotypes in many organs

such as the eye, brain, heart, and lung. STRA6 binds to RBP with high affinity and mediates vitamin A uptake into cells. This review summarizes the history of the RBP receptor research, its expression in the context of known functions of vitamin A in distinct human organs, structure/function analysis of this new type of membrane receptor, pertinent questions regarding its very existence, and its potential implication in treating human diseases.

Key Words: Vitamin A, Retinoid, RBP, STRA6, Membrane receptor, Retinol, Anophthalmia, Mental retardation.

1. Introduction

The molecular mechanism for vitamin A's physiological function was first elucidated for vision (Wald, 1968). Vitamin A's multitasking ability kept on surprising researchers starting almost a century ago. Today, biological functions of vitamin A have been discovered in almost every vertebrate organ system. In addition to vision, known biological functions of vitamin A include its roles in embryonic growth and development, immune competence, reproduction, maintenance of epithelial surfaces, and proper functioning of the adult brain (Drager, 2006; Duester, 2008; Mangelsdorf et al., 1993; Napoli, 1999; Ross and Gardner, 1994). Since vitamin A derivatives have profound effects on cellular growth and differentiation, vitamin A also plays positive or negative roles in a wide range of pathological conditions, such as visual disorders (Travis et al., 2006), cancer (Love and Gudas, 1994; Niles, 2004; Verma, 2003), infectious diseases (Stephensen, 2001), diabetes (Basu and Basualdo, 1997; Yang et al., 2005), teratogenicity (Nau et al., 1994), and skin diseases (Chivot, 2005; Orfanos et al., 1997; Zouboulis, 2001). Except for vision, which depends on the aldehyde form of vitamin A, most of these physiological or pathological functions can be ascribed to retinoic acid's effects on nuclear hormone receptors (Chambon, 1996; Evans, 1994). New biological functions are still being discovered for vitamin A derivatives. For example, it was recently discovered that retinal inhibits adipogenesis (Ziouzenkova et al., 2007).

Plasma retinol-binding protein (RBP), a high-affinity vitamin A binding protein, is the principal means of vitamin A transport in the blood, and is responsible for a well-regulated transport system that helps vertebrates adapt to fluctuations in vitamin A levels (Blomhoff et al., 1990). RBP specifically binds to vitamin A, effectively solubilizes it in aqueous solution, and protects it from enzymatic and oxidative damage (Goodman, 1984). In addition, RBP was recently discovered to play a role in insulin resistance (Yang et al., 2005). Using an unbiased strategy combining specific photo-cross-linking, high-affinity purification, and mass spectrometry, the high-affinity cell-surface

RBP receptor has been identified as STRA6, a protein with a multitransmembrane domain architecture typical of channels and transporters, but not homologous to any protein of known function. STRA6 binds to RBP with high affinity and mediates cellular uptake of vitamin A from the vitamin A/RBP complex (holo-RBP). Consistent with the diverse functions of vitamin A, human STRA6 mutations cause severe pathological phenotypes including the absence of eyes (anophthalmia), mental retardation, congenital heart defects, lung hyperplasia, and intrauterine growth retardation (Golzio et al., 2007; Pasutto et al., 2007).

In this review, we provide a summary of current knowledge of vitamin A and RBP, describing in detail our current knowledge of the RBP receptor including its identification, the unique features of its function both as a membrane receptor and a membrane transporter, and the relationships between its tissue distribution and the known organ specific functions of vitamin A. In addition, we provide answers to some pertinent questions related to the RBP receptor and its potential relationships with human diseases.

2. Diverse Physiological Functions of Vitamin A, a Molecule Essential for Vertebrate Survival

Vitamin A has alcohol, aldehyde, acid, and ester forms (Fig. 1.1). Some forms have direct biological activities and other forms serve as important reaction intermediates or as the storage form of vitamin A. Known functions of vitamin A and its derivatives and pathological effects of vitamin A deficiency are discussed in the following sections.

2.1. Alcohol forms of vitamin A

A main function of the alcohol form of vitamin A is to serve as the substrate for RBP for delivery in the blood. The fact that retinol evolved to be the major transport form of vitamin A is likely due to the fact that it is less toxic than retinal and retinoic acid. Free retinal has been shown to be highly toxic (Maeda et al., 2008, 2009). Retinoic acid is the most biologically active form of vitamin A and is also the most toxic form. In addition, retinol can be converted to retinal and retinoic acid.

Retinol also serves as an intermediate in the visual cycle for chromophore regeneration (Chen and Koutalos, 2010; Chen et al., 2005). In addition, the alcohol derivatives of vitamin A have biological activities distinct from retinoic acid. For example, they control the growth of B lymphocytes (Buck et al., 1990, 1991) and function as a survival factor in

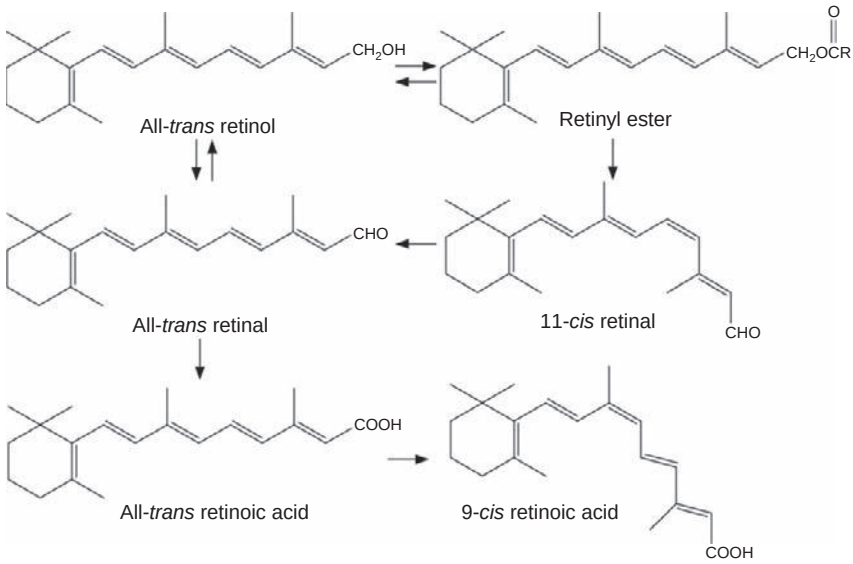

Figure 1.1 **Vitamin A and its major derivatives that have biological activities or serve as important intermediates.**

serum for fibroblasts (Chen et al., 1997). Anhydroretinol, another physiological metabolite of vitamin A, can induce cell death (Chen et al., 1999). Another known function of vitamin A is in the testis. Degeneration of germinal epithelium in testis caused by vitamin A deficiency can be reversed by vitamin A but not by retinoic acid (Griswold et al., 1989; Howell et al., 1963). One likely explanation for the distinction between retinol and retinoic acid is that retinol can be efficiently transported via RBP/retinol *in vivo* but retinoic acid cannot. In addition, retinol can also have biochemical functions distinct from the precursor to retinoic acid (Chen and Khillan, 2010; Hoyos et al., 2005). It was recently discovered that retinol, but not retinoic acid, regulates BMP4 expression in male germ line cells (Baleato et al., 2005). In addition, retinol, but not retinoic acid, prevents the differentiation and promotes the feeder-independent culture of embryonic stem cells (Chen and Khillan, 2008, 2010; Chen et al., 2007).

2.2. Aldehyde forms of vitamin A

Photoreceptor cells in the retina use 11-*cis* retinal as the chromophore (Dowling, 1966). In addition, the aldehyde form of vitamin A serves as an intermediate in the synthesis of retinoic acid, the vitamin A derivative with the most diverse biological functions. It was also recently discovered to inhibit adipogenesis (Ziouzenkova et al., 2007).

2.3. Acid forms of vitamin A

Retinoic acid (vitamin A acid) was initially known as a morphogen in development (Marshall et al., 1996; Reijntjes et al., 2005). Retinoic acid is essential in organogenesis (Maden, 1994). The nuclear receptors for retinoic acid were discovered in 1987 (Giguere et al., 1987; Petkovich et al., 1987). Nuclear retinoic acid receptors regulate the transcriptions of a large number of genes (Chambon, 1996; Evans, 1994). Retinoic acid can both stimulate or suppress mitogenesis depending on the cellular context (Chen and Gardner, 1998). In addition to gene transcription, retinoic acid may also mediate its effect by retinoylation of proteins (Takahashi and Breitman, 1994). Recently, retinoic acid was discovered to acutely regulate protein translation in neurons independent of its roles in regulating gene transcription (Aoto et al., 2008; Chen et al., 2008). In addition to its developmental roles, retinoic acid is also important in the function of many adult organs such as the nervous system, the immune system, the reproductive system, the respiratory system, and the skin. These functions will be discussed in detail in the context of the RBP receptor.

2.4. Ester forms of vitamin A

Retinyl ester is the major storage form of vitamin A inside cells (Batten et al., 2004; Liu and Gudas, 2005; O'Byrne et al., 2005; Ruiz et al., 2007) and is an alternative form for vitamin A delivery. However, vitamin A delivery through retinyl ester is associated with toxicity (Goodman, 1984; Smith and Goodman, 1976).

2.5. Pathological effects of vitamin A deficiency

Vitamin A deficiency affects a wide range of organ systems in vertebrates (West, 1994; Wolbach and Howe, 1925). For humans, the most well-known effects of vitamin A deficiency are night blindness (Dowling, 1966) and childhood mortality and morbidity (Sommer, 1997a). Even mild vitamin A deficiency (presymptomatic for ocular phenotypes) has a large impact on childhood mortality (Sommer, 1997a). This is due to vitamin A's role in boosting immunity (Semba, 1999; Stephensen, 2001). It was estimated that 140 million children and more than 7 million pregnant women suffer from vitamin A deficiency every year worldwide and 1.2–3 million children die each year due to vitamin A deficiency alone (Sommer and Davidson, 2002). Maternal vitamin A deficiency is associated with maternal mortality and congenital defects in multiple organs in newborn children. In adults, vitamin A deficiency can affect brain function. Experiments in animal models show that vitamin A deficiency can lead to profound impairment of hippocampal long-term potentiation and a virtual abolishment

of long-term depression (Misner et al., 2001). Consistently, vitamin A deficiency leads to impairment in special learning and memory (Cocco et al., 2002). In addition, vitamin A deficiency can lead to abnormal functions of the lung (Biesalski, 2003), the skin (Vahlquist, 1994), the thyroid (Morley et al., 1978), and the male and female reproductive systems (Livera et al., 2002; Wolbach and Howe, 1925).

3. Retinol-Binding Protein, the Specific Carrier of Vitamin A in Blood

Since mammals cannot synthesize vitamin A, the only sources of vitamin A are from the diet and maternal vitamin A (which is also ultimately from the diet). The majority of dietary vitamin A is stored in the liver. Vitamin A is insoluble in aqueous media, is chemically unstable, and is toxic to cells at low levels. Vitamin A transport to different cell types needs to be precisely regulated because too little or too much vitamin A can be detrimental both to cellular survival and function (Goodman, 1984). Plasma RBP is a member of lipocalin superfamily (Newcomer and Ong, 2000). RBP is the principal means of transporting vitamin A in the blood and is responsible for a well-regulated transport system that helps vertebrates to adapt to fluctuations in vitamin A level (e.g., during seasonal changes in natural environments; Blomhoff et al., 1990). RBP specifically binds to vitamin A and effectively solubilizes vitamin A in aqueous solution and allows for a plasma vitamin A concentration 1000-fold higher than would occur for free vitamin A. RBP also protects vitamin A from enzymatic and oxidative damage (Goodman, 1984). RBP helps to maintain a physiological range of vitamin A concentration so that vitamin A concentration is stable despite variable intake from food (Redondo et al., 2006). Another function of the RBP system is to decrease the toxicity associated with unregulated distribution of vitamin A (Dingle et al., 1972; Goodman, 1984). Experiments in rats (Mallia et al., 1975) and a study of human patients with hypervitaminosis A (Smith and Goodman, 1976) both suggested that more toxicity is associated with vitamin A delivery independent of RBP. An excessive dose of vitamin A is toxic *in vivo* only when the level of vitamin A in the circulation is presented to cells in a form other than bound to RBP, such as in retinyl esters (Goodman, 1984).

RBP in complex with vitamin A (holo-RBP) is mainly produced in the liver, but is also produced in many other organs. For example, RBP is highly expressed in adipose tissue (Makover et al., 1989). However, extrahepatic RBP cannot mobilize the vitamin A stores in liver (Quadro et al., 2004). The exact roles of RBP secreted by tissues other than liver are not clear. One exception is that RBP secreted by adipocytes has recently been

found to be an adipokine for insulin resistance (Tamori et al., 2006; Yang et al., 2005). This is a function of RBP other than vitamin A delivery. Holo-RBP in the blood is in complex with the thyroxine binding protein transthyretin (TTR), which is also called prealbumin. This complex increases the molecular weight of holo-RBP and reduces its loss through glomerular filtration in the kidney. The crystal structure of holo-RBP in complex with TTR has been determined (Fig. 1.2; Monaco et al., 1995; Naylor and Newcomer, 1999; Zanotti and Berni, 2004).

The main conclusion from studies of RBP knockout mice is that loss of RBP makes mice extremely sensitive to vitamin A deficiency. RBP knockout mice cannot mobilize the hepatic vitamin A store (Quadro et al., 1999). Even with a nutritionally complete diet, knockout mice have a dramatically lower serum vitamin A level, similar to the level in the later stages of vitamin A deficiency in humans. Given the role of vitamin A in immune regulation and the susceptibility of vitamin A-deficient children to infection before visual symptoms (Semba, 1998; Sommer, 1997a; Stephensen, 2001), it is likely that the immune system is also sensitive to RBP defect under vitamin A-sufficient conditions. Indeed, the circulating immunoglobulin level in RBP knockout mice is half of that in the wild-type mice even under vitamin A sufficiency (Quadro et al., 2000). It will be interesting to systematically study the effects of loss of RBP functions on mouse susceptibility to infection, which is well correlated with vitamin A status in humans (Semba, 1999; Sommer, 1997a; Stephensen, 2001). As demonstrated by a LacZ reporter system for retinoic acid level, there is a dramatic decrease in retinoic acid level in the developing brain of RBP knockout mice even under vitamin A-sufficient conditions (Quadro et al., 2005). Therefore, it will be interesting to test whether these mice have any cognitive defects in adulthood. In addition, RBP knockout mice have abnormal heart development (Wendler et al., 2003) and impaired vision (Quadro et al., 1999, 2003).

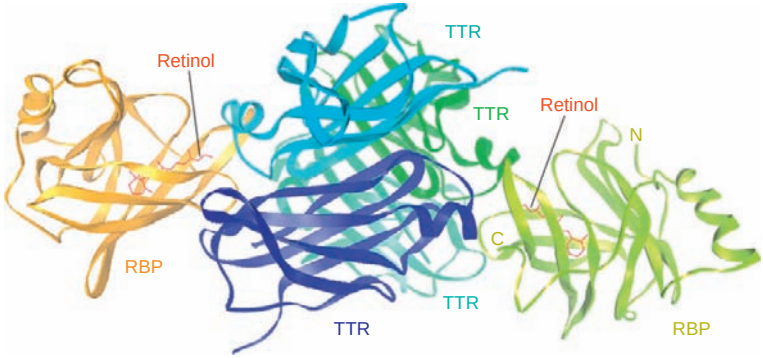

Figure 1.2 Crystal structure of holo-RBP and TTR complex.

Systematic study of several organ functions in RBP knockout mice demonstrated that RBP is essential for survival under vitamin A-deficient conditions (Ghyselinck et al., 2006; Quadro et al., 1999, 2005). Vitamin A-deficient conditions are common for most, if not all, animals living in natural environments. Studies of the RBP knockout mice subjected to different lengths of time of vitamin A deficiency revealed that RBP is the primary vitamin A source for fetal development (Quadro et al., 2005). Depending on the extent of dietary vitamin A deficiency, malformations in RBP knockout mice range from mild symptoms to complete fetal resorption. Under conditions of vitamin A deficiency, in which wild-type mice behave normally, RBP knockout mice have rapid vision loss in adults after merely a week of vitamin A deficiency (Quadro et al., 1999). In addition, these mice rapidly develop testicular defects (Ghyselinck et al., 2006).

4. Diverse Evidence for the Existence of an RBP Receptor that Mediates Vitamin A Uptake

Diverse experimental evidence accumulated since the 1970s from independent research groups supports the existence of a specific cell-surface RBP receptor that mediates vitamin A uptake (Table 1.1). It was first shown in the 1970s that there exists a specific cell-surface receptor for RBP on the retinal pigment epithelium (RPE) cells and mucosal epithelial cells (Bok and Heller, 1976; Chen and Heller, 1977; Heller, 1975; Heller and Bok, 1976; Maraini and Gozzoli, 1975; Rask and Peterson, 1976). During the past 30 years, there has been strong evidence for the existence of RBP receptors not only on RPE cells but also on other tissues including placenta (Sivaprasadarao and Findlay, 1988a; Sivaprasadarao et al., 1994; Smeland et al., 1995), choroid plexus (MacDonald et al., 1990; Smeland et al., 1995), Sertoli cells of the testis (Bhat and Cama, 1979; Bishop and Griswold, 1987; Shingleton et al., 1989; Smeland et al., 1995), and macrophages (Hagen et al., 1999). Evidence for a specific RBP receptor includes saturable binding of ^{125}I-RBP to cell membrane (Bhat and Cama, 1979; Heller, 1975; Pfeffer et al., 1986). Binding can be inhibited by an excess of unlabeled RBP (Bhat and Cama, 1979; Heller, 1975; Heller and Bok, 1976; Sivaprasadarao and Findlay, 1988a; Torma and Vahlquist, 1986), an antibody to RBP (Melhus et al., 1995; Rask and Peterson, 1976), or by a cysteine modification compound (Sivaprasadarao and Findlay, 1988a). When ^{125}I-RBP was injected into rat, specific labeling was observed on the basolateral membrane of the RPE (Bok and Heller, 1976) and in the choroid plexus (MacDonald et al., 1990). In a systematic comparison of RBP binding between different tissues and cell types, the highest RBP-binding activities were found in membranes from the RPE, the placenta, the bone marrow, the

Table 1.1 *In vitro* and *in vivo* experimental evidence (in chronological) for the existence of an RBP receptor on the RPE cell and other cell types and a mechanism for receptor-mediated vitamin A uptake from holo-RBP

References	Experimental systems	Experimental evidence
Heller (1975)	Bovine RPE cells	Binding of retinol-^{125}I-RBP to RPE cells is saturable and can be inhibited by an excess of unlabeled holo-RBP. Apo-RBP is less effective in displacing bound retinol-^{125}I-RBP
Bok and Heller (1976)	Live rats	Injection of ^{125}I-RBP into rat leads to specific labeling of the basal and lateral membrane of the RPE
Chen and Heller (1977)	Bovine RPE cells	RPE cells but not red blood cells or white blood cells efficiently take up ^{3}H-retinol from ^{3}H-retinol/RBP RPE cells cannot take up ^{3}H-retinol bound to BSA
Heller and Bok (1976)	Bovine RPE cells	Autoradiography of retinol-^{125}I-RBP bound to RPE cells showed specific labeling of the choroidal surface of the cells but not the retinal surface. The labeling can be abolished by an excess of unlabeled holo-RBP
Rask and Peterson (1976)	Monkey mucosal epithelial cells of small intestine	Uptake of ^{3}H-retinol from ^{3}H-retinol/RBP can be inhibited by unlabeled holo-RBP, apo-RBP, or antibody against RBP but not by the metabolite form of RBP
Bhat and Cama (1979)	Chicken testis membrane	Binding of ^{125}I-RBP is saturable and can be inhibited by an excess of unlabeled RBP
Rask et al. (1980)	Bovine cornea	Uptake of ^{3}H-retinol from ^{3}H-retinol/RBP is saturable and can be inhibited by unlabeled holo-RBP
Torma and Vahlquist (1984)	Human skin	Uptake of ^{3}H-retinol from ^{3}H-retinol/RBP is saturable and can be inhibited by an excess of unlabeled holo-RBP
Pfeffer et al. (1986)	Human RPE culture	Binding of retinol-^{125}I-RBP to RPE cells is saturable. Uptake of ^{3}H-retinol from ^{3}H-retinol/RBP can be inhibited by unlabeled holo-RBP
Torma and Vahlquist (1986)	Human placenta	Specific binding of ^{125}I-RBP and uptake of ^{3}H-retinol from ^{3}H-retinol/RBP, both are inhibited by an excess of cold RBP

Eriksson et al. (1986)	Embryonal carcinoma cell line F9	Differentiated, but not undifferentiated, F9 cells specifically bind to ^{125}I-RBP and take up ^{3}H-retinol from ^{3}H-retinol/RBP (both are inhibited by an excess of unlabeled RBP)
Ottonello et al. (1987)	Bovine RPE membrane	Isolated RPE membrane can specifically take up ^{3}H-retinol from ^{3}H-retinol/RBP. This uptake can be inhibited by an excess of unlabeled holo-RBP
Sivaprasadarao and Findlay (1988a,b)	Human placenta microvilli	Binding of ^{125}I-RBP can be inhibited by an excess of unlabeled RBP or TTR. PCMBS treatment of membrane can abolish ^{125}I-RBP binding
Sivaprasadarao and Findlay (1988a,b)	Human placenta membrane vesicles	Uptake of ^{3}H-retinol from ^{3}H-retinol/RBP can be inhibited by an excess of unlabeled holo-RBP, apo-RBP but not by serum albumin. PCMBS treatment of membrane can abolish uptake
Shingleton et al. (1989)	Sertoli cells of rat testis	Uptake of ^{3}H-retinol from ^{3}H-retinol/RBP is saturable and can be inhibited by an excess of unlabeled holo-RBP
Sivaprasadarao and Findlay (1994)	Human placenta membrane	Specfic mutations on the open end of the β-barrel of RBP can block RBP's interaction with its receptor
Melhus et al. (1995)	Bovine RPE membrane	A monoclonal antibody recognizing an entrance loop of RBP can specifically block its interaction with its receptor on RPE membrane
Smeland et al. (1995)	Membrane prepared from many organs	High binding activity of ^{125}I-RBP was found in placenta, RPE, bone marrow, and kidney and undifferentiated, but not differentiated, keratinocytes
Sundaram et al. (1998)	Human placenta membrane	Specific transfer of ^{3}H-retinol from ^{3}H-retinol/RBP to CRBP is dependent on the RBP receptor on placenta membrane. Serum albumin and β-lactoglobulin cannot substitute for RBP in this process
Vogel et al. (2002)	RBP knockout mouse model	The rate of retinol uptake from holo-RBP by the eye markedly exceeds all other tissues except for the kidney This suggests that the RPE can specifically recognize and efficiently absorb retinol when bound to RBP
Liden and Eriksson (2005)	A new retinol uptake assay in cell culture	A monoclonal antibody to RBP can block retinol uptake from holo-RBP by cells

choroid plexus, and undifferentiated keratinocytes (Smeland et al., 1995). The observed tissue distribution of the putative RBP receptor agrees well with what we know about vitamin A function and metabolism. For example, in order for vitamin A to exert its effect on adult brain, it must cross the choroid plexus, the blood–brain barrier, and therefore a high level of RBP receptor should be expressed in this tissue.

The receptor on RPE membrane can not only specifically bind to RBP but can also mediate vitamin A uptake from vitamin A-loaded RBP (holo-RBP) (Chen and Heller, 1977; Maraini and Gozzoli, 1975; Ottonello et al., 1987; Pfeffer et al., 1986). The uptake mechanism is highly specific because red blood cells do not take up vitamin A from RBP. In addition, the efficiency of vitamin A uptake from vitamin A bound to BSA is much less efficient than from vitamin A bound to RBP. Specific vitamin A uptake has also been demonstrated for mucosal epithelial cells (Rask and Peterson, 1976), human placenta (Sivaprasadarao and Findlay, 1988b; Sundaram et al., 1998; Torma and Vahlquist, 1986), Sertoli cells of the testis (Bishop and Griswold, 1987; Shingleton et al., 1989), human skin (Torma and Vahlquist, 1984), and macrophages (Hagen et al., 1999). Previous studies also showed that specific mutations in RBP or a monoclonal antibody against a specific region of RBP can abolish its interaction with the RBP receptor (Liden and Eriksson, 2005; Melhus et al., 1995; Sivaprasadarao and Findlay, 1994). Another strong piece of evidence that vitamin A uptake is mediated by a protein is that the uptake in human placenta membrane can be inhibited by a cysteine modification reagent (Sivaprasadarao and Findlay, 1988b). Not listed in Table 1.1 are indirect pieces of evidence for the existence of an RBP receptor. For example, in an unbiased search for a serum factor that stimulates the growth of B cells, it was found that holo-RBP is this factor (Buck et al., 1990). For lymphoblastoid cell lines Mou and BH, holo-RBP is much more potent than retinol itself in growth stimulation. These experiments also suggest the existence of a cell-surface receptor for holo-RBP on these cell types.

5. Identification of RBP Receptor

5.1. Identification of high-affinity RBP receptor as STRA6

Despite the large amount of evidence, the RBP receptor turned out to be very difficult to identify. Potential obstacles to purifying the RBP receptor include the fragility of the receptor protein and the transient nature of the binding of RBP to its receptor. These challenges likely prevented the purification of the receptor using traditional biochemical approaches. To overcome these two challenges, a strategy was designed to stabilize the RBP/receptor interaction and permit high-affinity purification of the

RBP/receptor complex without requiring the receptor to remain active during the purification (Kawaguchi et al., 2007). Another advantage of this strategy is that it permits stringent washing with high salt and urea, which can dissociate nonspecifically bound protein without causing membrane protein aggregation. Using this strategy, the RBP receptor was identified as STRA6, a multitransmembrane protein of previously unknown function (Kawaguchi et al., 2007). STRA6 binds to RBP with high affinity and specificity, and facilitates the release of vitamin A from holo-RBP and the transport of vitamin A into the cell (Kawaguchi et al., 2007).

STRA6 was first characterized as a retinoic acid induced gene in P19 embryonic carcinoma cells (Bouillet et al., 1995). STRA6 stands for "stimulated by retinoic acid 6." It was also identified as a gene induced by Wnt-1 and retinoic acid in mouse mammary epithelial cells (Szeto et al., 2001). The induction by Wnt-1 and retinoic acid is synergistic. Strikingly, STRA6 was found to be overexpressed up to 172-fold in 14 out of 14 human colorectal tumors relative to the normal tissue (Szeto et al., 2001). STRA6 is widely expressed during embryonic development and in adult organ systems. In development, STRA6 is widely expressed, consistent with the diverse roles of vitamin A in development. For example, its expression during mouse limb development suggested that STRA6 may participate in early dorsoventral limb patterning and in controlling endochondral ossification (Chazaud et al., 1996). In developing eye, it is expressed in the inner nuclear layer of the developing retina, and in developing RPE (Bouillet et al., 1997). Information on the distribution of STRA6 mRNA in adult tissues is available from the NCBI's comprehensive tissue EST (expressed sequence tag) profiler (Table 1.2) and from two tissue distribution studies of STRA6 (Bouillet et al., 1997; Chazaud et al., 1996). It was suggested that a high level of RBP receptors should be expressed in cells comprising blood–tissue barriers (MacDonald et al., 1990). Indeed, in adult tissues, STRA6 expression is enriched in blood–organ barriers such as the RPE (blood–retina barrier), the placenta (maternal–fetal barrier), the choroid plexus (blood–brain barrier), and the Sertoli cells of testis (blood–testis barrier), although STRA6's expression is not limited to blood–organ barriers. Correlations between STRA6 expression and known functions of vitamin A in adult organs are discussed in the following sections.

5.2. STRA6 in the eye

In the eye, STRA6 is abundantly expressed in the RPE. In contrast to its absence in the endothelial cells of the choriocapillaris, STRA6 is expressed in retinal blood vessels, although the signal is much weaker than that in the RPE (Kawaguchi et al., 2007). The retinal blood vessels are another location that constitutes a blood–retina barrier. As holo-RBP in choriocapillaris blood is the source of vitamin A for the RPE, holo-RBP from retinal blood vessels is a potential source of vitamin A for Müller cells in the retina (Mata et al., 2002).

Table 1.2 Correlation between STRA6's tissue expression and known functions of vitamin A, its derivatives, and RBP

Tissues	Transcripts per million	STRA6 EST/ total EST	Known functions of vitamin A, its derivatives, and RBP	References
Brain	51	48/933,463	Maintenance of synaptic plasticity and cortical synchronization during sleep	Misner et al. (2001), Maret et al. (2005), Drager (2006)
Connective tissue	27	3/107,437	Vitamin A promotes collagen synthesis	Anstead (1998)
Parathyroid	0	0/20,837		
Thyroid	72	4/54,920	VAD leads to hyperthyroidism and higher basal metabolic rate	Morley et al. (1978)
Pituitary gland	0	0/17,477		
Placenta	195	58/296,769	Vitamin A is essential for embryonic development	Maden (1994), Quadro et al. (2005)
Eye	28	6/207,123	Vitamin A is essential for light perception in vision and modulates neuronal signaling	Wald (1968), Weiler et al. (2001)
Embryonic tissue	60	12/199,222	Vitamin A is essential in almost all steps in organogenesis	Maden (1994), Reijntjes et al. (2005)
Abdominal cavity	0	0/40,397		
Cervix	20	1/48,034		
Ovary	28	3/106,265	Vitamin A promotes ovarian follicular growth and ooctye maturation	Ikeda et al. (2005)

Uterus	41	10/238,763	VAD leads to replacement of uterus mucosa by stratified, keratinizing epithelium	Wolbach and Howe (1925)
Prostate	6	1/154,822		
Testis	94	33/348,176	Vitamin A is essential for spermatogenesis	Livera et al. (2002), Chung and Wolgemuth (2004)
Bladder	0	0/30,298		
Kidney	42	9/212,609	Vitamin A has antifibrotic and cytoprotective effects on various renal cell types	Xu et al. (2004)
Tongue	75	5/66,626	Maintenance of normal taste bud function	Biesalski et al. (1985)
Larynx	32	1/30,370	VAD leads to replacement of respiratory mucosa by keratinizing epithelium	Wolbach and Howe (1925)
Pharynx	67	1/14,868	VAD leads to replacement of respiratory mucosa by keratinizing epithelium	Wolbach and Howe (1925)
Salivary gland	0	0/20,411		
Heart	11	1/89,584	Vitamin A has antigrowth activity in fully differentiated cardiac cells	Gardner and Chen (1999)
Lymph node	10	1/95,317	Regulation of hematopoiesis and maintenance of immune competence	Blomhoff and Smeland (1994), Stephensen (2001)

(*continued*)

Table 1.2 (*continued*)

Tissues	Transcripts per million	STRA6 EST/ total EST	Known functions of vitamin A, its derivatives, and RBP	References
Lymph	0	0/44,599		
Tonsil	0	0/17,168		
Spleen	18	1/52,804	Regulation of hematopoiesis and maintenance of immune competence	Blomhoff and Smeland (1994), Stephensen (2001)
Thymus	40	3/73,960	Regulation of hematopoiesis and maintenance of immune competence	Blomhoff and Smeland (1994), Stephensen (2001)
Mammary gland	17	3/170,913	Lactation transfers vitamin A from mother to infant for its growth and development	Ross and Gardner (1994)
Muscle	34	4/114,677	RBP is an adipokine that induces insulin resistance in muscle	Yang et al. (2005)
Lung	43	15/347,374	Vitamin A is involved in the maintenance of normal lung function.	Baybutt et al. (2000), Biesalski (2003)
Trachea	20	1/48,470	VAD leads to replacement of respiratory mucosa by keratinizing epithelium	Wolbach and Howe (1925)
Skin	15	3/187,916	Vitamin A is essential for the maintenance of normal skin	Vahlquist (1994), Varani et al. (2000)

VAD, vitamin A deficiency. The intensity of the ovals indicates the abundance of STRA6 message as suggested by EST (expressed sequence tag) counts. Tissue EST counts are recorded by the NCBI's EST profile Viewer for human STRA6. The relative abundance of STRA6 in different organs is similar but not identical between human and mouse. Expression from rare cell types, such as the RPE, may be underrepresented in this analysis. The sensitivity of this analysis is also affected by the total number of ESTs available per tissue.

In the RPE, STRA6 is largely localized to the basolateral membrane of the RPE cells. This localization is exactly what is expected for an RBP receptor, which should be localized to the basolateral membrane of the RPE facing the choroidal circulation (Heller and Bok, 1976; Pfeffer et al., 1986). STRA6, localized to the lateral membrane of the RPE, is in close proximity to the retinosome, a recently discovered RPE structure that stores retinyl esters (Imanishi et al., 2004). This suggests that vitamin A absorbed by STRA6 from holo-RBP is in close proximity to the cellular structures that store vitamin A. Interestingly, there are also STRA6 signals on distinct intracellular vesicles in the RPE. These vesicles may play a role in targeting STRA6 to the basolateral membrane or in recycling. Another major location in the eye that expresses STRA6 is the cornea (unpublished results). Consistently, it was known that RBP supplies vitamin A to the cornea (Rask et al., 1980).

5.3. STRA6 in the reproductive systems

Consistent with the essential role of vitamin A in both male and female reproductive functions, EST analysis shows that both male and female reproductive systems express STRA6 at high levels (Table 1.2). Vitamin A plays an essential role in spermatogenesis (Chung and Wolgemuth, 2004; Livera et al., 2002). In testis, STRA6 is expressed in the Sertoli cells (Bouillet et al., 1997). STRA6 protein has been localized to the plasma membrane of Sertoli cells (Bouillet et al., 1997), a localization consistent with Sertoli cells' RBP-binding activity (Smeland et al., 1995) and their ability to take up vitamin A from holo-RBP (Bishop and Griswold, 1987; Shingleton et al., 1989).

Consistent with the diverse roles of vitamin A in female reproductive functions (Clagett-Dame and DeLuca, 2002), STRA6 is highly expressed in several female reproductive organs such as placenta, uterus, ovary, and mammary gland (Table 1.2). Placenta has the highest level of STRA6 expression of all organs according to EST analysis (Table 1.2). This is in agreement with the facts that RBP is the most important source of vitamin A for embryos (Quadro et al., 2005), and placental membrane has one of the highest RBP-binding activities of any cell or tissue tested (Smeland et al., 1995). Human placental membrane has been used in the past as a model system to study RBP binding to the RBP receptor and vitamin A uptake (Sivaprasadarao and Findlay, 1988a,b; Sundaram et al., 1998).

5.4. STRA6 in the nervous system

Retinoic acid is a modulator of the nervous system (Drager, 2006; Lane and Bailey, 2005; Weiler et al., 2001). For example, retinoic acid plays an important role in maintaining synaptic plasticity in hippocampus (Misner et al., 2001) and cortical synchrony during sleep (Maret et al., 2005). Independent of its roles in regulating gene transcription, it also regulates

protein translation in neurons (Aoto et al., 2008; Chen et al., 2008). It also plays an important role in regenerative processes in the adult central nervous system (Maden, 2007; Vergara et al., 2005). Although retinoic acid is not preferentially transported from the blood to the brain, the brain has a high concentration of retinoic acid (Werner and Deluca, 2002). Highly abundant expression of STRA6 in the brain is consistent with the local absorption and conversion of vitamin A to retinoic acid. Consistent with a previous study (Bouillet et al., 1997), we observed strong STRA6 signals in the choroid plexus and meninges of the brain and weaker signals in a large subset of brain endothelial cells (unpublished results). Interestingly, these three sites constitute the blood–brain barriers (Abbott et al., 2006). Consistent with the strong expression of STRA6 in meninges, retinoic acid from the meninges has been shown to regulate cortical neuron generation (Siegenthaler et al., 2009). We also found that blood vessels negative for STRA6 were surrounded by astrocyte perivascular endfeet (Abbott et al., 2006) that are positive for STRA6 (Kawaguchi et al., 2007).

5.5. STRA6 in the lymphoid organs

Vitamin A plays important roles in hematopoiesis and in maintaining immunocompetence (Blomhoff and Smeland, 1994; Oren et al., 2003; Stephensen, 2001). For example, retinoic acid attenuates B cell proliferation to promote maturation and antibody production (Chen and Ross, 2005). In an unbiased search for a serum factor that stimulates the growth of B cells, holo-RBP was identified as this factor (Buck et al., 1990). Circulating immunoglobulin level in RBP knockout mice is half that in wild-type mice, even under vitamin A sufficiency (Quadro et al., 2000). Consistently, STRA6 is highly expressed in lymphoid organs such as thymus, spleen, and lymph nodes (Table 1.2). There are two possible mechanisms by which vitamin A is absorbed for immune regulation. One possibility is that specialized cell types in lymphoid organs other than leukocytes and their precursors absorb vitamin A from holo-RBP and generate retinoic acid locally for immune cells, similar to the role of the RPE cells in absorbing vitamin A and generating 11-*cis* retinal for photoreceptor cells. It was discovered as early as 1925 that vitamin A deficiency leads to thymus and spleen atrophy in rat (Wolbach and Howe, 1925). Another possible mechanism is that leukocytes and their precursors directly absorb vitamin A from holo-RBP and produce retinoic acid themselves. A combination of both mechanisms is also possible.

5.6. STRA6 in the skin

Vitamin A plays essential roles in maintaining normal skin (Vahlquist, 1994). Vitamin A treatment reduces matrix metalloproteinase expression and stimulates collagen synthesis in both naturally aged, sun-protected skin and

photoaged skin (Varani et al., 2000). Because of vitamin A's role in epithelial and bone formation, cellular differentiation, and immune regulation, vitamin A deficiency can impede wound healing (Anstead, 1998; MacKay and Miller, 2003). Human skin can specifically and efficiently take up vitamin A from holo-RBP (Torma and Vahlquist, 1984). Epidermis has much higher activity than dermis. Undifferentiated human skin keratinocytes were found to have the highest RBP-binding activity of any cell or tissue type tested, even higher than placenta and RPE cells (Smeland et al., 1995). Given the functions of vitamin A in skin and the ability of cells in the skin to take up vitamin A from holo-RBP, STRA6's relatively abundant expression in the skin makes physiological sense (Table 1.2).

5.7. STRA6 in the lung

Vitamin A plays important roles in maintaining the normal function of the lung (Biesalski, 2003). Vitamin A deficiency produces morphologic changes in the lung, impairs pneumocyte function (Baybutt et al., 2000), and potentiates hyperoxic lung injury (Veness-Meehan, 1997). Vitamin A also has a protective effect on respiratory status in patients with cystic fibrosis (Aird et al., 2006). STRA6 was originally identified as a retinoic acid stimulated gene (Bouillet et al., 1995). Consistent with the ability of retinoic acid to stimulate STRA6 expression, vitamin A combined with retinoic acid increases retinol uptake in the lung in a synergistic manner (Ross et al., 2006).

5.8. STRA6 in the kidney

Both EST analysis and Northern Blot analysis have shown that kidney expresses STRA6 at a fairly high level (Table 1.2). The detailed localization of STRA6 in kidney is unknown. STRA6 may function in vitamin A absorption for the kidney itself or in the recycling of vitamin A. Vitamin A is known to have an antifibrotic effect and a cytoprotective effect on various renal cell types (Xu et al., 2004).

5.9. STRA6 in the heart

STRA6 expression in the heart is seen in both EST analysis and Northern Blot analysis (Bouillet et al., 1997). Retinoids have antigrowth activity in fully differentiated cardiac cells. Thus, retinoids may be useful in the management of hypertrophic/hyperproliferative disorders of the heart and vascular wall (Gardner and Chen, 1999). In addition, vitamin A deficiency causes a significant decrease in contractile responsiveness of aortic smooth muscle as a result of a downregulation in the expression of contractile-related proteins (Wright et al., 2002). Retinoids have also been shown to regulate cardiac mitochondrial membrane potential (Korichneva et al., 2003).

6. Structure and Function Analysis of the RBP Receptor's Interaction with RBP

6.1. Transmembrane topology of STRA6

At the amino acid sequence level, STRA6 has no homology to any proteins to indicate its function. The transmembrane topology of STRA6 has been determined experimentally (Kawaguchi et al., 2008b). The topology model suggests that STRA6 has 19 distinct domains including five extracellular domains, nine transmembrane domains, and five intracellular domains (Fig. 1.3). Many membrane transporters have 8–12 transmembrane domains (Hediger et al., 2004). Although STRA6 represents a new membrane transport protein, its number of transmembrane domains lies within this range. Most proteins with more than seven transmembrane domains function as membrane transporters or channels. The large number of transmembrane domains potentially makes it more feasible to form a specific transmembrane pore, through which the ligand can pass through.

6.2. The RBP-binding domain in STRA6

Because STRA6 represents a new type of cell-surface receptor that is not homologous to any membrane receptor, transporter, or channel and has no obvious functional domains, we decided to use an unbiased strategy to study its structure and function. By creating and analyzing more than 900 random mutants of STRA6, an essential RBP-binding domain has been identified (Kawaguchi et al., 2008a). The locations of the three essential residues involved in RBP binding are indicated in the transmembrane topology model of STRA6 (Fig. 1.3). Mutations in any of the three essential residues in this domain can abolish the binding of STRA6 to RBP and its vitamin A uptake activity without affecting its cell-surface expression. The advantage of an unbiased screening approach is evident because one of the transmembrane domains predicted by computer softwares turned out to be the RBP-binding domain, which is an extracellular domain. Although this extracellular domain is most essential for RBP binding, other extracellular domains also contribute to RBP binding (Kawaguchi et al., 2008b).

6.3. STRA6 mutants associated with human disease

Human genetic studies found that mutations in STRA6 are associated with severe pathological phenotypes such as mental retardation, anophthalmia, congenital heart defects, lung hyperplasia, duodenal stenosis, pancreatic malformations, and intrauterine growth retardation (Golzio et al., 2007; Pasutto et al., 2007). More human genetic studies have further confirmed

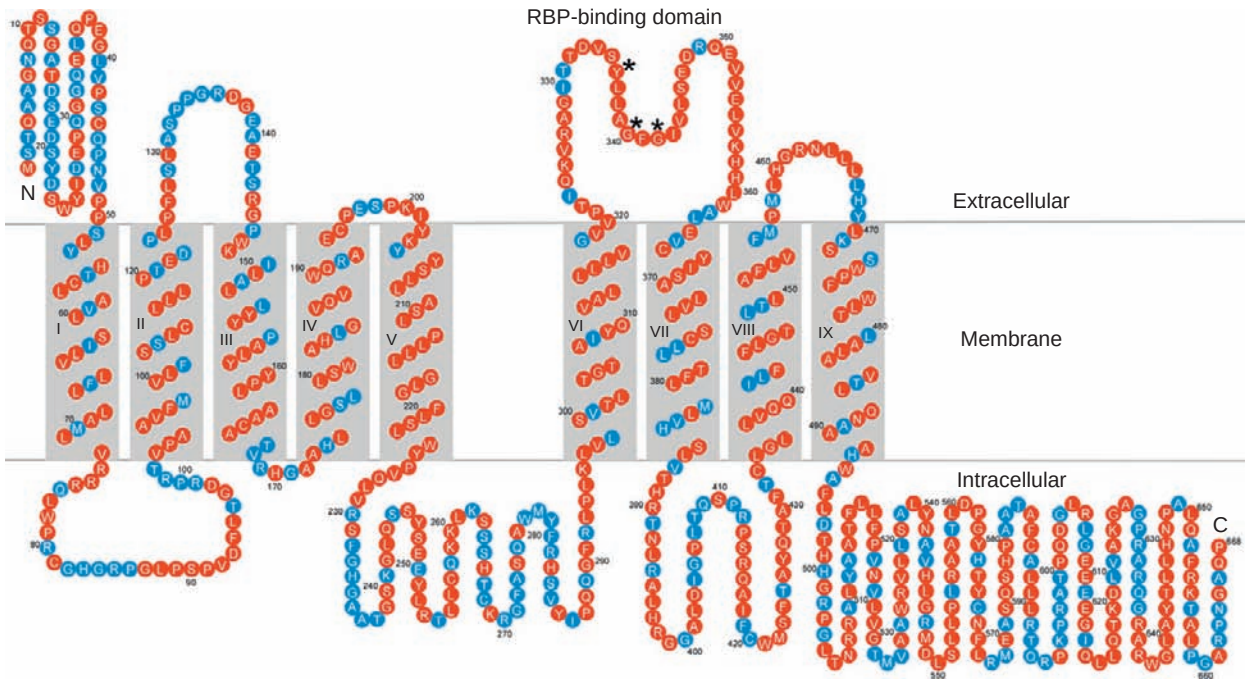

Figure 1.3 Transmembrane topology of STRA6. Bovine sequence is shown. Residues conserved between human, mouse, and bovine STRA6 are labeled in red. Residues essential for RBP binding are marked with asterisks.

the role of STRA6 mutations in malformations in humans (Chassaing et al., 2009; Segel et al., 2009; West et al., 2009; White et al., 2008). One of the most prominent features of loss of STRA6 function in human is the absence of the eye. Consistently, STRA6 knockdown also causes developmental defects in zebrafish (Isken et al., 2008). The loss of retinoid uptake in the eye due to loss of STRA6 has also been demonstrated in the zebrafish model (Isken et al., 2008).

Functional assays showed that the pathogenic missense mutations identified in the human genetic study abolish the vitamin A uptake activity of STRA6 (Kawaguchi et al., 2008a), consistent with the severe clinical phenotypes. The locations of these mutations are depicted in Fig. 1.3. As mentioned in a previous review (Niederreither and Dolle, 2008), this is the first example of a retinoid-signaling pathway mutation causing developmental abnormalities in humans.

6.4. RBP's interaction with its receptor

Three regions of RBP are potentially involved in binding to the RBP receptor (Fig. 1.4A and B). The first region (C-tail) was implicated in a study of urine RBP. It was found that there are two main forms of RBP in

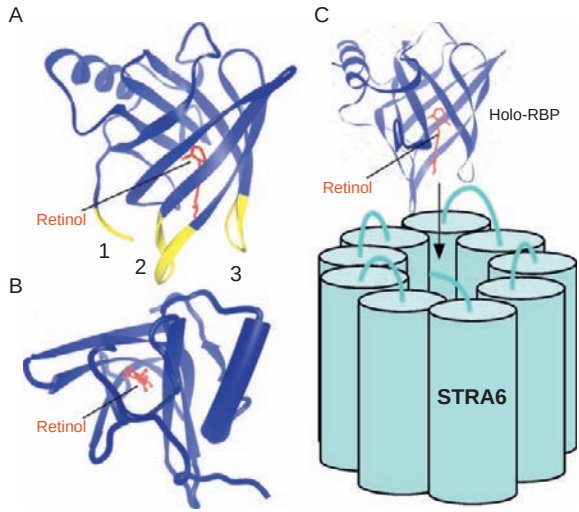

Figure 1.4 (A) Structure of holo-RBP. The three regions implicated in RPB receptor binding are indicated as 1, 2, and 3, respectively. These three regions (labeled in yellow) are all located on the vitamin A exit end of RBP. (B) Structure of holo-RBP showing the vitamin A exit end facing up. (C) A hypothetical model for RBP interacting with STRA6 via the vitamin A exit end.

human urine (Rask et al., 1971). One form has vitamin A bound and can bind to the RBP receptor. The second form has no vitamin A bound and cannot bind the RBP receptor (Rask and Peterson, 1976). The second form is missing a lysine residue at the C-terminus compared with the first form. Since the extreme C-terminal residue in full-length human RBP protein is not lysine, both products may represent RBP losing C-terminal residues with the second form having lost more. Given the fact that full-length apo-RBP can bind the RBP receptor, these studies suggest a role of the RBP C-terminus in interacting with the RBP receptor. In addition, alkaline phosphatase (AP) tagged at the N-terminus, but not the C-terminus, of RBP binds to the RBP receptor (Kawaguchi et al., 2007). The second region, a loop near the vitamin A exit site of RBP, was implicated by two independent studies. The first showed the effect of mutating this region (Sivaprasadarao and Findlay, 1994) and the second study found that a monoclonal antibody recognizing this region can block the binding of RBP with its receptor (Melhus et al., 1995). The third region, another loop near the vitamin A exit site of RBP, was implicated by a deletion experiment (Sivaprasadarao and Findlay, 1994). Consistent with the role of the RBP receptor in vitamin A release from holo-RBP, all three regions in RBP implicated in RBP receptor binding are located around the vitamin A exit end of the β-barrel (Fig. 1.4A and B). A hypothetical model based on the crystal structure of RBP is proposed that illustrates its interaction with its receptor (Fig. 1.4C).

7. Pertinent Questions Related to RBP Receptor

7.1. Retinol has the ability to diffuse through membranes. Why is it necessary to have a multitransmembrane domain protein (the RBP receptor) to assist its transport?

First, although free retinol has the ability to diffuse through membranes, retinol seldom exists in its free form under physiological conditions. During its transport in the blood, virtually all retinol in the blood is bound to the RBP/TTR complex, which makes retinol membrane impermeable. The RBP receptor serves both as a "homing" device of holo-RBP and mediates cellular uptake of retinol from holo-RBP.

Second, even for molecules that can diffuse through membrane, membrane transporters are known to facilitate their transport. For example, although urea and water can diffuse through membrane, urea transporters (Sachs et al., 2006; You et al., 1993) and water channels (Agre, 2004) greatly facilitate their transport. Even for free retinoids, there exist membrane transport systems that facilitate their transport across membrane. For example, biochemical evidence suggests that retinol uptake from the small

intestine is mediated by a membrane transporter (Dew and Ong, 1994). There is also strong evidence for the existence of a specific mechanism to transport 11-*cis* retinal in the RPE, and this mechanism depends on interphotoreceptor retinoid-binding protein (IRBP). Apo-IRBP is much more effective in promoting the release of 11-*cis* retinal from the RPE than the apo-forms of other RBPs (Carlson and Bok, 1992). In addition, apo-IRBP is only effective when it is present on the apical, but not basal, side of the RPE (Carlson and Bok, 1999). Another finding that challenges the assumptions about random diffusion is the identification of an ATP-dependent transporter (ABCR or ABCA4) that transports all-*trans* retinal released from bleached rhodopsin across membranes (Ahn et al., 2000; Sun et al., 1999; Weng et al., 1999). Prior to the surprising discovery of ABCR's role in retinoid transport, there was no biochemical or physiological evidence for the existence of such a transporter.

Third, free retinol diffusion is not efficient enough for tissues that demand a large quantity of vitamin A. Both our study and an earlier study (Maraini and Gozzoli, 1975) demonstrated that the RBP receptor-mediated vitamin A uptake is much more efficient than vitamin A uptake depending on the association of vitamin A with cellular membranes due to its hydrophobicity. In addition, a nonspecific diffusion mechanism would depend on free retinol's interaction with cell membrane, but free retinol is toxic to cell membranes. Specific delivery mediated by RBP would prevent cellular damage by free retinol (Goodman, 1984). The fact that retinol but not retinoic acid can prevent testicular degeneration (Howell et al. 1963) may be due to the efficient delivery of retinol by RBP and its receptor. Random retinoic acid diffusion may be insufficient to supply the amount of retinoid needed by the testis.

7.2. Why does RBP need a high-affinity receptor for vitamin A uptake?

Under normal physiological conditions of vitamin A sufficiency, the blood concentration of RBP is in the micromolar range (Mills et al., 2008). In contrast, STRA6 has high affinity (K_d = 59 nM) for RBP (Kawaguchi et al., 2007). Why does RBP need a high-affinity receptor? The first likely reason is to compete with TTR to bind to RBP in the blood. Holo-RBP is complexed with TTR in the blood. However, both TTR and the RBP receptor bind to the vitamin A exit end of RBP, as discussed above. Therefore, the RBP receptor needs to bind to RBP with higher affinity than TTR in order to absorb vitamin A from the blood despite the high concentration of RBP in the blood. Indeed, RBP's affinity for STRA6 (Kawaguchi et al., 2007) is higher than its affinity for TTR (Malpeli et al., 1996). Consistently, cells and tissues naturally absorb vitamin A from the holo-RBP/TTR complex in the blood, and STRA6 not only mediates

vitamin A uptake from purified holo-RBP but also from human serum (Kawaguchi et al., 2007). Although TTR blocks the vitamin A exit end of RBP (Monaco et al., 1995), a study using Sertoli cells showed that it only partially inhibits RBP receptor-mediated vitamin A uptake (Shingleton et al., 1989). A second likely function of the high-affinity interaction between RBP and its receptor is to help vertebrates survive vitamin A-deficient conditions, which can lower serum RBP level. Vitamin A-deficient conditions are common for most, if not all, vertebrates living in natural environments.

7.3. Why is the interaction between RBP and its receptor transient?

Transient interaction between RBP and its receptor is crucial for a vitamin A uptake mechanism that does not depend on endocytosis because each RBP protein only carries one retinol molecule, and stable interaction will prevent further retinol delivery by other holo-RBP complexes to the same RBP receptor. In a sense, the removal of apo-RBP from the RBP receptor is as important as the binding of holo-RBP to the receptor for vitamin A delivery to a cell. As discussed above, the transient nature of the interaction between RBP and its receptor was one of the major technical hurdles in the identification of the RBP receptor.

7.4. Why are the phenotypes of patients with RBP mutations different from patients with RBP receptor mutations?

Phenotypes associated with known human RBP mutations are different from phenotypes associated with known human STRA6 mutations. Two natural RBP mutations have been identified in humans, and they cause vision defects such as dystrophy of the RPE (Seeliger et al., 1999). This phenotype is consistent with the high expression levels of STRA6 in RPE cells, which need to absorb a large amount of vitamin A for proper visual functions. In contrast, known human STRA6 mutations cause severe and systemic phenotypes (Golzio et al., 2007; Pasutto et al., 2007). There are several likely reasons that can explain these differences. First, biochemical analysis showed that the only two natural mutations found in human RBP cause only a partial loss of RBP function (Folli et al., 2005). This is in sharp contrast to the complete or near-complete loss of STRA6 function caused by STRA6 mutations (Kawaguchi et al., 2008a). The human RBP mutants can still bind retinol (Folli et al., 2005). In addition, holo-RBP formed by the mutant RBP can still bind TTR like the wild-type RBP. The major defect identified is the relatively faster release of retinol in the presence of lipid membranes. The vision defect associated with human RBP mutations suggests that vision is most sensitive to the partial loss of RBP function.

RBP is highly conserved in evolution. The fact that human RBP mutations are so rarely identified (only two so far) is consistent with its essential function. Moreover, the detection limit of serum retinol/RBP in patients with RBP mutations in the previous study is 200 nM (Seeliger et al., 1999), which is still much higher than the K_d of the RBP/STRA6 interaction (Kawaguchi et al., 2007).

Further, even for complete nulls, the loss of RBP and the loss of RBP receptor do not necessarily generate the same phenotypes in the same species. For example, placental delivery of vitamin A from maternal RBP to the human embryo is very different between RBP null embryos and RBP receptor null embryos. A human embryo without the RBP receptor can be directly impacted by its loss of function in the placental absorption of vitamin A from maternal RBP. In contrast, a human embryo without RBP still has its functional RBP receptor and functional maternal RBP to ensure sufficient vitamin A delivery to the embryo through the placenta. Interestingly, RBP knockout mice have retinoic acid deficiency in the brain even under vitamin A-sufficient condition (Quadro et al., 2005) and may have cognitive defects. Consistently, STRA6 mutations in human are associated with mental retardation (Pasutto et al., 2007). Even human pathological phenotypes caused by STRA6 mutations are variable (Chassaing et al., 2009). The variability is likely caused by the variable degrees in the loss of STRA6 function and the variability in vitamin A intake of the affected individuals. If the RBP/STRA6 system of vitamin A delivery is lost, random diffusion of retinoid would become the primary route of vitamin A transport. When comparing human and mouse phenotypes, another source of variability is species differences in retinoid metabolism and transport. For example, the doses of retinoic acid needed to produce teratogenic effects have drastic species variation (Nau, 2001). Mouse and rat are about 100 times less sensitive to isotretinoin's teratogenic effect than human (Nau, 2001).

7.5. Retinoid has the ability to diffuse systemically. Why did such a complicated mechanism (RBP/STRA6) to deliver vitamin A to cells evolve?

As demonstrated by retinoid related drugs, retinoids can diffuse to most human organs without a special delivery system. What are the reasons for the existence of the special vitamin A delivery system mediated by RBP and STRA6? Compared with random diffusion, there are many advantages of specific vitamin A delivery. First, random retinoid distribution is associated with mild to severe toxic side effects. This is not surprising given the fact that vitamin A derivatives (retinoids) have profound effects on the growth and differentiation of diverse cell types by controlling the activities of their nuclear hormone receptors (Chambon, 1996; Evans, 1994). Retinoids can

both enhance and suppress gene expression. The potent biological effects of vitamin A are best illustrated during embryonic development. Both insufficient and excessive vitamin A can cause severe birth defects (Collins and Mao, 1999). The aldehyde form of vitamin A (retinal) has also been demonstrated to be highly toxic in adults (Maeda et al., 2008, 2009).

Retinoids, especially retinoic acid, have been used in the past to treat human diseases, especially in dermatology and oncology. However, retinoid therapy is often associated with undesirable side effects similar to the systemic toxicity found in hypervitaminosis A. The most well-known side effect of treatment by retinoic acid (e.g., Accutane) is teratogenicity (Adams, 1993; Nau, 2001; Nau et al., 1994). Excessive retinoic acid is more toxic than retinol (Adams, 1993). In addition, retinoid therapies in adults are generally associated with diverse side effects on mucocutaneous tissues, such as cheilitis, xerosis, desquamation, dryness of mucous membranes, ocular effects, hair loss, hypergranulation of tissue, bone toxicity, and serum lipid alterations (Shalita, 1987). Animal model studies found that chronic exposure to clinical doses of 13-*cis* retinoic acid suppresses hippocampal neurogenesis and disrupts hippocampal-dependent memory (Crandall et al., 2004). In addition, 13-*cis* retinoic acid causes night blindness (Sieving et al., 2001). The birth defects and widespread side effects on adult organs caused by retinoid drugs also demonstrate the danger of relying on random diffusion to distribute retinoid.

The RBP/STRA6 system is clearly designed to prevent random retinol diffusion. The specificity of this system in vitamin A delivery is achieved through at least two mechanisms. The first mechanism is the high affinity and low off rate in RBP's binding to retinol. The absence of vitamin A from abundant erythrocytes and serum albumin, which can bind vitamin A, argues against random release of retinol from the holo-RBP/TTR complex in the blood. The second mechanism is the specific binding of RBP to its high-affinity receptor STRA6, which acts as a "homing device" to target holo-RBP to specific cells.

Second, random retinoid distribution requires constant retinoid intake to supply target tissues. Although constant intake is routine for laboratory animals, it is impossible for most, if not all, animals living in natural environments and most people living in developing countries. Delivery of vitamin A to cells or tissues is analogous to delivery of water to a house. Although random mechanisms (e.g., rain or flooding) may achieve delivery, they cannot guarantee the appropriate quantity and are associated with undesired side effects (as described above). Random mechanisms also cannot provide a stable supply during times of insufficiency. RBP serves as a buffer to maintain stable vitamin A concentration in the blood. The buffering function is important given the adverse effect of both low and high retinoid on the growth and function of diverse organs. Without this buffering function, blood retinoid level would fluctuate dramatically depending on dietary intake.

Third, random retinoid distribution may not satisfy tissues that need a large amount of retinoid for proper function such as the eye and developing embryos. RBP solubilizes vitamin A, protects it against oxidative damage, and makes it possible to mobilize retinoid stored in the liver when there is low retinoid intake. As a special carrier for vitamin A, RBP efficiently and specifically delivers vitamin A to organs distant from the liver such as the eye, the brain, the lung, the testis, and the placenta. The efficient vitamin A uptake activity of the RBP receptor may be especially important for tissues or cell types demanding a large amount of vitamin A. As mentioned earlier, the efficient delivery of retinol by RBP is likely responsible for the ability of retinol, but not retinoic acid (which is not a natural ligand of RBP), to prevent testicular degeneration (Howell et al., 1963).

7.6. If the RBP/STRA6 system functions to specifically deliver vitamin A to target organs, why does excessive vitamin A uptake cause toxicity?

Like many essential things in life (e.g., water), too much vitamin A is as detrimental as too little (Penniston and Tanumihardjo, 2006). In normal physiological conditions, RBP mobilizes vitamin A stored in the liver and delivers vitamin A to target organs by specifically binding to STRA6 on target cells. Using water delivery as an analogy, excessive vitamin A intake would overwhelm this system, analogous to excessive water overflowing riverbanks. Experiments in rats (Mallia et al., 1975) and a study of human patients with hypervitaminosis A (Smith and Goodman, 1976) both suggested that more toxicity is associated with vitamin A delivery independent of RBP. An excessive dose of vitamin A is toxic *in vivo* only when the level of vitamin A in the circulation is presented to cells in a form other than bound to RBP, such as in retinyl esters (Goodman, 1984). An increase of 10% in retinyl ester is regarded as a sign of vitamin A overload.

