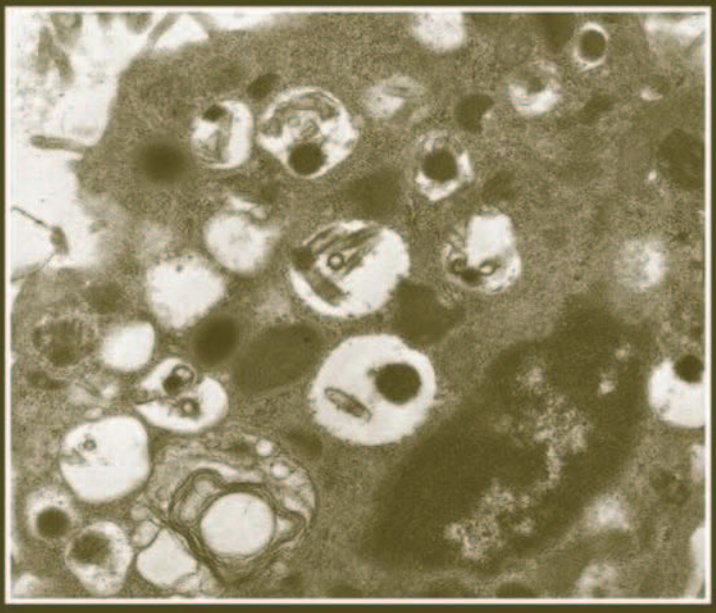
INTERNATIONAL REVIEW OF CELL AND MOLECULAR BIOLOGY

Edited by
Kwang W. Jeon



Volume 275

VOLUME TWO SEVENTY FIVE

International Review of CELL AND MOLECULAR BIOLOGY

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525 B Street, Suite 1900, San Diego, CA 92101-4495, USA
30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
32 Jamestown Road, London NW1 7BY, UK
Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands

First edition 2009

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

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ISBN: 978-0-12-374806-5

PRINTED AND BOUND IN USA
09 10 11 12 10 9 8 7 6 5 4 3 2 1

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CHAPTER ONE

Cell and Molecular Biology of Invadopodia

Giusi Caldieri, Inmaculada Ayala, Francesca Attanasio, *and* Roberto Buccione

Contents

Abstract

The controlled degradation of the extracellular matrix is crucial in physiological and pathological cell invasion alike. *In vitro*, degradation occurs at specific sites where invasive cells make contact with the extracellular matrix via specialized plasma membrane protrusions termed invadopodia. Considerable progress has been made in recent years toward understanding the basic molecular components and their ultrastructural features; generating substantial interest in invadopodia as a paradigm to study the complex interactions between the intracellular trafficking, signal transduction, and cytoskeleton regulation machineries. The next level will be to understand whether they may also represent valid biological targets to help advance the anticancer drug discovery process. Current knowledge will be reviewed here together with some of the most important open questions in invadopodia biology.

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International Review of Cell and Molecular Biology, Volume 275
ISSN 1937-6448, DOI: 10.1016/S1937-6448(09)75001-4

Key Words: Invadopodia, Cell adhesion, Cell invasion, Extracellular matrix degradation.

1. Introduction

The ability of cells to invade the extracellular matrix (ECM) is essential in the response to injury, pathogen infection, embryogenesis, differentiation, neoangiogenesis, and also during tumor cell invasion and metastasis (Basbaum and Werb, 1996). In particular, migration-associated proteolytic degradation of the ECM is a common feature of cancer cells (Wolf and Friedl, 2009).