7.7. What is the role of megalin in vitamin A uptake?

Megalin, a 600-kDa scavenger receptor in the renal proximal tubes, is a low-affinity nonspecific receptor for RBP that mediates the endocytosis and transcytosis of the RBP protein in the kidney for its recycling (Christensen et al., 1999; Marino et al., 2001). Megalin binds to a wide range of extracellular proteins including the highly abundant serum proteins albumin and hemoglobin (Christensen and Birn, 2002). For this reason, it is unlikely for a cell exposed to the blood to use megalin as a receptor to take up vitamin A from holo-RBP because megalin nonspecifically binds to most, if not all, serum proteins. Extremely abundant serum proteins like albumin will saturate its binding sites. Consistently, no study has shown that megalin can mediate vitamin A uptake from holo-RBP in the serum. Megalin's

extremely large extracellular domain may be responsible for the promiscuity in its ligand binding. This promiscuity is clearly important in the recycling of proteins in the kidney to prevent their loss in the urine. In contrast, STRA6 is a highly specific membrane receptor for RBP and can even distinguish RBP from other retinol binding proteins (Kawaguchi et al., 2007). This selectivity is essential for recognizing RBP in the complex mixture of proteins in the serum during vitamin A uptake. Consistently, STRA6 can mediate vitamin A uptake not only from purified holo-RBP but also from holo-RBP in the serum (Kawaguchi et al., 2007). A large number of studies have shown that the RBP receptor-mediated vitamin A uptake does not depend on endocytosis (Bok and Heller, 1976; Chen and Heller, 1977; Heller, 1975; Ottonello et al., 1987; Quadro et al., 2002; Rask and Peterson, 1976; Shingleton et al., 1989; Sivaprasadarao and Findlay, 1988b; Sundaram et al., 1998). These studies distinguished megalin from the specific RBP receptor even before its identification.

8. RBP Receptor as a Potential Target in Treating Human Diseases

Knowledge of RBP receptor may be used to design specific methods to increase or decrease tissue retinoid levels to treat human diseases and alleviate disease symptoms. Currently, the most common therapeutic uses of retinoids are in dermatology and oncology. Retinoids have been used to treat various types of cancer (Simoni and Tolomeo, 2001; Verma, 2003) and various skin diseases such as psoriasis and other hyperkeratotic and parakeratotic skin disorders, keratotic genodermatoses, severe acne, and acne-related dermatoses (Orfanos et al., 1997). Given the potent biological effects of retinoids, current retinoid treatment is associated with diverse toxic effects such as teratogenicity, bone toxicity, and increases in serum lipids.

The RBP receptor-mediated vitamin A uptake is a natural physiological mechanism for cellular absorption of vitamin A from the blood. Increasing retinoid level by stimulating RBP receptor activity using pharmacology or molecular biology methods can potentially avoid the toxic effects of systemic administration of retinoids. Upregulating RBP receptor activity will only increase uptake through a physiological system. An analogy of systemic administration of retinoid is to flood a city in drought with water in an uncontrolled manner. Upregulating RBP receptor activity in this analogy is to deliver more water to a city in drought through its natural water delivery system. When decreasing the retinoid level is desired in treating certain diseases, cell-specific or tissue-specific suppression of the RBP receptor activity is better than systemic lowering of vitamin A/RBP level in the blood.

Membrane transporters have been one of the most successful drug targets. For example, serotonin reuptake inhibitors including Prozac inhibit

monoamine transporters and are widely used as antidepressant. Given STRA6's function as a membrane transport protein for vitamin A uptake, it is possible to modulate tissue vitamin A level by specifically targeting this natural vitamin A uptake mechanism. This technique can avoid the use of retinoids, which are associated with a wide variety of toxic effects. Since STRA6 is not homologous to any membrane receptor, channels, and transporters, its uniqueness can also be an advantage in pharmacological targeting of this receptor to create specific drugs. Modulating tissue retinoid level by targeting this natural vitamin A uptake mechanism has the potential to treat or alleviate the symptoms of the following diseases.

8.1. Visual disorders

Vitamin A is essential for vision because it is the precursor to photoreceptor chromophore (Crouch et al., 1996; Wald, 1968). A variety of visual disorders has been associated with abnormal vitamin A metabolism in the eye (Thompson and Gal, 2003; Travis et al., 2006). Modulating vitamin A uptake is a known method to alleviate symptoms for some diseases such as Stargardt macular dystrophy (Radu et al., 2004, 2005). Therefore, STRA6 is a novel molecular target for treating visual disorders.

8.2. Cancer

STRA6 was originally identified as a cancer cell-surface marker (Bouillet et al., 1997; Szeto et al., 2001). STRA6 was overexpressed up to 172-fold in 14 out of 14 human colorectal tumors relative to normal colon tissue (Szeto et al., 2001). Since vitamin A is known to be required for cell proliferation in many contexts, these cancer cells likely use the enhanced retinoid level for proliferation. Thus, the identification of STRA6 as the RBP receptor makes it possible to inhibit cancer growth by inhibiting the RBP receptor. Retinoids have been used to treat many types of cancer (Verma, 2003), and targeting STRA6 is an alternative to systemic retinoid treatment.

8.3. Skin diseases

Vitamin A plays essential roles in maintaining normal skin (Vahlquist, 1994). Retinoid can reverse aging of the skin (Varani et al., 1998). At the molecular level, retinoid treatment reduces matrix metalloproteinase expression and stimulates collagen synthesis in both naturally aged skin and photoaged skin (Varani et al., 2000). Retinoids have been used for treatment of various skin diseases such as psoriasis and other hyperkeratotic and parakeratotic skin disorders, keratotic genodermatoses, severe acne, and acne-related dermatoses (Orfanos et al., 1997). Given the role of STRA6 as the natural mechanism of

vitamin A uptake, modulating STRA6 activity in the skin is an alternative and potentially less toxic method to regulating skin retinoid level.

8.4. Lung diseases

Vitamin A plays important roles in maintaining normal development and function of the lung (Biesalski, 2003). Consistently, STRA6 mutations cause lung hypoplasia (Golzio et al., 2007; Pasutto et al., 2007). Vitamin A deficiency produces morphologic changes in the lung, impairs pneumocyte function (Baybutt et al., 2000), and potentiates hyperoxic lung injury (Veness-Meehan, 1997). Vitamin A also has a protective effect on respiratory status in patients with cystic fibrosis (Aird et al., 2006). Therefore, modulating STRA6 activity can potentially improve lung function under pathological conditions.

8.5. Immune disorders

Vitamin A plays important roles in hematopoiesis and in maintaining immunocompetence (Blomhoff and Smeland, 1994; Oren et al., 2003; Sommer, 1997a,b; Stephensen, 2001). Since STRA6 is highly expressed in lymphoid organs such as thymus, spleen, and lymph nodes, modulating STRA6 activity has the potential to improve immune function.

8.6. Neurological disorders

Vitamin A is required for cognition, learning, and memory of adult brain because retinoic acid is a modulator of the nervous system (Drager, 2006; Lane and Bailey, 2005; Maden, 2007; Weiler et al., 2001). For example, retinoic acid plays an important role in maintaining synaptic plasticity in hippocampus (Misner et al., 2001) and cortical synchrony during sleep (Maret et al., 2005). It also plays an important role in regenerative processes in the adult central nervous system (Vergara et al., 2005). Highly abundant expression of STRA6 in the brain is consistent with vitamin A's function in the brain. Given the potential roles of retinoid signaling in depression, Parkinson disease, Huntington disease, neuronal regeneration, and Alzheimer disease (Goodman, 2006; Mey and McCaffery, 2004; Vergara et al., 2005), STRA6 is a potential target for treating or alleviating symptoms of neurological disorders.

8.7. Diabetes

RBP was recently discovered as a signal secreted by adipocytes for insulin resistance (Yang et al., 2005). STRA6 is the only known high-affinity receptor for RBP and is potentially involved in pathological events in insulin resistance.

9. Concluding Remarks

It is a surprise that evolution came up with a completely new type of cell-surface receptor to mediate cellular vitamin A uptake. This receptor is unlike any known membrane receptors, transporters, or channels, although functionally it is both as a receptor for RBP and a membrane transport protein that mediates vitamin A entry into the cell. How this multitransmembrane domain protein performs these functions are just beginning to be understood. Future studies of this receptor will shed light on the molecular mechanism of how cells take up vitamin A under physiological conditions and how this uptake process is regulated to maintain tissue retinoid homeostasis. This knowledge will help to develop new treatment for human diseases caused by insufficient or excessive retinoid levels.

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CHAPTER TWO

Vascular Smooth-Muscle-Cell Activation: Proteomics Point of View

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Abstract

Vascular smooth-muscle cells (VSMCs) are the main component of the artery medial layer. Thanks to their great plasticity, when stimulated by external inputs, VSMCs react by changing morphology and functions and activating new signaling pathways while switching others off. In this way, they are able to increase the cell proliferation, migration, and synthetic capacity significantly in response to vascular injury assuming a more dedifferentiated state. In different states of differentiation, VSMCs are characterized by various repertories of activated pathways and differentially expressed proteins. In this context, great interest is addressed to proteomics technology, in particular to differential proteomics. In recent years, many authors have investigated proteomics in order to identify the molecular factors putatively involved in VSMC phenotypic modulation, focusing on metabolic networks linking the differentially expressed proteins. Some of the identified proteins may be markers of pathology and become useful tools of diagnosis. These proteins could also represent appropriately validated targets and be useful either for prevention, if related to early events of atherosclerosis, or for treatment, if specific of the acute, mid, and late phases of the pathology. RNA-dependent gene silencing, obtained against the putative targets with high selective and specific molecular tools, might be able to reverse a pathological drift and be suitable candidates for innovative therapeutic approaches.

Key Words: Vascular smooth-muscle cells, Phenotype proteomics, Cardiovascular disorders, Biomarker discovery, Target validation, Gene therapy.

1. Introduction

Vascular smooth-muscle cells (VSMCs) are the main component of the artery medial layer where they are circumferentially aligned and interspaced by elastic fibers. They possess both contractile and secretory properties, they can switch from quiescent to proliferative states, and their contraction depends on the interaction between the smooth muscle (SM)-α-actin and β-myosin heavy chain. In addition to the contractile capacity, VSMCs also possess important secretory properties that ensure synthesis and repair of extracellular matrix components (ECMs) and regulate the structure of the vascular wall. Owing to these characteristics, VSMCs are basic structural and functional elements in the artery wall and are responsible for its homeostasis. An imbalance in their functions inevitably leads to wall dysfunction and vascular disease. For all these reasons, VSMCs are an interesting subject of study, either as cell model for basic biological research or as main accountable matter for clinical investigations in cardiovascular disorders.

2. Characteristics of Vascular Smooth-Muscle Cells

2.1. Defining vascular smooth-muscle cells

A major issue in defining VSMCs is that they exhibit a wide range of different phenotypes and are not terminally differentiated in adult organisms. VSMCs are able to modify their phenotypes in response to inflammatory mediators, growth factors or inhibitors, mechanical influences, cell–cell, and cell–matrix interactions (Owens et al., 2004).

Adult VSMCs are highly specialized cells committed to regulating blood vessel tone-diameter and consequently blood distribution and pressure. They proliferate at a very low rate and express a specific and unique repertoire of contractile proteins, ion channels, and signaling molecules that differentiate them from any other cell types. However, VSMCs retain considerable plasticity and can undergo significant and reversible phenotype changes. Thanks to these properties, they are able to significantly increase the rate of cell proliferation, migration, and synthetic capacity in response to vascular injury, thus playing a critical role in vascular repair. The other face of the coin of this high plasticity is that it prompts the cells to respond to environmental signals leading to the development of vascular diseases such as atherosclerosis, hypertension, and neointima formation. Complex changes in the differentiated state of VSMCs have been extensively described in the development and progression of cardiovascular diseases. These modifications include altered expression of contractile proteins, increased matrix production, expression of inflammatory cytokines and cell markers, and production of proteases. To define the wholeness of morphological and functional changes that VSMC can display in response to environmental signals, the term "phenotypic modulation" or "switching," originally coined by Chamley-Campbell et al. (1979), has been used worldwide.

Up until now, an enormous amount of papers has been published on VSMC phenotypic modulation, all related to the expression of proteins universally accepted as markers of a differentiated state, the great majority being isoforms of the contractile apparatus. SM-α-actin is the most widely exploited marker, but others are also used such as SM-MHC, h-calponin, SM-22α, h-caldesmon, metavinculin, telokin, and smoothelin (Owens et al., 2004). Generally speaking, quiescent VSMCs are smaller than the activated ones; they have spindle-shaped elongated morphology and express copiously contractile-specific proteins. They proliferate at a very low rate and do not migrate. The activated VSMCs demonstrate a decrease in the expression of specific contractile markers (Poliseno et al., 2006). They show increased production of the extracellular matrix, especially collagen III and fibronectin, together with matrix metalloproteinases (MMP-1 and MMP-3)

that facilitate the movement of cells by degrading the matrix (Gerthoffer, 2007). Osteopontin (OPN) is also preferentially expressed by synthetic VSMCs (Giachelli et al., 1995; Panda et al., 1997), and tropomyosin-4 has been reported to be a marker for the activated cells during atherosclerosis (Abouhamed et al., 2003).

At present, this panorama is further complicated by the widely accepted concept that VSMCs present phenotypic heterogeneity and by the evidence that different populations can be isolated from normal arterial media. At least two distinct cell clones have been described in a variety of species: the spindle-shaped VSMCs (s-VSMCs) and the epithelioid or rhomboid VSMCs (r-VSMCs; Fig. 2.1). These populations exhibit different features, the migratory capacity representing the main specific characteristic of the rhomboid cells (Hao et al., 2002). This activity is crucial for the isolation of

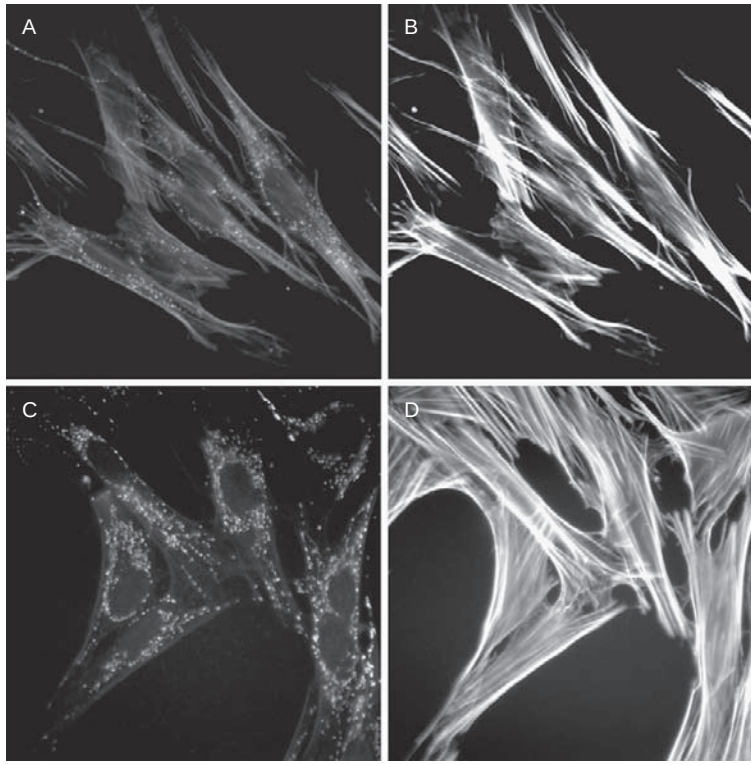

Figure 2.1 Spindle-shaped and rhomboid VSMC phenotypes. Upper panels (A) and (B) show quiescent spindle-shaped VSMCs obtained after 3 days of serum depleted cultures. Lower panels (C) and (D) show proliferating rhomboid VSMC cells. Fluorescent anti-Hsp60 antibodies allow the identification of mitochondria (panel A and C); phalloidin depicts actin cytoskeleton (panels B and D).

rhomboid VSMCs and explains why this population is only achieved by tissue explantation, while enzymatic digestion allows the separation of the spindle-shaped cells. Moreover, the rhomboid cells are localized preferentially in the abluminal side of the normal media (at least in the porcine model) and are recovered maximally in the stent-induced intima. They always exhibit enhanced proliferative capacity, high proteolytic activity, and low level of differentiation, as demonstrated by a decreased expression of cytoskeleton and contractile proteins such as desmin, SM-MHC, smoothelin. For all these reasons, they have been defined as atherome-prone cells in that they seem to represent a predisposed subpopulation of VSMCs, principal responsible for intimal thickening (Hao et al., 2003). To further complicate the scenery is the notion that s-VSMCs can switch to r-phenotype when stimulated by different factors such as platelet-derived growth factor-BB (PDGF-BB), fibroblast growth factor-2 (FGF-2), and co-culture with endothelial cells (ECs) (Brisset et al., 2007). A possible explanation for VSMC heterogeneity could be in embryologic vascular development, since VSMCs originate from different sources such as mesoderm, neurectoderm, and epicardium for coronaries (Gittenberger-de Groot et al., 1999). For example, from the aorta artery, it has been possible to isolate distinct populations of VSMCs with distinctive embryological origins, which respond differently to transforming growth factor-β (TGF-β) stimulation (Gadson et al., 1997).

In our opinion, it is not completely clear whether s- and r-VSMCs represent diverse populations of fully differentiated cells or correspond to clones of the same cell population in distinctive stages of differentiation. The main drawback in this issue derives from the problem of finding an exclusive and specific marker of the proliferative phenotype. So far, the only protein that has been described as r-VSMC marker is the S100A4 protein that increases its expression in the more proliferative, migrating VSMCs even if it is also present in the s-VSMCs (Brisset et al., 2007). In summary, defining VSMCs in a conclusive and precise way becomes a rather semantic decision, and for these reasons, we chose to generally name the migrating and proliferating, less differentiated cells as "activated." In parallel, the more differentiated contractile cells were called "quiescent."

2.2. Regulation of vascular smooth-muscle cell phenotypic diversity

The ability of contraction, proliferation, migration, and secretion in VSMCs is affected by a wide range of factors, including mechanical forces, contractile agonists such as angiotensin II (ANG-II), ECMs, reactive oxygen species (ROS), endothelial–VSMC interactions, PDGF, TGF-β1, and many other growth factors. Phenotypic modulation is thus dependent on this complex interaction of multiple environmental causes all together establishing a pattern of gene expression (Owens, 1995). More in general, the tight cross-talk

between extracellular and cellular factors is responsible for coordinated "outside-in" and "inside-out" signals generating conditions for the establishment of a given phenotypical status, as reported in Figs. 2.2 and 2.3.

Extracellular signals can be mechanical, physical, chemical, biochemical, and cellular, but all need to be transduced by elements present on the plasma membrane such as growth factors or integrin receptors, adhesion or connecting molecules, ion channels. The chemophysical characteristics of the membrane also play important functions in these mechanisms allowing, for example, the clustering of some factors. All these interface elements activate cascades of intracellular signals that end up with specific gene transcription and protein synthesis.

The intracellular effects of the previous "outside-in" signaling cascade concern a dramatic response at protein level mainly due to posttranslational modifications (PTMs) which trigger both alternative localizations and functions. They cause deep morphological changes depending on cytoskeletal remodeling and eventually, in the case of VSMCs, the phenotype switch. Due to their great plasticity, VSMCs are stimulated by a huge bulk of external inputs, react by changing morphology and functions, and activate new signaling pathways while switching others off. They synthesize new

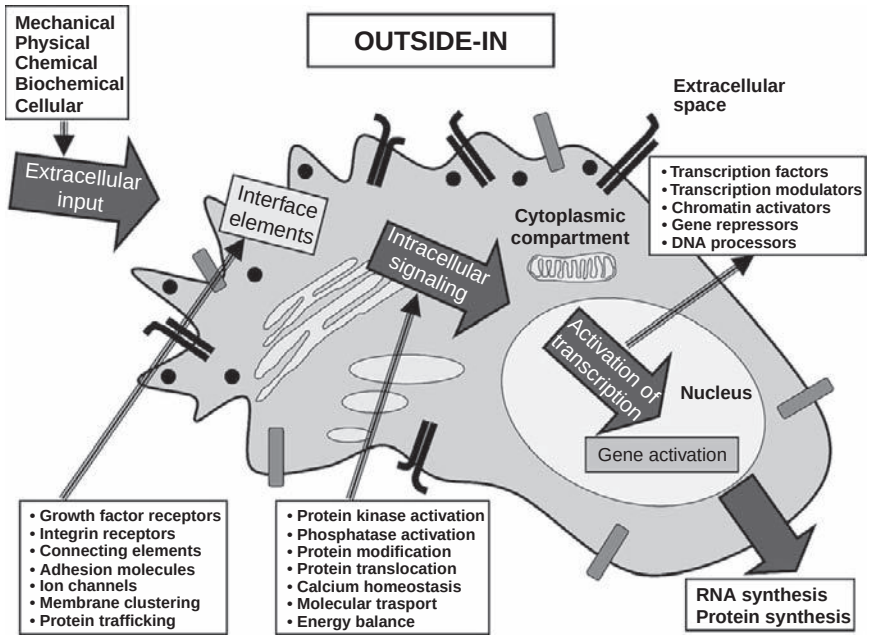

Figure 2.2 Extracellular stimuli-dependent cell activation. Cells sense the environment by means of dynamic surface elements whose response triggers a series of signaling mechanisms. Signals reach the nucleus where multiple genes are coordinately activated.

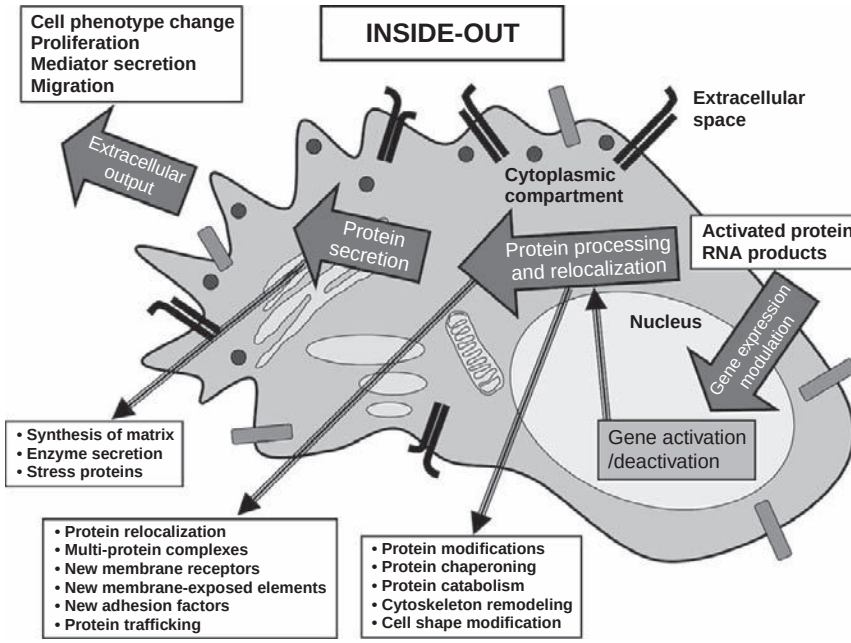

Figure 2.3 Cell reaction to the extracellular stimuli. Modified proteins and new synthesized proteins trigger dramatic changes. Activation of alternative metabolic pathways, cytoskeletal remodeling, exposure of a diverse repertoire of membrane proteins, and secretion of new factors contribute to the establishment of a different phenotype.

enzymes, membrane, and matrix proteins; interact with the external environment; and send signals via inside-out pathways preparing for migration. They produce growth factors and chemokines such as IGF-1, β-FGF, TGFβ, TNFα, HB-EGF, IL-1, and PDGF that exert autocrine–paracrine effects (Berk, 2001; Ross, 1993).

Many factors modulating VSMC activation could be associated with "outside-in" and "inside-out" cross-talk between the cells and surrounding environment. The above issues will only be briefly described below and we refer to more specific reviews for complete treatment. However, when reading this overview, it should be borne in mind that reported signaling family elements are intimately linked to each other as the results of an entangled network, still only partially elucidated, connecting the different factors both spatially and functionally.

2.2.1. Extracellular ground and integrin receptors

ECMs such as collagen, laminin, fibronectin, thrombospondin, and vitronectin (Gerthoffer, 2007) are all important in promoting VSMC migration, and among these extracellular proteins, OPN and thrombospondin-1

(TSP-1) are worth mentioning. Osteopontin has been reported to entail αvβ3 integrin signaling (Liaw et al., 1994; Panda et al., 1997). TSP-1 is a matricellular protein that stimulates VSMC proliferation and migration and contributes to the development of atherosclerosis (Raman et al., 2007). It is upregulated in diabetes (Panchatcharam et al., 2010), and in hyperglycemia, it alters the expression of many genes effecting several pathways (Maier et al., 2010).

Integrin-mediated cell signaling is often a positive regulator leading to cell proliferation; fibronectin, for example, promotes the synthetic phenotype through the activation of ERK MAP kinase pathways (Qin et al., 2000). However, α7β1 has been reported to negatively influence cell growth and to promote the VSMC contractile phenotype (Welser et al., 2007). Interestingly, α7β1 is a major laminin-binding receptor in VSMCs, and laminin has been demonstrated to maintain the differentiated state through p38 MAP kinase signaling.

External mechanical forces are also important to establish the VSMC phenotype. For example, it has been demonstrated that substrate stiffness affects VSMC behavior involvement in vascular pathogenesis. Changes in substrate stiffness can influence cellular responses to external stimuli through cross-talk between integrin and membrane receptors. These mechanisms involve membrane lipid rafts which play a central role in the signal transduction pathway of the surface receptors. Lipid rafts are stabilized, and PDGF receptors, for example, are recruited in this membrane domain (Brown et al., 2010).

Moreover, ECM/integrin/focal adhesion pathway is important to regulate VSMC gene transcription, differentiation, in response to the mechanical factors that are significant in arteries, subjected to the tensile stress due to blood pressure (Pyle and Young, 2010). In this context, actin cytoskeleton plays an important role (Gunst and Zhang, 2008; Tang and Anfinogenova, 2008), and its degradation is an early, key event inducing phenotypic modulation (Zheng et al., 2009). Therefore, actin cytoskeleton may be considered a mechanotransduction sensor intervening in transcriptional regulation in response to the mechanically induced signals from the extracellular matrix and cell surface to the nucleus (Wang et al., 2009).

2.2.2. Surrounding cell contacts

Communication between the endothelium and VSMCs is fundamental for the maintenance of vascular function and structure and even if *in vitro* studies usually involve single cell cultures, the influence of the ECs on the VSMC phenotype cannot be forgotten. Secretion of nitric oxide (NO; Palmer et al., 1988), prostacyclin (Moncada, 1982), and endothelin (Mawji and Marsden, 2003) acts on VSMC to regulate the vessel tone. This cross-talk critical for vascular homeostasis is bidirectional. For example, VSMCs inhibit EC endothelin 1 production (Di Luozzo et al., 2000). This issue has been studied since 1986 (Campbell and Campbell, 1986), and ECs have

been shown to promote VSMC differentiation from the synthetic to the contractile phenotype, as measured by cell morphology, differentiation protein marker expression, and cell contractility (Powell et al., 1998), through activation of the PI 3-kinase/Akt pathway (Brown et al., 2005).

2.2.3. Inflammatory mediators

Inflammatory responses are known to play a key role in the early onset of atherosclerosis and restenosis, and proinflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) are involved in the process (Tashiro et al., 2001). Cytokines stimulate VSMC migration through a reorganization of the cytoskeleton (Wang and Newman, 2003). Moreover, soluble cytokines together with cell–cell interactions activate matrix metalloproteinase (Matrixins), which contribute to intimal growth and vessel wall remodeling mostly by promoting VSMC migration (Newby, 2005; Wang et al., 2010a). In particular, TNFα is produced by VSMCs and influences the production of other cytokines and chemokines, thus triggering the recruitment and transmigration of monocytes and T cells across the vascular wall, inducing the migration of VSMCs. This last outcome has been demonstrated *in vivo* (Krishnaswamy et al., 1999) and in culture (Jovine et al., 1997) and entails the p38-MAPK/CREB/RAC1 pathway (Ono et al., 2004). If the proinflammatory effects of cytokines are well established, there can be anti-inflammatory effects. Recently, the anti-inflammatory role of IL-19 has been described. This cytokine is normally absent in VSMC, but is induced by other cytokines and by injury in the arteries (Tian et al., 2008). In VSMC, IL-19 reduces the proteins and RNAs of proliferative genes, probably inhibiting the expression and cytoplasmic translocation of the human antigen R (HuR), which has a destabilizing effect on proliferative and inflammatory mRNAs (Cuneo et al., 2010).

2.2.4. Receptor-mediated growth factors

PDGF-BB, produced by activated platelets and macrophages, has so far been the only factor demonstrated to selectively and directly promote phonotype switching (Owens et al., 2004). Twenty years ago, Blank and Owens (1990) and successively others (Li et al., 1997) demonstrated that treatment of VSMCs with PDGF-BB is associated with rapid downregulation of multiple differentiation marker genes. In summary, PDGF stimulates VSMC proliferation and migration (Ferns et al., 1991; Gerthoffer, 2007; Jawien et al., 1992). The role of PDGF receptors has been described in postinjury models (Davies et al., 2000) and detected in human coronary arteries following balloon angioplasty (Tanizawa et al., 1996). PDGF also acts indirectly inducing the synthesis of other growth factors such as EGF and FGF-2, thus causing longer-term activation (Pintucci et al., 2005).

Among the growth factors, TGF-β has shown to promote VSMC differentiation, displaying an effect that is opposite to that of PDGF

(Adam et al., 2000; Owens et al., 1988) and that could be protective in different pathological states (Lutgens et al., 2002; Mallat et al., 2001).

The data relative to the effect of the insulin-like growth factor-1 (IGF-1) on VSMC activation are contradictory (Poliseno et al., 2006). Interesting are the results obtained by Sobue et al. (Hayashi et al., 1999) who showed that VSMCs cultured on laminin maintain a differentiated phenotype when treated with IGF. According to the authors, the process is mediated through the IGF receptors (IGF-R) and subsequent activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway. IGF-1 would regulate phenotypic modulation through the insulin receptor substrate-1 (IRS-1)/PI3K/AKT pathway. Other authors demonstrated the effects of IGF-1-stimulating proliferation and migration of VSMCs through the PI-3 and MAP kinase pathways (Radhakrishnan et al., 2008). Moreover, in venous VSMCs, IGF-1/IGF-R signals activated by mechanical stretch through the mechanosensitive transcriptional factor Egr-1 (Wu et al., 2010) resulted in proliferation and neointima formation (Cheng and Du, 2007). A recent paper by Clemmons and colleagues (Radhakrishnan et al., 2010) seems to solve the controversy, indicating the level of glucose as determinant for the effect of IGF-1 on VSMC activation. According to the authors, exposure to high glucose attenuates IRS-1 signaling and, at the same time, stimulates SH2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1) phosphorylation, resulting in increased proliferation and migration. SHPS-1 is an integral membrane protein functioning as a scaffold for multiprotein signaling complexes that are assembled in response to IGF-1 (Shen et al., 2010). These data offer interesting elements for understanding the important linkage between cardiovascular diseases and diabetes. In this respect, there are evidences that VSMC activation is stimulated by hyperglycemia in an $\alpha V\beta 3$ integrin-dependent manner (Panchatcharam et al., 2010) and that cell growth is induced through the Rho pathway (Ishiko et al., 2010). Type 2 diabetes and metabolic syndrome are known to be associated with a high risk of atherosclerosis and restenosis, but the role of insulin in cardiovascular diseases is still highly controversial. In fact, increased insulin concentration has been identified as risk factor for cardiovascular disease (Yamada et al., 2002) and has recently demonstrated to have a crucial role in activating VSMC proliferation and migration (Wang et al., 2010a,b). For these reasons, some authors consider insulin a proatherogenic factor, but others believe that the evidenced atherogenic effects are due to insulin resistance that escorts both diabetes and metabolic syndrome (Breen and Giacca, 2010).

2.2.5. Tone agonists

ANG-II is a hormone with a clear role in vasoconstriction, but it also acts as a growth factor in VSMCs. Increased plasma concentration of ANG-II has been implicated in atherogenesis and is an important mediator of systemic

vascular remodeling. It is a potent inducer of VSMC dysfunction, as it promotes hyperplasia and/or hypertrophy of VSMCs *in vitro* as well as *in vivo* in the normal arterial wall (Kim and Iwao, 2000) and participates in the neointimal proliferation response to vascular injury (Daemen et al., 1991). ANG-II also stimulates migration of VSMCs both *in vitro* (Bell and Madri, 1990) and during restenosis formation after vascular injury (Nozawa et al., 1999). It also induces the expression of several proinflammatory cytokines and vasoactive eicosanoids.

2.2.6. Lipidic metabolism

Sphingolipids, derived from sphingomyelin metabolism, have been involved as potentially important mediators of cardiovascular function (Alewijnse et al., 2004; Takuwa et al., 2008). Basically, research has focused on the role of sphingosine-1-phosphate (S1P), formed via the actions of sphingosine kinase. S1P is released from activated platelets in the blood and acts on specialized receptors located on both ECs and VSMCs. In addition to the regulation of the vascular tone, sphingolipids may also affect the VSMC phenotype. In cultured VSMCs, S1P can activate intracellular pathways and gene expression typically associated with proliferation and migration (Spiegel and Milstien, 2003). S1P not only stimulates but can also inhibit VSMC migration depending on the expression pattern of individual S1P receptors. Recent data suggest that the S1P1 and S1P3 receptors are involved in stimulating S1P-induced cell migration. In contrast, the S1P2 receptor inhibits cell migration (Takuwa, 2002).

2.2.7. Intracellular signaling elements

The mammalian target of Rapamycin (mTOR) integrates intracellular and extracellular signals and is a central regulator of cell metabolism, growth, proliferation, and survival (Laplante and Sabatini, 2009). Its effects on VSMCs have been extensively studied since Rapamycin was first used on drug-eluting stents and was demonstrated to decrease the incidence of restenosis (Eisenberg and Konnyu, 2006). Rapamycin inhibits VSMC migration and proliferation *in vitro* and intimal hyperplasia *in vivo* and also induces differentiation in cultures of synthetic VSMCs (Martin et al., 2004). mTOR is a protein kinase crucial in regulating cell growth, proliferation, and migration in response to cellular environment, including nutrients, energy, oxygen supply (Sarbassov et al., 2005); moreover, it integrates growth factors and mitogen pathways (Hay and Sonenberg, 2004).

Among the intracellular pathways, also those involved in cAMP production are significant in the process of VSMC activation. In fact, it has been demonstrated that adenylyl cyclase 8 (AC-8), one of the principal enzymes controlling cAMP formation, is overexpressed in dedifferentiated VSMCs (Clement et al., 2006). Noteworthy, AC-8 allows the secretion of phospholipase A_2 type IIA, which is an inflammatory marker associated

with an increased risk of future artery disease (Mallat et al., 2005). More recently, it has been evidenced that there is a high level of AC8 in intimal VSMCs and moreover that this expression is related to increased migratory ability (Gueguen et al., 2010).

Owing to the contractile characteristics of VSMCs, calcium (Ca^{2+}) signaling plays a central role in their cell functions. Variations in Ca^{2+} intracellular concentration lead to contraction or relaxation of the cells and eventually of the vase. Cytoplasmic Ca^{2+} originates either from the outside through plasma membrane channels or from the inside, that is, from the sarcoplasmic reticulum. During contraction, membrane depolarization activates the L-type high voltage-gated Ca^{2+} channel, increasing Ca^{2+} entry, and consequently stimulating Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) as well as the Ryanodine receptor (RyR) responsible for Ca^{2+} release from sarcoplasmic reticulum. Conversely, relaxation is obtained through extrusion of Ca^{2+} via plasma membrane Ca^{2+}-ATPase (PMCA) or uptake into the sarcoplasmic by the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). A recent review (House et al., 2008) has summarized the changes in the expression of Ca^{2+} signaling proteins associated with VSMC phenotype switch and thus involved in vascular diseases. Loss of L-type and gain of T-type voltage-gated Ca^{2+} channels (Gollasch et al., 1998) are highlighted. Moreover, the *in vitro* switch from quiescent to activated VSMCs is characterized by RyR3 loss (Vallot et al., 2000), downregulation of SERCA (Lipskaia et al., 2005), and differential expression of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) isoforms (House et al., 2007). In conclusion, there is growing evidence that VSMC phenotypic switch is associated with changes in Ca^{2+} signaling molecules (ion channel, pumps, and Ca^{2+}-activated enzymes). The functional consequence is the acquisition of a "non-excitable-like phenotype," increased cytoplasmic Ca^{2+}, and subsequent proliferation.

2.2.8. Transcriptional activation

The transcriptional regulation of genes involved in controlling the VSMC phenotype is still poorly understood. Several transcription factors and co-regulators contribute to VSMC phenotype regulation. The GATA-6 transcription factor, a member of the GATA family of zinc finger DNA-binding domain proteins, exhibits the characteristics of a VSMC-specific master regulatory transcription factor. GATA-6 is expressed in quiescent VSMCs but is rapidly downregulated in response to mitogens or injury (Mano et al., 1999; Miano et al., 2007). GATA-6 induces a differentiated VSMC phenotype and promotes withdrawal from the cell cycle by induction of $p21^{cip1/WAF}$ (Morrisey, 2000). Several co-activators have shown to affect GATA-6 DNA binding and transactivation, and among these, a relevant role is played by myocardin. This factor is cardiac and smooth muscle-specific and is linked to the ubiquitous serum response factor (SRF; Pipes et al., 2006).

SRF binds to *cis* DNA regulatory elements called CArG boxes (CC(A/T-rich) 6GG), which are found in the promoters of muscle-specific and serum-inducible genes, such as c-*fos*, which regulate proliferation. Upon discovery of myocardin as a cell-type specific cofactor, it became apparent how SRF and these CArG motifs could mediate two opposing patterns of gene expression. In promoters such as c-*fos*, ternary complex factors such as Elk-1 are phosphorylated by ERK1/2 in response to serum growth factor stimulation. Phosphorylated Elk-1 binds to SRF, which is constitutively bound to DNA, and leads to promoter transactivation. In muscle-specific promoters, myocardin competes with Elk-1 for binding to SRF. Myocardin-binding to SRF promotes the expression of smooth muscle-specific genes. Using these mechanisms, myocardin and Elk-1 can act as binary transcriptional switches that may regulate the differentiated versus proliferative phenotypes in VSMCs.

2.2.9. Endogenous modulation of gene expression

We purposely conclude this section mentioning microRNAs, following the recent publication of several papers describing their involvement on VSMC differentiation and phenotypic modulation (Albinsson et al., 2010). MicroRNAs are endogenous small noncoding RNAs that regulate gene expression via degradation or translational inhibition of target RNAs. miRNA-145 is selectively expressed in vascular walls, its expression is downregulated during neointimal lesion formation and in cultured VSMCs; moreover, it is a phenotypic modulator through its target gene KLF5 (Cheng et al., 2009). miRNA-145 can be defined as the hippest VSMC phenotypic marker and has also been proposed as therapeutic target for vascular diseases (Zhang, 2009). Its features are similar to those of miR-143 since the *in vitro* overexpression of both miRNAs is sufficient to promote differentiation (Cordes et al., 2009), while their deficiency leads to VSMCs switching to the activated-synthetic state. It has been demonstrated that miR-143 binds the 3′ UTR of Elk-1 while myocardin, Klf4, and CamkIId are the targets of miR-145. In summary, the miR-143/145 cluster, controlling the concentration of the protein that regulates contractility, seems to be active in the acquisition and maintenance of the contractile phenotype (Boettger et al., 2009). miR-21 is one of the most studied miRNAs. It seems to play a role in hypoxia-induced pulmonary VSMC proliferation and migration (Sarkar et al., 2010). It is upregulated in the vascular wall after balloon injury, and it has been suggested that it may also have a role in proliferative vascular diseases (Scalbert and Bril, 2008; Zhang, 2008). Its inhibition in cultured VSMCs decreased proliferation and increased apoptosis and its postulated targets are PTEN and bcl-2 indirectly (Ji et al., 2007). PDGF induces the expression of miR-221 and miR-222, which decreases the expression of differentiated markers and triggers migration and proliferation (Davis et al., 2009).

At this point, it is clear that VSMCs are very dynamic cells in the middle of outside-in and inside-out pathways, subjected to external inputs but also

reactive to them and prone to exert their action toward the exterior. The balance among all these mingled interactions is delicate and critical and, when missed or dwindled, it can be the cause and eventually result in the onset and progression of pathological conditions.

2.3. Role of VSMC phenotypic modulation in restenosis and atherosclerosis development and progression

Despite the clear evidence that the phenotypic modulation of VSMCs plays a key role in vascular injury repair and in the development and/or progression of atherosclerosis, the extreme complexity of the mechanism still makes it difficult to understand satisfactorily how this process is regulated *in vivo*. Actually, we are just beginning to comprehend some of the molecular mechanisms and factors that control transitions in the phenotypic state of the VSMCs and little is known about what controls these transitions during the different stages of atherosclerosis development. Briefly, atherosclerosis is an inflammatory disease, whose first step is EC damage. The injury results in decreased production of NO, which inhibits VSMC growth and migration, as well as increased expression of ICAM-1 and VCAM-1. Alongside the production of chemokines and chemoattractant protein-1 (MCP-1), these adhesion molecules promote the attachment and migration of monocytes, macrophages, and platelets into the vascular wall. Macrophages internalize LDL becoming foam cells and then producing the fatty streaks (Glass and Witztum, 2001; Packard and Libby, 2008; Ross, 1999). Production of ROS, especially due to AD(P)H oxidase, has been correlated to atherosclerotic lesion development (Khatri et al., 2004; Stocker and Keaney, 2004). In this state, VSMCs migrate from the media to the intima, synthesize ECM, mingle in the area of inflammation, and interfere with the development of the lesion. Cyclic accumulation of monocytes, proliferation of VSMCs, and formation of fibrous tissue lead to an enlargement of the lesion, characterized by a fibrous cap and a necrotic core engulfed by lipids. This advanced complicated lesion may cause plaque rupture and a major cause of this phenomenon seems to be the thinning of the fibrous cap due to VSMC apoptosis. Thus, VSMCs may be involved not only in lesion growth but also in the process of plaque rupture.

According to more recent papers, VSMCs are responsible for promoting plaque stability in advanced lesions. Interferon-γ released by activated macrophages induces collagen synthesis by VSMCs, and this is important for the stabilization of the fibrous cap. Akt-1 demonstrates a pivotal role in promoting VSMC proliferation and migration, and in restraining oxidative stress-dependent apoptosis. Indeed, this function results to be beneficial in the later stages of atherosclerosis protecting against plaque vulnerability (Fenrandez-Hernando et al., 2009). The role of VSMC phenotypic modulation in atherosclerosis onset and development is thus controversial, and

this statement is also supported by the recent review of Delafontaine and colleagues (Higashi et al., 2010). Migratory and proliferative VSMC activities are either positively or negatively regulated since there are documented promoters such as PDGF, endothelin-1, thrombin, FGF, interferon-γ, IL-1, but also inhibitors such as heparin sulfates, NO, and TGF-β (Berk, 2001). If the role of growth factors in atherosclerosis has traditionally been thought to be permissive in stimulating VSMC migration and proliferation and promoting neointima formation, more recent papers have suggested the antiatherogenic effects of some growth factors. Both IGF-I and PDGF-BB seem to reduce inflammatory responses and atherosclerosis progression (Sukhanov et al., 2007; Tang et al., 2005).

Restenosis is a common complication affecting up to 30–40% of coronary angioplasties, a problem that has not yet been completely solved despite the technological advances of drug-eluting stents. Processes such as endothelial denudation, direct VSMC trauma, and subsequent release of multiple growth factors all play a role in promoting cell activation. As a consequence, VSMCs migrate to the subintimal space, proliferate, and secrete abundant amounts of extracellular matrix which forms the bulk of the intimal hyperplastic lesion contributing to restenosis. Stenting reduces vascular remodeling, but its long-term clinical efficacy is limited by neointima formation, late lumen loss (Ellis et al., 1992), and in-stent thrombosis (Ong and Serruys, 2005).

Although ECs (DeRuiter et al., 1997), adventitial fibroblast (Sartore et al., 2001), and bone marrow-derived cells (Sata et al., 2002; Simper et al., 2002) have proved to contribute to neointima formation, a long-standing dogma proposes that the intimal VSMCs originate primarily from preexisting medial VSMCs (Ross and Glomset, 1973). It was also unequivocally demonstrated that VSMCs within atherosclerotic lesions show morphological, biochemical, and physiological properties that differ according to the localization, characteristics, and stage of the pathologic condition (Dzau et al., 2002; Stintzing et al., 2009). The scenery is therefore extremely complicated and it is impossible to distinguish a "bad" pathological phenotype from a "good" physiological one. Several studies have been carried out to differentially characterize neointima VSMCs in comparison to the normal arterial VSMCs.

On this issue, many questions remain without a precise answer, the most important of which are the following: (1) What are the key environmental factors that induce phenotypic modulation after vessel injury? (2) What are the molecular mechanisms activated by these factors? (3) What are the molecular biomarkers truly specific and unique for a given VSMC phenotype? (4) Is it possible to hypothesize easy, quick, and noninvasive assays able to describe VSMCs state and thus to predict arterial wall condition? (5) Is it possible to develop therapeutic strategies using molecular tools specifically designed against key elements in the activation pathways or eventually capable of reverting the phenotypic switching?

In this field, proteomics can deal with problems that cannot be approached by genomic analysis, namely relative abundance of protein products, PTMs, compartmentalization, turnover, as well as interactions and functions. Proteomics is a complex discipline proposing the study of the entire cell-produced set of proteins and can bring new expectations for VSMC phenotype study. It is a hard fact that these cutting-edge technologies can help answer the fundamental questions listed above and provide not only the theoretical knowledge but also molecular tools necessary for new therapeutic approaches. The next sections are an overview of some of the existing literature written on these issues in the past years.

3. Proteomics Approaches for VSMC Proteome Mapping

Over the past few years, the genome has been recognized to represent only a first layer of complexity, since biological functions are not carried out by the genome but mainly by the dynamic population of proteins. For these reasons, there has been an increasing interest in the field of proteomics. The proteome was originally defined as the complete protein complement expressed by a genome. However, this definition does not take into account that the proteome is a highly dynamic entity that changes according to the cellular and extracellular conditions. There are indeed numerous instances of differential splicing and many posttranslational protein modifications (e.g., phosphorylation, glycosylation, ubiquitination, and methylation) that can govern the behavior of proteins. Protein functions depend on precise amino acid (AA) sequence, modifications (especially regulatory like phosphorylation), three-dimensional (3D) structure, protein concentration, association with other proteins, and extracellular environment. Accordingly, proteomics seeks to determine protein structure, modifications, localization, and protein–protein interactions in addition to protein expression levels.

Proteomic methods consist principally in separating polypeptide species according to their physical and chemical properties. The limitations of these techniques are that (a) the sample must be prepared in a way which is compatible with the separation and quantification techniques and (b) it is extremely difficult to analyze all the proteins or peptides present in a complex mixture simultaneously. This is due mainly to different ranges of expression levels and different protein behavior during the separation process. For these reasons, in order to obtain fruitful proteomic analysis, it is necessary to exploit the multidimensional separation techniques able to reduce the complexity of the whole proteome. The separation techniques can be divided into two main groups (see Fig. 2.1): (1) separation of entire proteins and (2) separation of digested proteins (peptides).

In the former group, the most used approach is two-dimensional electrophoresis (2-DE). It consists in separating proteins in the first dimension by isoelectrofocusing (separation according to the isoelectric point), followed by SDS-polyacrylamide gel electrophoresis in the second dimension (separation by relative molecular mass) (Celis and Gromov, 1999, 2003). Proteins are commonly visualized by Coomassie blue or the more sensitive silver staining. Detection of proteins by fluorescent stains is even more sensitive and provides a good dynamic range, but more appropriate and sophisticated equipment is necessary for capture to occur. Another approach consists in a differential solubility extraction procedure (IN-Sequence; Bizzarri et al., 2008; Neverova and Van Eyk, 2002), which allows separation of protein fractions according to their solubility at various pH levels. Specific antibodies can be used to enrich phosphoproteins by immunoprecipitation, and ever this method can be chosen to reduce complexity. Other emerging techniques for the separation of native proteins exist, such as reversed-phase high-performance liquid chromatography (HPLC; Opiteck et al., 1998), capillary zone electrophoresis (Manabet, 1999), and affinity chromatography (Lee and Lee, 2004).

In the latter group, the most commonly used approach is HPLC. Columns with different characteristics, also coupled together to ensure multidimensional separation (MudPIT approach), have been developed and standardized for peptide fractionation. Examples of different solid states are: reverse phase, strong cation exchanger, and hydrophilic interaction materials. To increase performance, other optimizations can be applied such as phase elution gradient, preconcentration columns, etc. Figure 2.4 summarizes the described approaches.

All these fractionation methods can eventually be coupled with mass spectrometry (MS) analysis for identification (Aebersold and Mann, 2003). In recent years, MS analysis has been integrated with precise and complete MS/MS analysis so as to overcome the poor accuracy of the peptide mass that could result in incorrect identifications. MS/MS analysis permits to obtain the AA sequence of proteins by fragmentation of selected peptides. Combined with the peptide map obtained by MS, the information on AA sequence gives a high probability of correct identification of the protein.

Some of these fractionation approaches have been used to map the VSMC proteome. The first map of VSMC proteins was published in 2001 (McGregor et al., 2001) and since then other papers have been produced (Bian et al., 2008; Brisset et al., 2007; Dupont et al., 2005; Padrò et al., 2007; Taurin et al., 2002). The most detailed 2D-PAGE (2D-polyacrylamide gel electrophoresis) map was presented by Mayr et al. (2005) who identified 235 proteins, corresponding to the 154 most abundant unique proteins in mouse aortic VSMC. Human VSMCs have been analyzed (Dupont et al., 2005) from a proteomic point of view. Proteome and secretome have also been investigated obtaining 121 and 40 intracellular

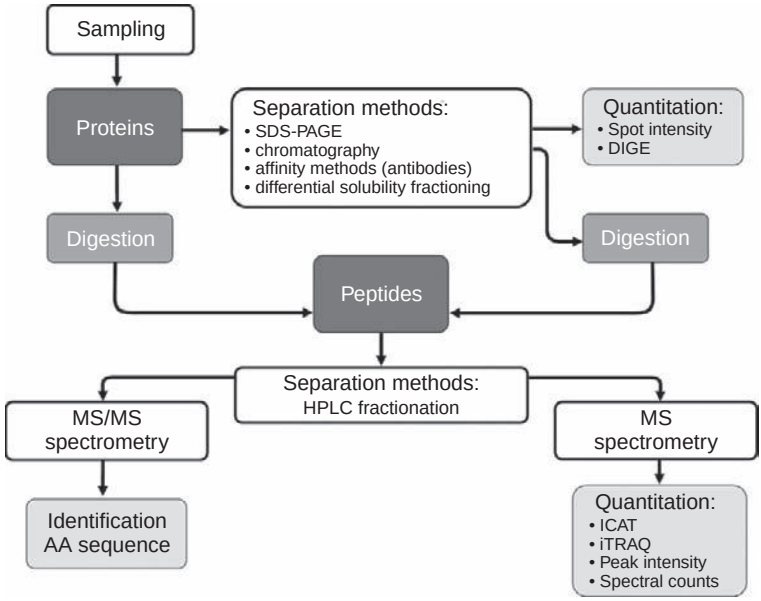

Figure 2.4 **Multiple approaches to proteomic studies. All the different strategies have been usefully applied, alone or combined, for protein quantification and identification.**

and secreted polypeptide spots, respectively, corresponding to 83 and 18 different proteins identified by MALDI/TOF/MS. 2D-electrophoresis for VSMC fractionation, coupled with MS for identification, was exploited in all these articles (Table 2.1). In 2008, Gao et al. and Sui et al. analyzed different effects induced on VSMC cultures stimulated by ANG-II and *S*- and *R*-enantiomers of atenolol, respectively, using LC/MS/MS approaches. The results of these studies have made it possible to identify 629 and 407 unique proteins in rats, the best VSMC proteome mapping ever obtained before. The first gel- and label-free approach for VSMC proteome mapping published this year (Rocchiccioli et al., 2010) has allowed to identify 815 nonredundant VSMC proteins in the pig. This approach combines solid phase extraction (SPE) techniques designed to obtain subproteomes from total extracts (first dimension of fractionation) with reversed-phase HPLC-MALDI/TOF/TOF (second dimension of fractionation) on the resulting peptide mixtures. The information stored in a database can be used to compare the effects of various stimuli on the level of these proteins in pathological conditions.

Table 2.1 reports authors who published a proteomics investigation of VSMC activation between 2001 and 2010. The first articles report bidimensional gel electrophoresis as fractionation method at the protein level, while HPLC fractionation techniques were introduced in 2008 to separate

Table 2.1 VSMC proteomics articles from 2001 to 2010

Author	No. of unique identified proteins	Species	Publication year	Technique	No. of not shared proteins[a]
Rocchiccioli et al.	812	Pig	2010	Gel-free, label-free	567 (70%)
Gao et al.	629	Rat	2008	PAGE-LC, label-free	279 (44%)
Sui et al.	407	Rat	2008	Gel-free, labeled	127 (31%)
Mayr et al.	154	Mouse	2005	2D-PAGE, silver	33 (21%)
McGregor et al.	129	Human	2001	2D-PAGE, silver	29 (22%)
Dupont et al.	83	Human	2005	2D-PAGE, silver	15 (18%)
Taurin et al.	24	Rat	2002	2D-PAGE, coomassie	8 (33%)
Wang et al.	13	Rat	2010	2D-PAGE, coomassie	4 (31%)

[a] Number of proteins exclusively identified by the author and not shared with the other reported authors.

digested proteins. The increase in the identification of the total protein extract has become significant in the past few years, but the overlap of identified proteins, as shown in Fig. 2.5, remains low (only 2%). It is frequent when authors use large and different datasets for protein identification and characterization.

3.1. Differential protein expression analysis: Insight in VSMC activation

As discussed in the previous section, VSMC phenotype changes are key events in the pathogenesis of vascular proliferative disorders initiated by a myriad of stimulating factors. VSMCs in different states of differentiation are characterized by various repertories of proteins, differentially expressed proteins, and activated pathways. The objective of differential proteomics is to separate, visualize, and quantify the proteins present in a complex mixture in order to reveal the differences of protein expression between two or more experimental conditions. In this framework, for many years, 2D-PAGE image analysis of cellular extracts has been considered the best approach to identify changes in specific proteins. After 2D-PAGE analysis, gels can be visualized using a variety of methods, and protein spots can be

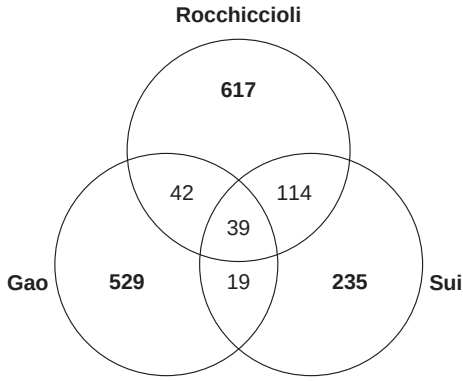

Figure 2.5 Venn's diagram comparing protein identification in LC/MS/MS approaches used for VSMC proteome mapping. The numbers represent the number of identified proteins, and of these, shared proteins are reported in the overlapping areas.

scanned and analyzed using appropriate software to search for changes in the protein levels. Various software packages for the analysis and processing of 2D gel protein images have been developed including PDQUEST (Biorad), MELANIE (SIB), ProXPRESS (PerkinElmer), and SameSpots (NonLinear Dynamics). These packages can include semiautomatic analysis of the comparison between gels, statistical analysis of differences in spot volume, and annotation of proteins with database information. Changes associated with either hyperplastic or hypertrophic growth have been analyzed by 2D-PAGE on extracts from quiescent aortic rat VSMCs and exposed to growth factors. Among the different upregulated proteins, there are mediators of protein folding and synthesis (Patton et al., 1995).

In another study, VSMCs were treated with TNFα in the presence or absence of alpha lipoic acid (ALA). In this work, the authors had the possibility of understanding the effects of double regulation using 2D-PAGE and of differentiating proteins upregulated by TNFα and downregulated by ALA as well as others downregulated by TNFα and upregulated by ALA (Jang et al., 2004).

Because atherosclerosis development is favored by local hemodynamic factors such as shear stress, changes in the expression pattern of VSMC proteins exposed to hemodynamic stress were analyzed identifying proteins able to bind actin filaments. Using this approach, many potential mediators of the stimulus have been found such as Gelsolin, HSP27, HSP60, PDI, Vimentin, Actin, CapZ, and others (McGregor et al., 2004). Oxidative stress is involved in mechanisms of plaque progression, and the application of ESI/MS/MS and Western Blot methods designed to study oxidative stress in VSMCs has led to the identification of HSP90 and cyclophilin B

secreted in the conditioned medium. These factors can be important mediators for the effects of ROS on vascular function (Liao et al., 2000).

In 2010, 2D-PAGE followed by MALDI/TOF was exploited to elucidate the role played by insulin in VSMC activation, resulting in the identification of 13 differentially expressed proteins (Wang et al., 2010b), which were mainly involved in cytoskeleton, glycometabolism, and post-translational processes. Among these proteins, OPN and matrix Gla protein were upregulated while SM-α-actin was downregulated. Despite these encouraging results, the problems associated with quantitative 2D-PAGE analysis are still numerous and especially related to the lack of reproducibility of PAGE technology and to the difficulties to properly recognize the spots as well as to normalize background and intensity. Relative quantification can be performed on proteins separated by 2D-PAGE incorporating a selective labeling approach called difference gel electrophoresis (DIGE; Unlu et al., 1997). However, the above restrictions imposed by the gel methods remain.

New techniques in comparative and quantitative proteomics for differential protein expression have been developed significantly in recent years. These include stable isotope labeling by amino acids in cell culture (SILAC; Ong et al., 2002), radio-labeled AA incorporation (Sirlin, 1958), isotope-coded affinity tags (ICAT; Gygi et al., 1999), and, more recently, isobaric tags for relative and absolute quantification (iTRAQ; Ross et al., 2004). Although very appealing, these label-based quantification approaches have important drawbacks, which comprise complex sample preparation, increased sample concentration, additional sample processing steps, and incomplete labeling. For these reasons, nonlabeled approaches have recently received considerable attention. These techniques comprise peptide match score summation (Allet et al., 2004), spectrum sampling (Liu et al., 2004), and ion intensity or peak area measurements which can be combined with statistical analysis to perform differential protein expression. Furthermore, it has been observed that electrospray ionization (ESI) as well as matrix-assisted laser desorption/ionization (MALDI) yield signal responses that linearly correlate with concentration (Chelius and Bondarenko, 2002; Hattan and Parker, 2006) when using an adequate number of shots/spot (Hattan and Parker, 2006). Label-free comparative analysis is often based on the correlation between each accurate mass measurement/corresponding retention time and signal response in one sample compared to another (Silva et al., 2005, 2006a,b). It has been demonstrated (Patel et al., 2009) that label-free techniques compared with labeled approaches are advantageous in terms of analysis time, reproducibility, experimental costs, limited number of samples, and technical replications. Moreover, label-free methods provide better information on protein coverage, single peptide identifications, and number of identified proteins.

A 4plex iTRAQ-coupled with 2D-LC/MS/MS approach was used in 2008 to investigate the proteins with differential protein expression levels

in the proteome of VSMCs incubated separately with individual enantiomers of atenolol, which is a β-selective drug. A total of 407 unique proteins were identified in three independent experiments (*S*-and *R*-enantiomer atenolol incubated and control cells). Protein profiles of cells incubated with individual enantiomers and labeled prior 2D-LC/MS/MS technique were analyzed, showing that some calcium-binding proteins were downregulated in cells incubated with *S*-enantiomer, while metabolism enzymes were upregulated according to the treatment of hypertension associated with atenolol (Sui et al., 2008). In the same year, Gao et al. used the label-free approach applied to MS analysis to quantify the changes in secreted protein abundance between control and ANG-II-stimulated VSMCs. They combined measurements of spectral counting, ion intensity, and peak area on 1D-PAGE-LC/MS/MS-based proteomics. The sensitiveness and accuracy of this algorithm were demonstrated using standard curves. Application to VSMC secretome allowed to identify 212 secreted proteins, 26 differentially expressed including the 18 proteins not previously reported (Gao et al., 2008).

Up to now, the reported differentially expressed proteins between activated and quiescent VSMCs are 95 (Table 2.2). These deserve a special attention because many of them could play important or even leading roles in the activation process. It is interesting to observe that a good percentage of them are represented by nonabundant factors with regulatory functions. As expected, many are cytoskeleton elements associated with contraction and migration, or cell cycle and differentiation regulators. Chaperones are not as numerous as we would have expected, but we believe that their role is critical especially in the earliest events of the phenotypic switch and thus their functions are regulated by posttransductional modifications more than through *ex novo* synthesis. Interestingly and somehow unexpectedly, the largest group is that represented by proteins involved in the management of the extracellular environment and in the outside-in or inside-out interactions. These are proteins that are eventually either secreted (secretome) or exposed on the cell surface and just for this reason, each of them (or others identified in future with the same characteristics) could represent putative marker of pathology easily detectable in body fluids.

3.2. Secretome analysis: A real prospective for clinical diagnosis and biomarker discovery

Proteomic analysis can be very informative and be performed on either protein extract or conditioned medium to obtain information on both intracellular proteins and secreted/released proteins. However, the primary culture of vascular cells involves changes in the phenotype, associated with the procedure of cell extraction and culture. Proteases used to dissociate VSMCs from an arterial wall, disrupt cell–cell and cell–extracellular matrix

Table 2.2 Differentially expressed proteins in VSMC activation

Differentially expressed proteins	Author
Migration	
Lysyl oxidase-like 2	Gao
Lysyl oxidase-like 3	Gao
Nestin	Sui
Osteopontin precursor	Gao
Protein S100-A4	Sui
Rho GDP dissociation inhibitor (GDI) alpha	Bian
Transforming protein RhoA precursor	Sui
Vinculin	Taurin
Cytoskeleton (contraction, cell shape)	
Beta-actin	Taurin
Alkali myosin light chain 1	Taurin
Caldesmon 1	Bian
Calmodulin	Taurin/Sui
Cell growth regulator with EF hand domain 1	Gao
Nonmuscle myosin alkali light chain	Bian
Tropomyosin	Sui
Tubulin alpha-2 chain	Sui
Vimentin	Taurin
Energy supply	
Alpha-enolase	Taurin
Aldehyde dehydrogenase	Taurin
Alpha-*N*-acetylgalactosaminidase precursor	Sui
Glyceraldehyde-3-phosphate dehydrogenase	Taurin
Similar to 6-phosphogluconolactonase	Bian
Chaperones and HSP	
47 kDa heat shock protein	Taurin
78 kDa glucose regulated protein	Taurin
Glutathione *S*-transferase P	Sui
Glutathione *S*-transferase Y-b subunit	Bian
Heat shock cognate 71 kDa protein	Taurin
Mitochondrial stress 70 protein (mortalin)	Taurin
Apoptosis and differentiation	
78 kDa glucose-regulated protein	Taurin
Calmodulin	Taurin/Sui
Clusterin precursor	Gao
Glutathione *S*-transferase P	Sui
Glutathione *S*-transferase Y-b subunit	Bian
Glyceraldehyde-3-phosphate dehydrogenase	Taurin
Inhibin beta A chain precursor	Gao

(*continued*)

Table 2.2 (*continued*)

Differentially expressed proteins	Author
Metalloproteinase inhibitor 1 precursor	Gao
Plasminogen activator inhibitor 1 precursor	Gao
Protein S100-A11	Sui/Gao
Rho GDP dissociation inhibitor (GDI) alpha	Bian
Similar to S100 calcium-binding protein A15	Bian
Transforming growth factor beta-1 precursor	Gao
Cell proliferation	
Growth-arrest-specific protein 6 precursor	Gao
Insulin-like growth factor binding protein 7	Gao
Mitochondrial stress-70 protein (mortalin)	Taurin
Nestin	Sui
Prohibitin (B-cell receptor-associated protein)	Taurin
Protein NOV homolog precursor	Gao
Similar to Mob4B protein	Bian
WAP four-disulfide core domain protein 1 precursor	Gao
14-3-3 protein gamma/delta	Sui
Extracellular matrix related, outside-in and inside-out pathways	
2;2-Macroglobulin receptor-associated protein	Taurin
47 kDa heat shock protein	Taurin
Annexin A6	Sui
Annexin I (lipocortin I)	Taurin
Cathepsin K precursor	Gao
Clusterin precursor	Gao
Collagen alpha-1(VI) chain precursor	Gao
Heat shock cognate 71 kDa protein	Taurin
Lysyl oxidase-like 2	Gao
Lysyl oxidase-like 3	Gao
Matrix Gla-protein precursor	Gao
Metalloproteinase inhibitor 1 precursor	Gao
Osteopontin precursor	Gao
Procollagen C-endopeptidase enhancer 1 precursor	Gao
Prolargin precursor	Gao
Protein CYR61 precursor	Gao
Protein NOV homolog precursor	Gao
Similar to lipoxygenase homology domains 1	Bian
Vinculin	Taurin
Housekeeping functions	
Aspartate aminotransferase, mitochondrial precursor	Sui
Cathepsin B precursor	Gao
Cathepsin K precursor	Gao
Histone H2b	Taurin

Table 2.2 (*continued*)

Differentially expressed proteins	Author
Histone H3.0	Taurin
Histone H4	Taurin
NADH-cytochrome b5 reductase	Sui
Novel serine protease-like protein	Gao
Nucleophosmin	Taurin
Ribosomal protein S12	Bian
Serine (or cysteine) peptidase inhibitor- clade C (Antithrombin)-member 1	Gao
Similar to ubiquitin C-terminal hydrolase X4	Bian
Ubiquitin-conjugating enzyme E2N (homologous to yeast UBC13)	Bian
Others	
48 kDa protein	Gao
90 kDa protein	Gao
Ab2-450	Gao
cDNA FLJ14844 fis clone PLACE1000133, human	Taurin
Haptoglobin	Bian
Hypothetical protein LOC498266	Bian
Mimecan precursor (Osteoglycin) isoform 1	Gao
Nucleobindin-1 precursor	Gao
PREDICTED: hypothetical protein XP_579818	Bian
PREDICTED: similar to Blvrb protein	Bian
PREDICTED: similar to osteoglycin precursor	Bian
Preprohaptoglobin	Bian
Spermatogenic-specific gene 1	Bian

interactions, and new and different adhesion molecules are expressed when the cells are plated in culture dishes. It is clear to everyone that when VSMCs are cultured in standard conditions with 10% FBS, they acquire a synthetic, proliferative phenotype that is different from the physiological one. This means that particular attention to the preanalytical and analytical steps is necessary, especially to explore the proteome of cultured cells, which could provide information on cell capacity so as to respond to a particular stimulus rather than to the original proteome expressed within the arterial wall.

Initiation of atherosclerosis is known to result from complex interactions of circulating factors and various cell types in the vessel wall including VSMCs. Most reviews largely discuss VSMCs in the context of late atherosclerosis when they migrate into the neointima and secrete matrix proteins to stabilize the plaque (Falk, 2006; Geng and Libby, 2002; Gronholdt et al., 1998; Lusis, 2000; Newby and Zaltsman, 1999; Owens et al., 2004).

Proximal biological fluids in contact with the plaque or with activated VSMCs may be enriched with proteins secreted or shed from cells. Such proteins could enter the circulation and be detected in body fluids such as blood. Accordingly, it is plausible to identify pathological biomarkers directly from the blood proteome, but the task is currently rather frustrating. The major technical obstacle comes from the evidence that the abundant blood proteins, such as albumin, immunoglobulin, fibrinogen, transferrin, etc., inevitably mask the less abundant proteins, which are usually potential biomarkers (Anderson, 2005, Omenn et al., 2005). Great efforts have been made (Ahmed et al., 2003, Bjorhall et al., 2005; Zolotarjova et al., 2005) to remove these abundant proteins before proteomic analysis. However, most procedures are currently far from effectiveness because of their inherent low screening efficiency associated with nonspecific binding. In this respect, the secretome of cells can be considered a fruitful pool of possible detectable biomarkers. The term "secretome" was introduced by Tjalsma et al. in a genome-wide study of the secreted proteins of *Bacillus subtilis* (Tjalsma et al., 2000) and includes proteins released by a cell, a tissue, or organism through different secretion mechanisms. These secreted proteins or their fragments always enter body fluids and can be measured by noninvasive assays, such as the ELISA test.

A comprehensive study of released markers in VSMC phenotype modulation can start from an *in vitro* analysis of cell secretion under different conditions of stimulation and activation. The first analysis of human VSMC secretome from internal mammary arteries obtained from patients undergoing coronary artery bypass was performed by Dupont et al., who identified 18 extracellular released proteins in a 2D-map (Dupont et al., 2005). After publication of this chapter, a secretome study of the differential protein expression of ANG-II stimulated VSMCs was performed by Gao et al. who detected 212 secreted proteins using a PAGE-LC/MS/MS approach (Gao et al., 2008). Of these proteins, 26 resulted to be differentially expressed after ANG-II stimulation, 22 proteins have not been previously reported, 21 proteins showed enhanced secretion by ANG-II stimulation, and 5 proteins displayed inhibited secretion. After annotation analysis, seven novel identified proteins (protein CYR61, clusterin, protein NOV, IGF-binding protein, growth differentiation factor-6, GAS-6, and twisted gastrulation homolog-1) related to cell proliferation were regulated by ANG-II. Using Gene Ontology analysis, 64 proteins were classified as extracellular matrix- and cell adhesion-related proteins and were secreted by VSMCs. Of these, periostin, collagen-1(VI), prolargin, OPN, CYR-61, TGF, and lysyl oxidase-like 2 were upregulated by ANG-II.

Investigation of the differential protein expression of VSMCs and/or entire tissues obtained from surgical explants of healthy and diseased subjects can be considered a real prospective for *in vivo* secretome analysis.

4. Posttranslational Modifications and Their Biological Functions in VSMCs

PTMs are enzymatic, covalent chemical modifications of proteins that typically occur after translation and perform crucial roles in regulating the biology of the cell, finely tuning the cellular functions of each protein. Diversification enabled by PTMs increases the protein molecular variants up to one or two orders of magnitude over the number of proteins encoded in a genome. These modifications are important because they can potentially change the protein physical or chemical properties, their activity, localization, or stability (Farley and Link, 2009). The need to understand the relationship between PTMs and functional changes has given rise to a new "omic" science, "posttranslatomics" (Seo and Lee, 2004), but identifying PTMs and characterizing their biological functions have so far appeared to be a rather ambitious task. Over 400 types of specific protein modifications have been identified, and more are likely to be identified (Farley and Link, 2009). A variety of chemical modifications have been observed in the proteins and these variations alone or in various combinations occur in time- and signal-dependent manner. Protein PTMs determine tertiary and quaternary structures and regulate activities and functions. PTMs serve as scaffolds for the assembly of multiprotein signaling complexes, such as adaptors, transcription factors, and signal pathway regulators (Table 2.3).

In VSMCs, the most studied PTM is phosphorylation, but other modifications have been considered and investigated. Protein–lipid modifications including myristoylation, palmitoylation, farnesylation, and prenylation have shown to have a role in protein–membrane interactions, protein trafficking, and enzyme activity (Linder and Deschenes, 2003). Mattingly and colleagues hypothesized that statins, which inhibit 3-hydroxy-3-methylglutaryl-CoA reductase and thus the synthesis of cholesterol which has a remarkable effect in the treatment of cardiovascular disease, prevent posttranslational prenylation, inhibiting the function of small GTPases, which are critical enzymes for VSMC phenotype modulation (Mattingly et al., 2002).

Zuckerbraun et al. investigated the inhibition mechanism of NO on VSMC proliferation. Their findings indicated that inactivation of RhoA plays a role in the NO-mediated VSMC antiproliferative activity, and that *S*-nitrosation is associated with decreased GTP binding of RhoA. Nitrosation of RhoA and other proteins is likely to contribute to cGMP-independent effects of NO (Zuckerbraun et al., 2007). Hung and colleagues applied redox proteomics to analyze the cellular mechanisms contributing to the attenuated proliferation of aortic smooth-muscle cells under the influence of oxidative stress factors such as homocysteine (Hcy; Hung et al., 2009). They also evaluated whether Salvia miltiorrhiza Bunge (Labiatae), a Chinese

Table 2.3 The most common PTMs encountered in mass spectrometry

PTM	Nominal mass shift[a] (Da)	Stability	Proposed biological function[a]	Reference (PubMed)
Phosphorylation				
pSer, pThr	+80	Very labile	Cellular signaling progress, enzyme activity, intermolecular interactions	178087
pTyr	+80	Moderately labile		
Glycosylation				
O-linked	203, >800	Moderately labile	Regulatory elements, O-GlcNAc	37345
N-linked	>800		Protein secretion, signaling	
Proteinaceus				
Ubiquitination	>1000	Stable	Protein degradation signal	7212
Sumoylation	>1000	Stable	Protein stability	1205
Nitrosative				
Nitrosylation, nSer, nCys	+29	Stable	Cell signaling	1102
Nitration, nTyr	+45	Stable	Oxidative damage	2732
Methylation	+14	Stable	Gene expression	51538
Acetylation	+42	Stable	Histone regulation, protein stability	19338
Sulfation, sTyr	+80	Very labile	Intermolecular interactions	3797
Deamidation	+1	Stable	Intermolecular interactions, sample handling artifacts	1080
Acylation				
Farnesyl	+204	Stable	Membrane tethering, intermolecular interactions, cell localization signals	2765
Myristoyl	+210	Stable		1053
Palmitoyl	+238	Moderately labile		1336
Alkylation, aCys	+57	Stable	Sample handling	51034
Oxidation, oMet	+16	Stable	Sample handling	9111
Disulfide bond	−2	Moderately labile	Protein structure and stability	7067

[a] Associated mass shifts, predicted MS stability, and proposed biological functions are included.

medicinal herb, widely used in folk medicine to treat a variety of human cardiovascular disorders, modulates the Hcy-promoted growth effect on aortic cells. They observed the inhibitory effect of the Radix Salvia miltiorrhiza aqueous extract on the Hcy-induced growth of cultured rat A10 cells. This effect is related to the PKC/p44/42-MAPK pathway, through carbonylation of three specific proteins in rat A10 cells.

Redox-dependent PTM of proteins is emerging as a key signaling system conserved through evolution, influencing many aspects of cellular homeostasis. This system exemplifies the dynamic regulation of the protein function by reversible modification of cysteine residues which, in turn, regulate many cellular processes such as proliferation, differentiation, and apoptosis. Cysteine oxidation, including disulfide bond formation, sulfenic- and sulfinic-acid formation, glutathionylation, is reversible and characterizes the cascades of protein modifications together with phosphorylation, acetylation, ubiquitination. One example of these cascades is the interplay between phosphorylation- and redox-dependent signaling at the level of phosphotyrosine phosphatase-mediated regulation of the receptor tyrosine kinases (RTKs; Chiarugi and Cirri, 2003; Van Montfort et al., 2003). Oxygen species-dependent signal transduction through reversible phosphotyrosine phosphatase inhibition represents a widespread and conserved component of the biochemical machinery triggered by RTKs. Reversible reduction can be produced by the peroxiredoxin and thioredoxin family of proteins (Woo et al., 2003; Yano et al., 2002).

4.1. Fractionation methods in posttranslational modification proteomics

PTM analyses are difficult to be accomplished for many reasons: first of all highly sensitive methods are required for detection, owing to low stoichiometry. Only 5–10% of a protein kinase substrate is phosphorylated, thus it is necessary to detect phosphorylated proteins at very low levels (<5–10 fmol). Secondly, since the covalent bond between the PTM and AA side chain is typically labile, it is often difficult to maintain the peptide in its modified state during sample preparation and subsequent ionization in MS. Thirdly, PTMs are frequently transient in dynamic homeostasis (Seo and Lee, 2004).

Classic approaches for the identification of PTMs involved Edman degradation and thin-layer chromatography (TLC). However, these methods are hampered by the requirement of significant amounts of starting material and inability to identify rare or substoichiometric PTMs. As most PTMs produce a change in the mass of the modified protein, methods able to detect changes in molecular mass, namely MS-based proteomics, are now routinely utilized to identify PTMs (Farley and Link, 2009). While the

identification of a given protein can be obtained by sequencing only a few peptides, PTM mapping requires complete coverage of the proteins.

Enrichment strategies to selectively isolate proteins or peptides with a desired PTM can be coupled with MS-based proteomics techniques. These techniques can ease the problems created by rare modifications. The different affinity enrichment strategies available can be grouped into two main categories. The former uses antibodies to recognize a specific PTM or uniquely modified peptide; for example, antiphosphotyrosine antibodies are employed to enrich peptides with phosphotyrosine residues (Zhang et al., 2005). The latter category is represented by the emerging technologies based on the chemical affinity of a modification for an immobilized resin. Such techniques include immobilized metal affinity chromatography (IMAC) for phosphorylations and lectin chromatography for glycosylations.

In recent years, many authors have used classical techniques for phosphorylation analysis such as 1D and 2D gel followed by Western Blot, but the enormous development of MS and its great sensitivity have made it the main choice in the study of phosphorylation. HPLC may be associated online with ESI-MS, or offline with MALDI. These strategies allow more complete mapping in less time and with greater efficiency. Tryptic digestion of complex mixtures can be successfully analyzed by HPLC–MS. However, the large population of unmodified peptides hides the mass spectrometric response to phosphopeptides, leading to the suppression of signals of interest (McLachlin and Chait, 2001). Suppression artifacts can be minimized by reducing the fraction of unmodified peptides relative to the PTM variants using any of a variety of enrichment strategies. One of these aimed at identifying phosphorylations consists in using strong cation exchange (SCX) resin to selectively isolate phosphopeptides. IMAC is the most widely employed method for selectively isolating phosphopeptides from complex mixtures of digested proteins. This method typically utilizes a metal chelating agent to bind trivalent metal cations, such as Fe^{3+} or Ga^{3+} (Sykora et al., 2007). The charged resin is subsequently used to bind the phosphorylated peptides. An analogous approach is the use of titanium dioxide (TiO_2) as a substitute for the metal chelating resin (Larsen et al., 2001, Thingholm et al., 2006). Studies have shown that, although these two methods use the same principle of affinity, they are complementary in that distinct phosphopeptides are detected by each of the two approaches (Cantin et al., 2007). IMAC is characterized by a higher affinity for multiply phosphorylated peptides while TiO_2 preferentially binds singly phosphorylated species (Bodenmiller et al., 2007), highlighting the utility of exploiting both approaches in a single investigation to obtain maximal coverage of the phosphoproteome. Recently, Larsen and coworkers have presented a new strategy called SIMAC (sequential elution from IMAC) for sequential separation of monophosphorylated peptides and multiply phosphorylated peptides from highly complex biological samples. The new survey approach

involves the use of various enrichment methods of mono- and polyphosphorylated peptides simultaneously (Thingholm et al., 2008).

4.2. Phosphoproteomics to disclose signaling networks in VSMC activation

Phosphorylation represents one of the most ubiquitous PTMs, and is the focus of many biochemical investigations. It has been estimated that 30% of the human proteome is phosphorylated. Protein phosphorylation signaling networks mediate cellular responses to a variety of stressors, growth factors, cytokines, and cellular interactions. Phosphorylation also influences a multitude of cellular processes such as proliferation, apoptosis, migration, transcription, and protein translation. Aberrant regulation of protein kinases and phosphatases has been involved in cancer, autoimmune diseases, metabolic disorders, and infectious diseases. Single phosphorylation events can have dramatic implications on the cellular processes (Farley and Link, 2009). Phosphorylation and dephosphorylation on S, T, Y, and H residues are the best known modifications involved in reversible activation and inactivation of enzyme activity and modulation of molecular interactions in signaling pathways (Pawson, 2002).

In VSMCs, several stimuli regulate both differentiation and activation through phosphorylation (Kawai-Kowase and Owens, 2007). Different signals triggering different pathways and signals from one cascade may inhibit a parallel cascade. IGF-1 causes the expression of genes associated with the contractile, differentiated phenotype through the PI3Kakt pathway while, at the same time, it blocks the Ras-MAPK pathway with the IPS-I/SHP2 complex. Despite the well known and important role that VSMC dedifferentiation and phenotype switching play in repairing vascular wall injury, very few factors and pathways have been identified that modulate these changes. The synthetic, activated phenotype is triggered by several growth factors, as described in the introduction. PDGF-BB is one of the few factors involved in VSMC phenotype switching by directly stimulating MAPK as well as by cleaving the IPS-I/SHP2 complex. MAPK transposition to the nucleus inhibits transcription of the genes associated with the contractile phenotype, and at the same time it stimulates the expression of the genes associated with growth (Fig. 2.6).

The same pathways involved in VSMC differentiation and/or phenotypic changes also play a major role in VSMC activation after vascular injury, triggered by growth factors, such as PDGF-BB and β-FGF. These factors stimulate the Ras-MAPK and PI3K Akt pathways that end up prompting cell proliferation, and/or the Rho kinase family pathways (Cdc42, Rac, and Rho), and thus cell migration. In this way, phenotype switching, migration, proliferation, and extracellular matrix synthesis are regulated. The cellular source of the factors that stimulate VSMCs is

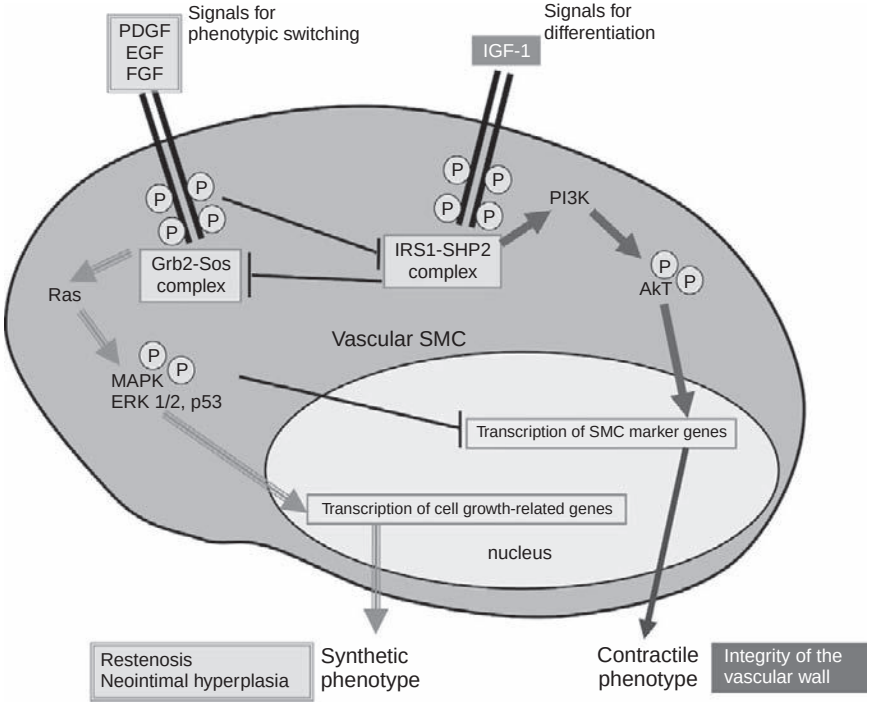

Figure 2.6 Signaling pathways involved in VSMC phenotype changes: activation (left) versus differentiation (right). IGF-1 triggers the signals for differentiation through the PI3K/Akt pathway while, at the same time, it blocks the Ras-MAPK pathway with the IPS-I/SHP2 complex. PDGF and other growth factors stimulate the synthetic, activated phenotype directly through MAPK and by cleaving the IPS-I/SHP2 complex. IGF-1: insulin-like growth factor-1; PDGF: platelet-derived growth factor; EGF: epidermal growth factor; FGF: fibroblast growth factor; PI3K: phosphoinositide 3-kinase; IRS-I: insulin receptor substrate-I; SHP2: Scr homology protein 2; MAPK: mitogen-activated protein kinase; p: phosphorylation.

not clear, as vascular wall injury can induce humoral, autocrine, and paracrine growth factors such as PDGF-BB, β-FGF, and HB-EGF from ECs, VSMCs, and invasive cells such as macrophages and platelets. Moreover, nonlaminar shear stress, that might be present after vessel injury, results in ERK1/2 activation through both PDGF-BB and interleukin-1α, leading to chemotaxis and proliferation. The MAPK/ERK cascade is the best well-known and studied pathway and it is responsible for VSMC proliferation and induction of additional growth factor secretion. To summarize, a protein-tyrosine kinase (PTK) receptor is activated by a growth factor and through adaptor proteins such as Grb2 and Shc activates the GTP-binding protein family Ras via the mammalian Son of Sevenless (mSOS; guanine nucleotide exchange factor (GEF)), and successively Raf

MAPKK/MEK and the downstream molecules p44 MAPK/p42 MAPK (ERK1/2). Phosphorylated MAPK enters the nucleus to form a complex with the transcriptional factors Elk-1 and Sap1 (an Ets family member), inducing transcription by binding to the SRE promoter of genes such as c-*fos*. This mechanism is thought to be critical in the regulation of the gene expression for proliferation, migration, differentiation, and phenotypic switching in VSMCs (Fig. 2.3). It is typical for many stimuli to activate multiple signal transduction cascades. For example, PTK receptors also activate the PI3K-Akt pathway, in addition to the MAPK pathway, when stimulated by signals that induce cell proliferation and cell survival. The phosphorylated PTK receptor activates PI3K that phosphorylates PI(3,4)P2 to PI(3,4,5)P3. The PH domain of Akt recruits PI(3,4,5)P3 on the cell membrane, and 3-phosphatidylinositol-dependent kinase (PDK) 1/2 then phosphorylates Akt on either Thr308 or Ser473. Phosphorylated Akt activates the target of rapamycin (mTOR)-raptor complex triggering cell growth, cell survival, NO production, cell proliferation, and cell cycling and delaying G1/S exit (Fig. 2.7).

4.3. Quantitative phosphoproteomics: Insight into VSMC activation and future prospectives

Despite the importance of labeled/dephosphorylation events, the fraction of phosphorylated proteins is generally very small ($<0.1\%$) *in vivo*, and the turnover of this modification is very fast. Therefore, specific techniques for the detection of phosphorylated peptides using labeled inhibitors are obligatory (Seo and Lee, 2004).

Multiple tools exist in proteomics to quantify the absolute or relative abundance of proteins and their specific PTMs (Dengjel et al., 2009; Paoletti and Washburn, 2006; Thingholm et al., 2009). *In vivo* and *in vitro* labeling methods have been developed in MS for quantifying PTMs and precisely measuring their changes (Goodlett et al., 2001; Gygi et al., 1999; Oda et al., 1999; Ong et al., 2002; Ross et al., 2004; Zhang et al., 2005). Quantification can be crucial in determining the impact of a given PTM, since simple identification of the presence of a modification may not provide sufficient biological information. A study by Mann and coworkers, performed on HeLa cells, found that 14% of the identified phosphorylation sites are modulated by epidermal growth factor stimulation, demonstrating the importance of quantifying PTMs (Farley and Link, 2009; Olsen et al., 2006). There are, however, several limitations that must be considered when using MS-based proteomics to identify PTMs. PTMs, such as phosphorylation of serine, tyrosine, and threonine, are labile, and preserving the modification during sample preparation can be difficult. If ineffective separation techniques are used, unmodified peptides or proteins can mask the modified ones during MS analysis.

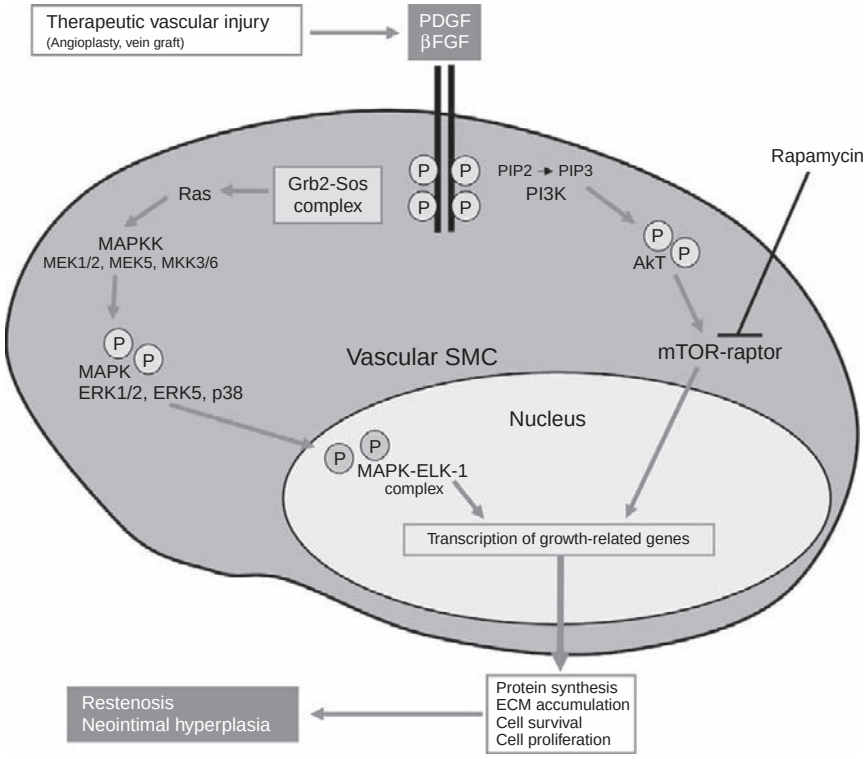

Figure 2.7 **Proliferative signaling during VSMC response to injury. The figure shows convergent signaling pathways resulting in protein synthesis and cell proliferation, thus causing restenosis and neointimal hyperplasia. PDGF-BB: platelet-derived growth factor-BB; bFGF: basic fibroblast growth factor; PI3K: phosphoinositide 3-kinase; PDK1: 3-phosphoinositide-dependent kinase 1; mTOR: mammalian target of rapamycin; MAPK: mitogen-activated protein kinase.**

In 2007, Boccardi et al. reported the first tyrosine-phosphoproteome of VSMCs. Using the classic 2D-PAGE followed by Western Blot analysis, they obtained a tyrosine-phosphoproteome map of VSMCs under physiological conditions and after growth factor activation. More than 20 proteins were found to be differentially modulated in tyrosine-phosphorylation after stimulation (Boccardi et al., 2007). Among these differentially phosphorylated proteins, we identified chaperons such as HSP60, peroxiredoxin, PDI A3, and HSP27. This last effector seems to be particularly interesting as some studies have suggested a protective role of HSP27 against atherosclerosis and transplant graft vasculopathy (Efthymiou et al., 2004; Hollander et al., 2004). In a recent work, Trott and colleagues performed 2D-DIGE showing that phosphorylated HSP27 is an important proliferation regulator in human vascular ECs and SMCs (Trott et al., 2009). More recently,

Garcia-Arguinzonis and colleagues confirmed the relation between HSP27 phosphorylation level and cardiovascular diseases. In particular, their results indicate that in VSMCs, LDL modulates HSP27 phosphorylation and its subcellular localization, affecting actin polymerization and cytoskeleton dynamics. 2D electrophoresis demonstrated that LDL modifies the proteomic profile of HSP27 (HSPB1). Western Blot analysis evidenced significant HSP27 dephosphorylation after the exposure of cells to native LDL (nLDL) and aggregated-LDL (agLDL) for 24 h (García-Arguinzonis et al., 2010). Still in 2010, Chen and colleagues investigated the signal transduction pathways of HSP27 phosphorylation in VSMCs after ANG-II and PDGF-BB stimulation (Chen et al., 2010). Evaluation of the HSP27 activity was performed by Western Blot using the specific labeled-HSP27 antibody. By means of specific protein inhibitors, they found that the P38MAPK and PI3K/Akt pathway contributes to HSP27 phosphorylation and therefore activates VSMC migration in response to Ang-II and PDGF-BB. ERK1/2 might be involved in HSP27 phosphorylation and VSMC migration provoked by Ang-II and PDGF-BB.

Similarly, Changui et al. observed the promotion of VSMC proliferation via the PI3K/Akt pathway induced by apelin-13 (apelin constitutes a novel endogenous peptide system involved in a broad range of physiological functions, such as cardiovascular function, heart development, control of fluid homeostasis, and obesity) (Changhui et al., 2010). The expression of PI3K, labeled-PI3K, labeled-Akt, ERK1/2, labeled-ERK1/2, and cyclin D1 was detected by Western Blot analysis. The results showed that apelin-13 promoted the expression of labeled-PI3K and labeled-Akt in dose- and time-dependent manner.

Several MS-based quantification methods have been implemented for phosphoproteomics, including stable-isotope labeled through chemical modification of peptides, SILAC, and label-free methods. In phosphoproteomic applications, the accuracy and reliability of the quantification approach is of key importance, in particular when individual peptide species are quantified to monitor site-specific phosphorylation changes. To minimize potential variations that can be introduced by parallel MS sample preparation, isotope encoding at an early stage, ideally already performed *in vivo* by metabolic incorporation, has considerable advantages. For metabolic isotope labeled, cells are cultured in a medium in which the natural form of an AA (typically arginine or lysine) is replaced with a stable-isotope form, so that proteins expressed by the cell incorporate the heavier version of this AA and therefore alter their molecular mass (see Ong and Mann, 2006 for a detailed, updated review of the method). This technique generally referred to as SILAC was devised by Mann and coworkers (Olsen et al., 2006; Ong and Mann, 2006; Ong et al., 2002) and can compare up to three samples in a single analysis. SILAC relies on the almost complete and specific incorporation of isotopic variants of essential AAs, which are

supplied with the cell-culture medium. To perform comprehensive phosphopeptide quantification, it is necessary for almost all peptides ultimately generated by proteolytic digestion to be labeled. Therefore, cultured cells are propagated in the presence of arginine (normal, $^{13}C_6$ or $^{13}C_6$–$^{15}N_4$-labeled) and/or lysine (normal, $^{2}H_4$ or $^{13}C_6$–$^{15}N_2$-labeled) variants for at least five cell doublings until full incorporation through protein biosynthesis has occurred (Ong and Mann, 2006).

We believe that the application of these techniques can significantly increase the detection of phosphorylation and that it should be applied to VSMCs to gain a significant increase in information concerning the modulation of phosphorylation involved in VSMC dedifferentiation. This is the first time we have applied SILAC to VSMCs, despite the difficulties in dealing with the primary cultures when using this technique, we have obtained a good incorporation rate (96.3%).

5. From Putative Markers to New Therapeutic Targets

The NIH definition for biomarker is: "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Atkinsons et al., 2001). A biomarker is a chemical, physical, or biological parameter that can be used to measure the progress of a disease or the effects of a treatment. Under a molecular point of view, biomarker is "the subset of markers that might be discovered using genomics, proteomics, or imaging technologies."

A different approach is applied to select therapeutic targets, which must be identified looking at the pathogenic mechanisms. So, while a biomarker is usually chosen considering primarily the phenotypical aspects of a pathological process, the selection of a therapeutic target concerns the mechanisms by which a pathological status is produced. Therefore, a therapeutic target does not coincide with a marker: a target is generally the cause of a pathological status, while the marker is the consequence of the target expression. Biomarkers should be exploited profitably to evaluate the significance of therapeutic targeting.

5.1. Marker validation: A decisive issue for the final acceptance of postulated mechanisms

Proteomic investigation has proved to be able to identify a large number of molecular factors putatively involved in the activation mechanisms of VSMCs. Proteomic studies will provide further insights into VSMC

phenotypic modulation focusing on the metabolic networks linking the differentially expressed proteins. The inferred role of a protein should be experimentally validated before being accepted and many different approaches can be implemented in this way. The actual role of a protein can be assessed through strong interference on its activity and absolute confidence that such interference is specifically circumscribed to the desired gene. The activity modulation can be obtained by either increasing or reducing the concentration of active species and verifying the biological, biochemical, and/or molecular responses. For instance, experiments performed transfecting specific vectors on cultured cells can provide the controlled modulation of specific proteins. Vectors expressing a protein or specific transcriptional activators may induce an overexpression of that protein, while vectors expressing antagonistic factors or specific transcriptional repressors may result in underexpression. Studies focused on the upregulation of a protein are less informative than focusing on the downregulation, since the hyperexpression of a given factor may induce global imbalance, triggering any unspecific stress responses masking the direct effect caused by the chosen protein.

Generally speaking, changes in protein concentration can be produced by: (1) specific drug administration, (2) specific antibody delivery, (3) selective gene "knock-out" (at developmental level), (4) local delivery of vectors able to express a therapeutic gene (at mature stage), (5) gene "knock-down" approaches using oligonucleotide technologies. The choice among the different protein modulators depends on three main factors: selectivity of the effects, applicability to the biological model, and availability of specific assays. Major drawbacks affecting all the described methods can be summarized as follows: (a) drug administration is frequently associated with unspecific effects (Edwards and Aronson, 2000); (b) antibodies cannot be easily delivered inside cells or tissues and may display lot-to-lot variable efficiencies (Dufour, 2004); (c) gene "knock-out" at the embryonic stage may result to be lethal or promote alternative pathways during organism development (Shlomi et al., 2005); (d) gene transfer (transient or persistent) by vectors is mainly coupled to viral vectors, characterized by low efficiency and may induce immune responses, toxicity, and, in some cases, oncogenicity (Howarth et al., 2010); (e) oligonucleotides utilized for gene "knock-down" strategies may produce unwanted off-target effects (Winkler et al., 2010). Here, we will briefly illustrate the molecular methods principally applied to cultured cells and, in particular, to VSMCs.

5.1.1. Endogenous expression of genes by engineered vectors

Genetic engineering allows the transfer of genetic information in test cells through a wide range of methods and strategies.

5.1.1.1. Transfection of exogenous expression vectors carrying the involved therapeutic genes: Target function modulation Molecular biology technologies enable the insertion of chosen genes in a variety of different expression vectors demonstrated to be successful. Commercially available plasmid vectors are commonly formed by different domains useful to carry and express exogenous genes. A large number of different investigations, mainly performed on cultured cells, have been reported. Sekiguchi et al. studied the promoter region of Smemb/NMHC-B, a nonmuscle myosin heavy chain, marker of dedifferentiated VSMC status (Sekiguchi et al., 2001). The authors were able to demonstrate the role played by the hex gene "homeodomain" in regulating Smemb/NMHC-B and to confirm *in vivo* that HEX is overexpressed in the balloon induced injury of neointima. Similarly, Hall et al. (2001) demonstrated the role of glucose metabolism by GUL-1 hyperexpression in the inhibition of apoptosis via suppression of GSK3-β kinase. Wada et al. (2002) demonstrated that the activating key role of the Calcineurin/GATA-6 pathway on the promoter of smooth-muscle myosin heavy chain (SM-MHC) in VSMCs. Ikeda et al. (2004) found that the membrane protein Vasorin can attenuate TGF-β signaling and diminish neointima formation after vascular injury. Morrow et al. (2005) could elucidate the inhibitory overexpression effects of Notch receptors (1 and 3) on important smooth-muscle markers such as α-actin, calponin, myosin, smoothelin. Also the hyperplasia suppressor gene (HSG) involved in the redifferentiation of dedifferentiated VSMCs induced a dramatic suppression of cell migration on HSG plasmid expression (Jiang et al., 2006); similarly, the hyperexpression of α-8-integrin reduces the migratory activity of dedifferentiated VSMCs, partially restoring the contractile phenotype (Zargham et al., 2007).

5.1.1.2. Persistent expression of exogenous genes by viral vector-mediated gene therapy To obtain a persistent expression of exogenous genes in target cells, viral vectors have been used extensively. They require a more expensive and laborious technique and seem less appropriate for *in vitro* studies on rapidly senescing VSMC cultured cells. On the contrary, they are more suitable tools for *in vivo* experiments. This type of technology displays several advantages over the previously described expression vectors because it allows a more controlled tissue tropism and a persistent sustained expression of the modulating agent enabling long-term investigations.

Three main tools have been developed: adenoviruses (Ad), adeno-associated viruses (AAV), and lentiviruses (mainly HIV). This issue has been recently reviewed by the Kingston group (Williams et al., 2010). Ad-vectors are the more experienced vectors historically employed in gene therapy (Volpers and Kochanek, 2004). Owing to their high infecting ability, the Ad-vectors may infect dividing and quiescent cells so that their action is to be suitably addressed to target cells by local delivery or

modifying capsid proteins with recognition elements specific for wanted cells (White et al., 2007). The proinflammatory nature of the virus confers a limited therapeutic effect as expressing cells are eliminated and viral particles are inactivated by the neutralizing antibodies (Zaiss et al., 2009).

The AAV-vectors appear to be more appealing because they are non-pathogenic and may exhibit a long-term expression since they show low inflammatory properties. The small packaging capacity (4.6 kb), however, greatly limits the kind of transgene to be inserted (Wu et al., 2009). Additionally, they present limited tropism (wild-type strain mainly infects the liver) and this fact reduces their applications (Ponnazhagan et al., 1997). Many efforts have been made to realize modified viral serotypes endowed with improved expression rate and specific cell tropism. Recently, an AAV-vector harboring a selected peptide on its capsid has been developed and proposed as vascular therapeutic tool for local gene delivery because it is able to transduce VSMCs without affecting the ECs (Lorraine et al., 2004).

Lentiviral vectors are not immunogenic and have very high packaging capacity up to 8 kb. Their potential in gene therapy lies in the ability to transduce nondividing cells, an attractive characteristic for cardiovascular disease (Williams et al., 2010). Their use is, however, hindered by the inherent oncogenic risks documented by trial reports (Hacein-Bey-Abina et al., 2003; Howe et al., 2008). A new generation of lentiviral vectors displaying reduced risks of both active virus recombination and oncogenesis is under development (Apolonia et al., 2007; Bayer et al., 2008).

5.1.2. "Gene knock-down" technology and its application to VSMCs

In cultured cell models as well as in *ex vivo* investigations, the oligonucleotide gene "knock-down" approaches seem more appropriate because they are efficient, fast, cheap, and reproducible. In addition, large population of cells can be targeted by the skilled nonviral vehicles available. Consequently, the gene "knock-down" technique may allow multiple, parallel investigations answering coordinated questions simultaneously.

There are many oligonucleotide inhibitors: antisense, siRNAs, ribozymes, DNA-decoys, aptamers; their inhibitory activity depending on rather different mechanisms. While antisense, siRNAs, and ribozymes deplete the biological function by RNA-dependent gene silencing, DNA-decoys act at the transcriptional level by distracting the transcription factors from the natural-binding site (Citti et al., 2002), and aptamers similar to antibodies act on the gene-product by inhibiting the protein activity (Keefe et al., 2010).

RNA-dependent gene silencing is the most studied and well-established approach. It works on the basis of complementary double helix interactions between inhibitor and target RNA and operates in the more accessible cytoplasmic compartment. The oligonucleotide-mRNA duplex triggers a functional block of the translation and impairs protein synthesis. To obtain

an appropriate inhibitory effect, two main conditions need to be satisfied, the target site must be accessible for binding and its sequence must not be shared with other mRNA sequences. The latter aspect is a crucial issue to be considered during oligonucleotide design because it affects target specificity and prevents any unwanted side effects (Fig. 2.8).

5.1.2.1. Applications of "knock-down" technology to VSMC studies The gene knock-down approach used to define the role of specific factors in VSMC activation pathways has been extensively applied to *in vitro* as well as *in vivo* models. In a paper appeared in 1998 (Morishita et al., 1998), a ribozyme selectively active against apolipoprotein-A (apo-A) but unable to affect the highly homologous mRNA of plasminogen was described. Because apo-A was reported to contribute to VSMC proliferation by inhibiting the plasminogen/plasmin pathway, the authors proposed ribozyme administration as therapeutic strategy for atherosclerosis alternative to

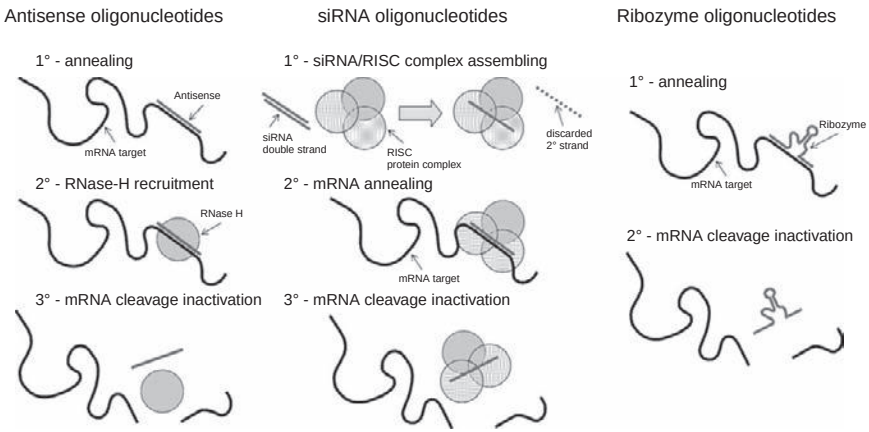

Figure 2.8 RNA-dependent gene silencing by different oligonucleotide inhibitors. *Antisense oligonucleotides* are DNA-based single-stranded molecules designed to target the mRNA to be depleted by producing a hybrid DNA/RNA duplex. This hybrid stretch is able to trigger the endogenous RNaseH ribonucleases which in turn cleave RNA moiety abolishing translation. *siRNA inhibitors* are double-stranded RNA stretches of about 20 base pairs whose activity is mediated by cell machinery globally described as RNA interference. Exogenously administrated siRNAs are incorporated into a multiprotein RISC complex which cooperates to the final effect by mediating the alignment of oligonucleotides to the target sequence. Once the target sequence has been captured, a nucleolytic moiety of the RISC complex catalyzes mRNA cleavage triggering its degradation. *Endo-nucleolytic ribozymes* are catalytic RNA sequences able to bind and cleave the sequence to be targeted. They act in a single step without the involvement of any cell machinery as they possess their own intrinsic catalytic properties. The hammerhead catalytic motif is the most studied and successfully applied ribozyme in gene "knock-down" applications.

LDL apheresis. Interestingly, the intrastent local delivery of a chimeric ribozyme addressed to proliferating cell nuclear antigen (PCNA), necessary for cell-cycle progression, reduced considerably neointima formation in a porcine restenosis model (Frimermann et al., 1999). Many other publications described the use of ribozymes to target different factors such as Bcl-2, to counteract hyperplasia and induce apoptosis of VSMCs in the neointima (Perlman et al., 2000), and TGF-β to inhibit the proliferation of VSMCs from spontaneously hypertensive rats and ANG-II-stimulated human cultures (Su et al., 2000; Teng et al., 2000). The exaggerated growth of VSMCs from spontaneous hypertensive rats was also investigated by targeting the PDGF-A. A chimeric DNA/RNA ribozyme tailored against rat PDGF-A successfully targeted VSMCs from spontaneous hypertensive rats by effectively inhibiting cell proliferation (Hu et al., 2001a). The same research team also developed the homologous human ribozyme addressed to human PDGF-A. They were able to record a significant inhibition of VSMC replication upon ribozyme administration even after stimulatory treatments with ANG-II or TGF-β (Hu et al., 2001b). The realization of ribozymes active against the cyclin-E and E2F1 transcription factors has been described. This approach concerns the proliferative signaling cascade where the final activation of transcription factor E2F1 regulates cell-cycle progression by activating the relevant cyclin-E. The activity of both ribozymes was tested and their biological activity assessed in cultured human VSMCs (Grassi et al., 2001).

More recently, researchers have focused on the use of siRNA to knock-down gene functions. A recent paper described the role of protein kinase A (PKA) and the prostacyclin receptor on VSMC differentiation in cardiovascular disease. The balance between thromboxane and prostacyclin is a recognized critical factor of vascular pathogenesis, the latter accounting for the contractile-quiescent phenotype. Signaling cascade, triggered by the prostacyclin receptor, activates PKA whose inhibition by siRNA administration opposes to maintain the quiescent phenotype (Fetalvero et al., 2006). Apoptosis is frequently triggered by ROS also in VSMCs. By analyzing cDNA expression libraries, Tchivilev et al. identified putative protective genes. Among the VSMC clones resistant to oxidative stress, they found the recurrence of protein phosphatase-1c-gamma1 (PP1Cgamma1). By using siRNA technology, they were able to demonstrate a correlation between PP1Cgamma1 levels and (a) activation of JNK1, (b) phosphorylation of p53, and (c) expression of Bax and consequent firing of the apoptosis process induced by ROS (Tchivilev et al., 2008). More recently, the role of the beta-PIX (β-PIX) function on the migration of VSMCs induced by ANG-II (Shin et al., 2009) was established by using siRNAs. β-PIX belongs to the GEFs family and is specific for activating Rac1/Cdc42 small Rho-GTPases. The knocking-down of β-PIX inhibited the ANG-II-

induced migration of rat VSMCs, so as to hypothesize which distinctive pathways are involved in this migration process.

Despite the widespread use of siRNA technology, important drawbacks, related to marker validation approaches exploiting RNA interference "knock-down," have brought in doubt the actual siRNA target specificity (Tedeschi et al., 2009). Because uncontrollable off-target consequences seem to considerably affect the results obtained with siRNAs, the use of ribozymes in marker validation studies is expected to increase in the next years.

5.2. Therapeutic targets and strategies

The development of new therapeutic approaches to vascular diseases related to the pathological activation of VSMC is an important issue. The proteomic studies and the technological opportunities described in this review can supply extensive knowledge and tools for specific experimental approaches to be performed *in vitro*, *ex vivo*, and *in vivo*. Actually, any provided representation of molecular mechanisms triggering the VSMC phenotypical switch enables to identify a series of key proteins involved in crucial steps of pathogenesis. Such proteins may be, in principle, useful targets of therapeutic action. However, as previously discussed for biomarkers, putative targets must also be experimentally validated. VSMC target selection depends on several aspects which should also be taken into account: (a) a target cannot be a vital factor for the cells because its inhibition by therapeutic agents would be lethal; (b) its function has to be specific for VSMCs in order to avoid any side effects of the therapy toward other cells and/or surrounding tissues; (c) the inhibition of the target should produce a partial or, even better, a complete reversion of the pathological phenotype; (d) therapeutic treatment should not be toxic.

To find and validate targets, a combined molecular strategy may be suggested in order to prevent time-consuming and expensive analyses. The already described "chemical omics approach" (Tedeschi et al., 2009) consists in a multistep study performed by alternating knock-down and proteomic analyses (Fig. 2.9).

As a first step, a selective gene inhibition will be obtained for each single putative crucial element of the pathological pathways. A subsequent proteomic study of knocked down cells will allow to verify the effects of single gene silencing on the phenotype, suggesting further elements involved in the process. Sequential mining of marker sets distinctive for each specific inhibition would provide a global vision on how cells respond to each single shot. This systematic study will elucidate the functional networking of the factors involved in the pathological mechanisms, and provide additional information on the possible side effects triggered by each knock-down step. A selection of the targets will then be possible according to the efficiency of inhibition combined with the least, if any, induction of side effects. Selected

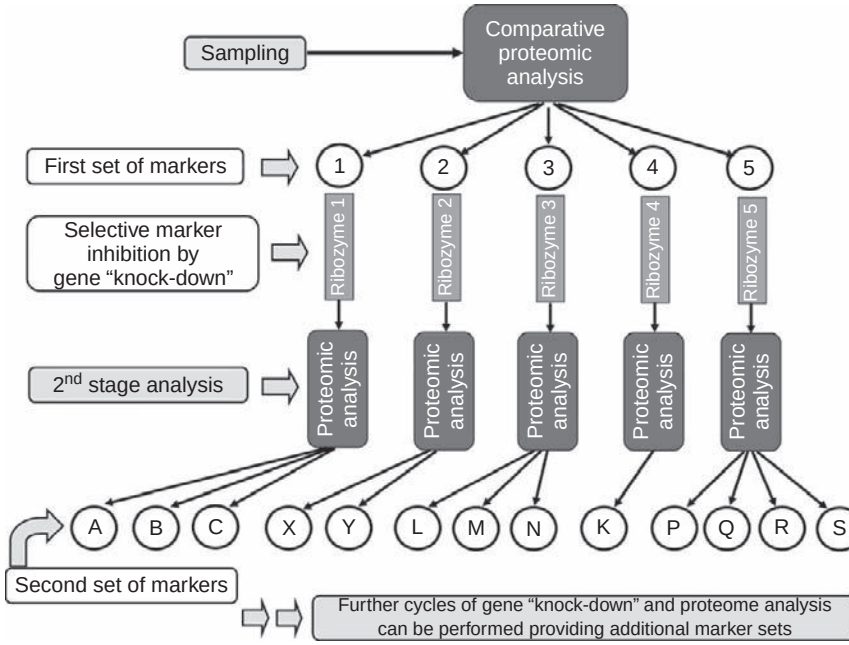

Figure 2.9 **"Chemical-omic" investigation approach. In a first step, a selective gene inhibition is performed for each single putative marker of a given pathological pathway suggested by the proteomics analyses. A subsequent proteomics study of knocked down cells will enable to verify the effects on the phenotype of single gene silencing and may highlight further elements involved in the process. The sequential mining of factors distinctive of each specific inhibition would provide a global vision on how cells respond to each single-targeted factor providing new insights on activation pathways networking.**

targets will be investigated experimentally *ex vivo* and *in vivo* to confirm their validity. We must consider that each molecular inhibitor (ribozyme, siRNA, or antibody) successfully used during target selection may represent a therapeutic agent potentially applicable to clinics. In this way, we expect the identification of a set of targets specific for early events of atherosclerosis useful for prevention, or other sets of targets related to acute, mid, and late phases of the pathology, whose inhibition would be differentially achieved after coronary bypass, surgical grafting, or percutaneous transluminal coronary angioplasty (with or without stent implantation). This approach should enable the development of therapeutic strategies utilizing molecular tools specifically designed against key elements of the activation pathways and able to revert the pathological drift.

In the past years, there has been a significant amount of literature in miRNAs and several authors have hypothesized potential therapeutic applications (Scalbert and Bril, 2008; Song and Li, 2010) for these molecules.

Specific miRNA can be modulated through genetic overexpression, exploiting synthetic miRNA mimics (Cordes and Srivastava, 2009), or silencing with, for example, antagomirs (single-stranded RNA complementary to miRNAs; Krützfeldt et al., 2005). The major advantage of miRNA application is the extreme druggability of these small molecules once the selection of miRNA candidates is clear. On the other hand, significant concerns derive from the fact that delivery of therapeutic doses of exogenous miRNA would partially saturate the RISC machinery necessary for miRNA activity and then induce a consistent imbalance among the other miRNAs endogenously expressed (uncontrolled general effects). Furthermore, each miRNA may target hundreds of genes with consequent ravaging off-target effects. In our opinion, miRNA therapeutic applications seem immature and deserve further investigations.

6. Concluding Remarks

At the end of this journey in the world of VSMC activation, the enormous amount of work accomplished on this subject in the past few years reveals the arousing interest of scientists from various fields and with different aims. The quantity of published data is amazing but, at the same time, the complexity of the signaling evidenced is frightening. For this reason, the idea of finding few key elements responsible for VSMC phenotypic modulation seems ambitious and the efforts to attain a mechanistic explanation starting from single elements or even pathways is an oversimplification. In our opinion, great help can derive from a comprehensive analysis of the biological systems, in other words from the "omics." These technologies, grown in parallel with bioinformatics, allow high-throughput analyses and will have a remarkable impact on the understanding of biological processes. Moreover, "omics" can be exploited for more accurate disease diagnosis and treatment. In particular, proteomics is likely to have a major impact on the understanding of the phenotypes of both normal and diseased cells. At present, it is also sufficiently advanced to allow the identification of less expressed factors. The novel challenge is to recognize disregulated or posttranslationally modified proteins, among which it is plausible to find putative biomarkers and/or targets. Up until now, chaperones and heat shock proteins have seemed to play a central role in the VSMC activation process, but the impression is that "hidden players" are still to be mined. In this view, the study of secreted proteins represents a powerful tool as, within the secretome, it is likely to find markers of pathology able to describe VSMCs and the state of the arterial wall. Most important elements, within the secretome, could be quickly and noninvasively validated due to their distribution in body fluids and/or plasma and,

consequently, proteomics could also become a diagnostic tool for patients and clinicians. The gene knock-down technology would exploit the data collected with the proteomic studies in order to design and develop new therapeutic strategies.

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CHAPTER THREE

Molecular Basis for Endothelial Lumen Formation and Tubulogenesis During Vasculogenesis and Angiogenic Sprouting

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Abstract

Many studies reveal a fundamental role for extracellular matrix-mediated signaling through integrins and Rho GTPases as well as matrix metalloproteinases (MMPs) in the molecular control of vascular tube morphogenesis in three-dimensional (3D) tissue environments. Recent work has defined an endothelial cell (EC) lumen signaling complex of proteins that controls these vascular morphogenic events. These findings reveal a signaling interdependence between Cdc42 and MT1-MMP to control the 3D matrix-specific process of EC tubulogenesis. The EC tube formation process results in the creation of a network of proteolytically generated vascular guidance tunnels in 3D matrices that are utilized to remodel EC-lined tubes through EC motility and could facilitate processes such as flow-induced remodeling and arteriovenous EC sorting and differentiation. Within vascular guidance tunnels, key dynamic interactions occur between ECs and pericytes to affect vessel remodeling, diameter, and vascular basement membrane matrix assembly, a fundamental process necessary for endothelial tube maturation and stabilization. Thus, the EC lumen and tube formation mechanism coordinates the concomitant establishment of a network of vascular tubes within tunnel spaces to allow for flow responsiveness, EC–mural cell interactions, and vascular extracellular matrix assembly to control the development of the functional microcirculation.

Key Words: Endothelial lumen formation, Tubulogenesis, Rho GTPases, Extracellular matrix, Collagen, MT1-MMP, Cdc42, Pericytes, Vascular basement membrane matrix assembly, Vascular guidance tunnels, Integrins.

1. Introduction

Considerable progress has been made in our understanding of molecular events underlying the development of the vasculature and how it is regulated in fetal and postnatal life (Adams and Alitalo, 2007; Carmeliet, 2005; Chappell and Bautch, 2010; Davis et al., 2007; Holderfield and Hughes, 2008; Iruela-Arispe and Davis, 2009; Lohela et al., 2009; Senger and Davis, 2010; Thomas and Augustin, 2009; Warren and Iruela-Arispe, 2010). Two major processes control vascular morphogenesis and they are: (i) vasculogenesis which represents *de novo* assembly of endothelial cells (ECs) in 3D matrices into tube networks (Figs. 3.1 and 3.2), and (ii) angiogenesis which represents EC sprouting and tube formation from preexisting vessels (Figs. 3.1 and 3.3; Adams and Alitalo, 2007; Chappell

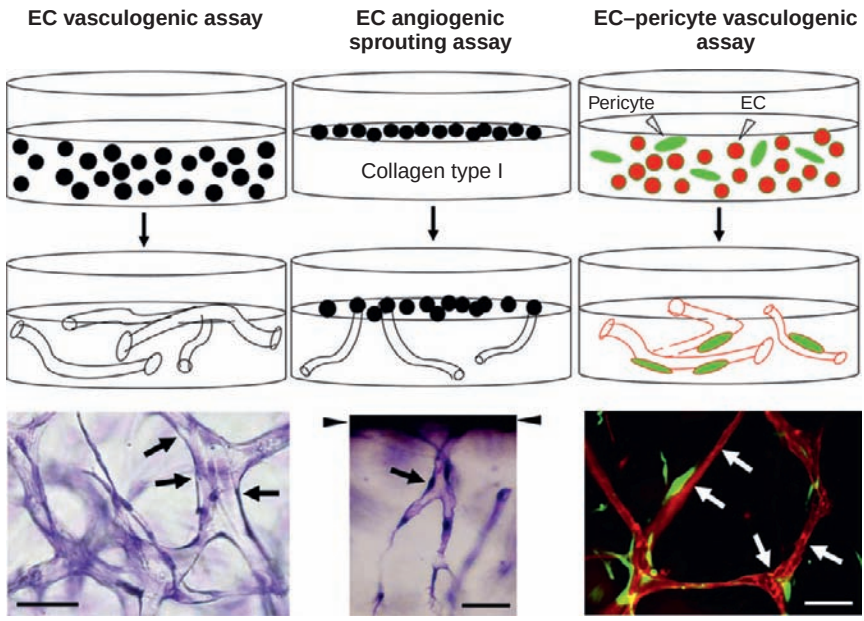

Figure 3.1 Microassay systems that model the processes of vasculogenesis, angiogenesis, and endothelial–pericyte tube coassembly events in 3D extracellular matrices. Three assay systems are illustrated that have been developed to mimic EC vasculogenic tube assembly, angiogenic sprouting from a monolayer surface and EC–pericyte tube coassembly. ECs are seeded as single cells in the EC vasculogenic assay and they assemble into multicellular tubes over time in 3D collagen matrices. Left lower panel: 72 h culture, arrows indicate an EC-lined tube. Bar equals 50 μm. ECs are seeded on a monolayer surface and EC invasion occurs in response to sphingosine-1-phosphate, stromal-derived factor-1α, or both in 3D collagen matrices. Middle lower panel: 24 h culture, a side cross-section is shown; arrowheads indicate the monolayer surface while the arrow indicates an invading EC tube. Bar equals 50 μm. ECs are seeded with GFP-labeled pericytes and are cocultured in 3D collagen matrices for various times. Right lower panel: 120 h culture, fixed cultures were stained with anti-CD31 antibodies (red) (arrows) while the pericytes carry a GFP label. Bar equals 50 μm.

and Bautch, 2010; Davis et al., 2007; Drake, 2003). The molecular control of the vasculature is affected by many factors and signals and, in this review, we will focus on the molecular basis for how EC lumens and tubes form and become stabilized in 3D extracellular matrix (ECM) environments (Davis and Senger, 2005; Davis et al., 2007; Holderfield and Hughes, 2008; Senger and Davis, 2010).