Invadopodia can be defined as stable actin-rich protrusions emanating from the ventral surface of invasive tumor or transformed cells, cultured on appropriate ECM substrates such as gelatin, fibronectin, collagen type I, collagen type IV, or laminin (Kelly *et al.*, 1994) and displaying focalized proteolytic activity toward the substrate (Chen, 1989; Mueller and Chen, 1991). Seminal work from the Chen and Mueller laboratories throughout the 1990s led to the identification of a number of molecular components including integrins, elements of signaling machineries, soluble and membrane-bound proteases (including matrix metalloproteases), and actin and actin-associated proteins such as cortactin and others (Bowden *et al.*, 1999; Chen, 1996; Monsky *et al.*, 1994; Mueller *et al.*, 1992; Nakahara *et al.*, 1997b). Considering the evidence accumulated to date, it appears that the biological function that can be specifically attributed to invadopodia is the degradation of ECM (Baldassarre *et al.*, 2003; Mizutani *et al.*, 2002; Nakahara *et al.*, 1997b; Yamaguchi *et al.*, 2005). Focal degradation of the ECM at invadopodia may thus very well recapitulate the initial steps of tumor cell invasion realized through the tight integration of the membrane remodeling, trafficking, and signaling machineries.

Invadopodia can be identified by the light microscope as roundish actin-rich structures at the ventral surface of cells (i.e., substrate face) that (1) are associated with sites of substrate degradation, (2) are not confined to the cell periphery, and (3) contain cortactin (or other actin related proteins, see below) and/or phosphotyrosine (Baldassarre *et al.*, 2006; Bowden *et al.*, 2006). Typical examples can be seen in Fig. 1.1. Other features that can be used to identify invadopodia, at least in some cell lines, include their location proximal to the Golgi complex (Baldassarre *et al.*, 2003), the central regulator for intracellular trafficking, and their extended half-life of up to 2 h or more (Baldassarre *et al.*, 2006; Yamaguchi *et al.*, 2005) as compared to related protrusive adhesions such as the podosomes (Linder, 2007; Linder and Aepfelbacher, 2003).