In general terms, the ECM represents a fundamental regulator of vascularization in that it presents a physical scaffold containing adhesive and growth factor modulatory signals which are required for both blood vessel morphogenesis and maturation (Arroyo and Iruela-Arispe, 2010; Davis and

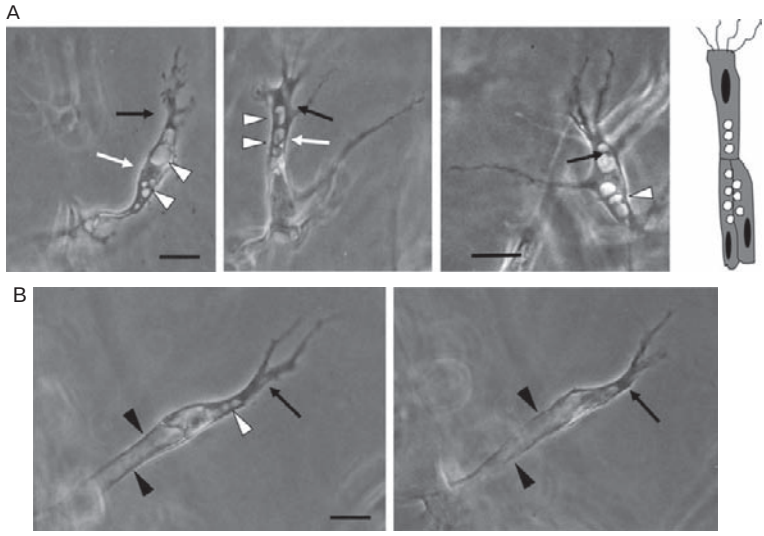

Figure 3.2 EC tip and stalk cells participate in EC lumen and tube formation and both cell types can generate intracellular vacuoles during this angiogenic sprouting process in 3D collagen matrices. ECs were seeded as confluent monolayers on top of collagen type I matrices containing sphingosine-1-phosphate to induce EC invasive responses. Imaging of the invasive front reveals both tip (black arrows) and stalk (white arrows) cells in these cultures. (A) Both tip and stalk ECs are able to vacuolate (white arrowheads). Intracellular vacuoles are observed particularly in the rear of tip cells and as other cells accumulate behind the tip cell, these cells; can also vacuolate and lumenize (schematic illustration). (B) As morphogenesis progresses, vacuole–vacuole fusion and MT1-MMP-dependent lumen expansion events occur concurrently to create lumen spaces (black arrowheads). White arrowhead: intracellular vacuoles; black arrow: EC tip cell. Bars equal 50 μm.

Senger, 2005, 2008; Germain et al., 2010; Hynes, 2007; Rhodes and Simons, 2007; Senger and Davis, 2010). ECM can present either stimulatory or inhibitory signals (Davis and Senger, 2005) and thus the ECM composition, the vascular cell types, and the biological context of the signaling control the nature of the cellular response. An important regulator of ECM structure and function are matrix metalloproteinases (MMPs) which can degrade matrix components (Baker et al., 2002; Davis and Saunders, 2006; Davis and Senger, 2008; Gill and Parks, 2008; Handsley and Edwards, 2005), and liberate factors such as cytokines, biologically active lipids, or peptides from these matrices to affect vascular cell behavior. Interestingly, MMPs have been reported to control both vascular morphogenesis and regression events (Davis and Saunders, 2006; Davis and Senger, 2008).

Within the vascular wall, heterotypic interactions of ECs and mural cells affect ECM production and deposition (Stratman et al., 2009a, 2010) as well as its ability to be degraded by MMPs (Saunders et al., 2006). Many new studies are focused on how mural cells affect EC behavior during

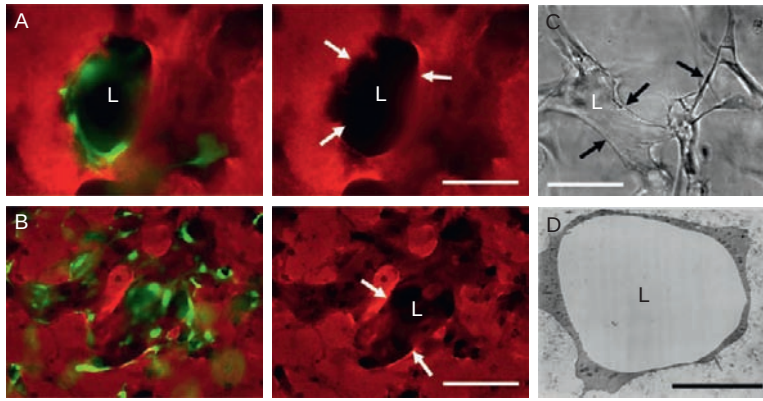

Figure 3.3 ECs concurrently generate lumens and tube networks as well as vascular guidance tunnels during vascular morphogenic events in 3D collagen matrices. (A, B) GFP-ECs were seeded within collagen matrices and allowed to form lumens and tube networks. Cultures were fixed at 72 h and immunostained using an anti-collagen type I monoclonal antibody that selectively recognizes native type I collagen. Representative fluorescent images are shown which illustrate that ECs undergo tube morphogenesis and create vascular guidance tunnels. Left panels:image overlays of GFP-ECs with the red-staining collagen type I. Middle panels: red staining collagen type I only. L indicates lumen. White arrows indicate the borders of the vascular guidance tunnels. Bar equals 50 μm (A) and 100 μm (B). (C) Representative light microscopic image of EC lumen and tubes in 3D collagen matrices. Arrows indicate the border of formed tube structure. Bar equals 50 μm. (D) Electron microscopic image of an EC lumen and tube. L indicates lumen. Bar equals 25 μm.

development and in various disease states such as cancer and diabetes (Armulik et al., 2005; Gaengel et al., 2009; Greenberg et al., 2008; Hughes, 2008; Saunders et al., 2006; Stratman et al., 2009a, 2010). In this review, we will discuss past and present work that addresses how vascular lumen formation is controlled at a molecular level and how this process affects later steps such as tube remodeling and maintenance, tube stabilization, and tube regression events to affect tissue vascularization responses.

2. Overview of Endothelial Lumen Formation and Tubulogenesis

2.1. Role of extracellular matrices and 3D matrix environments in endothelial lumen formation

ECM is a critical regulator of vascular tube morphogenesis that serves as a signaling scaffold to affect survival, proliferation, motility, invasion, and morphogenic events for ECs and mural cells, including pericytes and

vascular smooth muscle cells (Arroyo and Iruela-Arispe, 2010; Davis and Senger, 2005, 2008; Germain et al., 2010; Hynes, 2007; Rhodes and Simons, 2007; Senger and Davis, 2010). Different types of ECM have unique effects (i.e., basement membrane matrices vs. interstitial matrices vs. provisional matrices) depending on the biologic context with evidence for promorphogenic, prostabilization, and proregressive activities (Davis and Senger, 2005, 2008; Senger and Davis, 2010). Certain ECM environments such as vascular basement membrane matrices appear to present quiescence signals to vascular cells to facilitate vascular stabilization. In contrast, ECM alterations through proteolysis or conformational changes (i.e., which exposes matricryptic sites) can activate vascular cells. Thus, ECM is a critical regulator of how cells within the vascular wall sense an injurious stimulus (Davis, 2009; Davis et al., 2000) and then acutely respond by affecting processes such as vascular permeability, inflammation, angiogenesis, and thrombosis (Arroyo and Iruela-Arispe, 2010; Davis and Senger, 2005; Senger and Davis, 2010). The ECM is also a critical regulator of chronic EC responses to long-standing injurious stimuli such as that observed in diseases such as diabetes, atherosclerosis, and hypertension. In all of these latter cases, considerable ECM remodeling occurs that affects vascular cell function and behavior (Greene et al., 1989; Hill et al., 2007; Schiffrin, 2004). The transition from quiescent ECs to activated ECs (which then undergo morphogenesis) has been functionally linked to degradation of the vascular basement membrane matrix to induce angiogenesis in response to a variety of stimuli. In this instance, the signaling events are likely to be complex with loss of inhibitory signals, gain of activating signals, or both to induce ECs to undergo sprouting and tube morphogenesis.

The ECM scaffold not only possesses adhesive signals for cells by binding to cell surface adhesion receptors but it also presents specific growth factors to cells (Davis and Senger, 2005; Hynes, 2009; Somanath et al., 2009). A critical point is that different growth factors show affinity for unique ECM proteins present in the basement membrane matrix, interstitial matrix, or elastin-rich microfibrillar matrix (Hynes, 2009; Ramirez and Dietz, 2009; Senger and Davis, 2010). Furthermore, the ECM modulates the activation of specific growth factors and, thus, can modulate growth factor action to control vascular development or pathologic neovascularization (Hynes, 2009). Also, co-signaling between integrins and growth factor receptors are known to control vascularization responses during development and postnatal life (Hynes, 2009; Senger and Davis, 2010; Somanath et al., 2009). Interestingly, modulation of ECM through proteolysis or application of cellular mechanical force to ECM, which regulates matrix assembly and remodeling, may control the organization of growth factor and ECM interactions which then affect vascular signaling under normal or diseased conditions.

Particular ECM components are potent stimulators of vascular tube morphogenesis while others appear to be inhibitory. Collagen type I, the

most abundant ECM component particularly in adult animals, and fibrin are potent promorphogenic stimuli for vascular tube morphogenesis in 3D matrices (Figs. 3.1–3.3; Aplin et al., 2008; Bayless et al., 2000, 2009; Davis and Camarillo, 1996; Koh et al., 2008b; Nakatsu and Hughes, 2008; Whelan and Senger, 2003). The collagen-binding integrin, $\alpha 2\beta 1$ has been shown to control EC tube morphogenic events *in vitro* and *in vivo* in collagenous matrices (Bayless and Davis, 2003; Davis and Camarillo, 1996; Davis and Senger, 2005; Senger et al., 1997), while the fibrin/fibronectin-binding integrins, $\alpha 5\beta 1$ and $\alpha v\beta 3$, have been shown to control tube morphogenesis in fibrin matrices (Bayless and Davis, 2003; Bayless et al., 2000). Due to the strong promorphogenic influence of collagen and fibrin matrices, these have been primarily used to establish EC tube morphogenic models in 3D ECM environments (Figs. 3.1–3.3) (Aplin et al., 2008; Bayless et al., 2009; Koh et al., 2008b; Nakatsu and Hughes, 2008). Such models have strongly enhanced our knowledge concerning the molecular basis for EC tubulogenesis, sprouting, and tube maturation events. Overall, the ECM and integrin data demonstrate that vascular tube morphogenesis is directly connected to integrin-mediated recognition of these promorphogenic ECM components in 3D matrices.

2.2. Relevance of lumen formation mechanisms to vascular morphogenesis in development and disease

It is clear that the mechanisms underlying EC lumen and tube formation are necessary in order for EC networks to function as conduits to propel blood flow and to perform as exchangers for oxygen, carbon dioxide, nitric oxide, and nutrients (Fig. 3.4). This fundamental function of EC tubes is required for a closed circulatory system connected to a heart pump (Wagenseil and Mecham, 2009). Lumen formation mechanisms are also critical for the development of the lymphatic vasculature, although our understanding of these events is less developed (Adams and Alitalo, 2007). Also, the connection of blood EC vascular tubes with a pressurized and high flow system suggests that lumen remodeling events can occur when such flow responses are initiated or changed (Iruela-Arispe and Davis, 2009). This is a property of blood EC tubes that distinguishes it from other types of biological tubes such as epithelial tubes or less pressurized lymphatic EC tubes (Iruela-Arispe and Davis, 2009).

Recent work concerning the tumor vasculature is illustrative of how lumen formation mechanisms play a fundamental role in tissue perfusion that affects tumor cell growth and progression. The tumor cell vasculature is known to be abnormal and a functional microcirculation is absent within this microenvironment. Blood flow is abnormal, tissue interstitial pressure is elevated, and there is a fundamental lack of appropriate vascular remodeling events necessary to create a functional microcirculatory circuit (Jain, 2005).

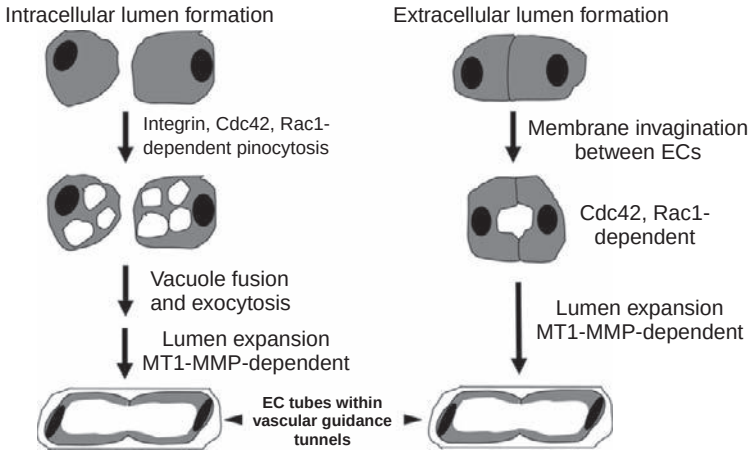

Figure 3.4 Mechanisms controlling EC lumen formation in 3D extracellular matrices. A diagrammatic representation of two mechanisms by which ECs can form lumens and tube structures *in vitro* and *in vivo* is shown. Intracellular lumen formation is characterized by a Cdc42/Rac1 and integrin-dependent pinocytic process that generates intracellular vacuoles which fuse together to form an intracellular lumen compartment which eventually and exocytoses with the plasma membrane so that multicellular lumen formation can occur. Lumen expansion occurs through an MT1-MMP-dependent event which also facilitates vacuole fusion events to promote lumen development and expansion. Extracellular lumen formation is instead characterized by membrane invagination events between adjacent ECs (in a manner still dependent on Cdc42 and Rac1) followed by lumen expansion events in an MT1-MMP-dependent manner. The membrane invagination events of both processes (intracellular vs. extracellular) and the molecular requirements appear also to be very similar. In one case, the invaginated membrane becomes internalized (to form the intracellular vacuole) while the other does not, which may be regulated by the fact that in the former case, the ECs are completely surrounded by ECM, while in the latter case, one face of the EC is in contact with an adjacent EC.

Interestingly, tumor cells tend to overproduce vascular endothelial growth factor (VEGF) which leads to increased expression of delta-like-4 (DLL4), a Notch inhibitor, on tumor ECs (Noguera-Troise et al., 2006). DLL4 has recently been found to control EC sprouting through its expression on EC tip cells (i.e., invasive cells at the leading front of sprouts) (Gerhardt et al., 2003; Gridley, 2010; Hellstrom et al., 2007; Holderfield and Hughes, 2008; Sainson et al., 2005; Figs. 3.1 and 3.2). When DLL4 is inhibited within the tumor microenvironment, there is a marked increase in angiogenic sprouting (Noguera-Troise et al., 2006). However, these treated tumors are

smaller despite the increase in vascularity (Noguera-Troise et al., 2006; Thurston et al., 2007). Thus, although sprouting is markedly increased, the tumor is very hypoxic and there appear to be few functional EC lumen and tube networks within these tumors (Noguera-Troise et al., 2006). This work suggests that inhibition of this molecule within the tumor microenvironment creates a functional disconnect or discoordination of EC tip cell formation from EC lumen formation mechanisms (Benedito et al., 2008; Hellstrom et al., 2007; Noguera-Troise et al., 2006; Thurston et al., 2007), suggesting that the signaling stimuli for these cellular events are distinct. Thus, the lack of a functional microcirculation within tumors is responsible in part for the inability of anti-tumor drugs to penetrate effectively into this microenvironment (Jain, 2005). These findings have lead to the concept of normalization of the tumor vasculature (Jain, 2005) which could increase tumor perfusion and the effectiveness of tumor treatment regimens. A very recent study demonstrates that modest inhibition of GSK-3β leads to increased vessel normalization by increasing microtubule stability within EC-lined tubes (Hoang et al., 2010c). Similarly, the same group demonstrated that delivery of either activated Cdc42 or separately, Rac1, leads to normalization of vessels in the face of chronic VEGF delivery within tissues (Hoang et al., 2010a,b), a significant problem within the tumor microenvironment. Thus, one strategy to normalize these abnormal tube networks is to modulate Rho GTPases by affecting the microtubule and actin cytoskeletons (Hoang et al., 2010a,b,c). Overall, the organization of the vasculature is fundamentally characterized as a connected series of vascular tubes whose establishment and maintenance depends on a variety of factors and mechanisms. These mechanisms include tube formation, flow responsiveness, and tube stabilization that involve homotypic EC–EC adhesion signaling, heterotypic EC–mural cell interactions, and EC interactions with vascular basement membrane matrices (Davis et al., 2007; Dejana et al., 2009; Iruela-Arispe and Davis, 2009; Lampugnani and Dejana, 2007; Mazzone et al., 2009; Senger and Davis, 2010; Wagenseil and Mecham, 2009; Warren and Iruela-Arispe, 2010). Thus, an understanding of the molecular mechanisms underlying these separate processes is critical to be able to appropriately manipulate vessels in a positive or negative direction for therapeutic applications in human disease.

2.3. Different model systems and species for investigating endothelial lumen and tube formation

A major effort of our laboratory as well as others has been to elucidate the molecule and signaling requirements for human ECs to form vascular tube networks in 3D matrices (which mimics vasculogenic tube assembly) (Davis and Senger, 2005; Davis et al., 2007; Holderfield and Hughes, 2008; Iruela-Arispe and Davis, 2009). Similarly, we have performed assays using

ECs attached to a monolayer surface which then sprout to form tubes in 3D matrices (Bayless and Davis, 2003; Koh et al., 2008b; Figs. 3.1–3.3). Other laboratories have developed related systems which complement those developed by our group (Alajati et al., 2008; Aplin et al., 2008; Bayless et al., 2009; Korff and Augustin, 1998; Nakatsu and Hughes, 2008; Whelan and Senger, 2003). In addition, a number of laboratories have developed EC–pericyte or EC–fibroblast coculture systems that mimic EC–mural cell interactions that control tube assembly, remodeling, and stabilization (Aplin et al., 2008; Bryan and D'Amore, 2008; Howson et al., 2005; Korff et al., 2001; Liu et al., 2009; Nakatsu and Hughes, 2008; Stratman et al., 2009a, 2010). Overall, these 3D matrix microassay systems have allowed for detailed analyses of these events.

Considerable advances in our understanding of the molecular basis for EC lumen and tube formation have been obtained using *in vitro* models of this process with human ECs in 3D matrices (Davis et al., 2007; Egginton and Gerritsen, 2003; Holderfield and Hughes, 2008; Iruela-Arispe and Davis, 2009). Also, work using a variety of *in vivo* models including Zebrafish, mouse, and quail has lead to important advances delineating these events as well (Drake et al., 1992; Egginton and Gerritsen, 2003; Iruela-Arispe and Davis, 2009; Kamei et al., 2006; Rupp et al., 2003; Sato et al., 2010; Strilic et al., 2009; Wang et al., 2010; Zovein et al., 2010). Lumen formation mechanisms have been investigated using 3D matrix models since EC lumen formation and tubulogenesis is a 3D matrix-specific process that does not occur on 2D matrix surfaces. Important advances have been made in the technologies necessary to approach such questions in terms of manipulating gene expression by transduction with viral vectors, suppression of gene expression using siRNA technology or morpholino oligonucleotides, performing signal transduction experiments in 3D matrix environments with primary human ECs, and performing time-lapse light and fluorescence microscopy to characterize the dynamic cellular morphologic changes that are necessary to form tube networks (Davis et al., 2007; Filla et al., 2004; Herbert et al., 2009; Iruela-Arispe and Davis, 2009; Kamei et al., 2006; Koh et al., 2008a, 2009; Larina et al., 2009; Lee et al., 2009; Lucitti et al., 2007; McKinney and Weinstein, 2008; Sacharidou et al., 2010; Saunders et al., 2006; Senger and Davis, 2010; Stratman et al., 2009a; Yaniv et al., 2006; Zamir et al., 2006). In addition, broad screening strategies using DNA microarray analyses and proteomic analyses as well as chemical modulatory screens are being used to understand vascular morphogenesis (Bell et al., 2001; Del Toro et al., 2010; Kalen et al., 2009; Lilly and Kennard, 2009; Sacharidou et al., 2010; Strasser et al., 2010; Su et al., 2008, 2010).

It is clear that these developing technologies have lead to rapid advances in our understanding of EC lumen formation and tubulogenesis in 3D matrix environments. This work led to the primary discovery that Cdc42

controls lumen formation which was first demonstrated in ECs (Bayless and Davis, 2002) (Fig. 3.5). In addition, Rac1, integrins such as α2β1 (in collagen matrices), α5β1 and αvβ3 (in fibrin matrices), MT1-MMP, Pak2, Pak4, Par3, Par6b, Jam-B, Jam-C, VE-cadherin, cerebral cavernous malformation protein 1 (CCM1), CCM2, atypical PKC (protein kinase C), PKCε, Src family kinases, B-Raf, C-Raf, and Erk kinases have all been shown to be required for EC lumen formation and tubulogenesis (Bayless and Davis, 2002; Kiosses et al., 2002; Koh et al., 2008a, 2009; Lampugnani et al., 2010; Sacharidou et al., 2010; Saunders et al., 2006; Stratman et al., 2009b; Whitehead et al., 2009; Yang et al., 1999) (Fig. 3.6). Of great interest is

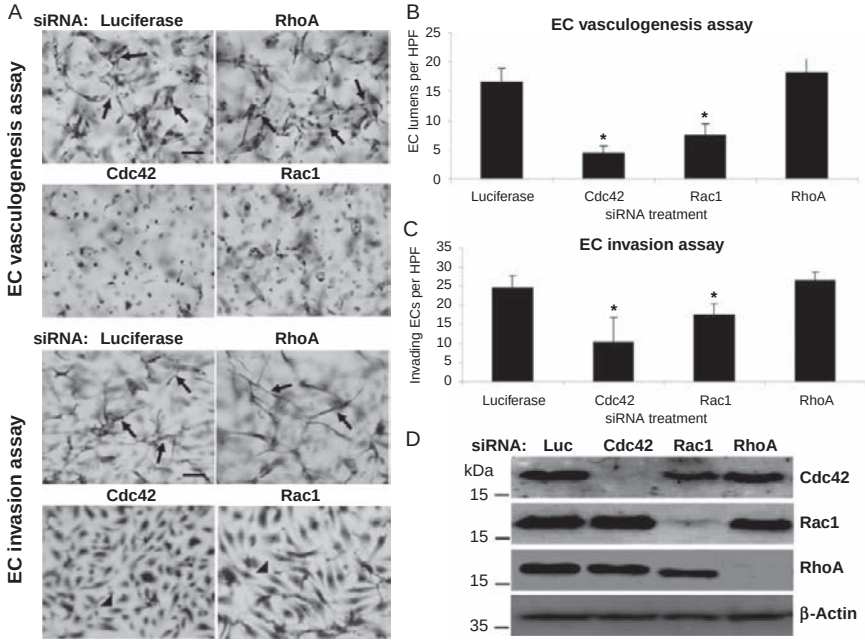

Figure 3.5 The Rho GTPases, Cdc42 and Rac-1, are required for EC lumen and tube formation and invasion in 3D collagen matrices. (A) Representative images from 3D cultures of ECs seeded within collagen type I matrices (EC vasculogenic assay) or seeded onto collagen matrices (EC invasion assay) are shown. ECs were transfected with the indicated siRNAs prior to setting up the assay. Cultures were fixed, stained, and photographed after 24 h. Arrows indicate EC tubular networks with a lumenal structure in the EC vasculogenic assay or indicate invading EC sprouts in the EC invasion assay. Arrowheads indicate ECs on the monolayer surface. Bars equal 50 μm. (B, C) Cultures shown in (A) were quantified for total lumens per HPF for the vasculogenic assay (B) and for EC sprouting (C). The data are shown as the mean values ± S.D., $p < 0.01$, $n \geq 10$ fields per condition from triplicate wells. (D) EC lysates were prepared from the indicated siRNA transfected ECs and Western blot analysis was performed to assess for the expression levels of Cdc42, Rac1, RhoA, and β-actin (loading control).

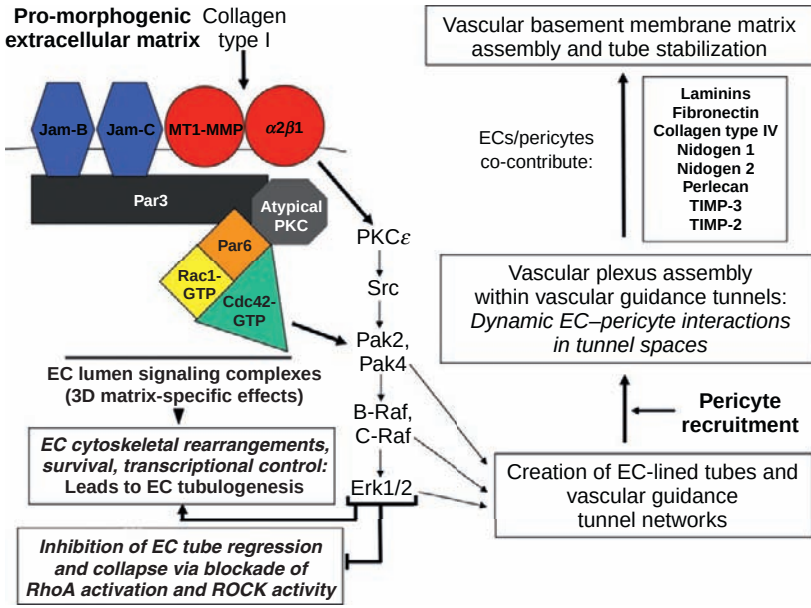

Figure 3.6 EC lumen signaling complexes coordinate lumen and tube formation in 3D extracellular matrices. A schematic diagram illustrates that ECs contain a complex of proteins termed lumen signaling complexes that stimulate the process of EC lumen and tube formation in 3D matrices. These complexes activate PKC, Src, Pak, Raf, and Erk dependent kinase cascade that regulates this process and controls EC cytoskeletal rearrangements, survival, and transcriptional controls necessary for tube formation. In addition, Erk and other upstream kinases block Rho/ROCK signaling which facilitate tube formation and decrease tube collapse mechanisms. The lumen and tube formation process leads to the formation of a network of vascular guidance tunnels which are utilized for EC tube remodeling and pericyte recruitment and where dynamic EC and pericyte interactions occur through motility events to control continuous vascular basement membrane matrix assembly. Both ECs and pericytes co-contribute basement membrane components which are necessary for this ECM remodeling process that controls vascular tube stabilization.

recent work that demonstrates the presence of an EC lumen signaling complex of proteins that are physically connected to control EC tubulogenic events in 3D matrices (Blobel, 2010; Sacharidou et al., 2010) (Fig. 3.6). These studies are examples of how rapidly the field of lumen formation in ECs has advanced over the last decade and this has been largely due to experiments performed using *in vitro* systems. It should also be pointed out that many new gene targets that control lumen formation such as CCM2 and CCM1 (and which are mutated in human diseases) have been demonstrated to play a role in lumen formation and vascular development (Gore et al., 2008; Kleaveland et al., 2009; Lampugnani et al., 2010; Liu et al., 2010; Whitehead et al., 2009). Knockouts of these genes in

mice or morpholino knockdown of genes in Zebrafish lead to lumen defects in developing vessels for both CCM2 and CCM1. Importantly, both have been demonstrated to be involved in human EC lumen formation in 3D collagen matrices further demonstrating the powerful technology that these assay systems bring (Lampugnani et al., 2010; Whitehead et al., 2009). Similarly, recent experiments in mice and Zebrafish also confirm the role of Pak2, Pak4, and Par3 (Galan Moya et al., 2009; Liu et al., 2007; Tian et al., 2009; Zovein et al., 2010), the latter two were first demonstrated using *in vitro* models in 3D collagen matrices (Koh et al., 2008a). p21-activated Kinase-2 (Pak2) was also demonstrated to play a critical role during these processes *in vitro* (Koh et al., 2008a). Thus, it is becoming increasingly clear that particular *in vitro* models are very accurately predicting the role of genes that control EC lumenogenesis *in vivo*. This was also the case with respect to the functional role of Notch signaling and EC sprouting and tip cell formation which was elegantly shown first using human ECs and an *in vitro* fibrin morphogenesis system (Sainson et al., 2005). Inhibition of Notch led to increased sprouting with marked increases in tip cell formation and increased EC proliferation (Sainson et al., 2005), which are also observed *in vivo* when Notch is inhibited (Leslie et al., 2007; Siekmann and Lawson, 2007; Uyttendaele et al., 1996). Later these studies were extended primarily using the *in vivo* postnatal retinal sprouting model or Zebrafish intersegmental vessels where DLL-4 was shown to be produced by tip cells and inhibit stalk cells through Notch signaling which trail the invading sprout (Hellstrom et al., 2007; Noguera-Troise et al., 2006; Siekmann and Lawson, 2007). Importantly, many of the fundamental concepts in these later studies were described first using the fibrin *in vitro* model system (Sainson et al., 2005). As more quality *in vitro* and *in vivo* systems are merged to investigate molecular mechanisms underlying vascular morphogenesis, the faster the advances in the field will proceed.

2.4. Relationships of EC lumen formation to epithelial lumen formation

It is apparent that both ECs and epithelial cells are able to make cell-lined tube networks, but these tubes are functionally very distinct. Both sets of tube networks serve as conduits for transport of materials, but interestingly, epithelial cells and their tubes are by their nature either secretory or absorptive and thus have very specialized apical and basolateral protein targeting mechanisms to control these events (Bryant and Mostov, 2008; Bryant et al., 2010; Hao et al., 2010; Jaffe et al., 2008; Lubarsky and Krasnow, 2003; Madrid et al., 2010; Martin-Belmonte et al., 2007). Epithelial cell–cell junctions are unique and are more specialized than typical EC–EC junctional contacts (Dejana et al., 2009; Iruela-Arispe and Davis, 2009; Lampugnani and Dejana, 2007). In general, the membrane contact

area for epithelial cell junctions is more extensive than for EC junctions which most likely plays a role in specializing these membranes for the apical–basolateral sorting that is required for selective secretion and absorptive functions (Bryant and Mostov, 2008; Bryant et al., 2010; Gassama-Diagne et al., 2006). Another critical difference is the fact that blood EC tubes are exposed to significant flow and blood pressure forces (Culver and Dickinson, 2010; Iruela-Arispe and Davis, 2009; Lucitti et al., 2007; Segal, 2005; Wagenseil and Mecham, 2009). Lymphatic EC tubes are exposed to lymph fluid and represent a lower pressure circuit. EC tubes are primarily conduits for plasma, blood cells, or lymph and are not typically secretory in nature and, thus, in combination with their exposure to high flow and blood pressure (i.e., in blood EC-lined tubes), suggests very different functions as it relates to their junctions as well as apical–basolateral polarity mechanisms. Another interesting difference is the presence of mural cells on the basal surface of most EC-lined tubes along with vascular basement membranes particularly in blood EC-lined tubes. The presence of mural cells as well as the vascular basement membrane matrix may be the defining feature that characterizes the basal versus apical surface of ECs (Stratman et al., 2009a, 2010). We have recently shown that pericyte recruitment to EC-lined tubes stimulates vascular basement membrane matrix assembly selectively on the basal surface, a surface that pericytes also occupy (Stratman et al., 2009a, 2010) (Figs. 3.1, 3.7, and 3.8). In addition, pericytes migrate along this surface during basement membrane assembly events (Stratman et al., 2009a). Thus, the EC apical and basal surfaces are distinct and can be easily distinguished during *in vitro* tubulogenesis when ECs and pericytes are cocultured in 3D matrices (Figs. 3.1, 3.7, and 3.8). These surfaces are also readily identifiable by the presence of ECM on the basal surface as well as a fluid compartment exposed to the apical surface (Figs. 3.1, 3.3, and 3.7). Thus, the lumen formation process rapidly creates such polarized surfaces in 3D matrices using the EC lumen signaling complexes and kinase signaling cascades mentioned above (Fig. 3.6).

There is considerable evidence that epithelial lumen formation involves apical targeting of proteins such as Cdc42, Par3, annexin II, PIP3, and PTEN (Bryant and Mostov, 2008; Bryant et al., 2010). In addition, there is apical targeting of membrane to expand the lumenal surface during tubulogenic events. More recent studies have identified a complex of proteins involving Rab11 and Rab8 which facilitate the targeting of vesicles to this developing apical surface in a Cdc42-dependent process (Bryant et al., 2010). Recent studies have also identified that two Cdc42 guanine exchange factors (GEFs), Tuba and Intersectin 2, play a role in formation of epithelial cell lumens and proper positioning of mitotic spindles in 3D matrices (Hao et al., 2010; Qin et al., 2010; Rodriguez-Fraticelli et al., 2010).

One question is how related EC lumen formation is with respect to these processes in epithelial cells. We have shown a critical functional role for Cdc42 in EC lumen formation as well as its effectors, Pak2, Pak4, and Par6b

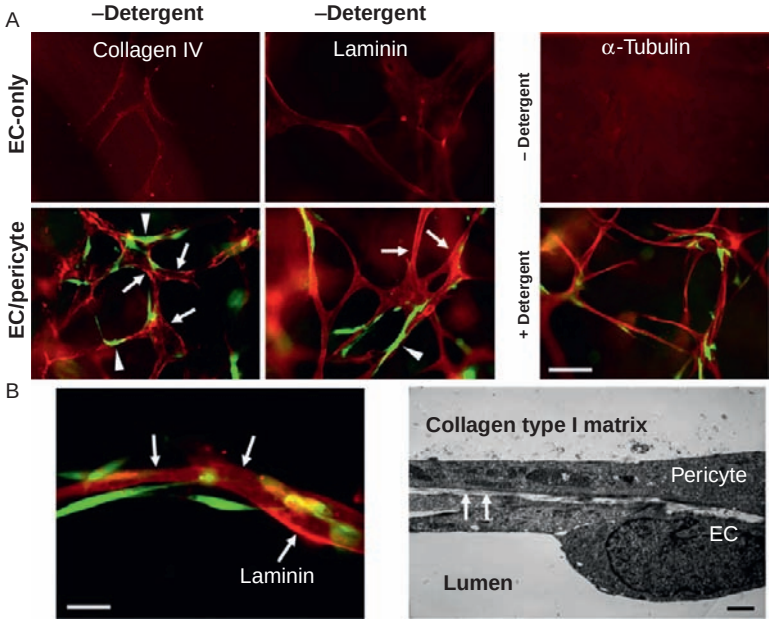

Figure 3.7 Pericyte recruitment to EC-lined tubes markedly stimulates vascular basement membrane matrix assembly and maintenance. EC-only versus EC–pericyte cocultures were established for 5 days in collagen type I matrices. Cultures were then fixed and immunostained using detergent-free conditions to recognize only extracellularly deposited proteins. (A) Detergent-free immunostaining of EC-only cultures reveals minimal deposition of extracellular matrix proteins (top); however, EC–pericyte cocultures show extensive deposition of both collagen IV (middle) and laminin (middle, bottom) using identical methodologies. Overlay images of the individual basement membrane proteins (red, indicated by white arrows) and GFP pericytes are shown (white arrowheads). Tubulin was only detected in cultures that were permeabilized with detergent (middle) and was not detected without detergent. Bar equals 50 μm. (B, left) High powered imaging of extracellular laminin deposition (red, white arrows) with the associated GFP pericyte overlay image. Bar equals 15 μm. (Right) Electron micrograph of 5-day EC–pericyte cocultures reveals the deposition and formation of a basement membrane between the two cell types (white arrows). Bar equals 0.5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this chapter.)

(Koh et al., 2008a; Sacharidou et al., 2010). Ongoing studies are focused on identifying the relevant Cdc42 GEFs that are involved in the controlling EC lumen and tube formation (Sacharidou and Davis, in preparation), but thus far these appear to be distinct from those utilized by epithelial cells. Previously, we implicated FGD4 (a Cdc42-selective GEF) (Ono et al., 2000) in this process due to the fact that an FGD4 siRNA disrupted lumen formation in a manner similar to siRNA suppression of Cdc42 (Davis et al., 2007). Finally, we have shown that Cdc42 targets to membrane surfaces including

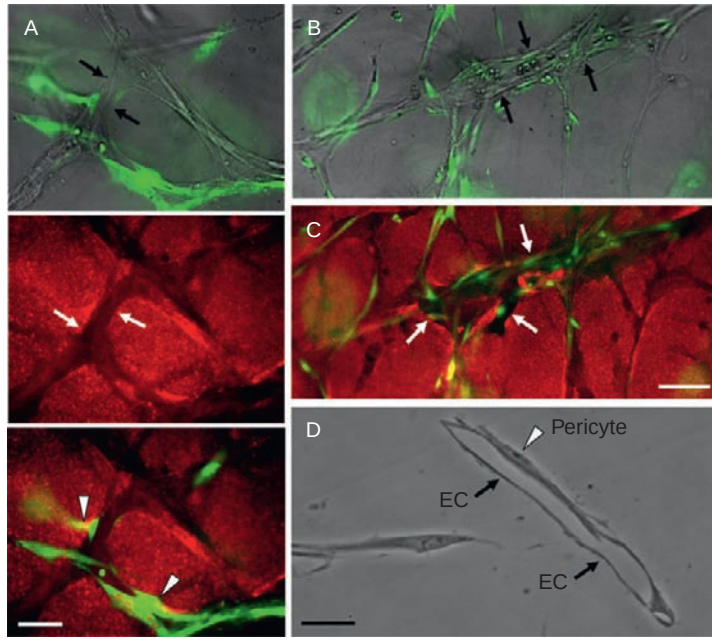

Figure 3.8 Pericytes recruit to EC tubes within vascular guidance tunnels, a necessary step to allow for direct EC–pericyte interactions and motility events controlling vascular tube remodeling and stabilization. EC- and GFP-pericyte cocultures were allowed to assemble for a period of 5 days. Cultures were then fixed and immunostained using anti-collagen type I antibodies (red) to recognize the native collagen type I matrix following tube assembly. (A, top) Bright field-GFP fluorescence overlay images of EC–pericyte cocultures were obtained after 5 days of tube assembly and pericyte recruitment events. (Middle) The corresponding anti-collagen type I image is shown (red), with the arrows indicating the borders of vascular guidance tunnels. (Bottom) Overlay images of the GFP pericytes onto the collagen type I image (red) reveals the presence of pericytes within the boundaries of the EC-generated vascular guidance tunnels. Arrowheads indicate GFP-labeled pericytes. Bright field-GFP pericyte (B) and GFP pericyte-collagen type I (red) (C) overlays reveal the association of pericytes with EC tubes (black arrows indicate EC tubes) and their presence within vascular guidance tunnels. Black arrows indicate EC tube borders, while white arrows indicate vascular guidance tunnel borders. (C, white arrows indicate the boarders of the vascular guidance tunnels). Bars equal 30 μm. (D) Plastic thin sectioning of 5-day EC–pericyte cocultures reveals the ablumenal association of pericytes (white arrowhead) with an EC-lined tube (black arrows). Bar equals 20 μm.

intracellular vacuoles that control EC lumen formation (Bayless and Davis, 2002; Kamei et al., 2006) and also Cdc42-GTP is a key component of EC lumen signaling complexes which links to Par6b, Par3, JamB, JamC, MT1-MMP, and $\alpha 2\beta 1$ and which together control EC lumen and tube formation (Sacharidou et al., 2010). Also, due to the fact that Rac1-GTP can also interact with Par6 isoforms (Brazil and Hemmings, 2000), it is likely that it

along with Cdc42-GTP mediates the function of this signaling complex to control EC tubulogenesis. Importantly, both Cdc42 and Rac1 control EC lumen formation and Rac1 appears to be a component of the EC lumen signaling complex that we have recently identified (Sacharidou and Davis, unpublished observations).

Despite the clear apical membrane targeting of specific proteins in epithelial cells during lumen formation, this has been much more difficult to demonstrate in ECs during these events. Past and recent studies demonstrate apical targeting of podocalyxin in EC tubes both *in vivo* and *in vitro* (Dekan et al., 1990; Lampugnani et al., 2010; Strilic et al., 2009). How critical this targeting event is for the lumen formation process remains unclear. Podocalyxin along with cortical actin, moesin, and other proteins are strongly targeted to apical surfaces during epithelial tube formation (Martin-Belmonte et al., 2007, 2008), but this is more difficult to demonstrate during EC tubulogenesis. One study showed that vascular endothelial (VE)-cadherin based adhesions play a role in podocalyxin targeting to the apical surface in a manner dependent on CCM1 (Lampugnani et al., 2010). Furthermore, a recent study demonstrated modest moesin targeting to apical membranes during Zebrafish intersegmental vessel tube formation (Wang et al., 2010). This study also revealed the presence of intracellular vacuoles (labeled with GFP-moesin) that were present and targeted to this apical surface (Wang et al., 2010). More work is necessary to understand how EC apical targeting of proteins is controlled and how it relates to EC junction formation during morphogenic events. Also, the question of whether EC–EC junctional contacts are primary stimulators of EC lumenogenesis has been raised in a few studies (Strilic et al., 2009; Wang et al., 2010) and, in our view, this remains an open question. Although it has been suggested to be a primary requirement for lumen formation (Strilic et al., 2009; Zeeb et al., 2010), this result is based on a central assumption that molecules such as VE-cadherin act solely or primarily as cell–cell adhesion molecules. In fact, single ECs can form lumen structures, without the need for contact with adjacent ECs (Davis and Camarillo, 1996). Junction adhesion molecule (Jam) proteins, which are known to participate in cell–cell adhesion of ECs (and of ECs and leukocytes), play a fundamental role in EC lumen formation from either single or aggregated ECs (Sacharidou et al., 2010). In fact, JamA has been reported to affect chemotactic responses of neutrophils migrating as single cells (Corada et al., 2005). Furthermore, it is clear that VE-cadherin has diverse effects on other EC functions including survival, maturation, proliferation, flow-induced EC cytoskeletal arrangements, and signal transduction (Dejana et al., 2009; Harris and Nelson, 2010; Lampugnani and Dejana, 2007; Tzima et al., 2005). Clearly, it is important to examine these issues in more detail and assess whether VE-cadherin may act independently of cell–cell junction assembly to influence EC lumen and tube formation. VE-cadherin has been more convincingly

linked to EC–EC junctional stability to maintain EC tubes that are formed (Crosby et al., 2005; Dejana et al., 2009; Montero-Balaguer et al., 2009; Rudini et al., 2008), and this function may also be particularly critical under flow and pressure conditions. Thus, it remains to be seen whether the vascular morphogenic defects observed in VE-cadherin mouse knockouts or morpholino knockdown Zebrafish are due to deficiencies in cell–cell adhesion that prevent lumen formation or actually represent defects in other critical signaling pathways that interfere with EC tube morphogenesis independently of effects on cell–cell adhesion.

3. Molecular Mechanisms Controlling Vascular Lumen Formation

3.1. Role of intracellular vacuoles and intercellular adhesion mechanisms in lumen formation

In a review on tubulogenesis a number of years ago, Krasnow and colleagues suggested five different mechanisms of lumen and tube formation including intracellular lumen formation and cord hollowing which appear to be the two major mechanisms controlling EC lumen formation (Lubarsky and Krasnow, 2003) (Fig. 3.4). These two primary mechanisms appear to play a role in both initiating and sustaining EC lumen and tube formation (Davis and Bayless, 2003; Davis et al., 2007; Iruela-Arispe and Davis, 2009). An important consequence of either mechanism by which ECs form tubes is that they will create physical spaces within the ECM, termed vascular guidance tunnels (Stratman et al., 2009b) (Figs. 3.3 and 3.8). Tunnel formation appears to be directly coupled to the signaling pathways that control tubulogenesis in 3D matrix environments (Sacharidou et al., 2010; Stratman et al., 2009b).

Recent controversies have arisen over whether intracellular lumen formation mechanisms are utilized by ECs to form lumens and tubes (Blum et al., 2008; Davis et al., 2002, 2007; Iruela-Arispe and Davis, 2009; Kamei et al., 2006; Strilic et al., 2009). In fact, some investigators have stated that they are either not involved or not relevant for lumen formation (Blum et al., 2008; Strilic et al., 2009; Zeeb et al., 2010). It is important to consider this issue by examining multiple studies from separate laboratories that involve both *in vitro* and *in vivo* analyses of these events. When this issue is examined critically and when both types of studies are considered, intracellular vacuoles clearly have been shown to play a role during EC lumenogenesis. Vacuoles have been demonstrated to be present during EC lumen formation both *in vitro* and *in vivo* (Bayless and Davis, 2002; Bayless et al., 2000; Davis and Bayless, 2003; Davis and Camarillo, 1996; Davis et al., 2002; Folkman and Haudenschild, 1980; Kamei et al., 2006;

Liu et al., 2010; Wang et al., 2010; Yang et al., 1999; Zovein et al., 2010). Disruption of either moesin or CCM1 in Zebrafish leads to a decrease in intracellular vacuoles within ECs *in vivo* and in both cases, these treatments result in marked deficiencies in EC lumen and tube formation (Liu et al., 2010; Wang et al., 2010). Also, blockade of intracellular vacuole formation in human ECs using anti-integrin antibodies or disruption of Cdc42 or Rac1 signaling markedly inhibits EC lumen formation in 3D matrices (Bayless and Davis, 2002; Bayless et al., 2000; Davis and Camarillo, 1996). It has also been demonstrated that either GFP-Cdc42 or GFP-moesin labels intracellular vacuoles and, furthermore, vacuoles can be demonstrated to dynamically form and fuse during lumen formation events in developing Zebrafish intersegmental vessels (Bayless and Davis, 2002; Kamei et al., 2006; Wang et al., 2010). Multiple independent groups have observed intracellular vacuoles during tube formation events in Zebrafish, mouse, and avian species. Furthermore, they have been demonstrated to play a critical role during lumen formation using human ECs in 3D matrix environments *in vitro*. Images of EC tubes during quail vascular development at E5 shows obvious evidence of intracellular vacuolation of ECs within tube walls and also with ECs that appear to be joining tubes during multicellular tube formation (Fig. 3.9). Thus, intracellular vacuoles are observed *in vitro* and *in vivo* during EC tube morphogenic events and clearly play a functional role during lumen formation and tube assembly.

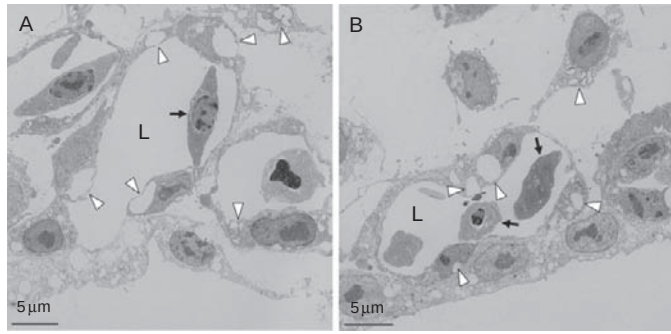

Figure 3.9 **EC intracellular vacuoles are present during vascular tube assembly *in vivo* during quail development. Quail eggs were incubated until embryonic day 5 and at that time the chorioallantoic membrane (CAM) was collected. Tissue was fixed for transmission electron microscopic (TEM) analysis and multiple cross sections were examined and analyzed. (A) A TEM image of embryonic day 5 CAM tissue from the quail reveals intracellular vacuoles within ECs, as indicated by the white arrowheads. The black arrow indicates a nucleated red blood cell within the blood vessel. (B) TEM images of assembled and nonassembled ECs in the quail CAM reveals intracellular vacuoles, as indicated by the white arrowheads. The black arrows indicate circulating red blood cells (L, vessel lumen). Bars equal 5 μm.**

Although vacuoles are present in ECs and play a role during these events, they are clearly not the sole mechanism which controls lumen and tube formation. For example, it has been demonstrated that EC lumen expansion from single or aggregated ECs involves cell surface MT1-MMP activity, a mechanism that is clearly distinct from the intracellular vacuole and coalescence mechanism (Davis and Saunders, 2006; Saunders et al., 2006; Stratman et al., 2009b). Interestingly, MT1-MMP activity appears to facilitate vacuole–vacuole fusion events to help expand the lumenal compartment during this process. This has been repeatedly shown in multiple real-time movies that have been published from our laboratory during vasculogenic tube assembly or EC sprouting events (Iruela-Arispe and Davis, 2009; Saunders et al., 2006; Stratman et al., 2009b). This latter mechanism is fundamental toward the creation of vascular guidance tunnel spaces within the ECM (Stratman et al., 2009b) and is likely to represent a primary regulator of the EC cord hollowing process. In order for solid cords or even vacuolating cords of ECs to form lumens, they will need to be able to create physical spaces within the 3D ECM (i.e., vascular guidance tunnels) (Figs. 3.3, 3.4, 3.6, and 3.8). In fact, we have demonstrated that MT1-MMP is required for EC aggregates to form lumens which are essentially forming through a cord hollowing or cell–cell aggregate hollowing mechanism (Davis and Saunders, 2006). In both cases, MT1-MMP activity is necessary to create spaces in the ECM so that the lumens and tubes can expand properly. We also observe intracellular vacuoles within these EC–EC aggregates; however, vacuoles are more obvious when ECs are suspended individually within 3D matrices, likely reflecting the dependence of intracellular vacuole formation on integrin-based EC adhesion in these matrices (Davis and Bayless, 2003; Davis and Camarillo, 1996; Davis et al., 2002). In support of this comment, we have previously shown that intracellular vacuole formation requires integrin–ECM interactions and different integrins control their formation depending on the ECM environment in which the ECs are suspended (i.e., $\alpha 2\beta 1$ requirement in collagen matrices, $\alpha v\beta 3$ and $\alpha 5\beta 1$ requirement in fibrin matrices) (Bayless et al., 2000; Davis and Camarillo, 1996).

It has recently been suggested that EC cord hollowing *in vivo* is mediated by cell mechanical mechanisms through Rho kinase (ROCK) to pull apart two opposing EC surfaces to form lumens (Strilic et al., 2009). In contrast, ROCK has been demonstrated by other groups to mediate tube collapse mechanisms that control vascular tube regression (Bayless and Davis, 2004; Im and Kazlauskas, 2007; Mavria et al., 2006) (Fig. 3.10). One distinction in the former study is that it addresses aortic tube formation and a central assumption of this work is that the effects observed are, in fact, EC autonomous, a question that was not directly addressed in this *in vivo* study. It is certainly possible that the addition of ROCK inhibitors might affect surrounding cells (i.e., non-ECs) as well as ECs so it is not possible to ascertain

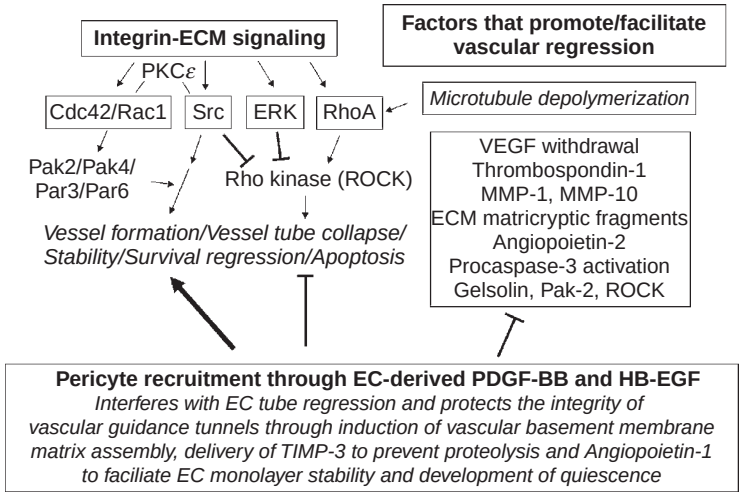

Figure 3.10 Molecular control of EC lumen and tubulogenesis requires coordinated signaling between Cdc42/Rac1, integrins, and proteinases to regulate promorphogenic versus proregressive signals and pericyte recruitment to tubes modulates these processes. The schematic diagram depicts a series of molecules and signaling pathways that have been identified, which control EC lumen formation and tubulogenesis, tube stabilization, and vascular regression. The process of pericyte recruitment to forming EC tubes regulates both promorphogenic and proregressive signals.

the relevant cellular target of the inhibitor. Developing aortic ECs are known to be in contact with other cell types including mesodermal cells and also definitive endoderm. Interestingly, siRNA suppression of RhoA (a critical activator of ROCK) or addition of dominant negative RhoA does not block lumen formation of human ECs in 3D matrices (Bayless and Davis, 2002; Koh et al., 2008a) (Fig. 3.5). Furthermore, addition of ROCK inhibitors such as Y-27632 (which blocks both ROCK-1 and ROCK-2) fails to block EC lumen formation from either single or aggregated ECs in 3D collagen matrices (Fig. 3.11). Thus, there is little support for the concept that RhoA and ROCK are primary regulators of EC lumen formation in 3D matrices through direct actions on ECs. In contrast, much of the evidence suggests that RhoA and ROCK need to be actively inhibited during EC lumen formation and tube maintenance events (Fig. 3.10). Interestingly, both Src and Erk kinases which are activated to promote EC lumen and tube formation, both participate in inhibiting Rho/ROCK signaling to maintain EC-lined tubes (Bayless and Davis, 2004; Im and Kazlauskas, 2007; Mavria et al., 2006) (Fig. 3.10). Also, there is considerable support for the role of Rho/ROCK in diassembly of EC–EC junctions and collapse of EC-lined tubes in 3D matrices *in vitro* and *in vivo*. Due to the ability of Rho/ROCK to cause EC junctional disassembly

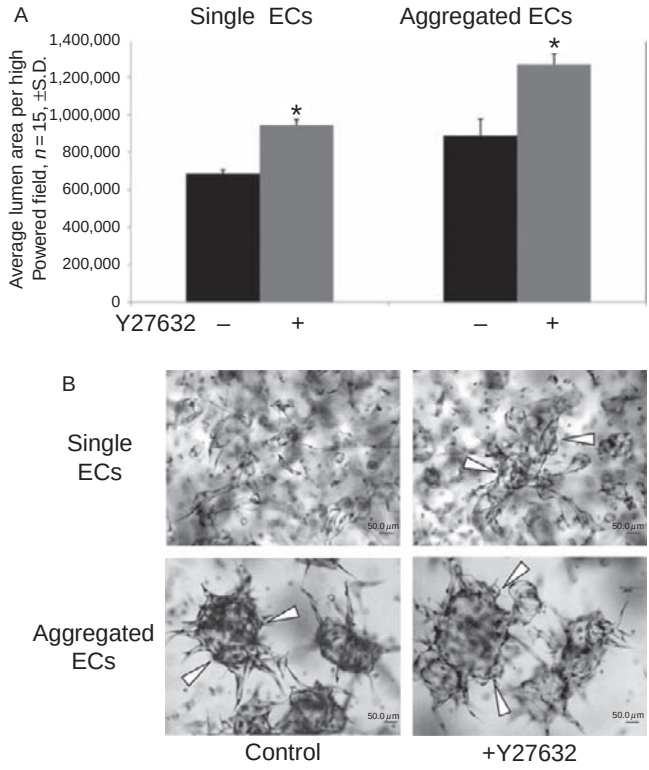

Figure 3.11 Rho kinase inhibitors enhance EC lumen formation of single and preaggregated ECs in 3D collagen matrices. ECs were either suspended within 3D collagen matrices as single cells or preaggregated cells in the presence or absence of 10 mM Y27632 to block Rho kinase activity. Cultures were fixed at 24 h and were either photographed (B) or quantitated for lumen and tube area (A). Arrowheads indicate EC lumen structures. $p < 0.01$, $n \geq 10$ fields from triplicate cultures.

(Liu and Senger, 2004; Wojciak-Stothard et al., 2001), it is possible that activation of this pathway could facilitate the transition of quiescent ECs to morphogenically active ECs (Liu and Senger, 2004).

Another issue that should be mentioned is the possibility that ECs within different vascular beds (i.e., aorta vs. small vessels) might form lumens and tubes by distinct mechanisms. Although this is certainly a possibility that needs to be investigated, it is also possible that external influences of other cell types on ECs may underlie these apparent differences. Interestingly, there is considerable evidence that embryo growth and motion are occurring during these stages of development which might exert mechanical forces on both cells and surrounding ECM to affect these cellular responses (Filla et al., 2004; Sato et al., 2010; Zamir et al., 2006). This is one good example of an *in vivo* phenomenon that would be very difficult to mimic

through *in vitro* morphogenesis models. As mentioned above, the developing aortic endothelium is in direct contact with underlying mesodermal cells as well as definitive endodermal cells; thus, it is possible that cell–cell or cell–ECM contacts which characterize these unique heterotypic interactions could play a major role in determining how aortic ECs assemble into lumen structures. Thus, the adhesive template for aortic EC lumen formation may be based on interactions with heterotypic cells, ECM, or both. These possibilities need to be strongly considered as possible reasons for the distinctions described in different studies. In contrast, ECs in the periphery, which assemble into small vessel capillary networks, are likely to have more direct contact with surrounding ECM, thus mimicking the behaviors observed in the *in vitro* models that have been developed. Overall, such issues need to be carefully considered to understand the molecular basis for EC lumen and tube formation in different vascular beds.

3.2. Role of cell polarity mechanisms in controlling EC lumen formation

As discussed above, EC lumen and tube formation results in networks of EC-lined structures that have a central lumen space exposed to fluid and a basal surface exposed to ECM. Similar events occur during epithelial lumen formation, except in the *Drosophila* tracheal system where the lumen and apical compartment contains abundant chitin (Devine et al., 2005). Thus, it is clear that EC and vertebrate epithelial cell lumen formation mechanisms must have an ability to create these unique apical fluid and basal ECM relationships. In addition, in the case of ECs, mural cells are specifically recruited to this basal surface and specialized ECM deposition occurs there (i.e., formation of the vascular basement membrane matrix) (Fig. 3.7). Interestingly, fluid and ion pumps have been shown to propel fluid into the lumen space but in order for this to stay within this compartment, appropriate cell–cell junctional contacts need to be in place such as tight junctions (Bryant and Mostov, 2008). This is particularly observed in epithelial cells where multicellular cell lumenal structures can be induced to acutely swell when treated with agents such as cholera toxin (to stimulate Cl^-, Na^+, and H_2O transport into the lumen space through the apical membrane) (Montesano et al., 2009). Interestingly, there is evidence for the involvement of CLIC Cl^- channels during intracellular vacuole formation and EC lumen formation suggesting that similar events could occur during vascular morphogenesis (Ulmasov et al., 2009). An important distinction is that EC tubes primarily propagate fluids being pumped from the heart, while many epithelial tubes are distinct and actively secrete fluids and ions from their specialized apical membrane domains.

The molecular basis for how EC tubes polarize is a question of great importance and there is little information that currently addresses this issue.

It is quite clear that epithelial cell polarity is distinct from EC polarity as the functions of the two types of cells as well as their apical versus basal domains are also distinct (Bryant and Mostov, 2008; Iruela-Arispe and Davis, 2009). In general, it appears that apical membrane protein targeting is much less obvious in ECs compared to that of epithelial cells. Thus, it is very difficult to identify membrane proteins that are uniquely targeted apically in ECs while there are many proteins and cytoskeletal elements that show strong apical targeting in epithelial cells. Despite this fact, it appears that EC tube formation occurs much more rapidly than does epithelial tube formation in 3D matrices *in vitro*. EC tube formation occurs within several days in EC 3D cultures while it generally takes weeks to form extensive tube networks for epithelial cell cultures (Koh et al., 2008b; O'Brien et al., 2006). Thus, EC tubulogenesis appears to occur much more rapidly and extensively when compared to epithelial cells despite the clear differences in apical membrane targeting of proteins which strongly favors epithelial cells. Thus, at least with respect to this property, this could suggest that apical membrane protein targeting mechanisms may not correlate well with the ability of cells to form lumen structures in 3D matrices. In contrast, a signaling system that is known to affect cell polarization such as Cdc42-GTP binding to Par6 is clearly involved in both systems and is required for both EC and epithelial lumen formation (Bryant et al., 2010; Koh et al., 2008a; Martin-Belmonte et al., 2007; Sacharidou et al., 2010). Perhaps the cytoskeleton as well as intracellular structures such as centrosomes and the Golgi apparatus need to be polarized to be able to form lumen structures. Centrosome reorientation and nuclear repositioning during cell motility events is known to be controlled by Cdc42 and its effectors (Gomes et al., 2005). Such issues like these are much more likely to be relevant during EC lumen formation than whether particular apical transmembrane proteins are targeted to the apical membrane surface. Clearly, much new information is necessary to understand how ECs polarize to form lumen structures in 3D matrix environments.

3.3. Mechanisms controlling lumen formation during EC sprouting events

A question that has not been addressed in detail concerns differences in lumen formation events during vasculogenic tube assembly or angiogenic sprouting. Of interest, particularly during the latter process, is the issue that lumen formation appears to occur selectively in the ECs that trail the invading EC tip cells (Sainson et al., 2005). However, we have observed intracellular vacuole formation at the rear of tip cells, and vacuoles are very clearly observed in the cells directly trailing the tip cell (Fig. 3.2). How lumen formation occurs in the cells trailing the EC tip cells is not understood and has not been investigated in detail at this point. Of great interest is

how EC tip cells interact with their trailing cells to control processes such as lumen formation. There is considerable evidence that DLL4–Notch signaling plays a role in defining the position of the tip cells and some suggestions have been made that this might control the process of lumen formation (Benedito et al., 2008; Hellstrom et al., 2007; Sainson et al., 2005; Siekmann and Lawson, 2007). Importantly, EC invasion depends on cell surface MMP activity and EC tip cells require such activity to invade into 3D collagen or fibrin matrices (Bayless and Davis, 2003; Saunders et al., 2006). Signals from molecules such as stromal-derived factor (SDF)-1α and sphingosine-1-phosphate (S1P) stimulate this process during EC invasion (Bayless and Davis, 2003; Saunders et al., 2006). Interestingly, lysophosphatidic acid (LPA) can stimulate invasion of tumor cells in 3D matrices by a process that also depends on cell surface MT-MMP activity (Fisher et al., 2006, 2009; Sabeh et al., 2009). In all cases, these invading cells create physical spaces during this process and leave such spaces behind them as they invade forward (Bayless and Davis, 2003; Fisher et al., 2009; Stratman et al., 2009b). In the case of ECs, the cells that follow tip cells already occupy a physical space that can contribute to the developing lumenal space, by flattening out within this space (Stratman et al., 2009b). In addition, the cells behind the tip cells can expand the lumenal space by accumulating intracellular vacuoles or expand through proteolytic events from lateral membrane surfaces (Figs. 3.2 and 3.4). This latter process is highly related to lumen formation that occurs from groups of aggregated cells (i.e., cord hollowing mechanisms) which also depend on cell surface MT-MMPs in 3D collagen matrices (Davis and Saunders, 2006). MT1-MMP regulates EC lumen formation during vasculogenic tube assembly from single cells or aggregated ECs that form spherical multicellular tubes (Davis and Saunders, 2006; Sacharidou et al., 2010; Saunders et al., 2006; Stratman et al., 2009b). In either case, they can sprout to connect with neighboring cells (or aggregates) to form multicellular lumen and tube structures as well as tube networks. This latter process also depends on MT1-MMP proteolytic activity in 3D collagen matrices. Blockade of MT1-MMP with inhibitors, such as TIMP-2, TIMP-3, or GM6001, using siRNA suppression or expression of a dominant negative MT1-MMP mutant completely attenuates EC lumen and tube formation in 3D collagen matrices (Sacharidou et al., 2010; Saunders et al., 2006; Stratman et al., 2009b).

3.4. Control mechanisms for remodeling and maintenance of EC-lined tube networks

The EC tubulogenic process is highly dynamic as revealed by real-time morphogenesis movies observed both *in vitro* and *in vivo* (Herbert et al., 2009; Iruela-Arispe and Davis, 2009; Kamei et al., 2006; Sato et al., 2010; Saunders et al., 2006; Stratman et al., 2009a,b; Wang et al., 2010; Yaniv

et al., 2006). Not only are tube formation and sprouting events very dynamic, subsequent remodeling events are equally dynamic. This is dramatically illustrated during mouse development where flow-induced changes through the initial vascular plexus (with very uniform vessel diameters) lead to dramatic remodeling where both large and small vessels are then observed (Lucitti et al., 2007). Thus, marked changes in lumen diameters and tube lengths occur to develop the characteristic features of the functional microcirculation including artery, arterioles, capillary, venules, and veins. In addition to flow-induced remodeling, a second major regulator of vascular tube remodeling is recruitment of mural cells (at later stages of vascular development). Mural cell recruitment, which involves both pericytes and vascular smooth muscle cells, is responsible for facilitating tube maturation and stabilization (Adams and Alitalo, 2007; Gaengel et al., 2009; Senger and Davis, 2010; Stratman et al., 2009a). Further, recruitment of vascular smooth muscle cells in particular to vascular tubes allows for lumen diameters of EC tubes to be regulated through smooth muscle contractility (i.e., via molecules such as nitric oxide to relax vessels and angiotensin II to constrict them) (Hill et al., 2007; Martinez-Lemus et al., 2003; Segal, 2005). This ability of vascular smooth muscle to dynamically control vessel lumen diameter is critical to the molecular control of blood pressure and blood flow to tissues (Hill et al., 2007; Segal, 2005). Interestingly, pericytes have also been shown to play a role in regulating vessel diameters in capillary beds, particularly in the central nervous system (Peppiatt et al., 2006). Alterations in such controls underlie diseases such as hypertension and diabetes where abnormalities in EC–mural cell interactions occur (Hammes, 2005). In hypertension or diabetes, vessel wall remodeling occurs which involves both cellular and ECM changes and that make treatment options more difficult, particularly with long-standing disease (Arroyo and Iruela-Arispe, 2010; Hill et al., 2007; Raffetto and Khalil, 2008; ten Dijke and Arthur, 2007; van den Akker et al., 2010).

Another issue that is not typically considered with respect to lumen and tube formation is how lumen and tube networks are maintained during the initial phases of the process (i.e., without regard to flow forces or contributions of other cell types such as mural cells). Our laboratory has shown, for example, that maintenance of EC tube structures depends on both the microtubule and actin cytoskeletons (Bayless and Davis, 2004). Microtubule depolymerizing factors (i.e., vinblastine, colchicine) dramatically cause collapse of EC-lined tubes in a manner dependent on the RhoA GTPase (Bayless and Davis, 2004) (Fig. 3.10). Interestingly, disruption of the actin cytoskeleton with cytochalasin B does not cause tube collapse. However, addition of cytochalasin B prior to addition of vinblastine or colchicine to diassemble microtubules blocks tube collapse, suggesting that actin-based cell contractility is necessary for this tube collapse mechanism (Bayless and

Davis, 2004). What is interesting is that there must be an important signaling pathway within ECs that maintains tube structures in a 3D matrix environment. When disrupted, EC tubes rapidly collapse and disassemble. This mechanism must involve a combination of cell–matrix adhesions as well as cell–cell adhesions that actively prevent tube collapse. The fact that tubes can rapidly collapse (within minutes) suggests that the tube networks may be under mechanical tension because this rapid collapse leads to clustering of groups of ECs together (Bayless and Davis, 2004; Stratman et al., 2009b). Following tube collapse, it becomes obvious that vascular guidance tunnels are present as physical spaces in which the tube structures are embedded (Stratman et al., 2009b). Furthermore, the tunnel spaces represent matrix conduits in which tube remodeling events can occur since ECs freely migrate within them. Interestingly, we have shown that EC motility responses within vascular guidance tunnels occurs in an MMP-independent manner (Stratman et al., 2009b), and can lead to tube remodeling events. It becomes easier to interpret important vascular responses such as flow-induced remodeling when it is realized that EC-lined tubes are present within vascular guidance tunnel networks that are created as a result of the tube morphogenic process. We also presented evidence that more tunnels are generated than are occupied by tubes at any given time (Stratman et al., 2009b) so this could account for rapid changes in tube structure and patterns in response to an applied stimulus such as flow or recruitment of mural cells. Since stimuli such as flow, fluid pressure, and ECM remodeling can regulate EC tube remodeling and stabilization events; tunnels may be important for such responses. A detailed molecular analysis of how tubes are maintained in a 3D matrix environment is particularly important from the standpoint of ECs, so that the influence of external stimuli can be examined and understood. Clearly, much further investigation is necessary in this area.

4. Functional Roles of Cdc42 and Rac1 in EC Lumen and Tube Formation

4.1. Evidence for roles of Cdc42 and Rac1 in lumen formation

Both Cdc42 and Rac1 have been shown to be required for EC lumen and tube formation and this has been demonstrated utilizing dominant negative mutants and siRNA suppression experiments (Figs. 3.5, 3.6, and 3.10) (Bayless and Davis, 2002; Davis et al., 2007; Koh et al., 2008a). In addition, Cdc42 and Rac1 control intracellular vacuole formation which plays a fundamental role in EC lumen formation, particularly when ECs are seeded as single cells in 3D matrices (Bayless and Davis, 2002). Other investigators have obtained similar results *in vitro* and interestingly, recent experiments demonstrate requirements for both Cdc42 and Rac1 in EC lumen

formation *in vivo* (Hoang et al., 2010a,b; Tan et al., 2008). Also, we have observed intracellular vacuole labeling with GFP-Rac1, GFP-Cdc42, and GFP-RalA (Bayless and Davis, 2002; Davis et al., 2007). GFP-Cdc42, when expressed from an EC-specific promoter in developing Zebrafish, revealed intracellular vacuole formation and fusion during the lumen formation process of intersegmental vessels (Kamei et al., 2006). In support of these findings, a recent study showed that a GFP-moesin construct also revealed EC intracellular vacuoles that form and fuse to control intersegmental vessel formation in Zebrafish embryos (Wang et al., 2010).

Over the past 15 years, many molecular requirements and signaling pathways have been revealed that control how human ECs form lumen and tube structures in 3D extracellular matrices (Figs. 3.6 and 3.10). Most of our studies have utilized collagen matrices and, thus, a major requirement for these events is the $\alpha 2\beta 1$ integrin, a collagen-binding integrin (Davis and Camarillo, 1996). Blocking antibodies directed to $\alpha 2\beta 1$ markedly block lumen formation (Fig. 3.12) as well as $\alpha 2$ integrin subunit siRNAs

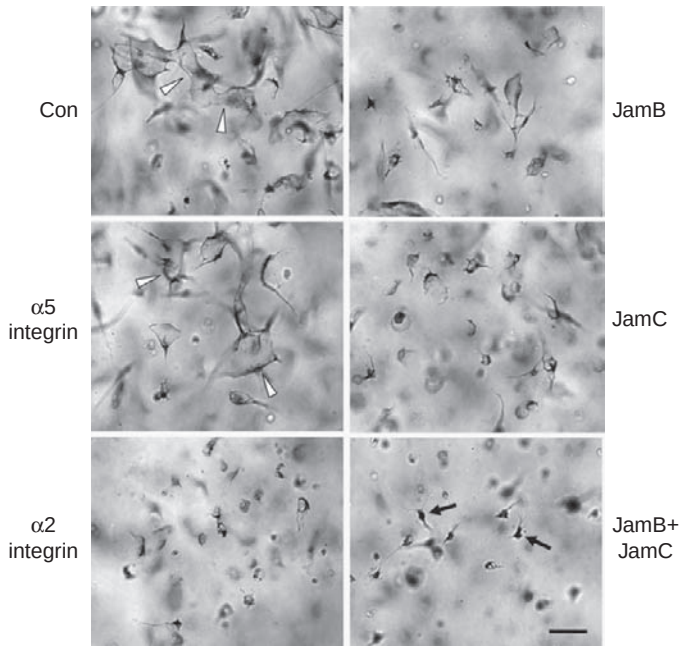

Figure 3.12 Blockade of JamB and JamC molecules using neutralizing antibodies markedly inhibits lumen and tube formation of endothelial cells. ECs were seeded within collagen type I matrices in the presence or absence of blocking antibodies to JamB, JamC either alone or blocking antibodies to $\alpha 5$ or $\alpha 2$ integrin subunits. Each antibody was added at 50 μg/ml and the cultures were fixed, stained, and photographed after 24 h. Arrowheads indicate EC lumenal structures, arrows indicate ECs without lumens. Bar equals 50 μm.

(Sacharidou et al., 2010). Blocking antibodies directed to other integrin subunits expressed by ECs, including α5β1, a fibronectin receptor, have no blocking influence in 3D collagen matrices with EC-only cultures (Fig. 3.12). Furthermore, considerable work has shown that α2β1 is important *in vivo* for both developmental and postnatal vascularization responses (San Antonio et al., 2009; Senger et al., 1997). In contrast, both αvβ3 and α5β1 control EC lumen and tube formation in 3D fibrin matrices (Bayless et al., 2000), while α2β1 was not shown to be involved. Importantly, these integrins have been shown to be involved during vessel formation during development and in postnatal mice (Astrof et al., 2007; Hynes, 2007; Senger et al., 1997; Somanath et al., 2009; Stupack and Cheresh, 2004; van der Flier et al., 2010). An important point is that data obtained *in vitro* models accurately predicted integrin requirements controlling vascular morphogenesis *in vivo*. Another key point is that *in vitro* model systems demonstrated first that multiple integrin chains can be utilized by ECs to regulate tube morphogenesis in promorphogenic ECM environments such as collagen and fibrin. The role of particular integrins in morphogenesis appears to be directly linked to the ECM environment (and the predominant ECM components) that are in contact with the ECs.

Integrins are known to activate Rho GTPases (Hall, 2005) and our laboratory first reported that Cdc42 was a critical GTPase controlling EC lumen formation (Bayless and Davis, 2002). Subsequent studies revealed that Cdc42 is a critical regulator of lumen formation for both ECs and epithelial cells (Bryant et al., 2010; Davis and Bayless, 2003; Jaffe et al., 2008; Koh et al., 2008a, 2009; Martin-Belmonte et al., 2007). We also reported a role for Rac1 in EC tubulogenesis (Bayless and Davis, 2002; Koh et al., 2008a), while RhoA had no apparent influence on these events. In contrast, we made the key observation that RhoA controlled EC tube collapse in response to depolymerization of microtubules in 3D matrices (Bayless and Davis, 2004) (Fig. 3.10). Both Cdc42 and Rac1 were shown to be activated during the morphogenic cascade in 3D collagen matrices (Koh et al., 2008a,b; Sacharidou et al., 2010). In a recent study, we demonstrated that Cdc42 activation in 3D matrices was dependent on Jam-B and Jam-C as well as MT1-MMP (Sacharidou et al., 2010; Fig. 3.6). Disruption of these three molecules led to marked decreases in EC Cdc42 activation in 3D collagen matrices (Sacharidou et al., 2010).

4.2. Roles of Cdc42 effectors, Pak-2, and Pak-4 in EC lumen formation

To identify downstream effectors that are responsible for the influence of Cdc42 and Rac1, a series of known effectors were screened using siRNA treatment of ECs (Koh et al., 2008a). Strong blocking phenotypes were observed using siRNAs to both Pak-2 and Pak-4 (Koh et al., 2008a).

Previous work suggested a potential role for Pak-1 in EC tube formation (Kiosses et al., 2002) and recent studies have shown that both Pak-2 and Pak-4 are involved in developmental vascularization events, which corroborate the *in vitro* findings (Galan Moya et al., 2009; Liu et al., 2007; Tian et al., 2009). Also, Pak-2 and Pak-4 siRNAs block EC sprouting into 3D collagen matrices (Koh et al., 2008a). Importantly, Pak-2 and Pak-4 activation (detected by phosphorylation) directly correlated with both Cdc42 and Rac1 activation and the EC lumen formation process (Koh et al., 2008a). Activated Pak-2 and Pak-4 could also be demonstrated to be associated with activated Cdc42 (i.e., Cdc42-GTP) during these events. Expression of dominant negative mutants of either Pak-2 or Pak-4 in ECs completely inhibited lumen and tube formation (Koh et al., 2008a). Interestingly, both Cdc42 and Rac1 are capable of activating Pak-2 while Cdc42 selectively activates Pak-4 (Bokoch, 2003; Galan Moya et al., 2009). Pak-2 can be activated in a Rho GTPase-independent manner by binding to GIT-1 (Loo et al., 2004), a protein that also binds to a known Cdc42 GEF and Cdc42 and Rac effector, β-PIX. Pak-2 is also known to be a substrate of caspase-3 and is cleaved during vascular tube collapse and regression when microtubules are disassembled (Bayless and Davis, 2004). Interestingly, Pak phosphorylation can lead to EC junctional disassembly (Orr et al., 2007; Stockton et al., 2004) and increased vascular permeability in the context of atherosclerotic lesions (Orr et al., 2007). Pak-2 is of particular interest in that it appears to be involved in both EC lumen and tube assembly but possibly also in tube disassembly. Thus, Pak-2 may represent a bimolecular switch that can participate in either the processes of tube assembly or disassembly and its regulation may be fundamental to controlling these events. Further studies will need to assess how Pak-2 can perform such distinct functions and determine which molecules work in conjunction with Pak-2 to affect these different events.

4.3. Roles of PKCε and Src family kinases in EC tube morphogenesis and subsequent Pak-2 and Pak-4 activation events

Kinases that are activated by cell–ECM interactions include PKC isoforms (i.e., conventional, novel, and atypical isoforms) and Src family kinases in addition to others (Fig. 3.6). In our studies of EC lumen formation in 3D collagen matrices, PKCε was shown to be involved but not PKCα or PKCδ (Koh et al., 2008a, 2009). Inhibition of PKCα or PKCβ using the chemical inhibitor, Go6976, fails to block EC lumen formation and, in fact, accelerates the process (Koh et al., 2009). Interestingly, this inhibitor shows great selectivity for these conventional isoforms and does not block PKCε or atypical PKC isoforms. In contrast, Go6983, which does block PKCε and atypical PKC isoforms, strongly inhibits EC lumen formation (Koh et al.,

2009). In further support for these findings, expression of dominant negative mutants of either PKCε or siRNA suppression of PKCε blocks lumen formation as well as downstream Src and Pak activation (Koh et al., 2009). Also, increased expression of PKCε markedly enhanced EC lumen and tube formation in 3D collagen matrices. This treatment further increased Src, Pak-2, and Pak-4 phosphorylation events that directly correlated with its morphogenic influence (Koh et al., 2009). Overall, these studies indicate a fundamental role for PKCε in EC lumen formation and, furthermore, that it appears to signal upstream of Src activation, while Src activation is upstream of Pak activation (Figs. 3.6 and 3.10) (Koh et al., 2009). Key future studies here include identifying PKCε phosphorylation targets that underlie its biological influence during these morphogenic processes.

Blockade of Src family kinases by siRNA suppression, increased expression of the Src inhibitor, Csk (i.e., C-terminal Src kinase), or treatment with chemical inhibitors with specificity for Src (e.g., PP2) markedly interferes with EC tube formation (Koh et al., 2009). Expression of a dominant negative Csk construct increased lumen formation, which further supports a key role for Src during EC lumen formation. Furthermore, Src and Pak kinases are known to activate Raf kinases to affect processes such as cell survival which have previously been demonstrated to effect angiogenesis *in vivo* (Alavi et al., 2003) and a B-Raf mouse knockout shows an embryonic lethal phenotype that is due to vascular abnormalities (Galabova-Kovacs et al., 2006). Our laboratory demonstrated that Raf kinase activation (i.e., both B-Raf and C-Raf) occurs downstream of Src and Pak activation to control EC tube formation in 3D collagen matrices (Koh et al., 2009). This is accompanied by downstream Erk1/2 activation which is also necessary for ECs to form tube networks. MKP-3 (also called DUSP-6), a serine/threonine phosphatase with selectivity for phospho-Erk1/2, markedly decreased Erk phosphorylation and completely abrogated lumen formation (Koh et al., 2009). A dominant negative MEK kinase inhibitor also blocks lumen formation as well as Erk1/2 phosphorylation events. Overall, this morphogenic signaling pathway is coupled to cytoskeletal rearrangements, cell surface-mediated proteolysis of ECM, survival and transcriptional events that are necessary to coordinately regulate EC lumen formation (Fig. 3.6).

4.4. Cdc42-mediated control of cell polarity pathways mediating EC lumen and tube formation

A key function of Cdc42 is its ability to affect cell polarity signaling by interfacing with the polarity proteins, Par6, Par3, and atypical PKC isoforms (Etienne-Manneville, 2004; Etienne-Manneville and Hall, 2003; Macara, 2004). Cell polarity signaling controls directional cell motility that

involves Cdc42 (Etienne-Manneville and Hall, 2003) and reorientation of the centrosome and positioning of the nucleus during these events (Gomes et al., 2005). In fact, active Cdc42 (i.e., Cdc42-GTP) binds directly to Par6 which then couples to Par3 (Macara, 2004). Rac1 also appears to interface with this pathway through its ability to interact with Par6 (Brazil and Hemmings, 2000). Par3 is known to interact with a variety of important regulatory proteins including Jams A, B, and C and VE-cadherin through their cytoplasmic tails and through the PDZ domains of Par3 (Ebnet et al., 2003, 2004). Par6 is known to interact directly with atypical PKC isoforms, such as PKCζ. Par6 is also a phosphorylation target of these kinases (Macara, 2004). We recently reported that Cdc42-dependent EC lumen and tube formation was dependent on Par6b, Par3, and atypical PKC (Koh et al., 2008a). Thus, this work reveals a fundamental role for Cdc42-dependent polarity signaling during EC tubulogenesis. Furthermore, we have recently reported that JamB and JamC associate with Par3 in ECs to control EC lumen formation in 3D collagen matrices (Sacharidou et al., 2010). Furthermore, blocking antibodies directed to Jams B and C markedly block EC lumen formation particularly when they are combined (Fig. 3.12). These Jam proteins co-assemble into a defined EC lumen signaling complex consisting of α2β1, MT1-MMP, Jam-C, Jam-B, Par3, Par6b, and Cdc42-GTP that together control the ability of ECs to form tubes in 3D collagen matrices (Sacharidou et al., 2010). These lumen signaling complexes are also directly coupled to the kinase cascade discussed above including PKCε, Src, Pak, Raf, and Erk 1/2 since disruption of these complexes completely interferes with the downstream signaling necessary to regulate vascular tube morphogenesis (Sacharidou et al., 2010). Cdc42 and Par3 have also been shown to control lumen formation in epithelial cells and a recent study shows that Par3 and β1 integrins co-regulate arteriolar lumen formation *in vivo* using a conditional β1 integrin subunit knockout mouse system (Zovein et al., 2010). Thus, this latter work again confirms our prior conclusions obtained *in vitro* showing that β1 integrins (i.e., α2β1), Cdc42, and polarity proteins control the lumen and tube formation process in 3D matrix environments (Davis et al., 2007; Koh et al., 2008a, 2009; Sacharidou et al., 2010).

4.5. Functional interdependence of Cdc42 and MT1-MMP-mediated signaling events controlling EC tube assembly in 3D collagen matrices

Several new findings are beginning to elucidate how Cdc42-dependent signaling events, which activate kinase cascades and interact with the cell polarity molecules (i.e., Par3, Par6, atypical PKC), intersect with MT1-MMP-dependent proteolytic events to create EC lumens and tubes in 3D extracellular matrices (Fig. 3.6) (Bayless and Davis, 2002; Ispanovic et al.,

2008; Koh et al., 2008a, 2009; Sacharidou et al., 2010; Stratman et al., 2009b). It is clear that EC lumen and tube formation is a 3D matrix-specific process (Davis et al., 2002) and, furthermore, that tubulogenesis does not occur on 2D matrix surfaces. Interestingly, EC motility can occur on 2D matrix surfaces and also in 3D matrices, but in the latter case, the motility response depends on MT1-MMP proteolytic events (Stratman et al., 2009b). These EC movement and proteolytic events are directly coupled during tube morphogenesis.

Since the lumen formation process in 3D collagen matrices requires both Cdc42-dependent and MT1-MMP-dependent activities, we addressed how these might be functionally coupled. Blockade of MT1-MMP activity using siRNA suppression or MT1-MMP inhibitors leads to marked interference with Cdc42 activation (a critical step necessary for activation of effectors such as Pak2, Pak4, and Par6 that lead to EC tubulogenesis) in 3D collagen matrices (Sacharidou et al., 2010). However, MT1-MMP blockade does not affect Cdc42 activation of ECs when they are seeded on 2D collagen surfaces, and random motility is also not affected (Sacharidou et al., 2010). Expression of a dominant negative MT1-MMP construct also markedly blocks lumen formation and Cdc42 activation (Sacharidou et al., 2010) in 3D collagen matrices. In contrast, activation of RhoA is not affected by blockade of Cdc42 or MT1-MMP. Overall, this data shows for the first time that MT1-MMP activity is directly coupled to Cdc42 activation in 3D, but not 2D matrices, to control the tube formation process (Sacharidou et al., 2010). The opposite is also true, in that blockade of Cdc42 using siRNA suppression or dominant negative mutants leads to marked decreases in both tube and vascular guidance tunnel formation, a consequence of inactivation of MT1-MMP-dependent proteolysis (Sacharidou et al., 2010). Thus, Cdc42 and MT1-MMP represent interdependent signaling molecules that control vascular tube morphogenic events specifically in 3D matrix environments.

4.6. Definition of an EC lumen signaling complex that controls vascular tube morphogenesis in 3D extracellular matrices

In order to explain how Cdc42 and MT1-MMP regulate each other during vascular tube morphogenesis, we wondered if they might be co-associated in a multimolecular signaling complex to control this process. During our analysis of Cdc42 activation from ECs undergoing morphogenesis in 3D matrices, we utilized Pak beads, a specific adsorbant for Cdc42-GTP and Rac1-GTP (the beads do not bind the GDP bound GTPases or RhoA-GTP). Eluates from these beads revealed Cdc42-GTP, but also contained MT1-MMP, the $\alpha 2\beta 1$ integrin, JamC, JamB, Par3, and Par6b (Sacharidou et al., 2010), suggesting the presence of a multimolecular protein complex

from ECs undergoing tube morphogenesis (Fig. 3.6). Furthermore, Cdc42, MT1-MMP, and JamB were each epitope tagged with an S-tag and when these proteins were expressed in ECs as recombinant proteins, we were able to demonstrate the co-association of the same molecules which now defines an EC lumen signaling complex that controls EC tubulogenesis in 3D collagen matrices (Sacharidou et al., 2010). Each of the molecules is required for lumen formation and disruption of any of these proteins leads to blockade of this process. Our evidence suggests that MT1-MMP and $\alpha 2\beta 1$ integrin are tightly associated and that they interact together with JamC (Fig. 3.6). siRNA suppression of either component eliminates their binding to JamC and dissociates them from the signaling complex suggesting that MT1-MMP and $\alpha 2\beta 1$ appear to be interdependent for their association with the lumen signaling complex. This makes considerable sense since they both bind collagen triple helices to perform their functions. It is also possible that binding of collagen by MT1-MMP and $\alpha 2\beta 1$ plays a role in the preparation of collagen type I for proteolysis that is critical for the lumen formation process. Collagenolytic MMPs do appear to play a role in facilitating unwinding of the collagen triple helix so that specific cleavage of collagen type I occurs (Chung et al., 2004; Lauer-Fields et al., 2002). JamC, which appears to associate with the MT1-MMP/$\alpha 2\beta 1$ complex, also shows affinity for JamB, a known binding partner for JamC. Both JamB and JamC bind through their cytoplasmic tails to Par3, a scaffolding protein with affinity for both Par6 and atypical PKC isoforms (Sacharidou et al., 2010). Par6 binds Cdc42-GTP to complete the signaling complex (Fig. 3.6). Interestingly, new data suggests that Rac1-GTP is also part of the EC lumen signaling complex, perhaps through its binding affinity for Par6 (Sacharidou et al., unpublished observations).

Expression of cytoplasmic tail deleted forms from either JamB or JamC completely inhibits EC lumen formation and Cdc42 activation (Sacharidou et al., 2010). Interestingly, these tail deleted mutants selectively block Cdc42 activation in 3D collagen matrices but not on 2D collagen surfaces. Also, the Jam tail mutants do not affect RhoA activation in 3D matrices or on 2D collagen surfaces. Interestingly, we observed that JamA appears to block EC lumen formation in that siRNA suppression increases lumen formation while increased expression of JamA blocks lumen formation and Cdc42 activation (Sacharidou et al., 2010). One obvious possibility is that increased expression of JamA blocks this process by competing for Par3 binding and thus interferes with the ability of JamB and JamC to bind. Further investigation is necessary to address this issue. Overall, our data indicates that interactions between JamB and JamC with Par3 are necessary for the lumen formation process. One question of great interest to us is to determine the identity of the Cdc42 GEF that are responsible for Cdc42 activation during these events. Also, we wish to determine how this Cdc42

GEF interacts with the EC lumen signaling complex since we hypothesize that it will show affinity for one of the components within the complex. Considerable future work will be necessary to identify other components of this signaling complex as well as to identify both positive and negative regulators of the complex that modulate these processes.

5. Roles of MT1-MMP and Vascular Guidance Tunnels in EC Lumen Formation and Tube Remodeling Events

MMPs are a family of zinc-dependent metalloendopeptidases that degrade a variety of substrates to affect the vasculature and other tissues (Arroyo and Iruela-Arispe, 2010; Davis and Saunders, 2006; Davis and Senger, 2008; Gill and Parks, 2008; Handsley and Edwards, 2005). There are also interesting data suggesting that some MMPs as well as tissue inhibitor of metalloproteinases (TIMPs) can act in a manner independent of their proteolytic or proteolytic inhibitory activity, respectively (Baker et al., 2002; Brew and Nagase, 2010; Gonzalo et al., 2010; Seo et al., 2003; Stetler-Stevenson and Seo, 2005). In terms of their proteolytic activity, MMP proteolytic targets include the ECM, cytokines, and cell surface receptors to affect cellular responses. A related set of metalloproteinases are the ADAM and ADAMTS family of enzymes which represent both cell surface (ADAM) and secreted proteins (ADAMTS) (Apte, 2009; Blobel, 2005; Edwards et al., 2008). These enzymes have a similar spectrum of proteolytic targets like the MMPs in terms of cell surface and ECM proteins. ADAM-10 and ADAM-17 are both known to shed a variety of proteins including cell surface expressed growth factors such as the EGF family of growth factors and TNF family isoforms (Blobel, 2005). ADAM-10 also plays a critical role in processing of Notch proteins (Hartmann et al., 2002) that play a fundamental role during vascularization responses.

5.1. MT1-MMP controls EC lumen formation and EC sprouting events in 3D matrices

Our work and that of others has demonstrated a role for MT1-MMP (i.e., MMP-14) in EC morphogenic events in 3D matrix environments (Chun et al., 2004; Haas et al., 1999; Lafleur et al., 2002; Saunders et al., 2006; Stratman et al., 2009b). MT1-MMP is a transmembrane protein and its cell surface expression is required for it to perform the localized ECM degradation necessary to control cell movement and morphogenic responses in 3D matrices (Fisher et al., 2006, 2009; Itoh and Seiki, 2004; Ota et al., 2009; Sabeh et al., 2009). This proteinase has been shown to play a crucial role

in cell invasive behavior in cross-linked ECM environments particularly in adult tissues (Sabeh et al., 2009). Its role during embryonic development is less clear although it could participate and work with other enzymes to facilitate invasive behavior in this context. More work here is necessary to understand these events by comparing invasion responses in embryos versus postnatal animals. MT2-MMP appears to have similar abilities compared to MT1-MMP (Ota et al., 2009; Sabeh et al., 2009) while MT3-MMP appears to be distinct in its functions. However, it may be capable of acting in conjunction with MT1-MMP, MT2-MMP, or both to control cell invasion. The degree of functional redundancy among MT-MMPs, other cell surface MMPs such as ADAMs or other proteinases remains an open question that needs to be investigated during developmental vascularization.

Considerable information suggests that membrane anchorage of the MMP is crucial to its ability to stimulate cell invasive behavior (also, a necessary function for EC tubulogenesis). For example, soluble MMP-1 is not able to regulate cell invasive behavior but when its catalytic domain is swapped in to replace the catalytic domain of MT1-MMP, it can promote cell invasion only if it is membrane tethered (Li et al., 2008). Knockout of MT1-MMP in mice is compatible with embryogenesis but the mice are very small and sickly and typically die within a month or two after birth (Zhou et al., 2000). It appears that there is a marked reduction of vessel formation within developing bones even in embryonic life (perhaps due to increased ECM density within this developing tissue). Attempts to induce angiogenesis in postnatal MT1-MMP knockout mice reveal that these responses do not occur (Zhou et al., 2000) and aortic ring assays show no sprouting in either 3D collagen or fibrin matrices compared to controls (Chun et al., 2004). Thus, MT1-MMP plays an important role in controlling vascular morphogenic events in 3D matrices and during *in vivo* angiogenic responses.

To elucidate molecular mechanisms underlying how MT1-MMP controls vascular tube morphogenesis and cellular invasive events, our laboratory has examined this question using both vasculogenesis and angiogenesis models (Fig. 3.13) (Bayless and Davis, 2003; Sacharidou et al., 2010; Saunders et al., 2006; Stratman et al., 2009b). EC sprouting in response to S1P (incorporated into the collagen matrices) was blocked by the broad spectrum inhibitor, GM6001, as well as TIMP-2, TIMP-3, and TIMP-4, but not TIMP-1 (Bayless and Davis, 2003). MT-MMPs are insensitive to TIMP-1, but the other inhibitors utilized block their activity. GM6001, TIMP-2, TIMP-3, and TIMP-4, all block EC lumen and tube formation completely, while TIMP-1 had no influence (Saunders et al., 2006; Stratman et al., 2009b; Fig. 3.13). One important distinction among the TIMPs is that TIMP-3 is able to block the activity of MT-MMPs but also many members of the ADAM family of metalloproteinases including ADAM-17 and ADAM-10 (Baker et al., 2002; Brew and Nagase, 2010).

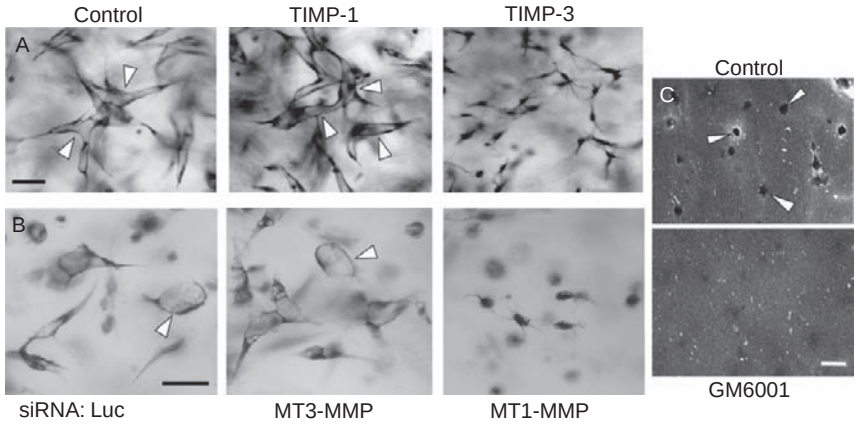

Figure 3.13 MT1-MMP activity is required for EC lumen and tube formation and generation of vascular guidance tunnels in 3D collagen matrices. (A) Representative images from 3D cultures of ECs seeded within collagen type I matrices in the presence or absence of recombinant TIMP-1 or TIMP-3 added at 5 μg/ml. Cultures were fixed, stained, and photographed at 24 h. White arrowheads indicate EC lumenal structures. Bar equals 50 μm. (B) Representative images from 3D cultures of ECs seeded within collagen type I matrices after having undergone treatment with the indicated siRNAs. Cultures were fixed, stained, and photographed at 24 h. White arrowheads indicate EC lumenal structures. Bar equals 50 μm. (C) Representative images of ECs seeded within FITC-labeled collagen type I matrices in the presence or absence of the proteinase inhibitor GM6001 added at 5 μM. Cultures were fixed and photographed using confocal microscopy at 24 h. White arrowheads indicate vascular guidance tunnels generated as a consequence of the EC lumen and tube formation. Bar equals 100 μm.

To functionally dissect which EC surface expressed metalloproteinases were relevant during lumen formation or EC sprouting, siRNA suppression analysis was performed. These results suggested that the dominant metalloproteinase controlling these events is MT1-MMP (Fig. 3.13), with a lesser influence of MT2-MMP during both sprouting and lumen formation (Sacharidou et al., 2010; Saunders et al., 2006; Stratman et al., 2009b). Also, a partial blocking effect of ADAM-15 siRNA knockdown in EC sprouting assays was observed using SDF-1α as the invasion stimulus (Saunders et al., 2006), in support of previous work showing a role for ADAM-15 in regulating angiogenic responses (Horiuchi et al., 2003). There was no influence of either MT3-MMP or ADAM-17 siRNAs in these invasion responses (Saunders et al., 2006). Further important support for a role for MT1-MMP during EC tubulogenesis is that increased expression of MT1-MMP using viral vectors leads to marked increases in lumen formation that depends on its MMP catalytic domain and activity since GM6001 addition completely inhibits the stimulatory influence of the

recombinant protein and a catalytically inactive recombinant protein fails to stimulate lumen formation (Stratman et al., 2009b). In very recent experiments, we have further shown that increasing the expression of wild-type, full-length MT1-MMP increases both the rate and extent of EC lumen formation in 3D collagen matrices (Sacharidou et al., 2010). Furthermore, we have generated a construct (an MT1-MMP active site mutant without its cytoplasmic tail) that behaves like a dominant negative mutant of MT1-MMP since its expression in the presence of wild-type MT1-MMP completely blocks the ability of ECs to form lumens and tubes (Sacharidou et al., 2010). It also completely inhibits the formation of vascular guidance tunnels, the carved out spaces of degraded ECM which characterize the lumen formation process. An additional finding is that expression of wild-type MT1-MMP without its cytoplasmic tail markedly stimulates the rate and extent of EC lumen formation even compared to increased expression of full-length wild-type MT1-MMP (Sacharidou et al., 2010). A number of studies suggest that the cytoplasmic tail of MT1-MMP plays a role in increased endocytic recycling (and possible turnover of MT1-MMP) and thus, deleting the tail increases cell surface expression. Overall, these data demonstrate that MT1-MMP is a major regulator of EC lumen and tube formation and that it works closely in conjunction with the collagen-binding integrin, $\alpha 2\beta 1$, as well as Cdc42- and Rac1-dependent signaling to control this process (Fig. 3.6).

5.2. MT1-MMP-dependent EC tubulogenesis leading to the formation of a network of physical spaces in 3D ECM

A critical role for MT1-MMP during EC lumen and tube formation is to create networks of physical spaces within the ECM that we term vascular guidance tunnels (Stratman et al., 2009b). These form as a result of MT1-MMP-mediated proteolysis of collagen matrices and can be demonstrated to be physical spaces by microinjection of silicone oil. In every instance that our laboratory has examined, there is a direct relationship between EC tube formation and the formation of vascular guidance tunnels (Stratman et al., 2009b). Thus, the lumen and tube formation mechanism must be integrated directly with cell surface proteolysis to create these physical spaces. For example, inhibitors that interfere with EC lumen and tube formation including anti-$\alpha 2$ and anti-$\beta 1$ integrin blocking antibodies, chemical inhibitors of PKCε and Src, as well as MT1-MMP inhibitors, completely abrogate the formation of vascular guidance tunnels (Stratman et al., 2009b). The presence of these tunnels raised the interesting possibility that vessel remodeling could occur through these preformed physical tunnel spaces which are generated as part of the EC morphogenic cascade in 3D matrices. Although MT1-MMP was necessary for the formation of vascular guidance tunnels and initial EC migratory events that are required to

assemble tubes, once tunnels were formed, MT1-MMP blockade did not affect the ability of ECs to migrate within the spaces (Stratman et al., 2009b). Thus, vascular guidance tunnel spaces are similar to 2D matrix surfaces where EC motility is insensitive to MT1-MMP inhibition (Stratman et al., 2009b). Interestingly, ECs have been shown to rapidly migrate *in vivo* during vascular development to regulate both tube assembly and vascular remodeling (Lucitti et al., 2007; Sato et al., 2010). Clearly, EC tube assembly is an obligate step for the formation of vascular guidance tunnels and thus these processes are directly linked. Several critical questions arise from these insights including how EC lumen and tube formation are functionally connected with the cell surface proteolytic machinery to create vascular guidance tunnels. Also, what is the functional purpose of vascular guidance tunnels during vascular tube assembly, remodeling, and maturation events?

5.3. Functional roles of EC-generated vascular guidance tunnels during vascular tube assembly and remodeling

ECs utilize a lumen signaling complex to form tubes in 3D matrices while at the same time generate networks of vascular guidance tunnel spaces. During early vascular development, there is evidence for marked tube remodeling that occurs following the onset of flow (Lucitti et al., 2007), and we hypothesize that this occurs due to the presence of vascular guidance tunnels which allows for ECs to rapidly rearrange to accommodate flow and pressure forces that are applied. Interestingly, addition of MMP inhibitors that block lumen formation (i.e., TIMP-3 and GM6001), but not TIMP-1, interfere with vascular remodeling events in rotating mouse embryo cultures suggesting that this flow-induced remodeling may also depend on MMP activity (Saunders et al., 2006). Increased vessel width, which occurs during this remodeling step *in vivo*, depends on MT1-MMP activity in our 3D collagen matrix cultures (Stratman et al., 2009b). It remains to be determined if MT1-MMP, other MMPs, ADAMs, or ADAMTS enzymes control these events during this developmental vascular remodeling event. In addition, at this stage of development, the ECM is likely to be more mechanically pliable and, thus, the applied forces generated could expand lumen or tunnel widths. We have also shown that groups of cells comprising a tube structure can migrate together through vascular guidance tunnels to connect with adjacent EC tubes to affect vascular remodeling (Stratman et al., 2009b). Interestingly, the EC lumen and tube formation process generates more vascular guidance tunnels than are utilized in 3D collagen matrices (Stratman et al., 2009b). If this occurs *in vivo*, the extra tunnels could allow for vascular remodeling events to rapidly occur when external forces (i.e., flow) are applied or other cell types such as pericytes are recruited so that a proper microcirculatory network matches the flow

patterns and oxygenation requirements of the developing tissues being perfused. Similar principles should apply in adult tissues following tissue injury or other pathologic events that lead to vascularization responses.

5.4. Vascular guidance tunnels as matrix conduits that can be utilized for vessel regrowth following vascular tube regression

Vascular guidance tunnels are also important to consider in the context of vascular tube regression and regrowth of vessels. We have previously reported that thrombin addition to networks of EC-lined tubes can induce rapid tube collapse, but when thrombin activity is reversed by addition of the thrombin inhibitor, hirudin, vessels can rapidly reform within the vascular guidance tunnel space that was previously lined by an intact vessel tube (Stratman et al., 2009b). Thus, vascular regrowth can clearly occur along preformed matrix conduits (i.e., vascular guidance tunnels) and if these were eliminated along with loss of the tubes, this would strongly decrease the possibility of vessel regrowth. We have previously reported that both EC-produced MMP-1 and MMP-10 play a role in vascular tube regression and together they are capable of collapsing both tubes and vascular guidance tunnel networks due to the fact that the collagen matrix scaffold is degraded during this process (Davis and Saunders, 2006; Davis et al., 2001; Saunders et al., 2005). Thus, such a regression mechanism would eliminate the possibility of vessel regrowth following a tissue injury response. The presence of pericytes, which blocks MMP-1- and MMP-10-dependent tube regression event in our *in vitro* model (Saunders et al., 2006), can thus protect the vascular tube structure but also the integrity of the vascular guidance tunnels through MMP inhibition (i.e., through EC TIMP-2 and pericyte TIMP-3) (Fig. 3.10).

Of related interest here is that tumor vessels are highly resistant to vascular regression. It is possible that this lack of regression is due to their production of regression inhibitors such as TIMP-1 and PAI-1 (Davis and Saunders, 2006). TIMP-1 is capable of protecting both the vessels and the tunnel spaces by blockade of MMP-1 and MMP-10 activity (Davis and Saunders, 2006; Saunders et al., 2005). Interestingly, when tumors are treated with vascular regression agents such as VEGFR2 antagonists, vessels regress, but they can rapidly regrow (following withdrawal of the regression agent) in a manner that appears to recapitulate the original vessel pattern (Mancuso et al., 2006). This may occur through vascular guidance tunnels that were previously generated during initial tumor vessel formation. So an important therapeutic consideration here in the context of tumor treatment would be to devise approaches to induce both vessel as well as vascular guidance tunnel regression within tumors. In this manner, vessel regrowth would be less likely to occur, allowing for a better therapeutic opportunity

to treat tumors, as well as its associated vasculature and matrix conduits (that may also facilitate local tumor invasive behavior) to affect vascular and tumor regrowth.

5.5. Potential role for vascular guidance tunnels in arteriovenous differentiation events

It is also important to consider how developmental events such as arteriovenous identity and sorting (Herbert et al., 2009; Kim et al., 2008; Swift and Weinstein, 2009; Wang et al., 1998) could be regulated by vascular guidance tunnels since they represent a 2D matrix surface in a 3D matrix environment (Stratman et al., 2009b). EphrinB2 (an arterial marker) and EphB4 (a venous marker) represent a repulsive signaling pair that appear to distinguish ECs that differentially express these arterial versus venous markers (Wang et al., 1998). These repulsive interactions allow for differential cell sorting when cells are in contact on a 2D matrix surface. Early in development, ECs expressing these different markers are intermixed, but with time they segregate to either the arterial or venous side (Herbert et al., 2009; Kim et al., 2008; Wang et al., 1998). Differential sorting is observed very early in development even at the level of initial cardinal vein formation due to sprouting from the aorta (Herbert et al., 2009). This process appears analogous to that described for lymphatic sprouting and development from the cardinal vein (Yaniv et al., 2006). Also, Notch signaling controls this phenomenon and when constitutively active Notch-4 is expressed in ECs, venous ECs inappropriately express ephrinB2 which contributes to the development of arteriovenous malformations (Kim et al., 2008). Interestingly, many of these lesions regress following withdrawal of activated Notch4. Repulsive ephrinB2–EphB4 interactions should occur within vascular guidance tunnels and the ability of ECs to segregate following such interactions requires their ability to migrate on 2D matrix surfaces within tunnels to assemble together with other arterial or venous ECs. Disruption of either arterial or venous differentiation leads to major vascular developmental defects or vascular malformations (Kim et al., 2008); so it is likely that EC–EC interactions within vascular guidance tunnels play a major role in controlling appropriate cell sorting and differentiation functions.

The concept of vascular guidance tunnels also extends to recruitment and sorting of mural cells within the vessel wall. For example, ephrinB2 is also expressed on vascular smooth muscle cells with selectivity in arteries, so similar repulsive interactions are likely to also control differential mural cell sorting in the arterial versus venous vasculature (Foo et al., 2006). Interestingly, mural cell recruitment to EC-lined tubes and within vascular tunnel spaces occurs in a polarized fashion exclusively on the EC ablumenal surface (Stratman et al., 2009a), which would be required in order for such mural

cell–mural cell repulsive interactions to allow them to be sorted similarly to ECs. Thus, mural cells could sort through repulsive interactions with each other but may also occur through repulsive interactions with ECs. The vascular guidance tunnel matrix conduit is a critical ECM structure that is necessary in order for these EC–EC, mural cell–mural cell, and EC–mural cell interactions to occur and to also allow for motility events required for proper sorting along vessels in a 3D matrix environment. In support of these possibilities are that ECs and mural cells are highly migratory during vascular tube assembly within vascular guidance tunnels during tube coassembly and maturation events (Stratman et al., 2009a, 2010).

5.6. Vascular guidance tunnels as matrix conduits that allow for dynamic EC–pericyte interactions necessary for vascular tube maturation and basement membrane matrix assembly events

Although pericytes were known to accumulate along the EC ablumenal tube surface, how this recruitment and how such interactions occurred in 3D tissue spaces were not previously well understood. Our new findings show that EC tubulogenesis leads to the generation of vascular guidance tunnel networks within 3D ECM and that these matrix conduits are physical spaces where ECs and pericytes interact to regulate EC tube remodeling, maturation, and stabilization events (Stratman et al., 2009a). Thus, pericytes that recruit to EC-lined tubes are actually within tunnel spaces (Fig. 3.8). These matrix spaces allow for rapid EC and pericyte motility within them to control these events (Stratman et al., 2009a). Interestingly, both EC and pericyte motility within these preformed spaces is MMP-independent. Real-time videos showed for the first time the ability of pericytes to actively recruit to tubes in 3D matrices but also to move along EC tube ablumenal surfaces to regulate tube maturation and vascular basement membrane matrix assembly (Stratman et al., 2009a). Pericyte recruitment to EC tubes in our defined serum-free system depends on EC-derived PDGF-BB and HB-EGF which controls directed pericyte motility and proliferation in 3D collagen matrices (Stratman et al., 2010). Also, this recruitment process leads to vascular basement membrane matrix assembly in between ECs and pericytes and within vascular guidance tunnels (Fig. 3.7). Blockade of PDGF-BB and HB-EGF interferes with pericyte accumulation within vascular guidance tunnels and along EC tubes and, importantly, vascular basement membrane assembly is blocked despite the close proximity of ECs and pericytes (Stratman et al., 2010). Thus, direct EC and pericyte contact within vascular guidance tunnels is necessary to affect basement membrane formation around EC tubes.

6. Mechanisms Controlling EC Lumen and Tube Stability

6.1. Pericyte recruitment to EC-lined tubes regulates vascular tube maturation and stabilization

Many studies indicate that microvessels covered with pericytes are more stable to proregressive stimuli and they show reduced vascular permeability indicative of tube stabilization. Interestingly, angiopoietin-1 is a growth factor that acts through EC Tie-2 to facilitate this tube stabilization process (Thomas and Augustin, 2009). Pericytes are believed to be a major source of angiopoietin-1 to act on ECs to facilitate tube stabilization in part through EC junction stability (Thomas and Augustin, 2009) (Fig. 3.14). Delivery of recombinant angiopoietin-1 *in vivo* has been shown to decrease vascular

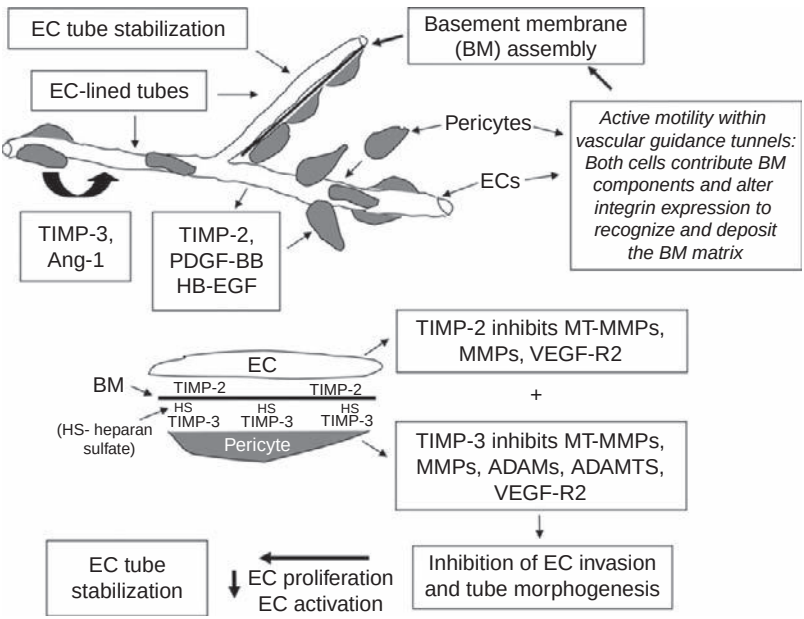

Figure 3.14 Mechanisms controlling of EC–pericyte tube stabilization in 3D extracellular matrices. A schematic diagram is shown that describes factors and mechanisms that control vascular tube stabilization in response to EC–pericyte interactions. Pericyte recruitment to tubes leads to vascular basement membrane matrix assembly which occurs within EC-generated vascular guidance tunnels. The pericyte-derived factors, TIMP-3 and angiopoietin-1, and the EC-derived factors, TIMP-2, PDGF-BB, and HB-EGF, play a role in both pericyte recruitment and stabilization events. TIMP-2 and TIMP-3 together block both EC tube formation as well as regression events and also promote stabilization by inhibition of VEGFR2.

permeability to angiogenic vessels (Thurston et al., 2000). Different vascular beds have varying pericyte numbers covering capillary networks although many have approximately 20–25% coverage of pericytes relative to ECs. Individual pericytes can span to touch multiple ECs which resemble other types of supporting cells such as glia in the nervous system interacting with multiple neurons. Tissues such as the central nervous system (including the retina) have a very high pericyte to EC ratio which approaches 1:1 (Armulik et al., 2005). Recent work suggests that pericyte recruitment to EC tubes plays a role in the development of the blood–brain barrier which shows strongly increased barrier functions with reduced vascular permeability relative to other vascular beds (Armulik et al., 2010; Daneman et al., 2010). Considerable work suggests that the high expression of VEGF within tumors is one reason why pericyte coverage is decreased compared to normal vascular beds (Greenberg et al., 2008; Jain, 2005) and why the vessels within the tumor microenvironment are highly permeable. Interestingly, treatment with VEGF antagonists leads to increased pericyte coverage which results in improved microcirculatory function (i.e., vascular normalization) with decreased permeability (Greenberg et al., 2008; Jain, 2005). This work has lead to a new therapeutic strategy to improve drug delivery into tumors.

6.2. Molecular mechanisms underlying why pericytes are able to stabilize EC-lined tube networks

A major question that has not been sufficiently addressed is why pericyte coverage stabilizes vessels and what their functions are when they arrive at the EC ablumenal surface. To address this question, we established novel EC–pericyte coculture models in 3D collagen matrices and have developed systems using either bovine retinal pericytes or human brain pericytes mixed with human ECs (typically HUVECs). Both pericyte populations express the pericyte markers, NG2 proteoglycan, PDGFRβ, 3G5 ganglioside, smooth muscle actin and desmin. Perhaps the most important known function of pericytes is to recruit to microvascular capillary beds which then regulates vascular tube maturation and stabilization. Using a model of EC vasculogenic tube assembly, we have developed a system whereby ECs and pericytes are randomly mixed at a 5:1 or 5:1.25 ratio (i.e., 20–25% pericytes compared to 100% of ECs) under serum-free defined conditions (Stratman et al., 2009a). Remarkably, ECs form tube networks which are then followed by pericyte recruitment to these tubes (Fig. 3.8). This ratio of ECs to pericytes is particularly optimal for tube assembly and subsequent pericyte recruitment but the reasons for this are currently not clear. Perhaps too many pericytes (through their production of TIMP-3) relative to ECs (Saunders et al., 2006) can interfere with morphogenesis, by inhibiting

MT1-MMP-dependent signaling, or by counteracting the ability of ECs to find other ECs to properly form multicellular tubes.

Furthermore, EC tube diameters from EC-only cultures eventually become much wider than EC tubes from EC–pericyte cocultures. Vascular tube diameters reached a range of 20–25 μm in EC–pericyte cocultures which are vessel diameters observed *in vivo* during vasculogenesis, while in EC-only cultures, diameters can reach 80–100 μm over a 5-day period (Stratman et al., 2009b). Thus, pericytes have the ability to negatively regulate vascular tube diameters which may have to do with the induction of TIMP-2 and TIMP-3 from ECs and pericytes, respectively. As discussed earlier, these TIMPs are induced in EC–pericyte cocultures (Fig. 3.14) (Saunders et al., 2006) and can restrict EC lumen diameters. Furthermore, pericyte recruitment leads to basement membrane assembly and laminins can inhibit tube morphogenesis and may contribute to vascular diameter as reported using laminin knockout embryoid body cultures where vessel formation occurs but with wider lumens than controls (Jakobsson et al., 2008). A number of studies indicate that vascular diameters are greater when pericyte recruitment is reduced or when ECM components such as fibronectin are knocked out of ECs during vasculogenesis *in vivo* (Astrof et al., 2007; Francis et al., 2002). We recently demonstrated the same phenomenon when PDGF-BB and HB-EGF are inhibited together which blocks pericyte recruitment to EC tubes, a condition leading to increased EC tube widths *in vitro* and *in vivo* (Stratman et al., 2010).

Previous work indicated that pericytes could be attracted to ECs on 2D plastic surfaces through PDGF-BB (Bjarnegard et al., 2004; Gaengel et al., 2009; Hirschi et al., 1998). Interestingly, EC-specific knockout of PDGF-BB leads to about a 50% decrease in pericyte coverage of vessels (Bjarnegard et al., 2004), although it is unclear whether this is due to effects on pericyte proliferation, recruitment, or both. Of interest is that these mice show primary defects in microvessel beds (where pericyte coverage is present) while larger vessels are much less affected. This microvascular deficiency phenotype manifests particularly in the kidney and central nervous system which strongly resembles that observed in diabetic microangiopathy (Bjarnegard et al., 2004). Loss of pericytes is a major pathogenic cause of this type of microvascular disease (Hammes, 2005). It is important to further understand the signaling mechanisms which underlie pericyte recruitment to EC-lined tubes to both identify other factors that regulate this recruitment as well as understand how pericytes invade 3D matrices to recruit to these tubes.

A new study by our group addresses these questions using our EC–pericyte tube coassembly model under defined serum-free conditions. The work reveals that EC-derived PDGF-BB and HB-EGF control pericyte recruitment to EC-lined tubes in 3D collagen matrices (Stratman et al., 2010). Under these conditions, pericyte motility in 3D collagen matrices

depends on the co-presence of ECs which produce both PDGF-BB and HB-EGF (Stratman et al., 2010). Without ECs, pericyte motility is minimal and shows no directional component. This was demonstrated using blocking antibodies to the two growth factors or using soluble receptor traps with affinity for these factors. To confirm these findings, chemical inhibitors directed to PDGF and EGF receptors or blocking antibodies to PDGF-BB and HB-EGF interfered with pericyte recruitment to EC tubes when administered *in vivo* to developing quail embryos (Stratman et al., 2010).

6.3. Pericyte recruitment to EC-lined tubes stimulates ECM remodeling events

Also, we sought to elucidate how pericytes, through their recruitment to tubes, affect vascular tube maturation and stabilization events. At different time points of tube coassembly, immunofluorescence microscopy and transmission electron microscopy were performed to examine if basement membrane matrix assembly occurred (Stratman et al., 2009a). In Fig. 3.7, we demonstrated immunostaining for the EC-specific marker, CD31, while the pericytes express green fluorescent protein (GFP), and were shown to be recruited to the EC tube ablumenal surface. This was further demonstrated in cross sections which showed both EC lumens and associated pericytes selectively on this ablumenal surface (Fig. 3.8). In addition, there was marked deposition of collagen type IV, a critical basement membrane matrix component along this ablumenal EC surface (Fig. 3.7). We recently reported that fibronectin, laminin, nidogen-1, nidogen-2, and perlecan were also strongly deposited around EC-lined tubes only when ECs and pericytes were cocultured (Stratman et al., 2009a). Furthermore, we demonstrated that basement membrane matrices were observed by transmission electron microscopy only when EC–pericytes were cocultured (Stratman et al., 2009a) (Fig. 3.7). In our work over many years of study, we have never observed basement membrane deposition around EC tubes in the absence of pericytes using electron microscopic analysis. Importantly, these results were confirmed *in vivo* showing that pericyte recruitment to developing quail EC tubes directly correlates with vascular basement membrane assembly at day 7 of embryonic development and this was also documented using both transmission electron microscopy and immunofluorescent staining (Stratman et al., 2009a). Prior to pericyte recruitment, no vascular basement membranes around EC tubes were observed *in vivo*. Also, we recently reported that blockade of pericyte recruitment *in vitro* or *in vivo* blocks vascular basement membrane assembly, despite the close proximity of both cell types (Stratman et al., 2010). This result is very intriguing and suggests that physical heterotypic cell–cell contacts and pericyte motility along tubes are necessary events for basement membranes to form.

The described immunostaining experiments were performed utilizing detergent-free conditions so that only ECM that is deposited extracellularly was examined (i.e., excluding intracellular ECM molecules) (Stratman et al., 2009a). We utilized this strategy for our *in vitro* 3D cultures, but also stained an *in vivo* tissue, the quail chorioallantoic membrane, in an identical manner (Stratman et al., 2009a). This is a central point because we demonstrated that extracellular deposition of vascular basement membrane matrix is markedly stimulated by pericyte recruitment both *in vitro* and *in vivo* (Stratman et al., 2009a).

Human EC and bovine pericyte cocultures were utilized to determine which cell types produce particular ECM components over time to regulate basement membrane matrix assembly (using species-specific RT-PCR primer sets). ECs were shown to increase their production of fibronectin selectively in the presence of pericytes (and not in their absence) and nidogen-1 was induced in pericytes selectively when ECs were present (Stratman et al., 2009a). Also, we observed induction of particular laminin isoforms as well as perlecan at the mRNA level that occurred in response to EC–pericyte interactions. Thus, EC–pericyte contacts during tube coassembly events affected mRNA and protein levels for key basement membrane matrix molecules (Stratman et al., 2009a). Interestingly, both fibronectin and nidogen-1 are known to bridge basement membrane matrix proteins together which are essential for the assembly of this specialized matrix (Davis and Senger, 2005; Miner and Yurchenco, 2004; Senger and Davis, 2010). For example, fibronectin binds collagen type IV and perlecan, while nidogen-1 binds collagen type IV and laminin isoforms. Thus, it is possible that fibronectin and nidogen-1 create a nidus which leads to assembly of the insoluble basement membrane matrix around EC-lined tubes. Most ECM proteins possess self-assembly functions but it is also known that they need to interact with one another to create the complex meshwork that characterizes an assembled basement membrane matrix (Miner and Yurchenco, 2004). Collagen type IV, a fundamental basement membrane component that is greatly responsible for its structural integrity, shows affinity for both fibronectin and nidogen-1, and this may be one reason why EC–pericyte interactions are necessary for this essential ECM remodeling event during vascular wall assembly (Stratman et al., 2009a). Interestingly, pericyte-induced fibronectin assembly around EC tubes appears also to directly affect collagen type IV assembly (Stratman et al., 2009a). To assess which cell types contributed to collagen type IV assembly during this process, siRNA suppression experiments were performed showing that ECs were the major source of deposited collagen type IV. Knockdown of collagen type IV in ECs strongly reduced collagen type IV deposition and this resulted in increased vascular tube width (Stratman et al., 2009a). Knockdown of collagen type IV in pericytes had a lesser influence, but nonetheless an inhibitory effect was detected. Thus, ECs and pericytes

both contribute collagen type IV during vascular basement membrane assembly in 3D matrices (Fig. 3.7).