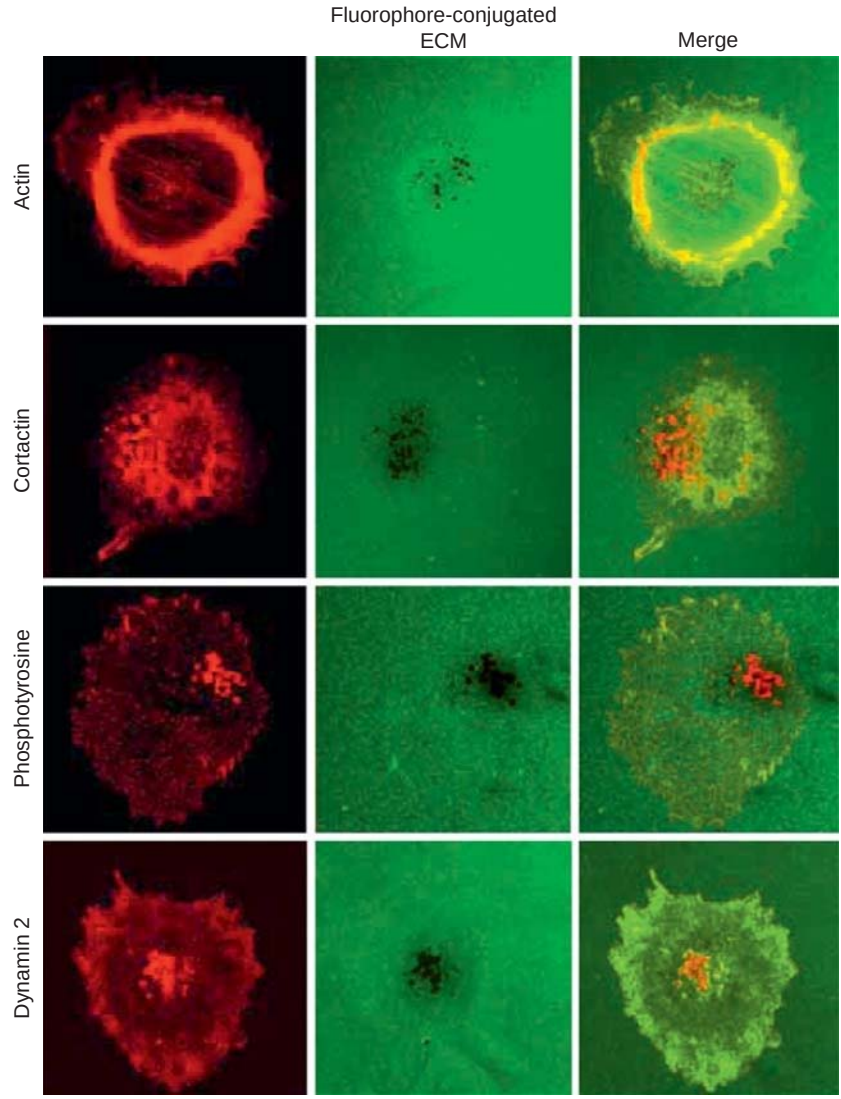

Figure 1.1 Identification of invadopodia. A375MM melanoma cells grown on FITC-conjugated gelatin (green) and then fixed and stained with Alexa 546-phalloidin and anti-cortactin, anti-phospho-tyrosine and anti-dynamin 2 antibodies (red). Invadopodia match with underlying areas of degradation. Merged images are also shown.

2. Biogenesis, Molecular Components, and Activity

A number of reviews have comprehensively listed and discussed the components and pathways underlying the biogenesis and function of invadopodia (Ayala *et al.*, 2006; Gimona *et al.*, 2008; Linder, 2007;

Weaver, 2006). This section provides an updated bird's eye view of current knowledge, while highlighting those players who are currently the better characterized and addressing some of the most urgent questions in invadopodia biology. These include understanding the molecular and physicochemical cues that trigger invadopodia biogenesis, the signaling cascades transducing those cues to the membrane and cytoskeleton remodeling machineries, and how focal degradation of the ECM is established at invadopodia. Remarkably little is known on many of these aspects, although the field is witnessing a significant acceleration that no doubt will lead to significant advances in the near future in these and many other aspects of invadopodia biology.

2.1. Structure

The description of the ultrastructural features of invadopodia is still rather incomplete. In fact, an initial transmission electron microscopy observation on transformed fibroblasts (Chen, 1989) suggested that they are thin protrusions extending from the plasma membrane into the underlying ECM; this was later confirmed on a breast cancer-derived cell line (Bowden *et al.*, 1999). No further progress was reported for some years until a detailed ultrastructural analysis was performed on the melanoma cell line A375MM, based on a correlative confocal light electron microscopy technique whereby individual areas of ECM degradation with matching invadopodia were first identified at the light microscope and the very same analyzed at the electron microscope and reconstructed in three dimensions. In this study, invadopodia were shown to be originated from profound invaginations of the ventral surface of the plasma membrane. In general, such invaginations averaged 8 μm in width and 2 μm in depth. From within, many surface protrusions originated with diameters ranging from hundreds of nanometers to a few micrometers, and averaging 500 nm in length, which sometimes penetrated into the matrix (Baldassarre *et al.*, 2003). These protrusions were consistent with the originally described ''invading'' structures (Chen, 1989) but seemed to be part of more complex superstructures (Fig. 1.2). More recently, electron microscopy tomography experiments analyzed the connections between invadopodial protrusions and the cell body, showing them to be quite narrow (Fig. 1.3; Baldassarre *et al.*, 2006). Furthermore, additional evidence obtained by generating sections perpendicular to the substrate have confirmed the initial reconstructions (Beznoussenko, Caldieri, Giacchetti, and Buccione, unpublished date).

A consistent pattern arising from observations at the light and electron microscopy levels is the polarization and juxtaposition of the Golgi complex, the central secretory pathway processing unit of the cell, toward the invadopodial area (Caldieri, Giacchetti, Beznoussenko and Buccione, unpublished date; Baldassarre *et al.*, 2003), suggesting a tight relationship between proteolytic activity and membrane/protein transport (to be discussed below).