We further addressed how continuous basement membrane assembly is accomplished along EC-lined tubes despite the fact that pericytes represent only one-fifth to one-fourth of the total number of ECs. Our data suggests that this occurs due to the dramatic motility of pericytes along the ablumenal EC tube surface (demonstrated by real-time videos of these events) which scan along the tubes to stimulate the deposition of the basement membrane in a continuous manner (Stratman et al., 2009a) (Fig. 3.7). We speculate that the movement of both pericytes and ECs along each other within vascular guidance tunnels exerts mechanical forces on newly deposited ECM components to facilitate basement membrane assembly. Thus, it is intriguing that fibronectin, a mechanosensitive ECM component and whose assembly is facilitated by cell-exerted tensional forces (Smith et al., 2007; Vogel, 2006; Zhong et al., 1998; Zhou et al., 2008), is a basement membrane component that strongly deposits along EC-lined tubes when pericytes are present *in vitro* and also *in vivo* (Stratman et al., 2009a). Disruption of pericyte recruitment *in vivo* leads to markedly decreased fibronectin deposition around EC tubes and fibronectin is known to play a fundamental role during cardiovascular development in mouse knockout studies (Francis et al., 2002; Hynes, 2007). Another interesting possibility is that the polarized presence of pericytes along the EC ablumenal surface stimulates the directional secretion and selective deposition of basement membrane components from both cell types toward the other. Thus, both mechanical forces and vectorial secretion mechanisms may account for how pericyte recruitment to EC-lined tubes leads to vascular basement membrane matrix assembly. Further work needs to define these issues in more detail and also examine whether production of particular ECM components or isoforms made by either ECs or pericytes might play crucial roles during this fundamental process.

6.4. Pericyte recruitment and vascular basement membrane matrix assembly alters integrin requirements

Again using a coculture system with human ECs and bovine pericytes, EC versus pericyte integrin expression was assessed during this process. mRNA levels were determined for both cell types and function-blocking experiments with anti-integrin monoclonal antibodies were performed at various times during the EC–pericyte tube coassembly process. Importantly, blocking antibodies to the fibronectin-binding $\alpha5\beta1$ integrin had function-blocking effects that selectively occurred in the EC–pericyte cocultures and not in EC-only cultures (Stratman et al., 2009a). Also, the EC $\alpha5$ integrin subunit mRNA was induced in EC–pericyte cocultures, and not in EC-only cultures where it was downregulated. Overall, an important

theme that was evident from these studies is that integrins with affinity for basement membrane components were upregulated in both ECs and pericytes in cocultures, but not in EC- or pericyte-only cultures (Stratman et al., 2009a). In contrast, the α2 integrin, which recognizes collagen type I, is downregulated in cocultures but is upregulated in EC-only cultures. Thus, as basement membranes assemble around EC tubes in EC–pericyte cocultures, the direct interaction of ECs with collagen type I decreases, while their contact with basement membrane matrix components increases. Concomitantly, increases in the expression of integrin α5, α3, and α6 from ECs were observed, which recognize fibronectin, nidogens, and laminin isoforms, while α5, α3, α6, and α1 integrin subunits were increased from pericytes which recognize fibronectin, nidogens, laminin isoforms, and collagen type IV (Stratman et al., 2009a). Also, functional deficits were observed when blocking antibodies to these integrins were administered to cocultures (but not EC-only cultures) (Stratman et al., 2009a). This data indicates that a major purpose of the multiple β1 integrins on the EC cell surface is to recognize key ECM components that they encounter at different stages of the tube morphogenic and later maturation process.

When exposed to collagen type I matrices, which serves as a strong agonist for tubulogenesis, ECs utilize collagen-binding integrins such as α2β1. However, when EC-lined tubes attract pericytes, vascular basement membranes form that are recognized by different sets of integrins such as α5β1 (a fibronectin receptor), α3β1 (a nidogen and laminin isoform receptor), α6β1 (a laminin isoform receptor), and α1β1 (a collagen type IV, collagen type I, and laminin receptor). Interestingly, α1β1 appears to be predominantly pericyte-derived during this tube coassembly process and, thus, the effects of blocking antibodies that have been observed may be due to a specific inhibitory influence on pericyte recognition of basement membrane matrices (Stratman et al., 2009a). Also, EC-dependence on α2β1 which is observed over time in EC-only cultures is lost with time in EC–pericyte cocultures as basement membrane matrix assembly occurs. Thus, exposure of ECs to collagen type I is strongly diminished while EC exposure to basement membrane proteins increases. These studies reveal that EC–pericyte interactions control vascular basement membrane matrix assembly and that critical changes occur in EC and pericyte integrin expression to recognize this newly remodeled matrix to facilitate further tube maturation events.

6.5. Pericyte-derived TIMP-3 controls vascular basement membrane matrix assembly and stability

Another important role of pericytes during these tube assembly processes is the delivery of TIMP-3, a basement membrane- and ECM-binding protein (Figs. 3.10 and 3.14). TIMP-3 has been previously shown to play a critical

role in pericyte-induced tube stabilization by blocking MMP-1, MMP-10, and ADAM-15, which together promote vascular regression events (Saunders et al., 2006) (Figs. 3.10 and 3.14). Also, TIMP-3 can inhibit further EC morphogenic events by blocking MT1-MMP function (Saunders et al., 2006). New data show that TIMP-3 plays as yet another role by facilitating collagen type IV assembly in EC–pericyte cocultures. siRNA suppression of pericyte TIMP-3 results in markedly decreased collagen type IV deposition around developing tubes in 3D collagen matrices (Stratman et al., 2009a). The mechanism by which TIMP-3 exerts its effects is not entirely clear but a major effect likely relates to its inhibition of MT1-MMP which can degrade type IV collagen. With decreased collagen type IV assembly around EC tubes when pericyte TIMP-3 expression is suppressed, there was a significant increase in vessel diameter (Stratman et al., 2009a). Thus, collagen type IV assembly around developing tubes may be a primary determinant of vessel diameter. It is intriguing to consider the fact that EC-only tubes, which are not surrounded by basement membrane matrices, become very wide during morphogenic events (secondary to continued morphogenesis) suggesting a lack of inhibitory signals. Those inhibitory signals are likely to be TIMP-3 and specific ECM components including laminin isoforms and collagen type IV that are delivered to ECs following interactions with pericytes (and which results in much narrower tube widths) (Stratman et al., 2009a). The marked differences in vessel diameter in these two situations demonstrate functional evidence for basement membrane assembly but also demonstrate that ECs recognize the deposited proteins and respond by restricting tube diameter. Thus, EC tube diameter is an important indicator of functional EC–pericyte interactions that lead to vascular stabilization.

7. Role for MMPs in the Molecular Control of Vascular Tube Regression Responses

An understudied area is the molecular basis for blood vessel regression under both physiologic and pathophysiologic situations. Physiologic regression occurs in the hyaloid vasculature (i.e., developing eye), during the menstrual cycle in the endometrium and ovaries, and vascular remodeling events during development (Davis and Saunders, 2006). Pathophysiologic regression occurs during wound repair and processes such as hypertension and diabetes where vessel densities decrease and particular vessels such as arterioles can be shown to be reduced in tissues (Greene et al., 1989; Schiffrin, 2004). Also, considerable efforts have been put forth to regress the tumor vasculature using a variety of agents including antagonists of VEGF, VEGFR2, and PDGF signaling as well as many other agents

including soluble VEGF traps (Bergers et al., 2003; Sela et al., 2008). An important point is that it is necessary to investigate and understand the molecular basis for vascular regression in much the same manner that prior studies investigating blood vessel formation have been performed.

7.1. MMP-1, MMP-10, and ADAM-15 control vascular tube regression responses

A variety of past and recent studies have identified MMPs which control vascular tube regression events (Davis et al., 2001; Saunders et al., 2005; Zhu et al., 2000). The secreted MMPs, MMP-1 and MMP-10, were shown to be highly induced during EC tube morphogenesis but, interestingly, neither enzyme could be shown to be involved in tube formation in 3D collagen matrices (Saunders et al., 2005). In contrast, when pro-MMP-1 and pro-MMP-10 are activated by plasmin, plasma kallikrein, or other serine proteinases, these MMPs fail to enhance tube formation, but dramatically cause EC tube network regression and collapse (Davis and Saunders, 2006; Davis et al., 2001; Saunders et al., 2005; Fig. 3.14). Importantly, the tube morphogenic systems that our laboratory has developed are performed in the absence of serum (Koh et al., 2008b) and thus there is no source of plasminogen or plasma prekallikrein which are typically supplied in serum and also produced by the liver *in vivo*. Interestingly, this indicates that EC tube formation as well as pericyte recruitment and tube stabilization in 3D collagen matrices does not require plasminogen or plasma prekallikrein addition. When plasminogen is added, it is converted to plasmin through plasminogen activators and this leads to activation of both pro-MMP-1 and pro-MMP-10. The addition of plasminogen does not affect tube formation, but interestingly, it induces dramatic tube regression phenomena following MMP-1 and MMP-10 activation. As expected, addition of MMP inhibitors such as TIMP-1 which can block MMP-1 and MMP-10, but not MT1-MMP (and thus does not block EC lumen and tube formation), can interfere with plasmin- and plasma kallikrein-dependent tube regression since the process is mediated through these two MMPs (Davis et al., 2001; Saunders et al., 2005). In support of these findings, siRNA suppression of either MMP-1 or MMP-10 had no effect on tube formation but markedly blocked tube regression following addition of plasminogen or plasma kallikrein (Saunders et al., 2005). Interestingly, a mouse knockout of histone deacetylase 7 (HDAC7) caused a vascular hemorrhage phenotype *in vivo* during vascular development leading to embryonic lethality (Chang et al., 2006). siRNA suppression of HDAC7 resulted in marked increases in MMP-10 and marked decreases in TIMP-1 expression which appear to control this vascular developmental regression response (Chang et al., 2006).

We also demonstrated that ADAM-15 plays a role during these events *in vitro*, although its specific role was not elucidated in terms of its molecular

targets. ADAM-15 is known to be expressed in EC junctional contacts and is also capable of inducing shedding of cell surface molecules (Ham et al., 2002). Recently, ADAM proteinases and MMPs have been shown to induce shedding of multiple EC surface proteins including VE-cadherin and VEGFR2, which could play a role in vessel regression events (Schulz et al., 2008; Swendeman et al., 2008). Also, MMPs and plasmin are known to cleave VEGF that controls its release from ECM (Lee et al., 2005) which might make it more susceptible to inhibition by soluble VEGF traps such as secreted forms of VEGFR1.

7.2. EC–pericyte interactions induce the expression of TIMP-2 and TIMP-3 to block MMP-1-, MMP-10-, and ADAM-15-dependent regression

Importantly, we have demonstrated that pericytes, when added to EC cultures, can interfere with these plasminogen-induced regression events (Figs. 3.10 and 3.14). Both EC-derived TIMP-2 and pericyte-derived TIMP-3 were shown to be involved in controlling the ability of EC–pericyte cocultures to resist this mechanism of tube regression (Saunders et al., 2006). This work was an important demonstration of how pericytes contribute to EC tube stabilization by preventing EC tube regression mechanisms. EC tube regression leads to EC tube collapse and EC apoptosis where caspase-dependent cleavage of molecules such as procaspase-3, gelsolin, and Pak-2 are observed (Bayless and Davis, 2004; Davis et al., 2001) (Fig. 3.14). It is well known that pericytes protect EC tubes from proregression stimuli (Benjamin et al., 1998, 1999), but little information previously existed to explain the molecular basis for such phenomena. Multiple factors appear to contribute to pericyte-induced EC tube stability including the induction of vascular basement membranes, presentation of cytokines such as angiopoietin-1 to oppose EC-derived angiopoietin-2 (Thomas and Augustin, 2009), and delivery of TIMP-2 and TIMP-3 to block proregressive MMPs and ADAMs (Saunders et al., 2006) (Figs. 3.10 and 3.14). Interestingly, both TIMP-2 and TIMP-3 are able to block VEGFR2-mediated signaling (Qi et al., 2003; Seo et al., 2003) (Fig. 3.14). Thus, EC–pericyte interactions can lead to vascular stabilization through inhibition of both vessel formation and regression mechanisms.

8. Conclusions and Future Directions

It is clear that considerable progress has been made over the past two decades on how EC lumen and tube networks form and become stabilized by a variety of mechanisms including flow-induced changes and mural cell

recruitment. These advances have come from major technological progress in developing *in vitro* and *in vivo* models to assess these questions. In general, a key point is the ability to regulate gene expression and perform signal transduction experiments in complex 3D matrix environments both *in vitro* and *in vivo*. In this way, particular molecules and signal transduction cascades can be identified that control vascular morphogenic events. A particularly effective strategy is to combine both *in vitro* and *in vivo* experimental approaches to address these complex questions. This appears to work best when investigators combine their expertise since it is becoming increasingly apparent that each of these systems requires considerable effort to establish and perform at the level that is necessary to address such issues. In conclusion, a balanced experimental strategy using both *in vitro* morphogenesis and *in vivo* assay systems is necessary to uncover the molecular mechanisms that regulate EC lumen and tube formation. We suggest that more studies that utilize the combined expertise of groups with such skills will result in greater and more rapid advances in our molecular understanding of these events.

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CHAPTER FOUR

SUMO and Its Role in Human Diseases

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Contents

Abstract

The covalent attachment of small ubiquition-like modifier (SUMO) polypeptides, or sumoylation, is an important regulator of the functional properties of many proteins. Among these are many proteins implicated in human diseases including cancer and Huntington's, Alzheimer's, and Parkinson's diseases, as well as spinocerebellar ataxia 1 and amyotrophic lateral sclerosis. The results of two more recent studies identify two additional human disease-associated proteins that are sumoylated, amyloid precursor protein (APP), and lamin A. APP sumoylation modulates Aβ peptide levels, suggesting a potential role in Alzheimer's disease, and decreased lamin A sumoylation due to mutations near its SUMO site has been implicated in causing some forms of familial dilated cardiomyopathy.

Key Words: Sumoylation, SUMO, SUMO-1, Cancer, DJ-1, Tau, α-Synuclein, SOD1, Amyotrophic lateral sclerosis, ALS, Parkinson's disease, Alzheimer's disease, APP, Lamin, Laminopathy, Heart.

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1. Introduction

Sumoylation regulates the functional properties of a large number of proteins and thereby plays a very important role in the normal functions of cells. Further supporting the importance of sumoylation are studies that have implicated a role for small ubiquitin-like modifier (SUMO) modification in human diseases. For example, a number of studies have demonstrated that among identified sumoylated proteins are a surprisingly large number that are known to be involved in human diseases, suggesting a role for sumoylation in the pathogenesis of these diseases. These diseases and their associated sumoylated proteins include Huntington's disease (huntingtin), spinocerebellar ataxia 1 (ataxin-1), Parkinson's disease (PD) (tau, α-synuclein, DJ-1), amyotrophic lateral sclerosis (SOD1), Alzheimer's disease (tau, APP), and familial dilated cardiomyopathy (lamin A). Other studies have supported the potential relationship between sumoylation and disease by demonstrating that a number of disease states are associated with changes in levels of proteins that are involved in mediating sumoylation or desumoylation. This review will focus on what is known about the sumoylation of human disease-associated proteins and the roles this modification may play in the etiology of these diseases. We will begin with a description of the enzymes and steps involved in the sumoylation cycle, from attachment of SUMO proteins to substrate polypeptides to their removal to begin a new cycle.

2. The Sumoylation Cycle

The covalent attachment of SUMO proteins to specific lysine residues in target proteins, or sumoylation, regulates many aspects of protein function, including subcellular localization, protein–protein interactions, and transcription factor transactivation (Anckar and Sistonen, 2007; Geiss-Friedlander and Melchior, 2007; Mukhopadhyay and Dasso, 2007; Ulrich, 2008; Yeh, 2009; Zhao, 2007). Cells express three major SUMO paralogs, SUMO-1, SUMO-2, and SUMO-3, with SUMO-2 and SUMO-3 being much more similar to each other than to SUMO-1. A gene encoding SUMO-4 has been described, but it is not yet clear if endogenous SUMO-4 protein is expressed in cells (Bohren et al., 2007). Some SUMO substrate polypeptides have been found to be preferentially modified by SUMO-2/SUMO-3 versus SUMO-1, which is mediated by interaction between the substrate protein and SUMO-2 or SUMO-3. This finding provides a mechanism for the increased modification of substrates by ubc9 enzymes carrying particular SUMO polypeptides (Meulmeester et al., 2008).

Like ubiquitin, SUMO attachment to polypeptides involves a numbers of different enzymes and accessory factors that perform their function in a series of steps (shown in Fig. 4.1). In the first step of the cycle, the SUMO protein is cleaved by the SUMO-specific carboxyl-terminal hydrolase activity of a SENP (*Sen*trin-specific *P*rotease) enzyme to produce a carboxyl-terminal diglycine motif. This so-called mature SUMO protein is then covalently attached via a thioester bond to a cysteine in the SAE2 (SUMO-activating enzyme subunit 2) subunit of the heterodimeric SUMO E1-activating enzyme in an ATP-dependent reaction (Desterro et al., 1999; Gong et al., 1999; Johnson et al., 1997; Okuma et al., 1999). The SUMO moiety is subsequently transferred from the E1 to UBC9, the SUMO E2 enzyme, which then attaches the SUMO to a lysine in the target protein that is typically, but not always, found within the consensus sequence

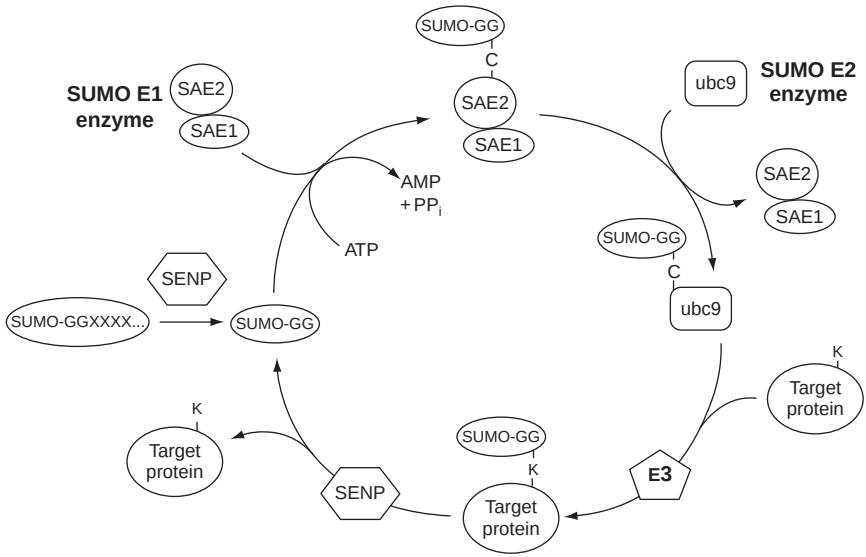

Figure 4.1 The SUMO modification cycle. Before attachment to proteins, SUMO proteins are cleaved into their mature forms by SENPs (*Sen*trin-specific *P*roteases), which remove 4, 11, and 2 amino acids from the C-terminal ends of SUMO-1, SUMO-2, and SUMO-3, respectively. The processed SUMO proteins are then activated by conjugation to the E1 heterodimer SAE1–SAE2, after which the SUMO is transferred to the E2 enzyme Ubc9. Finally, SUMO is ligated to substrate proteins by an isopeptide bond between the terminal glycine on SUMO and the ε-amino group of a lysine in the substrate. The efficiency of the ligation reaction is aided by SUMO ligase E3 proteins (E3) which directly interact with both target proteins and the E2 enzyme, thereby acting as bridging factors to increase the rate of the SUMO transfer. SUMO polypeptides are removed from target proteins by the action of SENPs, which recover the SUMO proteins for attachment to other proteins in another cycle of the sumoylation pathway.

ΨKXE (Ψ represents hydrophobic amino acids) (Desterro et al., 1997; Johnson and Blobel, 1997; Rodriguez et al., 2001; Sampson et al., 2001). Polypeptides called SUMO E3 proteins stimulate sumoylation by associating with both UBC9 and substrates, thereby acting as bridging factors to increase the efficiency of the SUMO attachment reaction (Geiss-Friedlander and Melchior, 2007; Mukhopadhyay and Dasso, 2007). The SUMO E3 proteins identified thus far include, but are not limited to, members of the PIAS (protein inhibitor of activated STAT) family of proteins (PIAS1, PIAS3, PIASx, and PIASy), the polycomb protein Pc2 (polycomb protein 2), and RANBP2 (Ran binding protein 2) (Johnson and Gupta, 2001; Kagey et al., 2003; Kahyo et al., 2001; Pichler et al., 2002; Takahashi et al., 2001b).

After SUMO groups are attached to proteins, they can be removed from them by enzymes called SENPs, of which there are six in human cells (Mukhopadhyay and Dasso, 2007; Yeh, 2009). The SENP1 and SENP2 enzymes are able to remove all three SUMO polypeptides from modified proteins, while the other SENPs appear to be specific for SUMO-2 and SUMO-3. The SENP1 and SENP2 enzymes also function as the C-terminal hydrolases shown in Fig. 4.1 that catalyze the removal of short stretches of C-terminal residues from the SUMO proteins, a step that must occur before they can be covalently attached to proteins.

3. Sumoylation and Cancer

Several lines of evidence implicate a role for the SUMO modification pathway in tumorigenesis (reviewed in Kim and Baek, 2006; Seeler et al., 2007). For example, increased levels of ubc9 have been found in a number of human cancers, and UBC9 overexpression is associated with increased cancer cell growth (McDoniels-Silvers et al., 2002; Mo et al., 2005). Also, the SUMO E3 protein PIAS3 (protein inhibitor of activated STAT3) is upregulated in a number of different cancer types (Wang and Banerjee, 2004), and elevated levels of the SUMO E1 enzyme are associated with lower survival rates in patients with hepatocellular carcinoma (Lee and Thorgeirsson, 2004). Further, sumoylation regulates the activities of important tumor suppressor proteins, including p53, pRB (retinoblastoma protein), p63, p73, and Mdm2 (murine double minute 2) (Seeler et al., 2007). Other studies indicated that levels of the SUMO protease SENP1 are upregulated in prostate and thyroid cancer, and that overexpression of this protein promotes the development of neoplasia in the prostate (Cheng et al., 2006; Jacques et al., 2005). These findings suggest that the relationship between cancer and sumoylation versus desumoylation is likely to be complex.

4. Sumoylation of Proteins Involved in Neurodegenerative Diseases

A number of polypeptides that play important roles in neurodegenerative diseases are covalently modified by SUMO proteins. These include proteins involved in Huntington's disease (huntingtin), spinocerebellar ataxia type 1 (ataxin-1), PD (tau, α-synuclein, DJ-1), amyotrophic lateral sclerosis (SOD1), and Alzheimer's disease (tau, APP).

4.1. Huntingtin

The neurodegenerative disorder, Huntington's disease, is caused by an increase in the size of the polyglutamine repeat located in the N-terminal region of the huntingtin protein, owing to an expansion of the trinucleotide CAG in the huntingtin gene (reviewed in Orr and Zoghbi, 2007; Walker, 2007). Expansion of the huntingtin polyglutamine repeat leads to a number of neurological defects, including decreased control of motor functions and deficits in cognitive abilities. Sumoylation has been observed at lysines 6, 9, and 15 of an N-terminal fragment of the huntingtin protein having an expanded (mutant) polyglutamine tract (Steffan et al., 2004). Sumoylation of these lysine residues was suggested to be associated with increased stability and reduced aggregation of this mutant huntingtin, thereby possibly increasing the levels of toxic intermediate poly-Q oligomers, as well as with an increase in its ability to repress transcription (Steffan et al., 2004). However, these same lysines are also ubiquitylated, making it difficult to be sure that the effects observed for these lysine mutants, except perhaps the alteration in stability, stem from a lack of sumoylation at these sites as opposed to a lack of ubiquitylation. In a *Drosophila melanogaster* model of neurodegeneration, in which all neurons express the huntingtin fragment containing a polyglutamine expansion, *smt3* (SUMO) heterozygotes exhibit reduced neurodegeneration, suggesting that sumoylation of huntingtin promotes the neurodegenerative process (Steffan et al., 2004). However, it is difficult to rule out the possibility of indirect effects, because reduced SMT3 levels would presumably also affect sumoylation of other cellular proteins.

A small G protein family called Rhes (Ras homolog enriched in striatum) has been found to stimulate the sumoylation of mutant huntingtin, but not wild-type huntingtin (Subramaniam et al., 2009). Rhes appears to mediate this effect by interacting with both mutant huntingtin and the SUMO E2 enzyme ubc9, thus acting like a SUMO E3 protein to stimulate the rate of the SUMO transfer. Rhes-stimulated sumoylation leads to increased disaggregation and cytotoxicity of mutant huntingtin (Subramaniam et al., 2009). Since Rhes is preferentially expressed in the corpus striatum,

Rhes-stimulated sumoylation and cytotoxicity of mutant huntingtin could provide an explanation for the tissue-selectivity exhibited in this disease.

4.2. Ataxin-1

Ataxin-1 is another polyglutamine-containing protein that has been found to be sumoylated. Polyglutamine expansion in this protein is the cause of spinocerebellar ataxia type 1 (SCA1), a disease that causes progressive loss of motor control in patients. Ataxin-1 is sumoylated at five different lysine residues, lysines 16, 194, 610, 697, and 746 (Riley et al., 2005). Sumoylation is decreased in mutant proteins containing the expanded polyglutamine repeat compared to those containing the wild-type polyglutamine repeats, but the mechanism responsible for this effect is not known. Phosphorylation of ataxin-1 negatively regulates its sumoylation, as evidenced by the finding that SUMO modification is greater in the S776A phosphosite mutant of this protein. In addition, ataxin-1 sumoylation is significantly decreased in the nuclear localization sequence mutant, K772T, suggesting that the ability of ataxin-1 to enter the nucleus is important for its ability to be sumoylated, or alternatively that nuclear localization prevents its desumoylation, or both. This finding is consistent with previous studies demonstrating that sumoylation machinery enzymes are localized to the nuclear pore complex (Hang and Dasso, 2002; Pichler et al., 2002; Zhang et al., 2002). Substitution of the sumoylation site lysines to nonsumoylatable arginines did not affect ataxin-1 nuclear localization or its ability to form inclusions (Riley et al., 2005).

The results of a more recent study showed that overexpression of SUMO-1 or the SUMO E2 enzyme ubc9 stimulates aggregation of ataxin-1 (Ryu et al., 2010). Since there is evidence suggesting that aggregation of mutant ataxin-1 may actually decrease its toxicity, it is possible that increased sumoylation of ataxin-1 may have potential as a therapeutic strategy for treatment of SCA1 (Zoghbi and Orr, 2009). The results of this study also indicated that oxidative stress increases sumoylation and aggregation of ataxin-1 (Ryu et al., 2010).

4.3. Tau

Tau is expressed at high levels in the brain and is associated with a number of neurodegenerative diseases including PD and Alzheimer's disease (reviewed in Ballatore et al., 2007). This protein is sumoylated, preferentially by SUMO-1, at lysine 340 (Dorval and Fraser, 2006). An increase in tau ubiquitylation caused by treatment with the proteasome inhibitor MG132, is associated with a significant decrease in tau sumoylation, suggesting that there could be competition between ubiquitylation and sumoylation for tau modification.

Treatment of cells with the phosphatase inhibitor okadaic acid also resulted in higher levels of tau sumoylation. This finding suggests that tau

sumoylation could be regulated by phosphorylation, although it is also possible that indirect effects, for example phosphorylation of other proteins, could be responsible for this result. Related to this possibility, SUMO-1 immunoreactivity colocalizes with phospho-tau in aggregates of neuritic plaques in *App* transgenic mice; however, again it is not clear whether this effect is indicative of phospho-tau sumoylation or the modification of other proteins present in these aggregates (Takahashi et al., 2008). Tau phosphorylation also negatively regulates its ability to interact with microtubules (Lindwall and Cole, 1984), and thus it is possible that the observed increase in tau sumoylation upon phosphatase indicates that the free soluble pool of tau is the primary sumoylation substrate. Consistent with this hypothesis, treatment with the microtubule depolymerizing drug colchicine is also associated with increased tau sumoylation (Dorval and Fraser, 2006).

4.4. α-Synuclein

Aggregation of the α-synuclein protein has been implicated in the pathogenesis of PD, and mutations in its gene are associated with PD, a condition that leads to difficulties in controlling motor function and speech, dementia, and mood disturbances (reviewed in Thomas and Beal, 2007). Sumoylation occurs on lysine 102 of α-synuclein, but it appears that this is not the only site because changing this residue to arginine decreased, but did not eliminate, its SUMO modification. Like tau, α-synuclein appears to be preferentially sumoylated by SUMO-1 compared to SUMO-2 or SUMO-3 (Dorval and Fraser, 2006). Unlike what was found for tau, however, α-synuclein is not affected by treatment with the proteasome inhibitor MG132, suggesting that SUMO modification of these two proteins is differentially regulated (Dorval and Fraser, 2006). The role sumoylation plays in regulating α-synuclein function is yet to be discovered.

4.5. DJ-1

The functions of the DJ-1 protein include acting as an antioxidant, transcriptional co-activator, and molecular chaperone. *PARK7* (*DJ-1*) mutations account for 1–2% of early onset cases of PD (reviewed in Thomas and Beal, 2007; Wang et al., 2006). DJ-1 can be sumoylated at lysine 130, and preventing sumoylation at this site by changing this lysine to arginine is associated with a decrease in DJ-1-mediated Ras-dependent transforming and cell growth-promoting activities (Shinbo et al., 2006). DJ-1 sumoylation increases upon UV irradiation and loss of this modification decreases its antiapoptotic function, suggesting that sumoylation of DJ-1 is important for its ability to protect cells from UV-induced apoptosis (Shinbo et al., 2006). Members of the PIAS family of proteins, which function as

SUMO E3 proteins, have been found to interact with DJ-1 and stimulate its sumoylation (Shinbo et al., 2006; Takahashi et al., 2001a).

Results from other studies suggest that DJ-1 also functions as a regulator of the sumoylation of other proteins, thereby modulating their activities (Zhong and Xu, 2008; Zhong et al., 2006). For example, DJ-1 inhibits the sumoylation of PSF (pyrimidine tract-binding protein-associated splicing factor), a transcriptional co-repressor; this inhibition has been suggested to be involved in the DJ-1-mediated transcriptional upregulation of the tyrosine hydroxylase and MnSOD (manganese superoxide dismutase, *SOD2*) genes. Thus, the role of SUMO modification with respect to DJ-1 activity could be complex, involving both sumoylation of DJ-1 itself as well as DJ-1-mediated regulation of the SUMO modification of other polypeptides.

4.6. SOD1

Amyotrophic Lateral Sclerosis, or ALS, is a disease that affects motor neurons in the brain and spinal cord, resulting in symptoms of muscle atrophy and paralysis (reviewed in Boillee et al., 2006; Gonzalez de Aguilar et al., 2007). A large number of mutations have been identified in the copper–zinc superoxide dismutase 1 (*SOD1*) gene, and these mutations are associated with at least 20% of the cases of familial ALS.

The results of a number of proteomic studies have demonstrated that the yeast SOD1 protein is sumoylated on multiple lysine residues, under both nonstress and stress conditions (Denison et al., 2005; Hannich et al., 2005; Wykoff and O'Shea, 2005; Zhou et al., 2004). Human SOD1 is also sumoylated but only at one site, lysine 75, and this modification is observed for both wildtype and ALS-associated mutant forms of SOD1 (Fei et al., 2006). Human SOD1 appears to be primarily modified by SUMO-1, with no detectable modification by SUMO-2 or SUMO-3. Overexpression of SUMO-1 protein leads to an increased percentage of cells exhibiting SOD1-containing aggregates, which also contain high levels of SUMO-1 (Fei et al., 2006). This suggests that sumoylation of SOD1 may promote its aggregation, although it is also possible that the stimulation of SOD1 aggregation by SUMO-1 could be mediated via increased sumoylation of other proteins. Since aggregation status of SOD1 is thought to play a role in causing cell defects that could contribute to ALS, an ability of sumoylation to regulate SOD1 aggregation could implicate a role for this modification in the pathogenesis of this disease (Rothstein, 2009).

4.7. APP

Alzheimer's disease, the most common aging-related human neurodegenerative disease, is a debilitating condition that progressively impairs cognitive function (Blennow et al., 2006; Goedert and Spillantini, 2006; LaFerla

et al., 2007). It is widely believed that amyloid-β (Aβ) protein, produced by APP processing via the amyloidogenic proteolytic processing pathway, plays a role in causing this disease.

APP was identified as a target of sumoylation by an *in vitro* expression cloning strategy, in which candidate sumoylation substrate proteins were identified by assaying successive subdivisions of cDNA pools using *in vitro* sumoylation reactions (Gocke et al., 2005). Lysines 587 and 595 of the APP protein, which are immediately N-terminal to the β-secretase cleavage site, are sites of covalent modification by both SUMO-1 and SUMO-2 (Fig. 4.2A; Zhang and Sarge, 2008a). Both of these lysine residues are surrounded by matches to the sumoylation consensus sequence ΨKXE/D. Preventing sumoylation at lysines 587 and/or 595 leads to increased Aβ protein aggregation, suggesting that this modification inhibits Aβ formation (Fig. 4.2B). Further, upregulating cellular levels of the

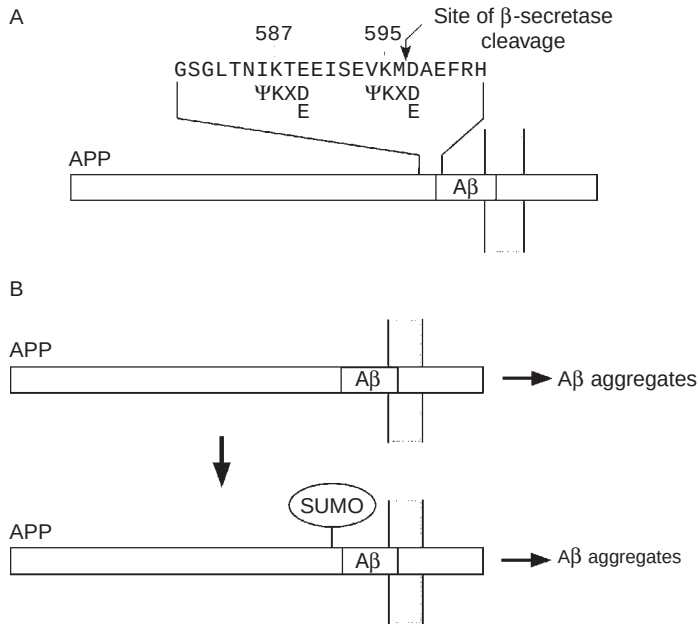

Figure 4.2 The location and function of APP sumoylation. APP SUMO modification occurs at two lysines immediately adjacent to the site of β-secretase cleavage, and is associated with decreased levels of Aβ aggregates. (A) This schematic shows the locations of the sumoylated lysine 587 and 595 residues in APP, their matches to the sumoylation site consensus sequence (ΨKXE/D), and proximity to β-secretase cleavage site and the Aβ peptide generated from APP processing. (B) Regulation of Aβ aggregate levels by APP sumoylation. Sumoylation of APP near the site of β-secretase cleavage is associated with a decrease in the amounts of Aβ aggregates.

SUMO E2 enzyme UBC9 resulted in elevated APP sumoylation and decreased Aβ protein aggregate levels. These results suggest that interventions that alter APP sumoylation could represent potential new approaches for combating Alzheimer's disease. Interestingly, one of the identified sumoylated APP residues, lysine 595, is substituted to asparagine in the previously characterized Swedish (KM-to-NL) APP mutant (Mullan et al., 1992). This suggests that an inability of APP to be sumoylated at lysine 595 could contribute to the increased Aβ production observed in the Swedish APP mutant.

Two previous studies indicated that SUMO-3 overexpression affects Aβ levels (Dorval et al., 2007; Li et al., 2003). However, these studies did not examine whether APP was sumoylated; moreover, the meaning of their results is not clear as the two studies observed opposite effects of SUMO protein overexpression on Aβ levels. Regardless, it appears that these effects of SUMO overexpression on Aβ are likely mediated by a mechanism different from that of direct APP sumoylation, because the effect was observed even when SUMO proteins incapable of covalent substrate attachment were utilized for the experiments (Dorval et al., 2007).

Before these findings were made, sumoylation of proteins that pass through the endoplasmic reticulum (ER) or other compartments of the secretory pathway had not been reported. However, the identification of SUMO modification of APP suggested that sumoylation could occur in one or more of these membrane-bound compartments. In support of this hypothesis, immunofluorescence analysis revealed co-localization between Calnexin and a portion of the cellular UBC9, suggesting that the SUMO E2 enzyme co-localizes with the ER (Zhang and Sarge, 2008a). This UBC9 staining pattern is consistent with the results of previous studies which showed that in addition to a nuclear-localized population, some UBC9 staining is found outside the nucleus in a pattern reminiscent of ER localization (Koldamova et al., 1998; Lee et al., 1998; Rodriguez et al., 2001). This data not only provides an explanation for how lysines 587 and 595 of APP can be sumoylated but also suggests the intriguing possibility that other proteins that enter the ER could also be targets of sumoylation. The mechanism by which UBC9 associates with the ER is not yet known, because UBC9 does not have an obvious signal sequence. However, the prediction program SecretomeP (Bendtsen et al., 2004) identifies UBC9 as a candidate nonclassical secretory pathway protein. Thus, one possibility is that UBC9 enters a membrane-bound compartment in the cell which then ultimately merges with the ER to deliver the SUMO E2 enzyme to this compartment. The presence of UBC9 within the ER suggests the possibility that sumoylation could play a role in other diseases associated with this cellular compartment.

5. Sumoylation and Heart Disease

Lamin A plays an important role in nuclear structure and function, and mutations in the lamin A gene cause a large number of different human diseases, including cardiomyopathies, muscular dystrophies, and Hutchinson–Gilford Progeria Syndrome (Broers et al., 2006; Capell and Collins, 2006; Mattout et al., 2006; Parnaik and Manju, 2006; Verstraeten et al., 2007). An interaction between lamin A and UBC9, the SUMO E2 enzyme, was discovered using a yeast two-hybrid screen, suggesting that lamin A could be a substrate for SUMO modification (Zhong et al., 2005). Analysis of the amino acid sequence of lamin A revealed a match to the sumoylation consensus sequence ΨKXE (MKEE) surrounding lysine 201 in the rod-containing domain, suggesting that sumoylation could occur at this site (Fig. 4.3A; Zhang and Sarge, 2008b). Subsequent experiments found that lamin A is indeed sumoylated at lysine 201 and that it is more efficiently

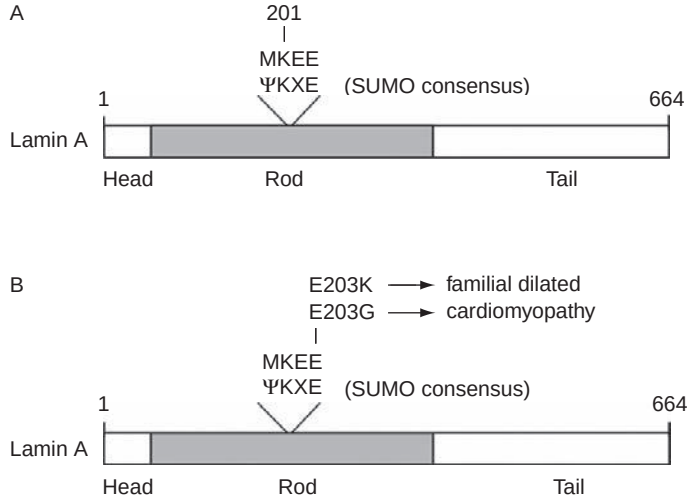

Figure 4.3 Lamin A sumoylation and familial dilated cardiomyopathy. Lamin A is sumoylated at lysine 201 in the rod domain, and mutations associated with familial dilated cardiomyopathy disrupt the lamin A SUMO consensus sequence. (A) Schematic showing the location of a match (MKEE) to the sumoylation site consensus sequence (ΨKXE) surrounding lysine 201 in the lamin A rod-containing domain. (B) The E203G and E203K substitutions in lamin A associated with familial dilated cardiomyopathy correspond to the conserved glutamic acid residue of the sumoylation site consensus sequence (ΨKXE) surrounding lysine 201. This glutamic acid position is important for sumoylation at the preceding lysine residue in the consensus sequence.

modified by SUMO-2 than SUMO-1 (Zhang and Sarge, 2008b). Lysine 201 sumoylation appears to be important for the normal subcellular localization of lamin A, as a nonsumoylatable mutant (K201R) exhibits an altered subcellular localization pattern of concentrating into foci, in contrast to the relatively continuous nuclear peripheral localization exhibited by the wild-type lamin A (Zhang and Sarge, 2008b).

Two different disease-causing substitutions of lamin A at glutamic acid residue 203 have been identified, E203G and E203K, which are associated with familial-dilated cardiomyopathy and conduction system disease (Fatkin et al., 1999; Jakobs et al., 2001). Intriguingly, this glutamic acid is only two residues C-terminal to the lamin A lysine 201 sumoylation site (Fig. 4.3B; Zhang and Sarge, 2008b), and in fact occupies the conserved glutamic acid position of the sumoylation consensus sequence ΨKXE (Desterro et al., 1997; Johnson and Blobel, 1997; Rodriguez et al., 2001; Sampson et al., 2001). The presence of an acidic residue at this position, in this case, glutamic acid, is known to be important for the efficiency of SUMO addition to the lysine in the consensus sequence (Rodriguez et al., 2001; Sampson et al., 2001). Consistent with this, the E203G and E203K mutant lamin A proteins both exhibit a significant decrease in sumoylation, compared to wild type, as does lamin A protein in skin fibroblasts from a patient harboring the E203K lamin A substitution (Zhang and Sarge, 2008b). E203G and E203K lamin A mutant proteins exhibit an altered pattern of lamin A subcellular localization very similar to that of the lamin A K201R SUMO attachment site mutant. E203K lamin A patient fibroblast cells also exhibit a significant increase in the percentage of cells showing abnormal lamin A localization/nuclear morphology, as well as increased cell death.

These results suggest that a defect in lamin A sumoylation could play an important role in the underlying molecular mechanism of the familial cardiomyopathies associated with the E203G and E203K lamin A substitutions. These substitutions in the lamin A proteins appear to provide the first examples of human disease-causing alterations that occur in a crucial residue of a sumoylation consensus sequence to cause decreased sumoylation of the mutant protein.

6. Concluding Remarks

The results of the studies described above indicate that sumoylation is not only an important regulator of the normal function of many vital cellular proteins but also plays a role in the pathogenesis of human disease states. These findings suggest that interventions, pharmacological or otherwise, that modulate protein sumoylation could represent new therapeutic approaches for treating the diseases discussed herein, and possibly also other

conditions yet to be discovered in which sumoylation plays a role. The SUMO E3 proteins responsible for regulating the sumoylation efficiency of disease-related proteins, such as those discussed in this review, could represent potentially desirable targets of such interventions because they would offer greater selectivity, as opposed to the global alterations in sumoylation that would occur if the SUMO E2 enzyme (UBC9) was targeted. Future studies are also warranted in determining the mechanisms by which aberrant sumoylation leads to disease states, because the results would not only increase the understanding of human disease pathogenesis, and hence possibly provide insights into potential new therapies, but also increase knowledge of the role that sumoylation plays in the normal functions of these proteins. Finally, it is likely that there are many other disease-associated proteins whose sumoylation is yet to be identified, and investigations into these new examples will likely also provide insight into the normal functions of these proteins, disease pathogenesis, and possibly new avenues for disease interventions.

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CHAPTER FIVE

Focal Adhesion Kinase: Exploring FAK Structure to Gain Insight into Function

Jessica E. Hall,* Wei Fu,* *and* Michael D. Schaller*,†,‡

Contents

Abstract

Focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2) are closely related nonreceptor protein tyrosine kinases. FAK can regulate cell proliferation, survival, and motility, and plays an essential role in development. Pyk2 shares some functions with FAK but is a nonessential gene product during

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development. Recent discoveries related to FAK and Pyk2 structure have provided important insights into the regulatory mechanisms of catalytic activity, molecular basis of assembly of signaling complexes, and the transmission of downstream signals. This chapter reviews these advances in FAK/Pyk2 structure/function, compares and contrasts features of these kinases, and discusses new drug discoveries in the context of molecular structure.

Key Words: FAK, Domain structure, FERM, Kinase, FAT domain, Autoinhibition, Therapeutics.

1. Introduction

Focal adhesion kinase (FAK—also known as protein tyrosine kinase 2, PTK2) and the closely related protein tyrosine kinase (Pyk2—also known as protein tyrosine kinase 2b, PTK2b) are nonreceptor tyrosine kinases that not only share structural features and some functions but also exhibit distinct properties. As kinases, an important role for each is phosphorylation of downstream substrates to transmit cellular signals, but in addition both proteins act as scaffolding proteins and play an integral part in the assembly of signaling complexes. Both enzymatic and scaffolding activities are critical for biological function. Multiple extracellular stimuli regulate the activation of FAK and Pyk2. FAK is activated through integrin signaling but is also activated in response to stimulation of growth factor receptors, cytokine receptors, and mechanical stimulation. Pyk2 can be activated in response to growth factors and cytokines, and interestingly the increase in cytosolic Ca^{2+} concentration induced by these ligands is important for subsequent Pyk2 activation.

Given the broad range of stimuli that activate these kinases, it is not surprising that they control important and general cell phenotypes. Physiologically, expression of the FAK gene is vital to development since $fak^{-/-}$ mice die during embryonic development. Pyk2 is more restricted in expression and function during development and $pyk2^{-/-}$ mice develop normally. Both Pyk2 and FAK have roles in human pathology. The best example is the significant role that FAK plays in cancer, where its functions in regulating tumor growth and metastasis make it a key target for new cancer therapies. The biological functions of these kinases are briefly reviewed below as a prelude to a discussion of structure/function relationships of these kinases.

Considerable structural information about FAK and Pyk2 has recently emerged. Crystal structures of the three major domains have been determined and additional NMR studies performed on one. These structures have provided important insight into the structural regulation of these

kinases as well as insight into the binding of various proteins to FAK, and its scaffolding function. In this review, the details of the molecular structure of FAK/Pyk2 will be discussed in the context of how structure impacts function. Additionally, this work toward targeting FAK and Pyk2 therapeutically will be presented from a structural perspective.

2. Biological and Physiological Significance of FAK

2.1. FAK's function in the cell

FAK has three major functions in the cell: controlling survival, proliferation, and motility. Integrin-dependent adhesion and signaling is vital for transmission of a survival signal that instructs the cell it is in the correct location. In the absence of this signal, the cells undergo apoptosis, due to the loss of this signal, a process that is called anoikis. In adherent cells, FAK transmits signals blocking anoikis. A number of different signaling pathways activated downstream of FAK can promote cell survival. These signaling events have been recently reviewed and will not be discussed in detail here (Gilmore et al., 2009; Lim et al., 2008b).

Integrin-dependent cell adhesion results in a cytoplasmic signal promoting cell cycle progression, which is responsive to changes in mechanical stimulation. FAK plays an important role in the regulation of adhesion-responsive signals to proliferate, initiating downstream signaling pathways, transcription factor activation, and expression of regulators of proliferation, for example, cyclins. This function is highlighted in a number of FAK reviews and will not be discussed further (Assoian and Klein, 2008; Cox et al., 2006).

FAK also plays an integral role in the regulation of the actin cytoskeleton, which underlies its role in controlling cell motility. Through direct interactions, or indirect interactions with a number of regulatory proteins that control the activation state of Rho family proteins, FAK functions in determining cell polarization, cellular protrusion, adhesion, and directional motility. These findings have also been recently reviewed and will not be elaborated in any detail beyond discussion of how new insight into FAK structure provides insight into molecular interactions and regulation of function (Schaller, 2010; Tomar and Schlaepfer, 2009).

Pyk2 is activated by an increase in cytoplasmic Ca^{2+} from stimulation by a ligand such as vasopressin or platelet-derived growth factor (PDGF) that triggers the phospholipase C pathway (Lev et al., 1995). Pyk2 plays a role in regulation of the cytoskeleton during cell repulsion, as well as cell attachment and spreading (Freitas et al., 2002; Hashido et al., 2006). Most

importantly, Pyk2 controls bone resorption and inhibits osteoprogenitor cells (Buckbinder et al., 2007).

2.2. FAK in development

FAK is essential for development since FAK-deficient mouse embryos do not develop past the E8.5 stage despite normal implantation. While FAK is expressed throughout the embryo, expression is highest in the mesoderm. Not surprisingly, the lack of FAK expression leads to mesodermal defects. These FAK-deficient embryos do not properly develop a head fold nor do they properly develop a neural tube or a heart tube (Furuta et al., 1995). The importance of FAK in the development of specific organ systems has been deduced through the development of conditional knockouts. Endothelial-specific FAK knockouts die between E9.5 and E11.5, indicating the importance of FAK in the development of the vasculature (Braren et al., 2006; Shen et al., 2005). Note that normal vasculogenesis occurs in the absence of FAK; however, embryonic angiogenesis fails resulting in lethality. There is a disagreement regarding the cellular defect responsible for the phenotype. Defects in proliferation, survival, and migration are reported in one knockout, while defects in spreading and migration are attributed to the phenotype in the other model.

Several studies show that FAK is important for cardiac development in addition to the development of the vasculature. In *Xenopus laevis*, FAK is essential for the development of a multichambered heart through enabling the proliferation of myocytes necessary for chamber outgrowth and facilitating the looping morphogenesis of the heart tube (Doherty et al., 2010). In mice, FAK is integral in the proper alignment of the outflow tract and development of the aortic arch (Hakim et al., 2007; Vadali et al., 2007). FAK is essential for proper cardiomyocyte migration and development of ventricular septation. These defects are similar to those observed in a congenital human heart defect called Tetralogy of Fallot. FAK and a dominant negative variant, FRNK, function in coordination to regulate the different stages of cardiac development. FRNK facilitates cell cycle withdrawal of cardiomyocytes, allowing them to terminally differentiate (DiMichele et al., 2009). A conditional knockout of FAK in neural crest cells also causes cardiovascular defects, in addition to craniofacial malformations, which are phenotypes very similar to those found in DiGeorge syndrome. This study shows that FAK is imperative for the differentiation and motility of neural crest cells and suggests that a defect in FAK signaling may be associated with this congenital defect, although this remains to be established (Vallejo-Illarramendi et al., 2009).

FAK also functions in the developing nervous system to control multiple processes including neuronal migration, morphogenesis, and myelination. In *X. laevis*, phosphorylated FAK is localized to neuronal growth cones and

aids axonal pathfinding (Worley and Holt, 1996). One molecular mechanism through which the process of axonal pathfinding may be regulated by FAK is downstream of netrin signaling. Several netrin receptors directly bind FAK; FAK is activated upon stimulation with netrin and is required for axonal outgrowth and directional growth of axons in response to netrins (Nikolopoulos and Giancotti, 2005). FAK also has been shown to be a negative regulator of axonal branching and synapse formation, leading to correct morphology of a given axonal pathway (Rico et al., 2004a). The mechanism of regulation of dendritic spine formation by FAK might be through inhibition of cofilin activity (Shi et al., 2009). A combination of conditional oligodendrocyte-specific and inducible FAK knockout mice was used to investigate the role of FAK in myelination. This study leads to the finding that FAK is involved in the early stages of myelination (Forrest et al., 2009).

Additional organ systems are also impaired by loss of FAK expression. FAK is essential for the differentiation of osteogenic cells (Ward et al., 2007), proper nephron tubulogenesis in the kidney (Wei et al., 2009), and mammary gland development during pregnancy (Nagy et al., 2007). These studies further illustrate the integral role played by FAK in the development of multiple organ systems.

Pyk2 has a significantly different role in development as compared to FAK. *pyk2*$^{-/-}$ mice are completely viable, fertile, and exhibit no visible malformations (Guinamard et al., 2000). However, these mice do exhibit some deficits in the immune system. The *pyk2*$^{-/-}$ mice have a reduced number of marginal zone B cells (found in the marginal zone of the spleen) and mount a defective humoral immune response (Guinamard et al., 2000). Pyk2-null CD8 positive T cells are not properly activated in response to T cell receptor and LFA1 costimulation (Beinke et al., 2010). The macrophages and osteoclasts in *pyk2*$^{-/-}$ mice are also defective. The macrophages show reduced infiltration toward inflammatory stimuli *in vivo* and exhibit polarization, contractility, and motility defects *in vitro* (Okigaki et al., 2003). *pyk2*$^{-/-}$ osteoclasts produce an abnormal sealing zone and a defect in bone resorption, leading to osteopetrosis (Gil-Henn et al., 2007). Interestingly, a second group attributes the increased bone mass in *pyk2*$^{-/-}$ mice to increased osteoblastogenesis and osteoblast activity, rather than differences in osteoclast activity (Buckbinder et al., 2007). These are clearly important mechanistic details that should be resolved. Finally, the *pyk2*$^{-/-}$ mice are also more prone to obesity and insulin resistance (Yu et al., 2005).

2.3. FAK and cancer

Studies designed to examine how the v-src oncogene and tyrosine kinase resulted in oncogenic transformation of fibroblasts led to the discovery of FAK as a substrate for v-src (Schaller et al., 1992). FAK was also

independently isolated as a tyrosine kinase expressed in breast carcinoma (Weiner et al., 1993). While two other groups also independently identified FAK in other contexts (Choi et al., 1993; Hanks et al., 1992), the two discoveries in the context of cancer suggested a potential role in oncogenesis and many studies have now confirmed the role of FAK in the development and metastatic spread of cancer. This conclusion is based upon three major observations: (1) FAK is overexpressed in many cancers, (2) aberrant FAK expression in some cancers is associated with poor prognosis, and (3) perturbation of FAK in a number of animal models blocks tumorigenesis and metastasis. The literature on this topic is extensive and is well covered in a number of recent reviews (Golubovskaya et al., 2009; Luo and Guan, 2010; Provenzano and Keely, 2009). Select studies will be discussed below to illustrate these points without attempting to cover the field in its entirety.

Initial studies to examine FAK in human cancer identified elevated FAK expression at the protein and/or RNA level in a variety of different tumor types. Interestingly, in melanoma, elevated phosphorylation of FAK without overexpression was observed (Kahana et al., 2002). In a number of tumors, FAK expression was elevated in advanced metastatic disease, and in melanoma, elevated phosphorylation was observed in metastatic but not primary tumors. These findings originally suggested that FAK might be associated with advanced disease. However, other studies have demonstrated that elevated FAK expression in early stages of cancer and thus is not strictly associated with advanced stages of cancer. In some types of cancer, FAK overexpression is prognostic. For example, FAK overexpression in ovarian cancer and esophageal squamous cell carcinoma is associated with poor outcome (Miyazaki et al., 2003; Sood et al., 2004). In breast cancer, FAK overexpression has not been linked to prognosis; however, amplification of the FAK gene is a poor prognostic indicator (Pylayeva et al., 2009). Not all studies have demonstrated a link between FAK expression/phosphorylation and disease outcome. Further analysis of some of these cancers might be warranted since the linkage of FAK expression to outcome may be more complex. For example, FAK overexpression is not prognostic in laryngeal cancer and neither is loss of E-cadherin expression. However, if FAK expression and E-cadherin expression are considered together, they do serve as prognostic indicators, that is, patients with tumors exhibiting elevated FAK expression and low E-cadherin expression exhibit significantly reduced survival (Rodrigo et al., 2007). These examples demonstrate FAK overexpression/amplification/phosphorylation in many tumor types and in some, but not all cases, this is indicative of poor prognosis.

Many experimental models have been employed to evaluate the role of FAK in tumorigenesis and metastasis. Antisense, dominant negative, and RNA interference strategies have been employed to investigate the role of FAK in xenograph and orthotopic tumor models and an experimental model of metastasis. The results of these studies have implicated FAK in

regulating the tumorigenic and metastatic properties of both murine and human cell line models of cancer, for example, in breast cancer models (Mitra et al., 2006a; van Nimwegen et al., 2005) and ovarian cancer models (Halder et al., 2006). Since the FAK knockout is embryonic lethal, conditional knockout approaches were used to evaluate the role of FAK in additional animal models of cancer. In a chemical carcinogenesis model of skin cancer, FAK was implicated in both the formation of papilloma and progression to carcinoma (McLean et al., 2004). In transgenic models of breast cancer, multiple groups have demonstrated a role for FAK in tumor formation and metastasis (Lahlou et al., 2007; Provenzano et al., 2008; Pylayeva et al., 2009). These studies establish an active role for FAK in controlling tumor formation and metastasis in animal models.

Based upon the expression of FAK in human tumors, the correlation of FAK expression or amplification with disease outcome, and the demonstration that FAK functions to promote tumorigenesis and metastasis in animal models, there is considerable interest in FAK's potential as a therapeutic target. Several groups have reported the development of small molecule inhibitors of FAK catalytic activity and others have suggested additional therapeutic strategies. These will be discussed in the last section of this review.

3. Domain Structure

The identification of domains within FAK was originally made based upon sequence homology and deletion analysis to identify functional regions of the protein. The general structure of FAK is very simple. There are four domains arranged in a linear fashion. At the N-terminus lays, the band 4.1, Ezrin, Radixin, Moesin (FERM) domain followed by the kinase domain. These two domains are separated from the C-terminal focal adhesion targeting (FAT) domain by a structurally uncharacterized proline-rich region (Fig. 5.1). PYK2's sequence is 46% identical to FAK, is 65% similar at the protein level, and exhibits the same domain organization and the structural characteristics as FAK.

FAK becomes phosphorylated in several locations (Calalb et al., 1995; Schaller et al., 1994). Tyr397, within the linker region between the FERM and kinase domains, is the major autophosphorylation site, and plays an important scaffolding role for SH2 domain-containing proteins, including Src (Fig. 5.2). The role of these proteins in FAK signaling and function has been extensively reviewed and will not be further discussed here (Hanks et al., 2003; Zhao and Guan, 2009). The other major phosphorylation sites Tyr577, 576, 861, and 925 are phosphorylated by Src (Calalb et al., 1995). Tyr576 and 577 are in the activation loop of the kinase domain and

Figure 5.1 The cartoon structure of FAK. The kinase and focal adhesion targeting (FAT) domains are highlighted. The F1, F2, and F3 subdomains of the FAK FERM domain are indicated, and the KAKTLRK basic sequence within the F2 subdomain is denoted by the dark blue box. Tyrosine phosphorylation sites are indicated in orange, serine phosphorylation sites in green, and "PxxP" motifs in red.

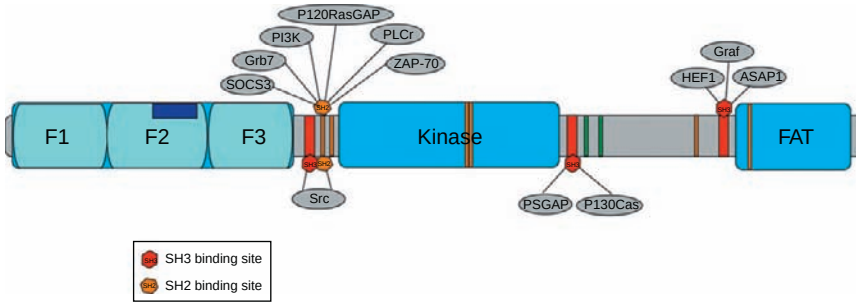

Figure 5.2 SH2- and SH3-mediated scaffolding interactions with FAK. The cartoon of FAK is shown with its major interaction partners that bind in the linker or the proline-rich region. These interactions are mediated through SH2 domains (orange hexagon) or SH3 domains (red hexagon).

phosphorylation promotes maximal catalytic activity. The structural basis for this regulatory mechanism is outlined in the following sections. In addition to tyrosine phosphorylation, several serine residues are phosphorylated in FAK. Phosphorylation of Ser722 is regulated by glycogen synthase kinase 3 (GSK3) and the PP1 phosphatase and has an inhibitory effect on FAK catalytic activity (Bianchi et al., 2005). Ser732 is phosphorylated by Cdk5 and is essential for cytoskeletal reorganization, neuronal migration, and centrosome function in mitosis and during neuronal migration (Park et al., 2009; Xie and Tsai, 2004; Xie et al., 2003). Thus, serine phosphorylation of FAK is also physiologically significant, although the molecular mechanisms remain to be established.

FAK also contains several proline-rich sequences that serve a scaffolding function by recruiting SH3 domain-containing proteins into complex (Fig. 5.2). Through this mechanism, Src binding is stabilized, and FAK

associates with other scaffolding proteins, p130cas and Hef1, and GTPase-activating proteins, ASAP1, PSGAP, and GRAF. These SH3 domain docking sites lie within unstructured regions of FAK and their roles in signaling downstream of FAK have been discussed in recent reviews (Guan, 2010).

The C-terminal noncatalytic domain of FAK, FAK-related nonkinase (FRNK), is expressed as a naturally occurring variant and functions as a dominant negative mutant to block FAK function (Gilmore and Romer, 1996; Richardson and Parsons, 1996; Schaller et al., 1993). The FRNK transcript is initiated from a second promoter within the FAK gene (Nolan et al., 1999). A similar variant of Pyk2 also exists (Xiong et al., 1998). FRNK consists of the C-terminal proline-rich and FAT domains of FAK and is missing the FERM and kinase domains. The dominant negative function is through competition with FAK for localization to focal adhesions and possibly by titrating away FAK binding partners important for function (Gilmore and Romer, 1996; Richardson and Parsons, 1996). Not only has FRNK been frequently used as an experimental tool to evaluate the role of FAK in controlling biological function, but it also seems to be a physiological regulator of FAK, for example, during heart development (DiMichele et al., 2009).