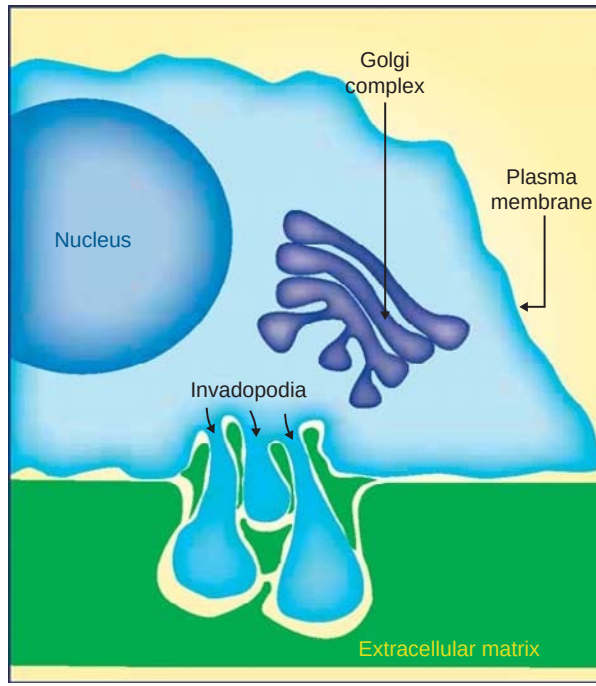

Figure 1.2 Schematic diagram of the invadopodial area. This is based on correlative light electron microscopy reconstructions on A375MM melanoma cells (Baldassarre *et al.*, 2003). Invadopodial protrusions originate from profound invaginations of the ventral surface of the plasma membrane; within the area delimited by the large invagination, large fragments of gelatin can often be seen. Also shown are the spatial relationships with the nucleus and the Golgi complex. Cartoon courtesy of Elena Fontana.

2.2. The cell–ECM interface

The engagement of cell surface integrins by substrate components is possibly the event that initiates invadopodia formation (Mueller *et al.*, 1999; Nakahara *et al.*, 1996, 1997b). The specific integrin combination that, when engaged, leads to invadopodia formation, might be cell-type dependent. In LOX melanoma cells, $\alpha_6\beta_1$ activation was found to promote Src-dependent tyrosine phosphorylation of p190RhoGAP, which in turn affected the actin cytoskeleton through the Rho family GTPases, thus activating membrane-protrusive and proteolytic activity, leading to invadopodia formation and cell invasion. The signaling pathways triggered by integrin engagement and leading to invadopodia formation and the molecular players involved in the cascade will be discussed later.

Integrins might also function as docking sites to spatially and temporally confine specific cellular activities and thus focalize the degradation process.

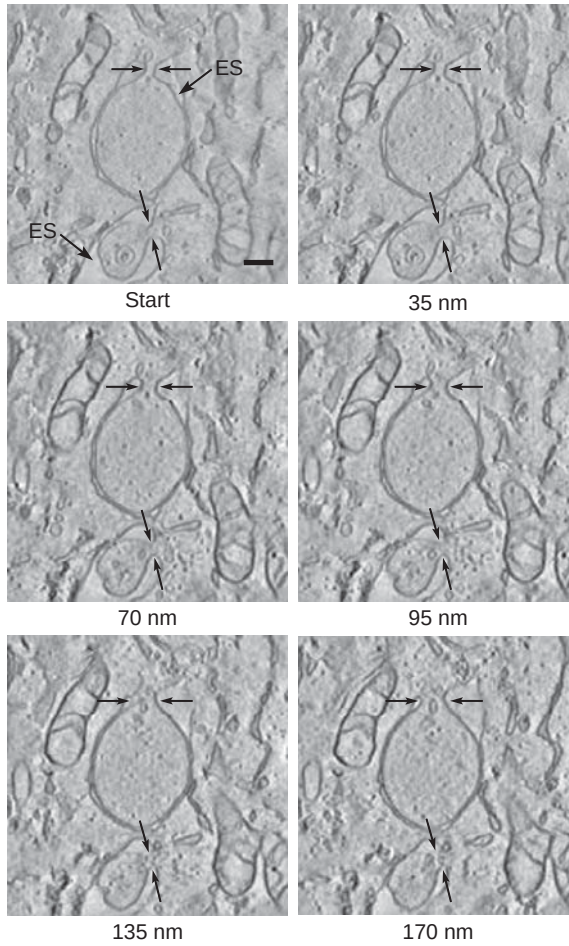

Figure 1.3 **Invadopodia ultrastructure. Frame shots of an electron microscopy tomographic reconstruction of an invadopodial complex. Displacement along the vertical (*z*) axis is indicated in nanometers. The arrows indicate the narrow connections between the two invadopodial protrusions and the cell body. ES: extracellular space. Size bar is 200 nm. This image (Baldassarre *et al.*, 2006) is reproduced with permission from *The European Journal of Cell Biology*.**