3.1. Structure of the FERM domain

FERM domains are trilobed, typically N-terminal domains (Chishti et al., 1998). They are commonly found in proteins that bind cytoplasmic regions of transmembrane proteins and often act as linkers between the cytoskeleton and plasma membrane. In addition to intermolecular interactions, FERM domains also frequently mediate intramolecular interactions, for example, the ezrin, radixin, and moesin FERM domains each bind their C-terminal domains (Hoeflich and Ikura, 2004). Hydrophobic cluster analysis showed that both FAK and Pyk2 have N-terminal FERM domains, although the FAK family FERM domains share only 12–15% identity with the sequences of other FERM domains (Girault et al., 1999). Recent crystal structures of the FAK FERM domain alone or in complex with the catalytic domain have been solved and have provided considerable new insight into FAK regulation and function (Ceccarelli et al., 2006; Lietha et al., 2007).

Like all FERM domains, the FAK FERM domain has three lobes: the F1, F2, and F3 subdomains (Ceccarelli et al., 2006; Fig. 5.3). The F1 subdomain spans residues 33–127 and consists of a five-strand β sheet capped by an α-helix. This subdomain exhibits an ubiquitin-like fold. The second α-helix is two turns longer than in most FERM domains. The β1–β2 and α2–β5 loop regions are also longer and contain 3_{10} helices, a feature unique among the FERM domain-containing proteins (Ceccarelli

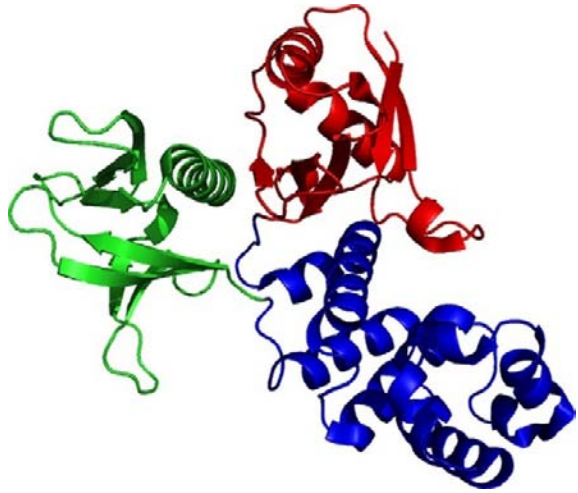

Figure 5.3 The structure of the FAK FERM domain. The three-lobed FERM domain of FAK is colored to denote the F1 subdomain in red, the F2 subdomain in blue, and the F3 subdomain in green. This figure was prepared using PDB file 2JOJ. All figures of structures were prepared using Pymol (DeLano, 2002).

et al., 2006). Each of these features lies on the surface of the FERM domains where structural differences potentially impact interactions with other proteins.

The F2 subdomain encompasses residues 128–253. The structure is entirely α-helical with a core similar to acyl-CoA-binding protein. There are several unique features of the F2 subdomain (Ceccarelli et al., 2006). Like the F1 subdomain, it has three segments containing 3_{10} helices and a nine-residue α2′ helix located between the α2 and α3 regions. This region of the protein forms a surface that docks with the catalytic domain in the autoinhibited conformation. The F2 subdomain also contains a basic patch on the apex of the domain that plays a significant role in FAK regulation. This patch contains the sequence KAKTLRK that is important for activation of FAK following cell adhesion and stimulation with growth factors (Chen and Chen, 2006; Dunty et al., 2004). These sequences are also important for phosphatidylinositol binding and association with a tyrosine-phosphorylated peptide from MET, which are ligands that regulate FAK activation (Cai et al., 2008; Chen and Chen, 2006).

The final subdomain covering residues 254–352 is the F3 subdomain. It is a β sandwich capped by a C-terminal α-helix. This structure resembles a PH (pleckstrin homology domain), a PTB (phosphotyrosine binding) domain, and an EVH1 (Ena/VASP homology) domain. PH domains contain a basic phospholipid binding pocket at the open end of the β sandwich

(DiNitto and Lambright, 2006). PTB domains contain basic residues that bind phosphotyrosine and stabilize alignment of tyrosine-phosphorylated peptides along the top of the β-strand next to the F3 α-helix (Zhou et al., 1995). EVH1 domains contain a peptide binding surface along the β-strands preceding the F3 α-helix (Beneken et al., 2000). While these binding surfaces are accessible in the F3 subdomain of FAK, the surface features are different in FAK (Ceccarelli et al., 2006). Thus the F3 subdomain of FAK lacks the structural features required to bind acidic phospholipids and tyrosine-phosphorylated peptides. Additional structural features are a 3_{10} helix within the β4–β5 turn. There are also an extra seven residues in the β5–β6 loop. As these differences are found near the surface of the subdomain, they potentially provide additional specificity for protein–protein interactions.

Taken individually, the subdomains of the FAK FERM domain are quite similar to those from other FERM-containing proteins. The orientation of the individual subdomains in relation to each other is a little bit different. The ERM proteins, such as radixin, have a very symmetrical arrangement of the three domains leading most ERM family members to structurally overlap each other with a root mean square of less than 1 Å. FAK FERM aligns with radixin with a root mean square around 1.5 Å, due to a shift of the domains in relation to each other. The most noticeable shift is in the orientation of the F3 subdomain. It is rotated in such a way that the α1 helix is 4 Å closer to the F1 subunit than in other FERM domains. The FERM domains of the ERM proteins have a phosphatidylinositol-binding site in a shallow basic cleft between F1 and F3. In the FAK FERM domain, the sequences between the F1 and F3 domains are not basic, the cleft between the two domains is narrower, and it is occupied by part of the linker between the FERM and catalytic domains, thus abrogating phosphatidylinositol binding via this mechanism.

The linker between the FERM and kinase domains spans residues 352–415 and contributes to the structural distinctiveness of FAK FERM domain. The FERM domain proximal portion of the linker forms an antiparallel β sheet that binds on a groove on the F3 subdomain. The linker, in this position, completely buries the site analogous to the phosphatidylinositol-binding site on other F3 subdomains. Two important features of the linker are Tyr397, which is the major autophosphorylation site and Src SH2 domain binding site, and the nearby "PxxP" motif that acts as an SH3 binding site for Src (Thomas et al., 1998). These features are unstructured in the FERM domain alone.

3.2. Structure of the kinase domain

The kinase domain of FAK has been crystallized in three states: with the activation loop fully phosphorylated and bound to a nonhydrolysable ATP analog (fully activated conformation) (PDB 2JOL) (Fig. 5.4), alone with an

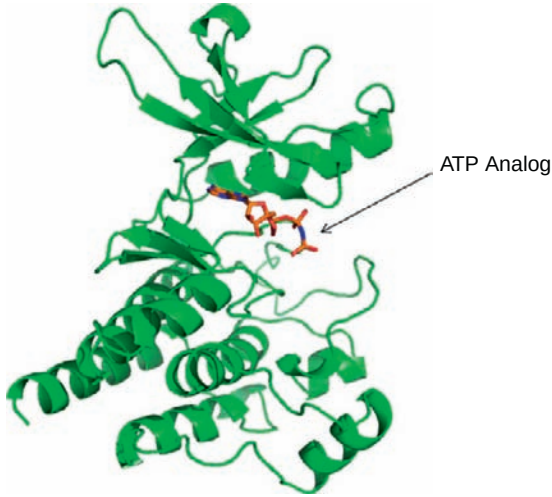

Figure 5.4 The structure of the FAK kinase domain. The crystal structure of the FAK kinase domain is shown with the small N-terminal lobe, with single α-helix and β-strands at the top and the α-helical larger C-terminal lobe at the bottom. The ATP binding site is centrally localized in the figure and is occupied by a bound nonhydrolyzable ATP analog (shown as sticks in the structure). This figure was prepared using PDB file 2JOL.

unphosphorylated activation loop (inactive conformation) (PDB 1MQB), and unphosphorylated and bound to the FERM domain (autoinhibited conformation) (PDB 2JOJ, 2JOK) (Lietha et al., 2007; Nowakowski et al., 2002). These structures demonstrate that like the catalytic domains of other protein kinases, the FAK kinase domain is bilobed, with the N-terminal lobe containing a single α-helix (the C α-helix) and a five-stranded β sheet, and the larger C-terminal lobe that is mostly α-helical. In FAK kinase, C α-helix is in the same position in the active and inactive conformations, indicating that C-helix displacement is not a method of regulation of FAK (Lietha et al., 2007). In the unphosphorylated structures, the activation loop was disordered. In the phosphorylated structure, the activation loop took on the β hairpin loop conformation seen in many other active kinases. In the unbound unphosphorylated kinase structure, the ATP binding site was a network of ordered water molecules, indicating a large amount of hydrogen binding in the active site. The hydrophobic pocket that contains the adenine base on ATP is bordered by Met499. Ribose binding involves a bifurcated hydrogen bond involving the 2′- and 3′-hydroxyl groups. The crystal structure of the unphosphorylated solitary kinase contains a disulfide bond in the N-lobe between Cys456 and Cys459 (Nowakowski et al., 2002). The cysteines are conserved in vertebrate FAKs and a regulatory role for this bond has been suggested, which is

intriguing as they reside in a loop immediately preceding the C α-helix. However, the subsequent crystal structures do not have this bond, and thus the physiological significance is uncertain (Lietha and Eck, 2008; Lietha et al., 2007). The crystal structure of the PYK2 kinase domain is very similar to that of the FAK kinase domain. There are cysteines at residues 459 and 463 in Pyk2, and like most of the solved FAK structures, the Pyk2 kinase domain lacks the originally reported disulfide bond (Han et al., 2009).

3.3. Structure of the FAT domain

The C-terminal ~135 amino acids of FAK comprise the FAT domain, which is responsible for FAK's discrete localization to focal adhesions (Hildebrand et al., 1993). There has been considerable interest in this domain to elucidate the mechanism of localization and due to the therapeutic potential of agents that could perturb the normal cellular distribution of FAK. The efforts of a number of groups have yielded both crystal structures and solution structures of the FAT domain (Arold et al., 2002; Hayashi et al., 2002; Liu et al., 2002; Prutzman et al., 2004). The domain contains four amphipathic α-helices that assemble into an antiparallel four-helix bundle with an up-down-up-down right-handed topology (Fig. 5.5). The crystal structure of the highly conserved FAT domain of Pyk2 has also been solved and this domain adopts a similar fold (Lulo et al., 2009). Interestingly, a C-terminal highly conserved region of Git1 and Git2, which are GTPase-activating proteins for the ARF GTPases, shows sequence homology with the FAT domain of FAK. A molecular model of this domain, based upon small angle X-ray scattering, was recently developed and the FAT homology domain of Git1 appears to form an antiparallel four-helix bundle similar to the FAT domain of FAK (Schmalzigaug

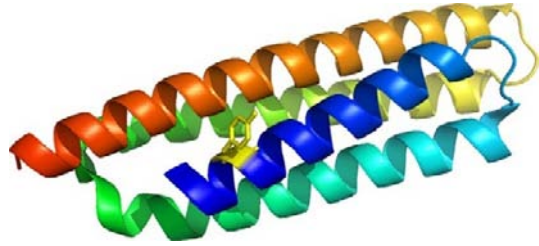

Figure 5.5 The structure of the FAT domain of FAK. The crystal structure of the FAT domain of FAK is shown. The four-helix bundle is colored, so cooler colors are at the N-terminus of the domain and warmer colors are toward the C-terminus. Tyr925 is shown as a stick representation and is colored yellow. This figure was prepared using PDB file 2RA7.

et al., 2007). There is similarity in the FAT domain structure and the structures of the C-terminal vinculin tail, α-catenin and apolipoprotein 3, since all contain a right-handed antiparallel four-helix bundles (Hayashi et al., 2002).

The striking features of the FAT domain of FAK are two hydrophobic patches on the surface of the domain flanked by basic residues. One of these lies at the interface of α-helices 1 and 4, and the other is on the opposite side of the molecule at the interface of α-helices 2 and 3. These hydrophobic patches were originally proposed as binding sites for FAK-associated proteins, which subsequent crystallographic and NMR studies have confirmed (Bertolucci et al., 2005; Gao et al., 2004; Hayashi et al., 2002; Hoellerer et al., 2003). Specifically, the two hydrophobic patches of the FAT domain engage leucine-rich domain (LD) motifs from paxillin. The LD motif is a peptide motif repeated multiple times in the N-terminal half of paxillin and the paxillin-related proteins, leupaxin and hic-5 (Brown et al., 1998; Schaller, 2001). The second and fourth LD motifs of paxillin function as FAK binding sites and each can form an amphipathic α-helix. The hydrophobic surface of these LD motifs docks with the hydrophobic patches of the FAT domain with acidic residues in the LD motifs interacting with the basic residues surrounding the hydrophobic patches on the FAT domain. While the conserved nature of the LD motifs and hydrophobic patches suggests interchangeable interactions, the docking of a peptide mimicking the LD2 motif to each binding site on the FAT domain exhibits different thermodynamic properties (Gao et al., 2004). Further, several experimental approaches demonstrate a preference of the LD4 motif for the hydrophobic patch formed at the interface of α-helices 2 and 3 in the FAT domain (Bertolucci et al., 2005; Scheswohl et al., 2008). Similar interactions facilitate binding of LD motifs to the FAT domain of Pyk2 and the FAT homology domain of Git1 (Lulo et al., 2009; Schmalzigaug et al., 2007).

Comparison of the structures of the free FAT domains of FAK and Pyk2 with LD motif-liganded structures reveals only minor differences that extend throughout the domain, but the addition of two α-helices to the four-helix bundle might stabilize the structure (Gao et al., 2004; Liu et al., 2002; Lulo et al., 2009; Prutzman et al., 2004). Stabilization of the four-helix bundle could significantly impact biological signaling. Tyr925 in FAK and Tyr881 in Pyk2 are sites of phosphorylation and mutation of these residues dramatically impairs biological function of the molecules implicating tyrosine phosphorylation and recruitment of Grb2 into complex as significant signaling events. These tyrosine residues reside within α-helix 1 of the FAT domain and this structure makes them poor substrates for kinases and poor recognition motifs for the Grb2 SH2 domain, which both recognize tyrosines in an extended peptide conformation and not an α-helix. Thus, for efficient phosphorylation and Grb2 binding, a conformation change in the FAT domain is envisioned. One crystal structure of the FAK FAT domain revealed a domain swapped dimer, where α-helix 1 of

two different molecules was exchanged to form a dimer (Arold et al., 2002). While this dimerization is not physiological, the result suggests conformational dynamics of the FAT domain during expression, purification, and crystallization. Hydrogen exchange experiments support the hypothesis that the FAT domain is a dynamic structure and discrete molecular dynamics analysis of the FAT domain structure, guided by the results of hydrogen exchange, further support the model that α-helix 1 can be displaced to facilitate transition from an α helical to extended conformation of the peptide sequence containing tyrosine 925/881 (Dixon et al., 2004; Zhou et al., 2006). These studies suggest that the FAT domain is dynamic, capable of adopting the four-helix bundle, and an altered conformation allowing phosphorylation of tyrosine 925/881 and Grb2 binding. Binding of the paxillin LD motifs to the FAT domain, particularly to the interface of α-helices 1 and 4, stabilizes the four-helix bundles and impairs tyrosine phosphorylation of these sites and Grb2 binding. Thus, paxillin binding may regulate FAK signaling by regulating FAT domain structure and promote specific signaling events while inhibiting others.

4. Autoinhibition

4.1. Mechanism of autoinhibition

The FERM domain was proposed as a negative regulatory element of FAK based upon several studies reporting that deletion of the FERM domain resulted in elevated catalytic activity and/or constitutive tyrosine phosphorylation in cells (Jacamo and Rozengurt, 2005; Schlaepfer and Hunter, 1996; Toutant et al., 2002). Based upon intramolecular autoinhibitory models of the ERM proteins, a similar autoinhibitory mechanism was proposed for FAK. The first experiments supporting this mechanism utilized an exogenous expression system where the FERM domain interacts with the kinase domain in *trans*, and this interaction has a negative effect upon FAK phosphorylation *in vivo* and catalytic activity *in vitro* (Cooper et al., 2003). Additionally, expression of individual F1 and F2 subdomains inhibits phosphorylation, indicating that these two subdomains are key for kinase inhibition in FAK (Cohen and Guan, 2005).

A fragment of FAK containing both the FERM and kinase domains was crystallized (Lietha et al., 2007), and the structure showed that the FERM and kinase domains did indeed interact (PDB 2JOJ) (Fig. 5.6). There are two points of contact between the two domains. The most extensive is between the F2 subdomain of the FERM domain and the C-lobe of the kinase. This interaction leads to a 649 $Å^2$ area of the kinase domain being buried. This area is not large; however, it is highly conserved among various species. This interaction is critical for autoinhibition since mutation of a

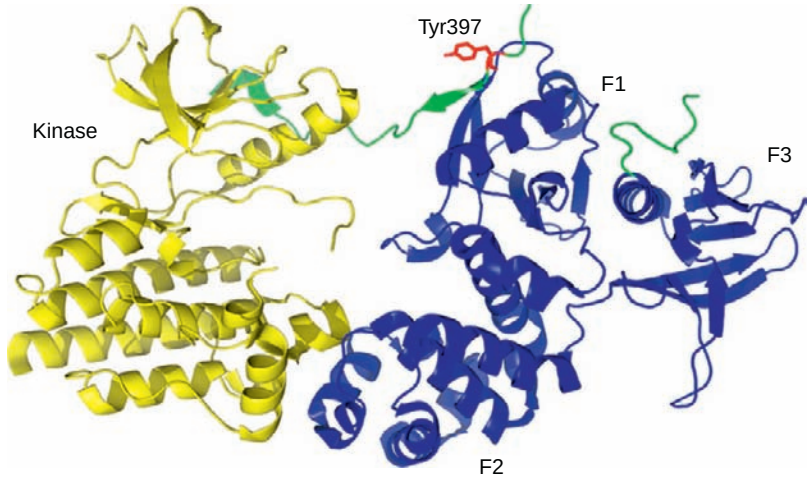

Figure 5.6 The structure of the FAK FERM and kinase domains in the autoinhibited conformation. The complex of the FERM domain and kinase domains in the autoinhibited conformation is shown. The FERM domain is colored blue, the linker segment is colored green, and the kinase domain is shown in yellow. The F1, F2, and F3 subdomains of the FERM domain are labeled. Tyr397, the major site of autophosphorylation, is in the linker and is represented by sticks colored in red. Note the distance between Tyr397 and the active site of the kinase domain in this autoinhibited conformation. This figure was prepared using PDB file 2JOJ.

number of different residues within the buried area creates constitutively active variants of FAK (Lietha et al., 2007). The second, less intimate, point of contact is between the F1 subdomain and the N-lobe of the kinase by way of the linker. This interaction may also be important in stabilizing the autoinhibited conformation, since mutation of Lys38, an F1 subdomain residue that appears to interact with the linker, results in a constitutively active mutant (Cohen and Guan, 2005). When the FERM domain is bound to the kinase domain, the linker rests against the F1 subdomain in an antiparallel β strand interaction. This binding keeps Tyr397 approximately 35 Å away from the catalytic cleft of the kinase domain, preventing intramolecular autophosphorylation. There are several splice variants of FAK that create a longer linker region (Burgaya et al., 1997; Derkinderen et al., 2001). These variants show elevated phosphorylation and catalytic activity (Toutant et al., 2002). Further, these variants may exhibit an altered mechanism of autophosphorylation, via an intramolecular rather than *trans* phosphorylation mechanism (Toutant et al., 2002). Destabilization of the autoinhibited conformation by the altered linker could explain both constitutive activation and mode of autophosphorylation, although this hypothesis has not been tested.

In the autoinhibited conformation, the activation loop of the kinase domain is disordered (Lietha et al., 2007). Based upon the topology of the autoinhibited conformation, Tyr576 and Tyr577 are somewhere in the cleft between the active site and the F1 and F2 subdomains of the FAK FERM domain. In this position, these residues are sequestered and unavailable as substrates for Src. In the crystal structure of the phosphorylated, active kinase domain, the phosphorylated activation loop takes on a β-hairpin conformation that is stabilized by hydrogen bonds and electrostatic interactions between the phosphate group on pTyr577 and residues Ser580, Ala579, and Arg569 (Fig. 5.7). Several residues in the activation loop in the phosphorylated kinase crystal structure, including pTyr576, occupy space where the FERM domain sits in the autoinhibited structure, suggesting activation loop phosphorylation blocks the FERM domain interactions with the catalytic domain. Thus, restoration of the autoinhibited state is dependent on phosphatase activity. Mutation within the activation loop, that is, of two lysines next to glutamic acids, creates a constitutively active mutant, consistent with a role for the activation loop in promoting catalytic activity (Gabarra-Niecko et al., 2002).

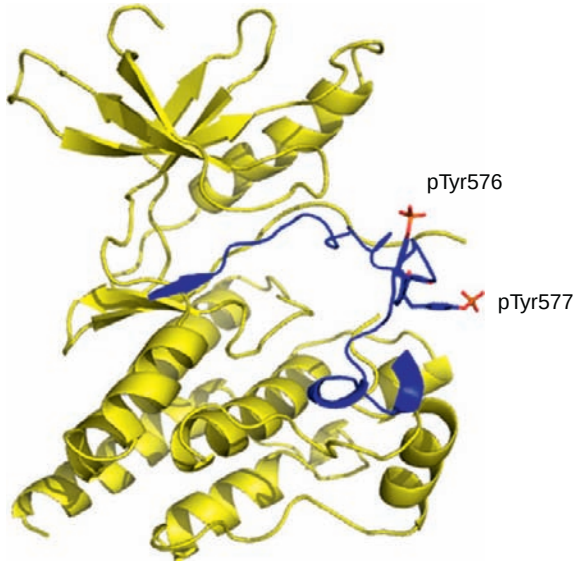

Figure 5.7 The activation loop of FAK stabilizes upon phosphorylation. The structure of unphosphorylated FAK kinase is shown in yellow. Note that the activation loop is disordered. The phosphorylated activation loop is superimposed in blue. Note that the phosphorylated activation loop is highly ordered. This figure was prepared using PDB files 2JOl and 3FZS.

Pyk2 is presumed to fold into a similar autoinhibitory conformation, based upon the conservation of key residues that mediate the FERM/kinase domain interaction. Calcium is an important second messenger controlling the activation of Pyk2 and a number of signaling mechanisms have been proposed to link calcium flux to Pyk2 activation. Two mechanisms involve protein–protein interactions with the Pyk2 FERM domain. First, Ca^{2+}/calmodulin is proposed to bind to the F2 lobe of the FERM domain and release autoinhibition (Kohno et al., 2008). Considerable structural changes in the FERM domain may be required to facilitate this interaction if the binding occurs via the molecular mechanism proposed, which is based predominantly upon sequence conservation between Pyk2 and a calmodulin binding partner. The second potential mechanism for release of autoinhibition is by dimerization of the Pyk2 FERM domains, resulting in displacement of the kinase domain (Kohno et al., 2008; Riggs et al., 2011). Additional studies are required to fully elucidate the mechanism of autoinhibition relief downstream of calcium.

Based upon these structural studies and other avenues of investigation, the following model of FAK regulation has emerged (Fig. 5.8). A ligand, an acidic phospholipid or tyrosine-phosphorylated peptide (Cai et al., 2008; Chen and Chen, 2006), binds the KAKTLRK sequence in the F2 subdomain triggering a conformation change. The molecular details of the conformation change are unknown, but are necessary for substrate access to the FAK active site and activation loop phosphorylation by Src. Autophosphorylation at Tyr397 in the linker occurs. The effect of phosphorylation upon conformation is unknown. Autophosphorylation creates a binding site for SH2 domain-containing signaling proteins, and a number of scaffolded complexes could form. Src also binds to this site and phosphorylates the activation loop, fully activating FAK catalytic activity. At this point, ligand binding to the F2 subdomain is no longer required since the phosphorylated activation loop blocks the FERM/catalytic domain interaction. Dissociation of Src would allow additional Tyr397-mediated scaffolding activities. The action of protein tyrosine phosphatases is required to allow FAK to reconfigure into the autoinhibited conformation.

4.2. Measuring FAK activation using biosensors

Several FRET-based biosensors to measure Tyr397 phosphorylation and conformational change in FAK in living cells have been engineered. A Tyr397 phosphorylation biosensor consists of a FAK molecule labeled on the N-terminus with CFP and a citrine-labeled Src SH2 domain construct. Since phosphorylation of Tyr397 is necessary for Src binding, increased FRET signal indicates phosphorylation (Ballestrem et al., 2006; Cai et al., 2008). Two conformational biosensors have been created. One consists of CFP fused to the N-terminus of FAK and citrine inserted at the kinase

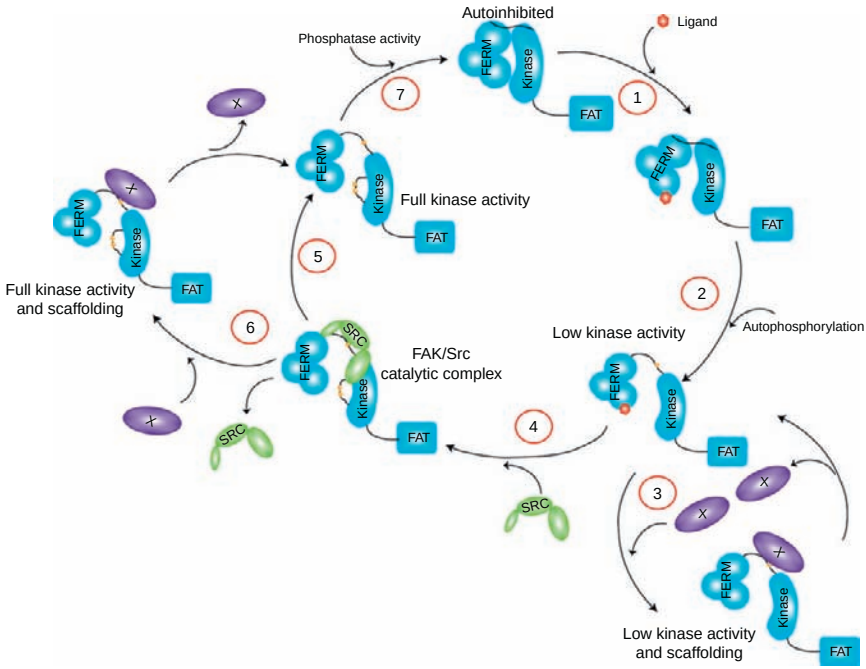

Figure 5.8 The cycle of FAK activation/inactivation. The autoinhibited conformation of FAK is shown with the FERM and kinase domains forming a direct interaction blocking access of ATP and substrate to the active site. (1) The first step of activation requires ligand binding releasing autoinhibition. (2) Autophosphorylation of FAK creates an SH2 binding site at Tyr397. Phosphorylation of this site could further destabilize the autoinhibitory conformation through disruption of the interaction between the linker and the F1 subdomain, although this remains to be determined. (3) The Tyr397 SH2 binding site can act as a scaffold to recruit various signaling molecules into complex. (4) This same site is the binding site for Src, which is responsible for phosphorylation of the activation loop resulting in maximal activation of FAK catalytic activity. The FAK/Src complex represents the most active enzyme complex in the cycle. (5) When Src is released, FAK retains maximal activity due to phosphorylation of the activation loop. (6) After release of Src, the fully active FAK kinase may scaffold other SH2 domain-containing proteins. (7) Return to the autoinhibited conformation requires phosphatase activity. The details of this step have yet to be elucidated.

domain proximal end of the linker at Arg413. This probe has been validated using activating mutations and exhibits higher FRET in the autoinhibited conformation and leads to a reduction in FRET signal upon conformational activation (Cai et al., 2008). The second conformational biosensor contains YFP at the N-terminus of FAK and a CFP inserted in the FERM domain proximal end of the linker at Val391, switching the positions of the donor and acceptor molecules for FRET. Interestingly, positioning of the

fluorescent molecules in this alternate conformation results in increased FRET upon FAK activation (Papusheva et al., 2009). This is a powerful set of probes to study conformational changes in living cells, since the fluorescent responses of the two probes to physiological stimuli should be reciprocal (Cai et al., 2008; Papusheva et al., 2009). They are also important tools to elucidate the mechanism of conformational change upon FAK activation.

5. FAK Binding Partners

5.1. FERM domain-mediated interactions

5.1.1. Growth factor receptors

A broad range of extracellular stimuli induce FAK tyrosine phosphorylation and activation, including growth factors. FAK acts as a bridging adaptor protein that links growth factor receptor and integrin signaling pathways to support cell motility. FAK is required in PDGF- and EGF-induced cell migration, and the N-terminal 402 amino acids of FAK form a complex containing activated growth factor receptors (Sieg et al., 2000a) (Fig. 5.9A). Phosphorylation of FAK at Tyr397 is not required for the formation of this complex; however, the EGF receptor promotes FAK phosphorylation at Tyr397. Overexpression of FRNK disrupts the interaction of FAK with

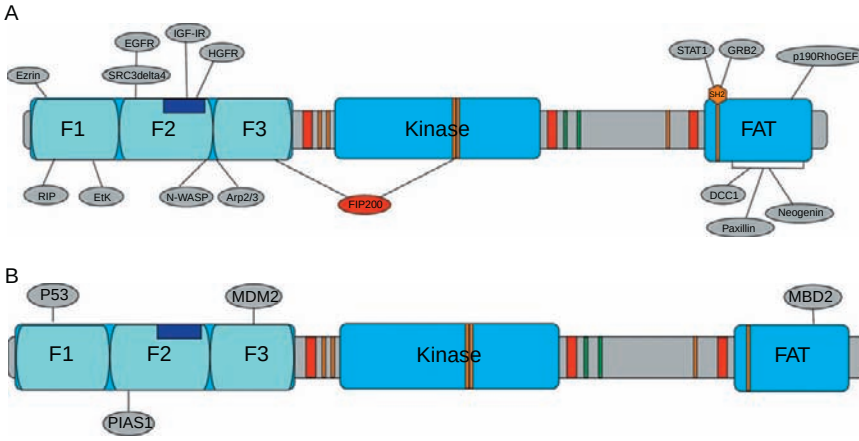

Figure 5.9 FAK is involved in many intermolecular interactions. (A) The cartoon of FAK is shown with its major interaction partners that bind the FERM, kinase, and FAT domains in the cytoplasm. The bracket along the FAT domain represents LD motif binding. (B) The cartoon of FAK is shown with its major interaction partners that bind FAK in the nucleus.

activated EGF receptors, and since FRNK does not directly mediate receptor binding, cell adhesion-dependent activation of FAK may be required for interaction with these growth factor receptors. Despite its conservation of sequence, Pyk2 does not associate with these activated growth factor receptors (Sieg et al., 2000). A recent study suggests a completely unanticipated mechanism of interaction between the EGF receptor and FAK mediated by SRC-3Δ4 (SRC-3 is the steroid receptor coactivator 3 and should not be confused with the Src tyrosine kinase) (Long et al., 2010). SRC-3Δ4 is an alternatively spliced variant of SRC-3 lacking the nuclear localization sequence and thus localizing in the cytoplasm. Endogenous SRC-3Δ4 coimmunoprecipitates with FAK and the EGF receptor. siRNA-mediated knockdown of SRC-3Δ4 partially reduces the coimmunoprecipitation of EGF receptor with FAK, suggesting that SRC-3Δ4 may mediate this interaction, although there may be additional molecular mechanisms of interaction between these two kinases. Interestingly, serine and threonine phosphorylation of SRC-3Δ4 by PAK is required for binding to both EGFR and FAK. The proposed EGFR/SRC-3Δ4/FAK complex seems important for cell migration *in vitro* and metastasis *in vivo*.

The Met receptor mediates hepatocyte growth factor (HGF) signaling in cells. Stimulation of cells with HGF promotes FAK tyrosine phosphorylation (Matsumoto et al., 1994). The phosphorylation of Tyr1349 and Tyr1356 of Met receptor β-chain is required for its biological activity in promoting cell motility and invasion (Zhu et al., 1994). A direct interaction between the FERM domain of FAK and the Met receptor, when Met is phosphorylated on tyrosine residues 1349 and 1356, has been reported. This tyrosine-phosphorylated peptide is proposed to directly bind several basic residues (216KAKTLRK222) on the surface of the FERM domain of FAK (Chen and Chen, 2006). As a consequence of this interaction, tyrosine-phosphorylated Met relieves FERM-domain-mediated autoinhibition of FAK, resulting in FAK activation. This interaction is important for promoting HGF-induced cell invasion (Pongchairerk et al., 2005).

The insulin-like growth factor I receptor (IGF-IR) is a transmembrane receptor that initiates a cascade of events that include mitogenic and anti-apoptotic responses (Kulik et al., 1997; Obberghen-Schilling and Pouyssegur, 1983). The relationship between FAK and IGF1 signaling is complex and likely dependent upon cell context. Different reports demonstrate that IGF1 induces FAK dephosphorylation through recruiting SHP2 or has no effect upon FAK phosphorylation (Manes et al., 1999; Zhang et al., 2005). Others report FAK is important for IGF1 responses. FAK and IGF-IR were found in the same complex in pancreatic adenocarcinoma cells (Liu et al., 2008). This interaction is direct and occurs between the catalytic domain of the IGF-IR and the F2 subdomain of the FAK FERM domain (Zheng et al., 2009). Additional molecular details of the interaction and characterization of the consequences of binding on the catalytic activity

of IGF-IR and FAK have not been fully elucidated. Cell proliferation stimulated by IGF-1 is dependent on the presence of FAK. Dual inhibition of both FAK and IGF-IR using dominant negative, siRNA, and pharmacological approaches decreased cell viability and increased apoptosis in cancer cells (Liu et al., 2008). There is a small molecule tyrosine kinase inhibitor, TAE226, which inhibits both FAK and IGF-IR activity. This compound causes cellular apoptosis in pancreatic cancers and impairs tumor formation in a number of models (Halder et al., 2007; Liu et al., 2007, 2008).

5.1.2. Other binding partners

It was reported that the N-terminal domain of Ezrin interacts with amino acids 1–376 in the FERM domain of FAK. This interaction may promote activity of FAK through increasing Tyr397 phosphorylation. Ezrin-dependent activation of FAK is not linked to cell–extracellular matrix adhesion (Poullet et al., 2001). The molecular details of this interaction and significance of this mechanism of regulation are currently unknown.

FAK also interacts with the PH domain of Etk directly through the FERM domain to promote endothelial cell or epithelial cell migration. Activated FAK may play a critical role in increasing the activity of Etk since the FAK Y397F mutant lacks the ability to active Etk. The FAK sequence mediating the interaction is unknown, but is presumably unique to FAK since Pyk2 fails to bind Etk. The Tyr40 and Glu42 residues in the PH domain of Etk are critical for Etk activation by FAK (Chen et al., 2001). Glu42 is involved in the Etk/FAK interaction because the Glu42Lys mutant exhibits weak binding and is not activated by FAK. The Tyr40Phe mutant binds FAK but exhibits significantly diminished FAK-induced tyrosine phosphorylation of Etk. The regulation of Etk by FAK is independent of the lipid binding activity of the PH domain, as the key residue for lipid binding, Arg29, is not required. Tyr40 is buried in the lipid binding pocket and Glu42 is on the surface of the PH domain in the proximity of the lipid binding site (based upon the structure of the highly related Btk PH domain). Under some scenarios, signaling via other tyrosine kinases might promote the association of FAK and Etk. He et al., for example, demonstrate that the tyrosine kinase inhibitor, AG879, which is an inhibitor specific for ErbB2 and VEGF receptor FLK-1, inhibits the association of FAK and Etk through the activation of PAK1 (He et al., 2004). While this is an intriguing mechanism of regulation, there are several outstanding questions including the relationship between lipid binding and FERM domain binding, and if lipid- and FERM-mediated activation occurs via similar mechanisms.

RIP is a family of proteins named receptor interacting proteins. They are serine/threonine kinases that contain an N-terminal kinase domain and a C-terminal death domain. The death domain of RIP1 interacts with a number of receptors that function in the induction of apoptosis. RIP1 acts

downstream of TNF-R1 to control nuclear factor (NF)-κB activation and thus plays a role in determining whether cells survive or undergo apoptosis (Martinon et al., 2000). It was originally demonstrated that the death domain of RIP also interacts with the N-terminal domain of FAK both *in vivo* and *in vitro* (Kurenova et al., 2004). The interaction may be more complex, as the N-terminal domain of RIP (lacking the death domain) can also bind FAK, although the docking site on FAK is unknown (Kamarajan et al., 2010). RIP provides proapoptotic signals in attached cells treated with staurosporine and in detached cells undergoing anoikis because $RIP^{-/-}$ cells were resistant to cell death under these conditions (Kamarajan et al., 2010; Kurenova et al., 2004). Induction of apoptosis and anoikis results in the release of RIP from FAK, and in the latter case promotes the formation of a FAS/RIP complex, resulting in a proapoptotic signal. Based upon these observations, the assembly of a complex with RIP is postulated as one mechanism used by FAK to generate a strong survival signal in tumor cells (Kurenova et al., 2004).

Kadare et al. found that the protein inhibitor of activated STAT1 (PIAS1) interacts with the N-terminal domain of FAK in a yeast two-hybrid screen (Kadare et al., 2003). They identified residues 403–651 in the PIAS1 C-terminal domain as the FAK binding site. This interaction will induce sumoylation of FAK at Lys152 and increase the phosphorylation level of FAK at Tyr397 in cells. Lys152 is buried in the structure of the FAK FERM domain, in the groove between the F1 and F2 subdomains. In the auto-inhibited FAK conformation, Lys152 lies between the FERM and kinase domains and is further inaccessible for sumoylation in this conformation. Considerable structural rearrangement would be required to facilitate FAK sumoylation at this site, but the SUMO moiety would certainly interfere with assembly of the autoinhibited conformation, consistent with the observed elevation in phosphorylation in the sumoylated population of FAK.

5.1.3. FERM domain binding partners that regulate the cytoskeleton

Wiskott–Aldrich syndrome (WAS) protein is encoded by WASL gene. The WAS family of proteins shares similar structure and is involved in signaling pathways that regulate the actin cytoskeleton. N-WASP is one family member that is an effector of Cdc42-mediated actin cytoskeleton regulation in cells. FAK interacts with N-WASP and binding occurs between the N-terminal domain of FAK (residues 1–400) and N-WASP residues 148–273 (Wu et al., 2004). This interaction promotes phosphorylation of N-WASP at Tyr256 by FAK, and this phosphorylation can regulate the subcellular location of N-WASP. FAK-mediated N-WASP phosphorylation is important in promoting cell migration (Wu et al., 2004). Cell migration requires dynamic regulation of actin filaments and focal adhesion complexes in

lamellipodia. In these structures, actin filament polymerization is controlled by the Arp2/3 complex, which is regulated by Rac1 and Cdc42 through WASP proteins. The FERM domain of FAK is also believed to interact with the Arp2/3 complex, and thus may scaffold N-WASP and the Arp2/3 complex to control actin polymerization in lamellipodia (Serrels et al., 2007). Interestingly, phosphorylation of Tyr397 of FAK impairs the interaction of FAK with Arp2 and Arp3, suggesting that FAK autophosphorylation triggers the release of the Arp2/3 complex, which promotes initiation of nascent lamellipodia formation and cell spreading.

5.1.4. FERM domain interactions in the nucleus

Although FAK was reported to localize to the nucleus in several early studies, the nuclear role of FAK remained obscure until more recently. As the FERM domain alone could enter the nucleus, one mechanism of localization is FERM domain mediated. Basic sequences within the F2 subdomain of FAK are important for nuclear localization, although these sequences are also critical for interaction with other cytoplasmic/membrane located ligands (Lim et al., 2008a). Golubovskaya et al. found that FAK could directly interact with p53 *in vitro* and documented the interaction *in vivo* (Fig. 5.9B). The N-terminal transactivation domain of p53 interacts with FERM domain of FAK in tumor cell lines and in normal human fibroblasts. The FAK binding site on p53 was further narrowed to the seven amino acids extending from residues 65 to 71, which is located in a conserved proline-rich region of human p53 (Golubovskaya et al., 2008a). Binding to FAK inhibits p53-induced transcription and p53-induced apoptosis by altering MDM2 and Bax activity in cells (Golubovskaya et al., 2005, 2008a). Further studies of FAK and p53 suggest that FAK functions to scaffold MDM2 and p53. The F1 domain of the FAK FERM domain binds p53 and the F3 domain of the FAK FERM domain binds MDM2. By promoting the association of MDM2 and p53, FAK is proposed to promote the ubiquitination and turnover of nuclear p53 (Lim et al., 2008a).

5.2. FIP200 binds multiple FAK domains

FIP200 is the FAK family kinase-interacting protein of 200 kDa (Ueda et al., 2000). It was originally identified in a yeast two-hybrid screen for Pyk2 interacting proteins. The C-terminal domain of FIP200 binds to the catalytic domain of Pyk2 and impairs the activity of Pyk2 three- to fivefold *in vitro* (Fig. 5.9A) (Ueda et al., 2000). In transient expression assays, FIP200 inhibited Pyk2 phosphorylation and the induction of apoptosis, suggesting FIP200 can regulate biological responses to Pyk2. FIP200 also binds FAK. The C-terminal domain of FIP200 binds the N-terminal domain of FAK, while the middle and N-terminal domains of FIP200 both bind the catalytic domain of FAK (Abbi et al., 2002). The interaction impairs kinase activity

in vitro. FIP200 complexes with FAK in cells in suspension and appears to dissociate upon integrin-dependent cell adhesion, particularly to fibronectin. Interestingly, FIP200 appears to inhibit FAK-dependent phosphorylation of paxillin and Shc, but does not affect FAK-dependent phosphorylation of p130cas and Grb7, in transiently transfected cells (Abbi et al., 2002). For a number of reasons, FIP200 is an intriguing binding partner of FAK and Pyk2, but the molecular details of the interaction and mechanisms controlling biochemical and biological events have yet to be determined.

5.3. FAT domain binding partners

Growth factor receptor bound protein 2 (Grb2) contains an SH2 domain and a pair of SH3 domains (Schlaepfer et al., 1994). The binding of Grb2 to FAK is dependent on phosphorylation of FAK at Tyr925, which creates a consensus Grb2 SH2 domain binding site. As discussed above, this phosphorylation/binding site resides in the first α-helix of the FAT domain and conformational change of the FAT domain must precede phosphorylation and Grb2 binding. The FAK/Grb2 interaction is induced by integrin receptors and is coupled to activation of the Ras signaling pathway and downstream MAPK (Schlaepfer et al., 1994). In breast cancer cells, this pathway may be critical for the induction of VEGF expression (Mitra et al., 2006b). In addition to regulating RAS/MAPK signaling, phosphorylation of FAK at Tyr925 also functions to recruit a Grb2/dynamin complex. This complex is postulated to promote internalization of focal adhesion components when microtubules engage focal adhesions (Ezratty et al., 2005).

There is another group of proteins related to a transcription signaling pathway that is regulated by FAK activity. Overexpression of FAK causes activation of STAT1, which is one of the signal transducer and activator of transcription (STAT) pathway proteins. STAT1 interacts with the C terminus of FAK directly (Xie et al., 2001). The consequences of STAT1 binding are apparently a reduction in cell adhesion but an increase in cell motility.

As described in detail above, FAK binds to two peptide motifs (LD motifs) on paxillin and in turn has two binding sites for paxillin in the FAT domain. Paxillin binding is one mechanism by which FAK is localized to focal adhesions, and talin binding is proposed as a second, although the details of interaction are not established. Paxillin is also a substrate for FAK, and tyrosine phosphorylation of paxillin recruits additional signaling molecules into complex to activate downstream signaling. Paxillin also scaffolds signaling molecules in a phosphotyrosine-independent manner, including PKL/Git2 (paxillin kinase linker) and β-pix (a guanine nucleotide exchange factor for Cdc42 and scaffold for Rac and PAK). The FAK/paxillin complex also facilitates tyrosine phosphorylation of PKL/Git2 and β-pix (Chang et al., 2007; Yu et al., 2009). Tyrosine phosphorylation of β-pix (Chang

et al., 2007) impacts Rac recruitment and tyrosine phosphorylation of PKL regulates cell polarization, lamellipodia stability, and directional persistence of migrating cells (Yu et al., 2009).

The endocytosis motif in the cytoplasmic domain of CD4 has some similarity to the LD motifs of paxillin. Notably, the signature aspartic acid is not found in CD4, but the endocytosis motif is predicted to form an amphipathic α-helix similar to the LD motifs. A peptide mimicking the CD4 motif (amino acids 405–417) binds the FAT domain of FAK with similar thermodynamic properties as the paxillin LD4 motif. Both interactions show two binding sites on the FAT domain, are entropy driven, and exhibit similar affinities (Garron et al., 2008). Mutation of the LD sequence in the CD4 endocytosis motif abolishes binding. The structure of this CD4 peptide in complex with the FAT domain of FAK has been solved. The amphipathic α-helix docks with two hydrophobic patches of the FAT domain generally reminiscent of the interaction of paxillin LD motifs (Garron et al., 2008). Interestingly, CD4 residues contacting the FAT domain are also critical for CD4 interactions with Lck, which binds with significantly higher affinity. It is proposed that disruption of the CD4/Lck complex, for example, by cellular stimulation with the HIV gp120 protein, is a prerequisite for the assembly of a CD4/FAK complex (Garron et al., 2008). In light of this interaction, it is interesting that gp120 stimulation of cells can induce tyrosine phosphorylation of FAK (Cicala et al., 2000). The functional significance of this signaling event is unknown.

Convergent lines of investigation led to the discovery that FAK plays a role in neurons to regulate axonal outgrowth and guidance, and in oligodendrocytes to control branching. FAK directly associates with the cytoplasmic domain of neogenin and DCC (deleted in colon cancer) (Li et al., 2004; Liu et al., 2004; Ren et al., 2004), which serve as receptors for netrin 1, an extracellular ligand controlling axonal pathfinding and stimulating oligodendrocyte branching. The FAT domain of FAK mediates this interaction with an LD-like motif in the C-terminal domain of neogenin/DCC (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). Intriguingly, these receptors bind FAK but not Pyk2 (Ren et al., 2004), but the basis of selective binding is unknown. This receptor/FAK interaction is essential for netrin 1-induced FAK tyrosine phosphorylation and FAK is required for netrin 1-induced axonal outgrowth and guidance (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). FAK may also be required for netrin 1-induced oligodendrocyte branching (Rajasekharan et al., 2009). In addition to controlling neuronal outgrowth, netrin 1 also promotes specific translation of the κ-opioid receptor in dorsal root ganglion neurons. FAK and the associated adaptor protein, Grb7, have been identified as components of this signaling pathway (Tsai et al., 2006, 2007). FAK has also been implicated downstream of other netrins to control biological responses of other cell types. The absence of neogenin in skeletal muscle results in reduced

myofiber size, which is correlated with reduced FAK phosphorylation *in vivo*. Further, stimulation of cultured myoblasts with netrin 2 promotes FAK phosphorylation in a neogenin-dependent manner (Bae et al., 2009).

p190RhoGEF was identified as a FAK FAT domain binding partner in a yeast two-hybrid screen, and endogenous p190RhoGEF coimmunoprecipitates with endogenous FAK (Zhai et al., 2003). Pyk2 presumably interacts through the same mechanism as it also coimmunoprecipitates with p190RhoGEF (Lim et al., 2008c). The p190RhoGEF sequence extending from residues 1292 to 1301 is required for FAK binding, but the extent of the binding site has not been defined (Zhai et al., 2003). This sequence is not similar to the LD motifs of paxillin and thus binding apparently occurs via a unique mechanism, which remains to be determined. Cell adhesion to laminin or fibronectin induces tyrosine phosphorylation of p190RhoGEF, which correlates with activation of Rho, and both of these signaling events are dependent upon FAK (Lim et al., 2008c; Zhai et al., 2003). This signaling pathway is proposed to regulate axonal branching in Purkinje and hippocampal neurons by impairing branching and promoting axonal retraction via activation of Rho (Rico et al., 2004b).

Gelsolin is an intriguing FAT domain binding partner, since it associates with the FAT domain of Pyk2 but not FAK (Wang et al., 2003). Pyk2 and gelsolin bind in a yeast two-hybrid system, when exogenously expressed in mammalian cells, and endogenous Pyk2 and gelsolin coimmunoprecipitate from lysates of osteoclasts. Deletions that impinge on the FAT domain abrogate gelsolin binding and mutations in an LD motif peptide sequence in the C-terminal part of gelsolin abolish Pyk2 binding (Wang et al., 2003). Thus it would appear that gelsolin associates with Pyk2 through a similar mechanism as paxillin family proteins, but it is totally unclear how selective binding to Pyk2 is achieved. This interaction may promote Pyk2-dependent phosphorylation of gelsolin. Expression of Pyk2 apparently reduces the monomeric actin binding activity of gelsolin and increases $PI(4,5)P_2$ activity, which may reduce capping activity in cells (Wang et al., 2003). The molecular mechanism of Pyk2-dependent regulation of gelsolin activity remains to be elucidated.

Methyl-CpG-binding protein 2 (MBD2) was also initially isolated in a yeast two-hybrid screen using the Pyk2 C-terminal domain (Luo et al., 2009). The binding site in Pyk2 was subsequently identified as the FAT domain and FAK was also shown to bind MBD2 (Fig. 5.9B). The molecular details of this interaction remain to be fully determined, but binding site on MBD2 does not appear to be a simple peptide motif. MBD2 binds methylated sequences of DNA and histone deacetylase 1 (HDAC1) to regulate histone acetylation and chromatin structure. FAK binding to MBD2 appears to reduce HDAC1 binding and thus could impact the regulation of chromatin structure and consequently gene expression (Luo et al., 2009).

6. Development of FAK Therapeutics

Given the FAK's role in cancer development and angiogenesis, it has emerged as an excellent therapeutic target, since drugs might impact both tumor function and tumor-induced angiogenesis. Several techniques have been used to inhibit FAK, including dominant negative approaches, shRNA interference, and antisense oligonucleotide approaches (Jiang et al., 2006; Wu et al., 2006). While each has been useful in testing the significance of FAK in models of cancer, limitations in drug delivery systems for these reagents preclude their use therapeutically. At present, most promising FAK inhibition strategies are small molecule inhibitors that target kinase activity.

The Genomics Institute of the Novartis Research Foundation and the Novartis Institute for BioMedical Research used rational drug design to produce compound 1 (Choi et al., 2006b). This pyrrolo[2,3-*d*]pyrimidine uses a five-membered ring to bridge the four and five phosphates of the pyrimidine. The results of structural analysis and molecular modeling suggested that improvements to specificity and inhibition could be made by modifying the side groups (Choi et al., 2006a). The modifications resulted in compound 32 which contains a 2-pyrimidine ring and extended carboxyl group. This carboxyl group forms a salt bridge with Lys454 and reduces the IC50 to 4 nM in an *in vitro* assay. Further development of this compound has not been reported.

Novartis has developed TAE226, a bis-anilino pyrimidine that interacts with the kinase hinge region (Lietha and Eck, 2008). It has an IC50 in *in vitro* assays of 5.5 nM for FAK and TAE226 is equipotent for Pyk2 (Liu et al., 2007). Cys502 forms two hydrogen bonds with the pyrimidine and 2-methoxyaniline groups of TAE226. Additional hydrophobic contacts between the pyrimidine and Ala452 and Leu553, as well as interactions between the 2-methoxyaniline and Ile428 and Gly505, hold the inhibitor securely in place. The binding of the inhibitor in this way stabilizes the DFG motif, within the activation loop, in an unusual helical conformation (Fig. 5.10A). The flexibility to adopt this conformation is due to a glycine preceding the DFG motif. While this glycine is highly conserved in FAK (and Pyk2), it is not conserved in most other kinases (Lietha and Eck, 2008). One exception where this glycine is present is in IGF-IR and TAE226 effectively inhibits IGF-1R as well. TAE226 increases survival rates in rats with glioma and ovarian cancer xenografts; however, because it also inhibits IGF-IR, it is currently unsuitable for trial in human subjects and requires additional development (Halder et al., 2007; Liu et al., 2007).

Pfizer has developed several FAK inhibitors including PF 573,228 and PF 562,271. These compounds were discovered through a combination of

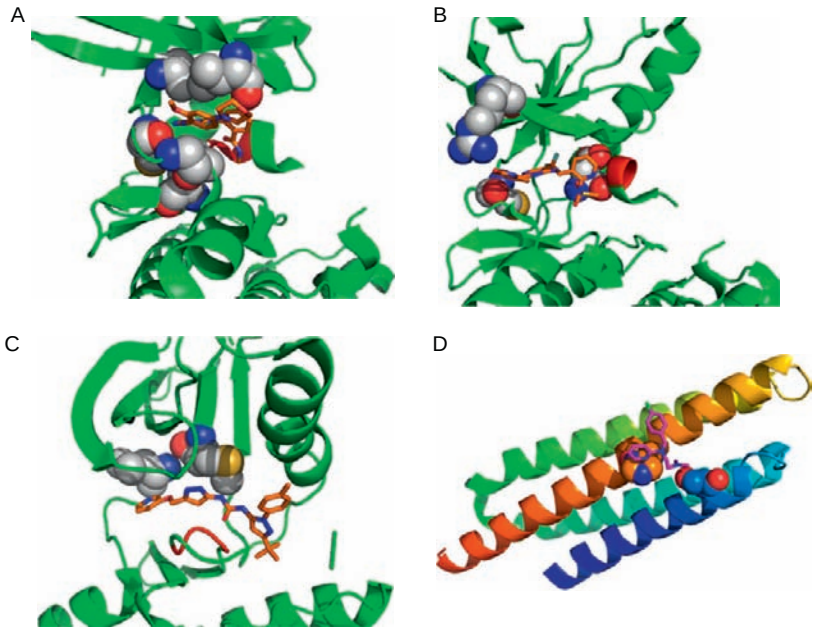

Figure 5.10 Structures of drugs bound FAK and Pyk2. (A) The structure of the kinase domain of FAK in complex with TAE226. Ile428, Ala452, Cys502, Gly505, and Leu553 are shown as spheres and the compound as a stick figure. Note the DGF motif forms a short α-helix (red). This figure was prepared using PDB file 2JKK. (B) The kinase domain of FAK in complex with PF 562,271. Arg426, Cys502, and Asp564 are shown as spheres and the compound as a stick figure. Again note the short α-helix formed by the DFG sequence (red). This figure was prepared using PDB file 3BZ3. (C) The kinase domain of Pyk2 in complex with PF 461,8433. Lue504 and Met502 are shown as spheres. The DFG motif is shown in red. This figure was prepared using PDB file 3FZR. (D) The structure of the FAT domain in complex with *N*-(4-chlorobenzyl)-*N*′, *N*′-dimethyl-*N*-pyridin-2-ylethane-1,2-diamine. Ser939 and His1025 are shown as spheres and the compound as a stick figure. This figure was prepared using PDB file 2RA7.

high-throughput screening, structure-based design, and conventional medicinal chemistry and both are methane sulfonamide diaminopyrimidines (Roberts et al., 2008; Slack-Davis et al., 2007). PF 573,228 has an IC50 of 4 nM for FAK and 1 μM for Pyk2 and is very specific to these two kinases (Slack-Davis et al., 2007). PF 573,228 inhibited cell spreading and migration as well as reduced cell growth. The crystal structure of FAK in complex with PF 562,271 has been solved (PDB ID 3BZ3) (Roberts et al., 2008; Fig. 5.10B). PF 562,271 forms three hydrogen bonds with FAK, two with Cys502, and one with Arg426. The sulfonamide group interacts with the DFG motif, with the oxygen–hydrogen binding to Asp564 (Roberts

et al., 2008). This interaction results in the movement of Asp564 upward and away from the ATP binding site, and creates the same helical DFG motif conformation seen in FAK bound to TAE226 (Lietha and Eck, 2008; Roberts et al., 2008). PF 562,271 is effective in a number of preclinical models and inhibits FAK phosphorylation in tumors as well as increases apoptosis of tumor cells (Roberts *et al.*, 2008). This compound has an IC50 of 1.5 nM against FAK, impairs the growth of breast cancer cells when injected into bone to model colonization at a metastatic site, and impairs tumor-induced bone loss. The former was attributed to inhibition of FAK in the tumor cells and the latter to inhibition of Pyk2 in osteoclasts (Bagi et al., 2008). Similar results were seen with a combined dosage of PF 562,271 and the antiangiogenic drug, sunitinib, in a rat xenograft model involving subcutaneous injection of human hepatocellular carcinoma cells (Bagi et al., 2009).

The newest FAK kinase inhibitor is PND1186, which has an IC50 of 1.5 nM for FAK (Tanjoni et al., 2010). PND1186 increases apoptosis in breast and ovarian carcinomas, and reduces tumor-associated inflammation (Tanjoni et al., 2010; Walsh et al., 2010). A twice daily oral dose of 150 mg/kg prevented tumor growth and metastasis to the lung in breast cancer in an orthotopic mouse model (Walsh et al., 2010).

Due to the role of Pyk2 in bone homeostasis, Pfizer was interested in designing a Pyk2 specific inhibitor. PF 431,396, which effectively inhibits both FAK and Pyk2 (IC50 for Pyk2 of 31 nM and an IC50 of 1 nM for FAK), reduces bone loss in ovariectomized rats (Buckbinder et al., 2007; Walker et al., 2008). In fact, in this model, PF 431,396 is more effective at preventing bone loss than estrogen replacement, which is the current treatment for bone loss in postmenopausal women (Buckbinder et al., 2007). SAR studies failed to produce a compound with increased specificity toward Pyk2 (Walker et al., 2008). Structure-based studies suggested that Pyk2 could adopt at DFG-out conformation which has been key to the development of successful kinase inhibitors such as Gleevec (Han et al., 2009). BIRB796 (a p38 kinase DFG-out inhibitor) can inhibit Pyk2 with an IC50 of 1.5 μM, suggesting the strategy might be feasible (Han et al., 2009). Through modification of BIRB796, Pfizer derived PF 461,8433. This compound pushes Pyk2 into a catalytically incompetent DFG-out conformation in which Leu504 blocks the ATP binding site. In addition, this compound takes advantage of the unique Met502 gatekeeper between the N-terminus and the hinge loop to improve specificity (PDB ID 3FZT) (Han et al., 2009; Fig. 5.10C).

Additional small molecules that inhibit activity/function of FAK have come out of *in silico* screens (Golubovskaya et al., 2008b). Screening the National Cancer Institute's compound structural database against FAK structures has resulted in the identification of a number of compounds. Compound **14**, 1,2,4,5-benzenetetramine tetrachloride, was identified in a

screen for compounds that would bind to FAK adjacent to the major autophosphorylation site. It has an IC50 of 1 μM for FAK. *In vitro* studies showed that compound **14** impairs phosphorylation of Tyr397, and that treatment of cells with the compound reduces cell adhesion and cell viability. In an animal model of breast cancer, compound **14** impaired the growth of tumors (Golubovskaya et al., 2008b).

Since the FAT domain alone is also a potent dominant negative mutant (Ilic et al., 1998), this domain is considered a potentially significant therapeutic target to impair FAK signaling. A phage display strategy to identify peptides that bind to the C-terminal domain of FAK resulted in discovery of the AV3 peptide. This peptide can displace FAK from focal adhesions and impair FAK function (Garces et al., 2006). These studies were extended using an *in silico* screen for compounds that bind to the FAT domain at the AV3 binding site. The compound, chloropyramin hydrochloride [*N*-(4-chlorobenzyl)-*N*′,*N*′-dimethyl-*N*-pyridin-2-ylethane-1,2-diamine], impaired FAK signaling in cells, inhibited proliferation, and induced apoptosis (Kurenova et al., 2009). The crystal structure of this compound bound to the FAT domain has been solved (PDB ID 2RA7) (Fig. 5.10D). The compound docks to the α-helix 1/4 side of the FAT domain, making extensive hydrophobic contacts and H-bonding to Ser939 (in helix 1) and His1025 (in helix 4). The drug binding site partially overlaps the paxillin LD motif binding site, and His 1025 is one of the basic residues flanking the hydrophobic patch on this side of the FAT domain. Thus this compound potentially perturbs paxillin binding and might effectively displace FAK from focal adhesions, since the paxillin binding site on the helix 1/4 surface of the FAT domain appears most important for localization (Scheswohl et al., 2008). These hypotheses have yet to be addressed.

7. Future Directions

While recent studies have provided abundant structural information about FAK/Pyk2, providing important novel insight into function and molecular mechanisms of regulation, important structural questions remain outstanding. While a large portion of the FAK molecule has been crystallized, the proline-rich region between the catalytic and FAT domains remains uncharacterized. Structural determination is an important objective given its critical scaffolding function. Lack of structural information could be due to the fact that the region is unstructured in the absence of ligands and ligand binding could cause the region to adopt a structure amenable to crystallization. Solution of the structure of the entire FAK protein is still an important goal since it would provide insight into the three-dimensional configuration of the domains as a whole. Future structural studies may

reveal strategies for novel therapeutic approaches to target FAK and will also enable the modification of existing drugs to improve specificity.