For example, collagen-induced $\alpha_3\beta_1$ association with the serine protease seprase was shown to drive the degradative activity of this gelatinolytic enzyme specifically at the tip of invadopodia (Artym *et al.*, 2002). An inhibitory anti-β_1 integrin antibody prevented the association between uPAR and seprase at invadopodia, suggesting a fundamental role for β_1 in the organization and targeting of proteases at sites of ECM degradation (Artym *et al.*, 2002). Integrins can also cooperate with the membrane-type 1

matrix metalloprotease (MT1-MMP) to localize and enhance proteolysis through the activation of matrix metalloprotease-2 (MMP2) (Deryugina *et al.*, 2001). Further, integrins might act at invadopodia by facilitating the clearing of partially degraded ECM by phagocytosis (Coopman *et al.*, 1998). In conclusion integrins are fundamental organizing centers to deploy the activity of various components at invadopodia. Research into the roles of integrins in invadopodia biology, given also potential implications for translational research, has been inexplicably stagnating for the last few years.

Recent research has shown that invadopodia formation can also be triggered/enhanced by soluble factors. For example, engagement of the EGF receptor with subsequent triggering of the signaling cascade leads to activation of the actin polymerization machinery (Yamaguchi *et al.*, 2005). At present a unifying model integrating ECM- and soluble ligand-derived activation is still lacking; furthermore, invadopodia formation does not necessarily require EGF receptor activation in many cell models.

2.3. Actin-remodeling machinery

2.3.1. Actin organization at invadopodia

The actin polymerization molecular machine based on the actin nucleator Arp2/3 and its activator N-WASP (Goley and Welch, 2006; Stradal and Scita, 2006) has been shown to be fundamental in the initiation and progression of the protrusive process leading to invadopodia formation. Hence, similar to lamellipodia, invadopodia rely on an Arp2/3-mediated branched actin meshwork, regulated and stabilized by cortactin. In general, however, the mechanisms generating the forces behind membrane remodeling and protrusion at invadopodia still need to be defined.

Two main hypotheses have recently been discussed (Vignjevic and Montagnac, 2008). In one, the constant growth of the branched actin meshwork would propel invadopodia into the underlying matrix, a mechanism similar to lamellipodia protrusions. Alternatively, following activation of the N-WASP/Arp2/3 system, and through the recruitment of actin bundling proteins, actin bundles could originate from the branched network, to win the stiffness of the substrate and to allow invadopodia protrusion, akin to filopodia formation. The evidence is still sparse and presents some inconsistencies possibly due to a combination of different experimental approaches and cell models. For instance, a FRET-based study showed that N-WASP is active at the base of the invadopodial protrusions in a rat mammary carcinoma cell line (Lorenz *et al.*, 2004). In another study, based on the A375MM human melanoma cell model, actin in actively degrading invadopodia was revealed as organized in very dynamic structures in which "head" (i.e., roundish, thicker) and "tail" (i.e., thinner, longer) sections were distinguishable (Baldassarre *et al.*, 2006), so that they explicitly resembled the actin-rich propelling structures associated with invading bacteria