The mechanism for releasing autoinhibition and activating FAK has yet to be completely elucidated. Major outstanding questions include how ligand binding is translated into destabilization of the FERM/catalytic domain interaction, how these domains move from autoinhibited conformation into the activated conformation, and the role of linker domain phosphorylation and ligand binding in the release of the autoinhibitory conformation. Future structural, biophysical, and biochemical studies using probes like the conformational biosensors will provide the solution to these outstanding problems.

One major unresolved issue regarding FAK binding partners and signaling is the identification of the different components in higher order signaling complexes. To date, most FAK/binding partner interactions have been studied as bipartite pairs. Some of the molecular details determining specificity of interactions are unknown, for example, how do LD motifs discriminate between two binding sites on FAK or between FAK and Pyk2. In addition, higher order complexes certainly exist *in vivo* and each unique complex will produce a different physiological outcome. For example, the signaling output from a FAK/Src/p190RhoGEF complex would differ from a FAK/p120RasGAP[p190RhoGAP]/p190RhoGEF complex. Related to the issue of complex formation is the fact that FAK binds with many different proteins at a relatively small number of binding sites. With multiple partners for each binding site on FAK, additional mechanisms for regulation of assembly of different complexes must exist. In higher complexes, how association of one binding partner with FAK impacts recruitment of subsequent interacting proteins at different sites remains unknown. Finally, the spatial and temporal regulation of the formation of FAK complexes is largely unknown and is an important direction for future research.

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CHAPTER SIX

Roles of Small Ubiquitin-Related Modifiers in Male Reproductive Function

Margarita Vigodner

Contents

Abstract

Spermatogenesis consists of the mitotic division of spermatogonia, meiosis of spermatocytes, and postmeiotic differentiation of spermatids, processes tightly controlled by hormones and growth factors secreted by testicular somatic cells. The events during spermatogenesis are precisely regulated by the sequential appearance of different proteins and their posttranslational modifications. Sumoylation (covalent modification by small ubiquitin-like modifiers; SUMO proteins) has emerged as an important regulatory mechanism in different cell types, and data obtained from studies on germ cells imply that SUMO proteins

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are involved in multiple aspects of spermatogenesis. Although progress has been made in the initial characterization of sumoylated proteins during spermatogenesis, the targets of sumoylation, their corresponding pathways in the testis, are mostly unknown. In this chapter, I review what we know about sumoylation in somatic cells, summarize the expression patterns, suggest possible functions of SUMO proteins in testicular cells, and discuss some difficulties and perspectives on the studies of sumoylation during spermatogenesis.

Key Words: Spermatogenesis, Sumoylation, Spermatogonia, Spermatocytes, Spermatids, Sertoli cells, Leydig cells, Myoepithelial cells.

1. Introduction

Spermatogenesis is the complex process of spermatozoa formation, which continues throughout a male's life from puberty until old age. It consists of spermatogonial proliferation, meiosis of spermatocytes and post-meiotic maturation of spermatids (spermiogenesis). During the initial wave of spermatogenesis in mice, spermatogonia arise around day 6 postpartum (dpp) and undergo several rounds of proliferation and differentiation (Bellve et al., 1977). The last mitotic division gives rise to preleptotene primary spermatocytes, where the final DNA synthesis occurs. At approximately day 10, the cells enter the prolonged prophase of the first meiotic division, a time when homologous chromosomes synapse and meiotic recombination takes place. After the rapid meta-, ana-, and telophase stages, the first meiotic division is complete, and the resulting secondary spermatocytes immediately undergo the second meiotic division without preceding DNA synthesis (Cohen and Pollard, 2001). The resulting round haploid spermatids, which are evident at around day 20 postpartum, undergo cellular and nuclear reorganization, resulting in the formation of testicular sperm (Bellve et al., 1977). Further differentiation of the spermatozoa takes place in the epididymis and is completed in the female reproductive tract by a process known as capacitation (Visconti, 2009). Successful progression through spermatogenesis is crucial for normal gamete formation. Unfortunately, in humans, infertility affects approximately 15% of couples worldwide (Nishimune and Tanaka, 2006) and about 8% of couples in the United States (Leke et al., 1993). The male partner is responsible for infertility in at least half of all cases, and about 39% of infertile men have idiopathic infertility (infertility with an unknown cause or origin) (De Kretser and Baker, 1999). These facts emphasize the need for the better understanding of spermatogenesis and its regulation, particularly by new molecules and novel protein modifications that have not previously been described in the testis but are known to regulate important pathways in other tissues.

Posttranslational modification refers to the enzyme-mediated addition of different chemical groups to proteins after the proteins have already been synthesized. Such modifications can change protein activity, stability, and the ability to bind other proteins and either stay in the cytoplasm or move to the nucleus. Translational and posttranslational controls have unique features in germ cells because the transcriptional machinery is inactive at the late stages of spermatogenesis. Transcription factors, enzymes, and structural proteins have been identified as targets of phosphorylation, acetylation, ubiquitination, glycosylation, and other posttranslational modifications in male germ cells. For example, the acetylation of α-tubulin might be associated with the positioning of spermatogonia on the basement membrane (Luo et al., 2010), phosphorylation by c-kit protein kinase regulates signaling pathways in spermatogonia (Mauduit et al., 1999), and numerous phosphorylation events regulate sperm motility and capacitation (Visconti, 2009). The posttranslational modification of histones has emerged as an additional way to regulate the structure of chromatin and, therefore, the transcription of specific genes (Jenuwein and Allis, 2001). Histone modifications have been extensively studied during spermatogenesis and are either common (also found in mitotic cells) or represent unique, germ cell-specific events. For example, histone H3 phosphorylation is associated with the condensation of both mitotic and meiotic chromosomes (Cobb et al., 1999); H2AX phosphorylation marks the sites of DNA breaks in mitotic and meiotic cells but is specifically accumulated over the X and Y chromosomes in spermatocytes during meiotic sex chromosome inactivation (Turner et al., 2004); and massive histone acetylation takes place in elongated spermatids, where it apparently has a specific function in the replacement of histones by transition proteins and then by protamines (Hazzouri et al., 2000).

There is also extensive crosstalk among different posttranslational modifications, which means that one modification can inhibit or activate another. For example, because both histone acetylation and ubiquitination occur on lysine residues in spermatids, there might be competition between these modifications for the same residue (Baarends et al., 1999). Further complicating matters, another type of posttranslational modification on lysine, mediated by small ubiquitine-like modifiers (SUMO proteins), known as sumoylation, has emerged as an important regulatory mechanism in different cell types, including germ cells. We and others have recently localized sumoylated proteins to specific domains in germ and somatic testicular cells and initiated studies on the functional role of sumoylation during spermatogenesis (Brown et al., 2008; La Salle et al., 2008; Metzler-Guillemain et al., 2008; Rogers et al., 2004; Vigodner and Morris, 2005). The findings obtained from these studies are consistent with the diverse roles that SUMO proteins play during testicular function, although the targets of sumoylation and their corresponding pathways are not yet well characterized. The purpose of this chapter is to briefly review the major facts about sumoylation in somatic cells, summarize the expression patterns and

possible functions of SUMO proteins in different types of testicular cells, and discuss the difficulties, barriers to progress, and further perspectives in the study of sumoylation during spermatogenesis.

2. Regulation of Protein Functions by Sumoylation: Lessons from Other Cell Types

SUMO proteins were discovered based on their homologies with ubiquitin. Although the primary sequences of SUMO and ubiquitin share only limited homology, their three-dimensional structures are very similar (Bayer et al., 1998; Kerscher et al., 2006). Since the 1996 discovery of SUMO1 as a regulator of Ran GTPase-activating protein 1 (RanGAP1), which is involved in the regulation of protein transport across the nuclear pore complex, other members of the SUMO superfamily have been identified and extensively studied (Matunis et al., 1998). There are four SUMO paralogs in mammals: SUMO1, which shares about 50% homology with SUMO2 and 3 (often termed SUMO2/3 because of their 95% sequence identity), and SUMO4 (Dohmen, 2004; Geiss-Friedlander and Melchior, 2007; Gill, 2005; Hannoun et al., 2010; Wang and Dasso, 2009). While SUMO1, 2, and 3 are abundantly expressed in different tissues, SUMO4 is restricted to the kidney, liver, and lymph nodes (Bohren et al., 2004; Dohmen, 2004; Li et al., 2005).

2.1. The sumoylation–desumoylation cycle and major enzymes

Similar to ubiquitination, sumoylation (covalent modification by SUMO) involves the formation of isopeptide bonds on the lysine residues of target proteins. This process involves maturation, activation, conjugation, and ligation steps that are mediated by specific protein complexes (E1, E2, and E3). While the pathway is conserved across different eukaryotes, I will use the vertebrate terminology in this review; the corresponding yeast terminology can be found in other reviews about SUMO (Wang and Dasso, 2009). Following the proteolytic cleavage of the SUMO precursor by sentrin-specific proteases (SENPs), mature SUMO forms a thioester bond with SUMO-activating enzyme (SAE), which is composed of two subunits (SAE1/SAE2; Fig. 6.1). While numerous ubiquitin-conjugating enzymes have been identified, Ubc9 is the only SUMO-conjugating E2 enzyme (Wang and Dasso, 2009; Wilkinson and Henley, 2010; Yeh, 2009; Yeh et al., 2000). Ubc9 forms a thioester bond with activated SUMO and catalyzes its transfer to the target protein. The last ligation step is mediated by SUMO ligases, whose functional relevance were initially questioned because high concentrations of UBC9 are sufficient for the *in vitro*

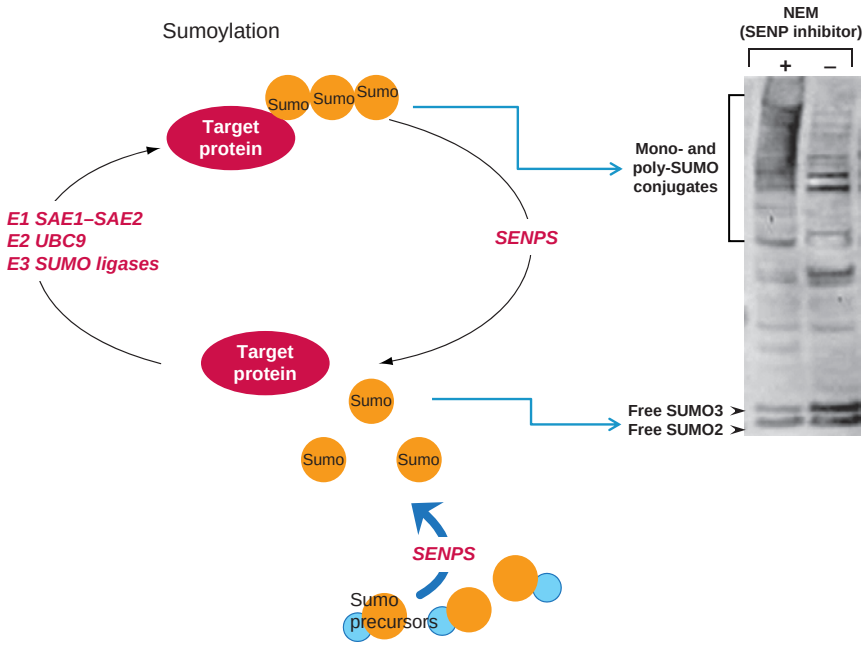

Figure 6.1 The sumoylation–desumoylation cycle. Following the proteolytic cleavage of the SUMO precursor by sentrin-specific proteases (SENPs), mature SUMO is activated by SUMO-activating enzyme (SAE), which is composed of two subunits (SAE1/SAE2). Ubc9 enzyme conjugates SUMO to target proteins with the help of SUMO ligases. Mono- or polysumoylation is reversed by the action of SENPs that cleave the isopeptide bond between SUMO and its substrate. SUMO is detected either as a free isoform or as a conjugate on other different molecular weight proteins. SUMO2 and 3 are 95% identical and are detected with the same antibody. The two isoforms have a slightly different molecular weight and can be resolved on a gel. An addition of the isopeptidase inhibitor NEM during a cell lysis procedure is critical to prevent desumoylation of high-molecular-weight SUMO conjugates.

sumoylation of numerous targets (Sarge and Park-Sarge, 2009a). New experimental data, however, suggest that different ligases may determine or enhance the specificity of sumoylation by acting as scaffolds and interacting with the target protein and SUMO–UBC9 complex (Palvimo, 2007).

There are several families of SUMO ligases. The largest is the family of PIASs (protein inhibitors of activated STAT [signal transducer and activator of transcription]), which were discovered based on their ability to bind and modulate the transcriptional activities of STATs. This group is characterized by the presence of a RING motif, which binds UBC9 and possesses E3 SUMO ligase activity (Liu et al., 1998; Tan et al., 2000). In vertebrates, PIAS proteins are encoded by four genes (*Pias1*, *Piasx* [*Pias2*], *Pias3*, and *Piasy* [*Pias4*]), which produce five PIAS proteins (PIAS1, PIAS3, PIASxα,

PIASxβ, and PIASy). Different PIAS family members have particular specificities for SUMO1 versus SUMO2/3 ligation. It has been shown that many substrates can be modified by more than one PIAS protein, suggesting some redundancy in their interactions (Palvimo, 2007; Wilkinson and Henley, 2010). PIAS family members have been implicated in various cellular functions that can be dependent or independent of their SUMO-ligase activities (Liu et al., 1998; Palvimo, 2007; Tan et al., 2002).

Another SUMO ligase, which contains a RING motif known as TOPORS (topoisomerase I binding, arginine/serine-rich), is quite unique because it can also act as a ubiquitin ligase (Rajendra et al., 2004; Weger et al., 2005; Wilkinson and Henley, 2010). There are also several SUMO ligases that lack RING domains, including nuclear pore complex Ran-binding protein 2 (Ranbp2), which functions in mitosis (although the role of sumoylation in its mitotic function is unclear), and polycomb protein 2 (Pc2), which induces the translocation of the transcriptional repressor CtBP (C-terminal-binding protein) from the cytoplasm to the nucleus (Agrawal and Banerjee, 2008; Dasso, 2008; Kirsh et al., 2002; Lin et al., 2006; Saitoh et al., 1997). A surprising finding is that histone deacetylases (HDACs) 4 and 7 also possess SUMO-ligase activities that are independent of their deacetylating activities (Geiss-Friedlander and Melchior, 2007; Wang and Dasso, 2009; Wilkinson and Henley, 2010).

Sumoylation often occurs on a target lysine found within the consensus sequence ψKxD/E, where ψ is a hydrophobic amino acid, x is any amino acid, and D/E are any acidic residues, but many proteins are sumoylated outside this sequence, and not all consensus motifs are sumoylated. Because SUMO2/3 has an internal consensus motif for sumoylation, it can form polySUMO chains. SUMO1 lacks such a motif and mostly conjugates to proteins as a monomer or as a polySUMO2/3 chain terminator (Rodriguez et al., 2001). Interestingly, a phosphorylation-dependent sumoylation motif has been discovered in which phosphorylation takes place a few residues away from the consensus motif and increases sumoylation (Hietakangas et al., 2006).

Sumoylation is a dynamic modification and is reversed through the action of SENPs that cleave the isopeptide bond between SUMO and its substrate (Fig. 6.1). Mammals have sex SENP proteins: SENP1, SENP2, SENP3, SENP5, SENP6, and SENP7 (Hannoun et al., 2010; Mukhopadhyay and Dasso, 2007; Wilkinson and Henley, 2010; Yeh, 2009). While SENP1 and SENP2 reverse both SUMO1 and SUMO2/3 modifications, SENP3, 5, 6, and 7 preferentially act on SUMO2/3. SENP6 and 7 are also able to modify polySUMO2/3 chains. As noted above, the same SENPs (mostly SENP1 and 2) are also responsible for the proteolytic cleavage of SUMO precursors to produce mature SUMO (Hannoun et al., 2010; Mukhopadhyay and Dasso, 2007; Wilkinson and Henley, 2010; Yeh, 2009). Importantly, some previous studies prepared cell lysates for studies on sumoylation using standard protease inhibitors but without a specific

inhibitor for isopeptidases, enzymes that, when active, cleave SUMO from its substrate, resulting in the loss of most of the high-molecular-weight SUMO conjugates. In our experiments, a freshly prepared isopeptidase inhibitor, *N*-ethylmaleimide (NEM), is always added to the lysis buffer (Fig. 6.1).

In addition to the numerous proteins regulated through the sumoylation cascade, many proteins bind SUMO noncovalently through a SUMO-interacting motif (SIM) (Chupreta et al., 2005; Kerscher, 2007; Lin et al., 2006; Song et al., 2004, 2005). Interestingly, various components of sumoylation pathways, including UBC9 and some PIAS proteins, can either be sumoylated or contain SIMs, further complicating regulation by sumoylation (Rytinki et al., 2009).

2.2. Knockout studies of SUMO and SUMO-modifying enzymes

Several studies have suggested nonoverlapping functions of the different SUMO isoforms within the cells because some cellular targets are preferentially modified by SUMO1, while others are preferentially modified by SUMO2/3 (Saitoh and Hinchey, 2000). However, recent knockout studies have challenged this paradigm. Several developmental processes, including spermatogenesis, have recently been studied in SUMO1 knockout mice (Zhang et al., 2008a). In contrast to a previously published study that reported abnormal palate development in SUMO1 knockouts (Alkuraya et al., 2006), this study found no abnormalities in mouse development, suggesting that SUMO2/3 can compensate for SUMO1's functions in SUMO1 knockout animals. Although these results require further evaluation, they suggest that the SUMO-conjugating machinery, and not an individual SUMO isoform, should be the target of future experiments that aim to inhibit sumoylation. Unfortunately, UBC9 (SUMO-conjugating enzyme) knockout mice exhibit early embryonic lethality with severe disruptions in mitosis, a finding that supports the overall biological importance of SUMOylation in mitotic progression (Nacerddine et al., 2005).

Studies in mice with a deletion of *Pias* gene revealed only mild developmental abnormalities with no significant changes in the overall sumoylation pattern (Roth et al., 2004; Santti et al., 2005; Wong et al., 2004). These results suggest considerable overlapping functions between the different mammalian PIAS proteins.

2.3. Strategies for the identification of sumoylated proteins

While mass spectrometry (MS) is a powerful tool to identify the sites of different posttranslational modifications, including phosphorylation and ubiquitination, sumoylated sites are difficult to identify using the same approach. SUMO cleavage by trypsin produces a long peptide conjugated

to its target proteins, which makes the identification very complex (Jeram et al., 2009). New approaches to identify sumoylated sites using MS are under development but require further validation (Hsiao et al., 2009).

Several programs have been developed to predict sumoylated sites on proteins of interest. While the SUMOplot™ analysis program by Abgent (http://www.abgent.com.cn/doc/sumoplot) predicts the sumoylated sites based on the presence of only the consensus sequence, the development of the site-specific predictor SUMOsp2 was based on the analysis of hundreds of reported proteins that are sumoylated in both consequence and non-consequence motifs (Ren et al., 2009).

For identifying sumoylated proteins in somatic cells, an epitope-tagged SUMO (such as 6His-SUMO) is transiently or stably transfected into cell lines (Tatham et al., 2009). In many cases, a stimulus that increases sumoylation, such as heat stress, is applied. The tagged sumoylated proteins are purified from protein mixtures by affinity chromatography or immunoprecipitation using an antibody against the epitope tag, followed by protein identification using MS. Because only a small portion of a protein is often modified by SUMO at a given time, immunoprecipitation of endogenous proteins followed by protein identification via MS is rather challenging but has been successfully used to identify SUMO targets (Matafora et al., 2009). Two-dimensional gel electrophoresis followed by MS has also been employed to analyze SUMO targets in melanoma cells (Ganesan et al., 2007).

Another advanced approach to quantify and compare the sumoylated proteomes of cells cultured under two different experimental conditions is stable isotope labeling of amino acids in cell culture (SILAC; Andersen et al., 2009; Golebiowski et al., 2009). Cells from two different experimental conditions or two different cell lines are grown in media containing either "light" or "heavy" amino acids. "Heavy" refers to the amino acid (e.g., lysine or arginine) containing a heavier stable isotopic form of carbon or nitrogen, ^{13}C and/or ^{15}N. Once the growing cells fully incorporate the "heavy"-labeled amino acid, the cells from the two conditions are mixed in a 1:1 ratio and lysed. The cell lysate is then used for immunoprecipitation analysis using anti-SUMO (or epitope tag) antibodies. SDS-PAGE is followed by MS analysis, resulting in data that contain tryptic peptides from both the "light" and the "heavy" proteins that have either undergone no change in protein levels or up- or downregulated protein levels. This approach (with some improved technical features and a novel data processing analysis) has recently been used successfully for the identification of 766 putative SUMO substrates of before and after heat stress induction (Golebiowski et al., 2009).

After identifying the proteins, the targets of interests are confirmed by coimmunoprecipitation experiments using an anti-SUMO antibody and antibodies against the identified proteins. Another method involves an *in vitro* sumoylation assay using a recombinant protein and sumoylation

enzymes followed by Western blot analysis to detect both the unmodified and sumoylated forms of the proteins (Sarge and Park-Sarge, 2009a). Immunofluorescence studies can also be performed to examine whether the proteins colocalize with SUMO in a certain region of the cell. If the sumoylation sites of the selected proteins have not been identified, then site-directed mutagenesis is usually employed to change candidate lysine residues to arginine and to determine if this substitution eliminates the sumoylated isoform(s) on a Western blot. This is performed after either cotransfecting the cells with plasmids carrying SUMO and the mutated gene or, again, using an *in vitro* sumoylation assay (Sarge and Park-Sarge, 2009a). It has been suggested that such identification should be accompanied by studies of a mutation in another amino acid in the identified consensus sequence, which should provide similar results (Tatham et al., 2008).

2.4. Functions of sumoylation within cells

A diverse set of SUMO target proteins has been identified, including factors that regulate transcription, replication, DNA repair, RNA metabolism, translation, transport, and stress responses. Interestingly, the sumoylation of mammalian histones has been recently reported (Golebiowski et al., 2009; Shiio and Eisenman, 2003). Modification by SUMO proteins is thought to provide new binding sites that interact with other proteins. Such interactions can affect protein localization, activity, and stability (Dohmen, 2004; Geiss-Friedlander and Melchior, 2007; Gill, 2005).

Many SUMO targets are transcription factors. Sumoylation modifies the ability of transcription factors to bind DNA and often correlates with transcriptional repression through the recruitment of chromatin silencing complex components, such as histone deacetylases (HDACs) (Gill, 2005). However, there are some proteins whose transcriptional activities are stimulated by sumoylation (Lyst and Stancheva, 2007; Wilkinson and Henley, 2010). An interesting model has recently been suggested to explain how a profound effect within cells is achieved by only a small proportion of sumoylated protein. In many cases, the effect of sumoylation persists even after SUMO has been removed from the substrate. For example, if a sumoylated transcriptional factor has been recruited to a domain of heterochromatin, it will stay inactive in this repressive environment for some time, even after the SUMO tag has been already removed (Wilkinson and Henley, 2010).

While ubiquitin is mostly involved in proteolytic pathways within cells, SUMOs had not been initially associated with protein degradation. However, later studies found that polysumoylation can act as an indirect signal, recruiting a number of SIM-containing ubiquitin ligases, followed by protein ubiquitination and degradation (Geoffroy and Hay, 2009; Jadhav and Wooten, 2009; Wilkinson and Henley, 2010). The sumoylation of

some ubiquitin ligases, however, can inhibit their activities. Furthermore, sumoylation, ubiquitination, and acetylation can compete for the same lysine residue, providing an additional level of crosstalk among these post-translational modifications. As noted above, phosphorylation can regulate sumoylation through a phosphorylation-dependent sumoylation motif (PDSM; Hietakangas et al., 2006). There is also a complex interplay between sumoylation and kinase regulation (Yang and Sharrocks, 2006).

Although the majority of the identified targets of SUMO are nuclear proteins, new studies provide evidence of sumoylation playing a role in other cellular compartments, including mitochondria, the endoplasmic reticulum, and the plasma membrane (de Brito and Scorrano, 2008; Neuspiel et al., 2008).

Cell-specific regulation by sumoylation has been demonstrated in different cell types, including neurons, liver cells, cardiomyocytes, and connective tissue cells (Chen et al., 2010; Guo et al., 2010; Scheschonka et al., 2007; Wang and Schwartz, 2010; Yan et al., 2010). Consequently, impaired sumoylation has been associated with multiple diseases, including neurodegenerative disorders, cancers, and arthritis (Bartek and Hodny, 2010; Dorval and Fraser, 2007; Sarge and Park-Sarge, 2009b; Wang and Schwartz, 2010; Yan et al., 2010). Interestingly, the overexpression of SUMO2/3 causes premature cell senescence, a finding that opens a new avenue for age-related research on SUMO in different cell types (Li et al., 2006).

3. Unique Aspects of Sumoylation in Male Germ Cells

In nontesticular cells, knowledge about the role of sumoylation has grown rapidly. Similar to phosphorylation, sumoylation acts in different cellular compartments and regulates multiple pathways. Sumoylation has been implicated in various events, including the modulation of transcriptional activity, chromosome integrity and function, DNA repair processes, nuclear–cytoplasmic transport, stress responses, and apoptosis. Spermatogenesis is a unique process in which all these events occur in a precisely coordinated manner and is, therefore, a unique *in vivo* model to study sumoylation. We and others have been recently studying SUMO1 and SUMO2/3 during mouse and human spermatogenesis. From the accumulated data, it is apparent that sumoylation is involved in the regulation of every step of germ cell development. In the following sections, we summarize the published and unpublished experimental data regarding the expression patterns and the possible roles of SUMO proteins in different types of testicular cells as well as the difficulties and future perspectives in the studies of sumoylation during spermatogenesis.

3.1. SUMO proteins in spermatogonia

In the mouse, the proliferative phase of spermatogenesis begins around dpp with the mitotic division of spermatogonia stem cells (A_{single} spermatogonia), which can either retain stem cell properties or enter a series of mitotic divisions to become A_{paired}, $A_{aligned}$, differentiated A_1–A_4, intermediate (In), and type B spermatogonia (de Rooij, 2001). Apparently, the progenitor cells up to the $A_{aligned}$ stage still have stem cell potential, as demonstrated by a transplantation assay (Dym et al., 2009; Golestaneh et al., 2009). This has also been suggested to be a feedback mechanism between the number of advanced spermatogonia and the stem cell proliferative activity. Studies on spermatogonia are important for understanding the regulation of the first steps of spermatogenesis, which are critical for fertility, and the overall biology of stem-like progenitors. The manipulation of spermatogonia has the potential to improve the techniques used for germ cell transplantation and identify novel approaches for the creation of transgenic animals (He et al., 2010; Takehashi et al., 2010).

The connection between sumoylation and mitosis has been well established in various species and experimental systems. In yeast, mutants lacking different SUMO pathway components exhibit severe disruptions in mitosis (Dasso, 2008). In the *Xenopus* egg, sumoylation has important roles in chromosome cohesion and centromere/kinetochore function. Downregulation of sumoylation enzymes in mammalian cells also results in aberrant mitosis (Dasso, 2008; Yeh, 2009). Strikingly, UBC-9-null mice have early embryonic lethality and impaired mitosis, a finding supporting an indispensable role for sumoylation in mitotic division (Nacerddine et al., 2005). Our initial study of sumoylation found SUMO in the nuclear periphery of spermatogonia (Sg), but new data obtained using several anti-SUMO antibodies provide evidence of both nuclear and perinuclear SUMO localization (Fig. 6.2A and B). Preliminary analysis suggests that SUMO localizes to the nucleus in all types of spermatogonia, but more focused studies are needed to follow stage-specific patterns of SUMO expression.

Due to their mitotic nature, spermatogonia may have many targets of sumoylation that are common to those identified in other somatic cells. To this end, different mitotic targets of SUMO, including RanGap1, condensin and cohesin complexes, topoisomerases, and other centromere/kinetochore proteins (such as CENPE), are interesting to study in spermatogonia (Table 6.1) (Dasso, 2008; Golebiowski et al., 2009; Matunis et al., 1998; Zhang et al., 2008b). SUMO targets in spermatogonia stem cells may be common to those that play roles in other stem/progenitor cells. For example, a modification by SUMO to the transcription factor OCT4 is important for the self-renewal and pluripotency of embryonic stem cells (Hannoun et al., 2010; Wei et al., 2007). OCT4 is also important for spermatogonia proliferation (Dann et al., 2008). Another example is the

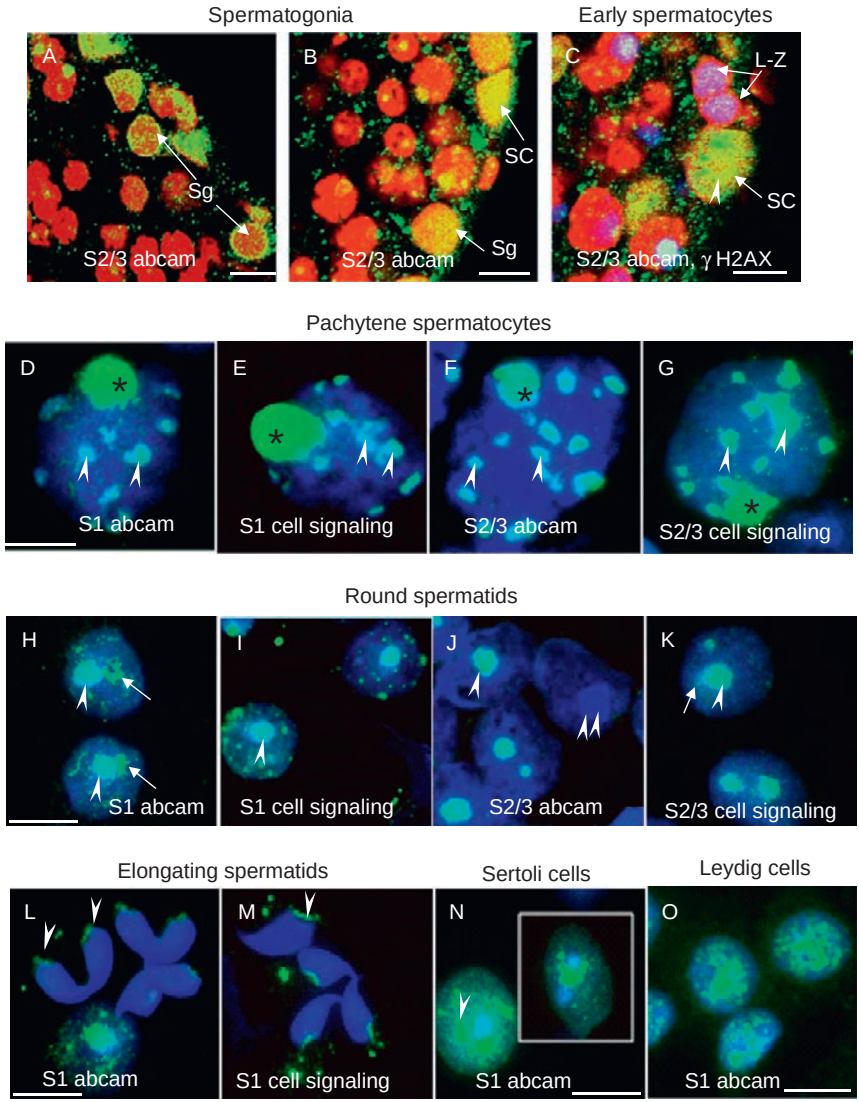

Figure 6.2 The expression pattern of SUMO1 and SUMO2/3 during mouse spermatogenesis as detected using different anti-SUMO antibodies. (A, B) Confocal microscopy images showing spermatogonia (Sg; A and B) and Sertoli cells (SC) in the peripheral layer of the seminiferous tubules. (C) Leptotene–zygotene spermatocytes (L–Z) are identified based on the γH2AX staining in the whole nucleus (purple). DNA is stained using propidium iodide (red). (D–O) Single-cell bioimaging. In mouse spermatocytes, SUMO is concentrated in the XY body (D–G, asterisk), and centromeric heterochromatin (D–G, arrowheads). In spermatids, SUMO signal is detected in the chromocenters (H–K, arrowheads) and XY domains (H and K, arrows). In elongating spermatids, SUMO localizes to the neck of the spermatids (L and M,

interplay between the MAP kinase and sumoylation pathways, including, for example, the extracellular signal-regulated kinase ERK (Yang and Sharrocks, 2006). MAP kinases (and specifically ERK) have been suggested to play roles in the proliferation of differentiated spermatogonia (Table 6.1) (Dolci et al., 2001).

The global identification of sumoylated targets in spermatogonia needs to be performed. Unlike other germ cells, spermatogonia can be maintained *in vitro*. Furthermore, spermatogonia-derived cell lines can be utilized to supplement sumoylation studies in spermatogonia. Therefore, some of the methods used in the study of sumoylation in somatic cells can be uniquely applied to spermatogonia. For example, the targets of SUMO and cellular phenotypes can be studied following the up- or downregulation of sumoylation by either SUMO overexpression or the inhibition of sumoylation enzymes by inhibitory RNAs in spermatogonia and the derived cell lines. Cell lines represent additional, powerful tools for the identification of the relative amount of sumoylated proteins in different types of spermatogonia using SILAC, without any limitations on cell number. The C18-4 cell line is derived from type A spermatogonia at day 6 and exhibits several markers of stem-like progenitor cells (Hofmann et al., 2005). GC-1 spermatogonia are derived from the more differentiated type B spermatogonia and express several specific spermatogonia markers. With larger cell numbers, a higher potential yield of sumoylated proteins is possible and may result in the identification of more targets. The results obtained using cell lines can be validated further (e.g., by using isolated spermatogonia after enrichment for specific protein types [e.g., nuclear, basic, etc.]). The information obtained in spermatogonia can be further studied in other germ cells because some of the SUMO-regulated pathways will be common to different testicular cells.

The aberrant expression of signaling molecules in spermatogonia may result in an enhanced risk of testicular cancer and other tumors. Sumoylation has been implicated in the regulation of mitosis and in the development and progression of cancer (Alarcon-Vargas and Ronai, 2002; Baek, 2006; Morris, 2010a). Our preliminary data provide evidence that SUMO expression is a prominent feature of human germ cell tumors (unpublished data) and, therefore, the study of sumoylation in spermatogonia may lead to a better understanding of the etiology of the disease.

arrowheads). In Sertoli and Leydig cells, anti-SUMO antibodies produce signals in the nucleus and the nucleolus (N and O). In Sertoli cells, SUMO is also concentrated adjacent to the nucleolar heterochromatin bodies containing centromeric heterochromatin (arrowhead). DNA is stained using DAPI (blue); scale bar is 10 μm.

Table 6.1 Possible targets of sumoylation in different types of testicular cells

		Spermatocytes					
	Spermatogonia	Meiotic DNA breaks	Sex body	Centromeres	Synaptonemal complex	Spermatids	Somatic cells
Centromere/kinetochore proteins (including topoisomerases, CENPE, RanGap1) (Dasso, 2008; Golebiowski et al., 2009; Matunis et al., 1998; Shrivastava et al., 2010; Zhang et al., 2008a)	+			+			
Proteins of synaptonemal complex (Brown et al., 2008)					+		
Members of MAP kinase pathway (e.g., ERK) (Yang and Sharrocks, 2006)	+						
OCT4 (Dann et al., 2008; Hannoun et al., 2010; Wei et al., 2007)							
Proteins involved in the formation and repair of DNA strand breaks (e.g., components of origin recognition and minichromosome maintenance complexes, homologous recombination repair (HRR) factors, topoisomerases, BRCA-1, and 53BP1 (p53-binding protein). (Golebiowski et al., 2009; Morris,	+	+	+	+		+	

2010a,b; Morris et al., 2009; Prudden et al., 2007)							
Histones (Nathan et al., 2006; Shiio and Eisenman, 2003)	+		+	+	+	+	+
Proteins involved in the formation of heterochromatin (including histone deacetylases [HDACs], methyltransferases and HP1 proteins) (Golebiowski et al., 2009; Ling et al., 2004)	+			+	+	+	+
Nuclear steroid receptors (Janne et al., 2000; Palvimo, 2007; Poukka et al., 2000a,b)							+
Proteins involved in stress response and apoptosis (Golebiowski et al., 2009; Shrivastava et al., 2010)	+		+	+	+	+	+

3.2. Possible roles of sumoylation in meiotic spermatocytes

The proliferation phase of spermatogenesis is terminated by the division of type B spermatogonia into preleptotene spermatocytes that enter meiosis. Two sequential meiotic divisions aim to successfully segregate the maternal and paternal genetic material into the daughter cells and produce haploid gametes. During the prolonged prophase of the first meiotic division, preleptotene spermatocytes pass through leptotene, zygotene, pachytene, and diplotene stages, which are followed by diakinesis (Cohen and Pollard, 2001). In leptotene spermatocytes, histone H2AX is phosphorylated by ataxia telangiectasia-mutated (ATM) kinase at the sites of massive DNA double-strand breaks, which precede the synapsis of the homologous chromosomes and meiotic recombination (Fernandez-Capetillo et al., 2003; Mahadevaiah et al., 2001; Turner, 2007; Turner et al., 2004, 2005). During the zygotene stage, the autosomes synapse, and a protein structure (called the synaptonemal complex) is formed and associates with the axes of the homologous chromosomes. Simultaneously, the X and Y chromosomes are aligned along a small region of sequence homology, inactivated, and form a heterochromatin domain called the sex body (Handel, 2004; Turner, 2007). Meiotic sex chromosome inactivation (MSCI) coincides with the disappearance of γH2AX from the entire nucleus and its concentration across the sex chromosomes. Aside from γH2AX, ataxia telangiectasia and Rad-3-related (ATR) kinase and BRCA-1 have also been identified as major players in MSCI. Furthermore, it has also been demonstrated that any unsynapsed chromatin during male and female meiosis is inactivated by the same mechanism, which is known as meiotic silencing of unsynapsed chromatin (MSUC; Baarends et al., 2005; Burgoyne et al., 2009; Turner, 2007; Turner et al., 2005).

To better understand the possible roles of sumoylation during meiosis, we and others have localized SUMO proteins in mouse and human spermatocytes using both isolated cell bioimaging and confocal microscopy of seminiferous tubules (Brown et al., 2008; Metzler-Guillemain et al., 2008; Rogers et al., 2004; Vigodner and Morris, 2005; Vigodner et al., 2006). Recently, using more sensitive antibodies, the localization data have been confirmed and extended in our laboratory. It should be noted that immunofluorescence techniques are based on different fixation methods and use various antibodies and, therefore, have limitations. From the experimental data accumulated in our laboratory, we noted that various antibodies against the same SUMO isoform can differ in their sensitivities and produce slightly different localization patterns within cells. Similarly, the apparent difference between the SUMO1 and SUMO2/3 expression patterns can sometimes be attributed to differences in the sensitivities of the antibodies raised against these specific isoforms and not to real differences in their localizations. Therefore, the results of the localization studies should be confirmed

using different antibodies and other cell analysis techniques. Based on the knockout studies that suggest that SUMO2/3 can compensate for the loss of SUMO1 function during spermatogenesis, in this chapter, I will focus on the overall localization patterns and possible roles of SUMO proteins during meiosis without focusing on the slight differences between SUMO1 and SUMO2/3 reported by our and other groups. These differences cannot be excluded but, in my opinion, require further evaluation.

3.2.1. SUMO during meiotic recombination and in the sex body

In mouse leptotene–zygotene (L–Z; Fig. 6.2C) spermatocytes, SUMO localizes to the sites of the double-strand DNA breaks during meiotic recombination and is then concentrated across the sex chromosomes from the start of meiotic inactivation (Fig. 6.2D–G) (Shrivastava et al., 2010; Vigodner, 2009). Therefore, SUMO is a likely candidate for a protein that is involved in meiotic recombination and the inactivation of the X and Y chromosomes.

The role of sumoylation at the DNA double-strand breaks has been actively studied over the past decade. The components of the sumoylation pathway were identified through a large-scale proteomic analysis of the proteins that are phosphorylated in response to DNA damage by ATM and ATR kinases (Matsuoka et al., 2007). In somatic cells, numerous proteins that are involved in the formation and repair of DNA strand breaks are regulated by sumoylation, including components of origin recognition and minichromosome maintenance complexes, homologous recombination repair (HRR) factors, BRCA-1, and 53BP1 (p53-binding protein). These proteins may have similar functions during meiotic recombination (Table 6.1) (Golebiowski et al., 2009; Morris, 2010a,b; Morris et al., 2009; Prudden et al., 2007). The connection between MSCI and DNA damage response is poorly understood but similar to SUMO, several other proteins were localized both to the sites of DNA double-strand breaks and to the sex body, although their regulation can be different at the two sites (Turner et al., 2004). For example, the massive H2AX phosphorylation in the leptotene stage is directed by ATM kinase but phosphorylation on the sex chromosomes is under the control of ATR kinase (Turner et al., 2004). Therefore, both common and unique SUMO targets may potentially function during meiotic recombination and MSCI. Interestingly, BRCA-1 is sumoylated at the sites of DNA breaks in somatic cells and, similar to SUMO, is localized to the sex body area from the time of MSCI. Thus, BRCA-1 is an attractive candidate for sumoylation during meiotic recombination and MSCI. γH2AX is another protein closely associated with SUMO in the sex body area and at the sites of meiotic recombination. Interestingly, our preliminary unpublished data suggest that γH2AX and other histones can be potentially sumoylated in germ cells (Table 6.1).

While ATR, γH2AX, and BRCA-1 are involved in MSCI, several proteins are detectable on the sex chromatin after MSCI has occurred and may be involved in the additional condensation of the sex chromatin that takes place through the pachytene stage (Escalier and Garchon, 2000; Hoyer-Fender et al., 2004; Motzkus et al., 1999). Our study showed that the SUMO signal intensified in the sex body of early- to mid-pachytene spermatocytes, suggesting that the proteins involved in the additional condensation and maintenance of XY heterochromatin can also be sumoylated (Vigodner and Morris, 2005).

Ret finger protein (RFP) is another interesting protein that has been studied in the sex body area (Matsuura et al., 2005). In somatic cells, RFP is sumoylated and implicated in transcriptional repression through its interaction with a member of the polycomb group of proteins and HDAC1. RFP was initially localized to the sex body area (Matsuura et al., 2005) with SUMIO and PIASy SUMO ligase. However, these findings have been recently questioned (Gillot et al., 2009). It was suggested that RFP serves as a specific attachment point for the sex chromosomes to the nuclear lamina and is not localized to the SUMO- and γH2AX-positive area. These data were obtained using immunofluorescence and electron microscopy studies with particular anti-SUMO and anti-γH2AX antibodies and, thus, require further evaluation.

3.2.2. SUMO and centromeric chromatin

In mouse spermatocytes, aside from the XY body (Fig. 6.2D–G, asterisk), SUMO was also concentrated in centromeric chromatin (Fig. 6.2D–G, arrowheads). The centromere is a chromosome region responsible for the attachment of the sister chromatids and the assembly of the kinetochore multiprotein complex, which, in turn, regulates chromosome segregation during mitosis and meiosis. The centromere is surrounded by pericentromeric heterochromatin, which is important for sister chromatin cohesion and kinetochore function (Amor et al., 2004; Dasso, 2008; Guenatri et al., 2004). Therefore, SUMO in the centromeric and pericentromeric chromatin could have a role in heterochromatin organization and meiotic centromere function. Several centromere/kinetochore proteins have been identified as targets of sumoylation (Azuma et al., 2003; Dasso, 2008). One of the major mitotic targets is topoisomerase II (TOP2), a protein that regulates chromatin structure and is required for centromeric function, apparently for resolving catenated DNA-linking sister chromatids (Azuma et al., 2003; Ryu et al., 2010). During meiotic prophase, topoisomerase has been shown to be important for chromosome condensation (Cobb et al., 1997, 1999). One of the isoforms of topoisomerase II, TOP2α, is concentrated in the region of the centromeric heterochromatin and is detectable at a low level in whole spermatocyte nuclei. Our group has recently identified TOP2α as a sumoylation target in germ cells (Cobb et al., 1999; Shrivastava

et al., 2010). Further studies are needed to characterize the functional significance of TOP2α sumoylation in germ cells, but based on the studies in mitotic cells, it may be important for targeting the protein to the chromatin and proper chromosome segregation (Azuma et al., 2003; Lee and Bachant, 2009; Ryu et al., 2010). Sumoylation may also be important for the recruitment and proper localization of other centromere/kinetochore proteins, such as CENPE and RanGAP1 (Dasso, 2008).

The localization of SUMO to the pericentromeric and XY heterochromatin is consistent with the previously described role for sumoylation in transcription repression and in the formation of inactive chromatin domains, such as PML nuclear bodies (David et al., 2002; Gill, 2005; Johnson, 2004). Other proteins involved in chromatin silencing have been localized to pericentromeric and XY chromatin, similar to SUMO (Cowell et al., 2002; Metzler-Guillemain et al., 2008; Motzkus et al., 1999; O'Carroll et al., 2000). Interestingly, numerous proteins involved in the formation of heterochromatin (including HDACs, methyltransferases, and HP1 proteins) have been recently identified as targets of sumoylation in somatic cells (Golebiowski et al., 2009; Ling et al., 2004). Histone deacetylation and methylation and the recruitment of HP1 proteins have been detected in centromeric and XY chromatin and, therefore, can be regulated by sumoylation during meiosis. Furthermore, histone sumoylation has recently been discovered and implicated in transcription repression (Nathan et al., 2006; Shiio and Eisenman, 2003). This modification might be involved in a complex crosstalk among various histone modifications in germ cells (Table 6.1).

3.2.3. Synaptonemal complex and sumoylation

During meiosis in *Saccharomyces cerevisiae*, sumoylation controls the assembly of the synaptonemal complex (Cheng et al., 2006; Sacher et al., 2006). In mouse spermatocytes, only SUMO-modified enzymes (but not SUMO) have been reported along the synaptonemal complex. This inability to detect SUMO has been attributed to the limited resolution of microscopic detection (La Salle et al., 2008). Our images indeed support this view; when the SUMO signal in the sex body and centromeres is intentionally overexposed, a specific signal can be detected by some antibodies in the remainder of the nucleus (2D, E, and G). These results suggest that SUMO may potentially have a role in the assembly and function of synaptonemal complexes and may modify other chromatin-associated proteins during meiosis in the mouse (Table 6.1).

In human spermatocytes, SUMO was initially localized to peri- and centromeric chromatin and XY bodies, but its expression pattern across sex chromosomes appeared to be variable, suggesting species-specific differences (Vigodner et al., 2006). Another study detected SUMO in the centromeric regions but questioned the presence of SUMO in the sex

bodies of human spermatocytes (Metzler-Guillemain et al., 2008). As discussed above, low detection levels and differences in the antibodies' abilities to detect SUMO can cause such discrepancies in the results. In addition to these previously reported findings, SUMO has recently been shown to modify synaptonemal complex proteins in human spermatocytes, a finding that supports the idea that the particular antibodies and detection techniques can significantly affect the level of detection of sumoylated proteins (Brown et al., 2008). The specific targets of sumoylation and its regulation in spermatocytes have yet to be determined. The identification techniques should focus on the purification/enrichment of spermatocytes from animals of different ages that are undergoing the initial wave of spermatogenesis and from the adult mouse testis. Immunoprecipitation with anti-SUMO antibodies can be followed by mass spectrometry protein identification and bioinformatic analysis.

The identification of SUMO targets in spermatocytes will lead to a better understanding of the specific role of sumoylation during meiosis. Paternally derived meiotic errors account for nearly 50% of XXY chromosome abnormalities and trisomy 21 and about 5–10% of most other trisomies (Hassold and Hunt, 2001). Most of these errors have been linked to abnormal meiotic recombination, including unrepaired DNA breaks, defects in chromosome pairing, and misregulated centromere function. Despite these correlations, the molecular mechanisms responsible for the meiotic errors are not well understood, especially in males. Strikingly, SUMO has been detected at the sites of meiotic DNA breaks during meiosis in the centromeres and on the X and Y chromosomes. This localization pattern suggests that the identification of sumoylated proteins during meiosis will provide important new information about the regulation of the major events in spermatocytes, which, when misregulated, can be directly linked to the formation of abnormal gametes and aneuploidy. Furthermore, spermatocytes and oocytes can share the same SUMO targets at DNA breaks and at centromeres, and, therefore, our experimental results obtained from this project may serve as an important foundation for further studies in oocytes.

3.3. SUMO during spermiogenesis

Meiosis results in the production of round haploid spermatids, which undergo further differentiation to become mature spermatozoa. The process is called spermiogenesis and includes acrosome formation, changes in chromatin structure and composition, the development of the flagellum, and the loss of much of the cytoplasm. The most important events that determine the nuclear status of spermatozoa are the replacement of histones by transition proteins and the later by protamines (Hazzouri et al., 2000). This process causes additional compaction of the chromatin, changes in

nuclear shape, and the cessation of transcription. When sperm leave the testis, they pass through the epididymis, where additional maturation takes place and the ability to move forward is acquired. The final maturation of spermatozoa takes place in the female reproductive tract under the process known as capacitation (Visconti, 2009).

In both mice and humans, a prominent SUMO signal has been detected in the chromocenters of round spermatids, sites where pericentromeric chromatin is clustered (Fig. 6.2H–K, arrowheads) (Vigodner and Morris, 2005; Vigodner et al., 2006). Interestingly, the staining intensities of round spermatids can vary from very high to almost undetectable, as has been observed using several anti-SUMO antibodies (Fig. 6.2J, arrowheads). This finding should be further evaluated but can suggest either stage-specific differences in SUMO localization or different amounts of SUMO in X- and Y-bearing spermatids. Possible SUMO targets at the centromeric heterochromatic sites were discussed in the spermatocyte section. One can expect to find some common targets of sumoylation in the centromeric chromatin of spermatocytes and spermatids (Table 6.1).

A more detailed analysis of immunofluorescence staining obtained by several (but not all) anti-SUMO antibodies reveals that sumoylated proteins are detectable at a low level outside of the areas of centromeric heterochromatin, similar to spermatocytes (Fig. 6.2H and I). Furthermore, it has been recently demonstrated that, contrary to previous assumptions, the X and Y chromosomes remain silent in spermatids, a phenomenon identified as postmeiotic sex chromatin silencing (Namekawa et al., 2006; Turner et al., 2006). Indeed, certain antibodies stain the areas adjacent to the chromocenters, sites that have been identified as XY domains (Fig. 6.2H and K, arrows) (Turner et al., 2006). Once the specific SUMO targets involved in XY inactivation during meiosis are identified, one must keep in mind that these proteins can still associate with XY chromatin in spermatids.

In elongating mouse spermatids (Vigodner and Morris, 2005), SUMO was initially reported in the perinuclear ring and in the neck/centrosome area of the spermatids. Our new data, obtained with several anti-SUMO1 antibodies, confirmed the localization of the sumoylated proteins in the neck of the spermatids (Fig. 6.2L and M, arrowheads). However, only one antibody detected SUMO in the perinuclear ring, questioning the specificity of the signal. Contrary to what has been suggested previously, the SUMO signal does not disappear during spermiogenesis but is detectable at a low level in the neck areas of both mouse and human mature sperm, suggesting an important evolutionarily conserved role for sumoylation in this region. In the neck area of the sperm, SUMO may have a role in protein turnover during disposal from the nucleus to cytoplasmic droplets (e.g., during histone–protamine replacement). In a similar manner, ubiquitinated proteins have been localized to the neck region of sperm, suggesting that

SUMO may also regulate other posttranslational modifications (Haraguchi et al., 2007).

A new study has suggested a role for sumoylation in the regulation of human sperm motility, but the underlying mechanism is not yet clear (Marchiani et al., 2010). Experimental data obtained by the author using electron microscopy suggest that SUMO is also found within the chromatin of human sperm. Based on these results and that immunofluorescence techniques are rarely sensitive enough to detect proteins in densely packed chromatin, the presence of sumoylated proteins in chromatin of mouse spermatids and sperm cannot be excluded. SUMO may be involved in various functions in the nuclei. For example, new studies have provided evidence of the formation of double-strand DNA breaks in elongating spermatids, to which both topoisomerase and γH2AX proteins (possible targets of sumoylation) have been localized (Leduc et al., 2008).

Future efforts should focus on identification of SUMO targets in spermatids and sperm. These studies will lead to an improved understanding of SUMO's proposed functions in the differentiation of spermatids into mature, fertile sperm. The role of sumoylation during spermiogenesis was originally suggested based on mouse studies, but the new data also support its role in human sperm function, which requires further characterization.

3.4. Sumoylation in testicular somatic cells

Germ cell development is tightly regulated by hormones and growth factors secreted by testicular somatic cells. Sertoli cells subdivide the seminiferous epithelium into basal and adluminal compartments by establishing a diffusion barrier (blood–testes barrier) formed by tight junctions between adjacent Sertoli cells (Mruk and Cheng, 2004; Zhang et al., 2005). They provide the proper environment for differentiating germ cells by secreting several paracrine factors. Sertoli cells proliferate during pubertal testicular development, whereas their number is constant in the mature testis. Leydig cells are found within testicular interstitial tissue and produce androgens under the influence of luteinizing hormone (LH). These hormones, in turn, bind the androgen receptors (APs) of myoepithelial and Sertoli cells, regulating their activities through a poorly defined mechanism (Grootegoed et al., 2000; Weinbauer and Wessels, 1999). Myoepithelial cells are responsible for contractile motion and sperm transport; they also produce several factors affecting Sertoli cell function (Albrecht, 2009; Skinner and Fritz, 1985).

Mouse Sertoli (SC), Leydig, and myoepithelial cells have a prominent nuclear and perinuclear SUMO signal and a weak cytoplasmic signal (Fig. 6.2B, C, N, and O). In Sertoli and myoepithelial cells, changes in SUMO protein expression can be stage-specific and should be further evaluated. In both Sertoli and Leydig cells, some antibodies produce intense SUMO signals in the nucleolus. Interestingly, SENP3 and SENP5 are

predominately localized in the nucleolus (Kim and Baek, 2009) and may be involved in the regulation of sumoylation in Sertoli and Leydig cells. In Sertoli cells, SUMO is also concentrated adjacent to the nucleolus in heterochromatic bodies containing centromeric chromatin (Fig. 6.2N, arrowhead; Vigodner and Morris, 2005).

A recent study in HeLa cells identified more than 700 targets of SUMO (Golebiowski et al., 2009). Many of these SUMO-modified proteins can also potentially function in testicular somatic cells. However, studies on steroid nuclear receptors would be of special interest because they are extensively modified by SUMO in other cell types that are regulated by steroid hormones. In nontesticular cells, the AR has been one of the most-studied targets of sumoylation (Janne et al., 2000; Poukka et al., 2000a,b). Depending on the cell type, the sumoylation of AR can either activate or suppress its transcription activity. Interestingly, even within the same cell type, the interaction of AR with SUMO or PIAS SUMO ligases can differentially attenuate its receptor activity, suggesting regulation at multiple levels (Palvimo, 2007). PIAS1, PIAS3, and PIASxα are highly expressed in testicular somatic cells and, therefore, might be involved in the regulation of androgen effects on spermatogenesis (La Salle et al., 2008; Moilanen et al., 1999; Tan et al., 2002).

In human Sertoli cells, SUMO is mostly localized in the heterochromatic regions of the nucleoli, a pattern that correlates with the low nuclear level of AR in these cells. By contrast, a low to undetectable cytoplasmic SUMO signal correlated with high level of AR (Vigodner et al., 2006). Interestingly, in a similar manner, in rat immature Sertoli cells, which represent yet another system to study sumoylation, SUMO and AR signals never produced an overlapping signal; AR-positive cells were SUMO negative and vice versa (Vigodner et al., 2006). Whether there is a connection between AR and SUMO expression should be confirmed using immunostaining with other antibodies and other techniques for studying protein interactions, but the results imply either the suppression of AR transcription by sumoylation or an inability of the particular anti-AR antibody to recognize a sumoylated form of the protein. In infertile patients with spermatogenic arrest and Sertoli-only syndrome, the levels of SUMO and AR are decreased, suggesting impaired cellular activity, but more samples and further analysis are required to better understand the functional relevance of these findings (Vigodner et al., 2006). Similar to the findings in the mouse, human peritubular myoid and Leydig cells had nuclear and perinuclear SUMO signals. In those cells, sumoylation can regulate androgen-dependent and androgen-independent cellular functions.

Aside from AR, other steroid nuclear receptors are regulated by sumoylation, including progesterone and glucocorticoid receptors (PR and GR) (Kotaja et al., 2002; Shao et al., 2004). PR and GR are differentially expressed in testicular somatic cells, where they may be regulated by

SUMO proteins (Luetjens et al., 2006). Interesting new data have recently been published on the effect of retinoic acid on the subcellular localization of SUMO2/3. It has been suggested that retinoic acid affects the nuclear versus the cytoplasmic localization of SUMO2/3 in both Sertoli and germ cells during testicular development (Zhu et al., 2010). However, some of the author's findings contradict previous data and are difficult to explain. For example, the author reports the localization of SUMO2/3 in the nuclei of germ and Sertoli cells only before the initiation of meiosis and exclusively in the cytoplasm afterward. Previous publications by several groups have reported that SUMO is clearly localized to the nuclei of germ and somatic testicular cells, including adult Sertoli and postmeiotic cells (Fig. 6.2) (Rogers et al., 2004; Vigodner and Morris, 2005). Stage-specific differences in SUMO localization and the effects of retinoic acid at different stages of spermatogenesis are important and should be evaluated further.

3.5. Sumoylation and stress responses in the testis

The role of sumoylation in stress responses has been well characterized in somatic cells. Changes in the levels of high-molecular-weight SUMO conjugates have been described after the induction of various types of cell stress, including heat and oxidative stresses (Saitoh and Hinchey, 2000; Tempe et al., 2008). A new recent study analyzed more than 700 SUMO targets before and after the induction of heat stress and revealed global changes in the sumoylation of numerous proteins involved in major cellular pathways. These findings suggest that SUMO initiates important early responses against cellular stress through the modification of hundreds of molecules (Golebiowski et al., 2009).

In our recent study, changes in the levels of both free SUMO isoforms and high-molecular-weight SUMO conjugates were monitored before and after the induction of different types of cellular stress (Shrivastava et al., 2010). Using both cell lines and primary cells freshly isolated from mouse testes, significant changes were detected in the levels of SUMO1 and SUMO2/3 conjugates after a brief cellular exposure to heat and oxidative stresses. Immunofluorescence studies localized SUMO to the sites of DNA double-strand breaks in stressed germ cells. To study the effect of oxidative stress *in vivo*, animals were exposed to tobacco smoke for 12 weeks. Changes in the sumoylation of high-molecular-weight proteins were consistent with oxidative damage in the tobacco-exposed mice (Shrivastava et al., 2010). Although oxidative stresses, including tobacco- and age-related stresses, have been associated with the formation of abnormal sperm, the underlying mechanisms are not well understood (Aitken and Roman, 2008). Our results show that stress has a dramatic effect on sumoylation in male germ cells, even before other pathways are activated. Given the abundant expression of SUMO and its suggested importance in testicular cells, stress-induced

changes in sumoylation may adversely affect spermatogenesis. Therefore, the identification of cell-specific sumoylation targets during normal spermatogenesis and under stress may provide a better understanding of the molecular mechanisms responsible for stress responses in normal germ cells and in certain instances of unexplained infertility.

4. Concluding Remarks and Future Perspectives

Although the studies reviewed above provide important initial information regarding the possible roles that sumoylation plays during spermatogenesis, little progress has been made in understanding how SUMO regulates its suggested functions. As has been shown in somatic cells, the identification of sumoylation targets is a critical step toward understanding its cellular functions (Andersen et al., 2009; Sarge and Park-Sarge, 2009a; Tatham et al., 2009). However, although hundreds of SUMO targets have been identified in somatic cells (Golebiowski et al., 2009), they remain uncharacterized in the testis, with the exception of Top2A and SC proteins (SCP-1 and SCP-2), which have been coimmunoprecipitated with SUMO from a testicular lysate (Brown et al., 2008; Shrivastava et al., 2010). One of the reasons for this gap is that, in contrast to the cell lines that have mostly been used for the identification of SUMO targets in somatic cells, testicular tissue is complex and multicellular in nature. Therefore, the identification of cell-specific sumoylation targets is required. Furthermore, certain SUMO conjugates are dynamic, unstable and/or found at low levels in cells. In somatic cell lines, SUMO has mainly been overexpressed before its targets have been identified, but such an approach cannot be used for germ cells because of their difficulty/inability to be maintained and transfected *in vitro*.

In my perspective, the most promising direction would be the identification of cell-specific SUMO targets using well-validated cell separation techniques followed by immunoprecipitation using anti-SUMO antibodies and mass spectrometry protein identification. After a positive confirmation, promising targets should be chosen for further characterization. If the sumoylation sites of the selected proteins are not identified, then site-directed mutagenesis should be used to change candidate lysine residues to arginine and determine if these substitutions cause a disappearance of the sumoylated isoform(s), as determined by Western blotting. This should be done after the cells are cotransfected with plasmids carrying SUMO and the mutated gene, or an *in vitro* sumoylation assay. After the identification of the acceptor lysine residue(s), specific antibodies can be produced against the sumoylated forms of the proteins, which may be used for localization and interaction studies. The functional consequences of the mutations in the sumoylated sites of the identified proteins and other aspects of impaired

sumoylation in germ cells will then be addressed both *in vitro* and *in vivo* through the generation of transgenic animals and through the direct screening of testicular biopsies that are obtained from males suffering from idiopathic sterility or testicular cancer.

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