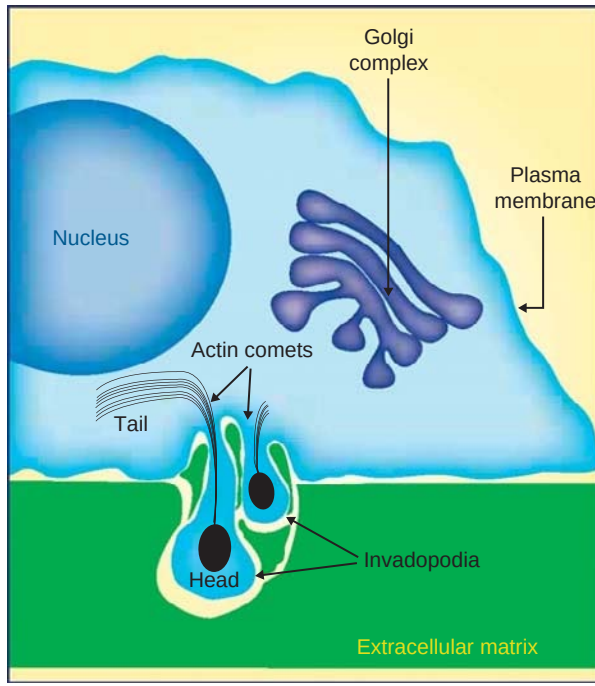

Figure 1.4 **Actin comets at invadopodia. Graphical representation of actin tail structures contained within invadopodial protrusions. This is inferred from the previously published morphological descriptions of invadopodia (Baldassarre *et al.*, 2003, 2006). Where the nucleus "constrains" the structures, invadopodia-associated actin tails bend over and allow detection of a quasi-rotatory motion. Cartoon courtesy of Elena Fontana.**

(i.e., actin comets or tails) (Cudmore *et al.*, 1995; Gouin *et al.*, 2005) with the striking difference that at invadopodia, the head sections remained stationary while the tails moved around continuously in a quasi-circular motion (Fig. 1.4). In this study, Arp2/3 and N-WASP were localized to the whole actin tail structure and to the ''head'' section, respectively, suggesting actin branching at the tip of the invadopodial protrusion (Baldassarre *et al.*, 2006). The main components of the actin polymerization machinery acting at invadopodia will be discussed below.

2.3.2. The Arp2/3 complex

Arp2/3 is a seven-protein complex that contains two related proteins (Arp2 and Arp3) and five unique polypeptides (ARPC1-5) (Goley and Welch, 2006). The complex presents little biochemical activity on its own, but when activated, initiates the nucleation of a new actin filament that emerges from an existing one in a y-branch configuration with a regular 70° angle

(Mullins *et al.*, 1998). As such, Arp2/3 is responsible for the actin rearrangements that govern the formation of lamellipodia and filopodia and, in general, cell motility (Goley *et al.*, 2004).

Arp2/3 has been repeatedly localized at invadopodia (Baldassarre *et al.*, 2006; Yamaguchi *et al.*, 2005). Also, the downregulation of a single subunit by siRNA and the overexpression of N-WASP mutants (see below) that lack the sequence required for Arp2/3-binding or the CA domain of N-WASP that competes for the binding to Arp2/3, resulted in inhibition of invadopodia formation (Yamaguchi *et al.*, 2005). Altogether the results provide evidence of the requirement of Arp2/3 for invadopodia biogenesis and ECM degradation. A dysfunction in the Arp2/3 complex might also be associated with cancer metastasis, which relies on the ability of cells to migrate away from the primary tumor. Indeed, the expression of Arp2/3 together with N-WASP and other factors related with cell motility is upregulated in some tumor tissues and invasive cells (Otsubo *et al.*, 2004; Semba *et al.*, 2006).

2.3.3. N-WASP

The WASP family of proteins includes hematopoietic WASP, ubiquitous N-WASP, and WASP family verprolin homologous (WAVE) proteins (WAVE1, WAVE2, and WAVE 3) (Bompard and Caron, 2004). WASP was originally identified as the causative gene product for the hereditary X-linked Wiskott–Aldrich syndrome, characterized by thrombocytopenia and immunodeficiency. WASP and N-WASP are direct effectors of Cdc42, whereas WAVE proteins play a major role in Rac-induced actin dynamics. All these proteins are implicated in a variety of cellular processes such as formation of membrane protrusions, vesicular trafficking and motility of pathogens. The family possesses a common C-terminal catalytic verprolin homology, cofilin homology or central, acidic (VCA) domain for the activation of the Arp2/3 complex, which induces rapid actin polymerization and generates a branched network of actin filaments (Mullins *et al.*, 1998; Welch *et al.*, 1998).

The expression of dominant negative mutants of N-WASP, unable to activate the Arp2/3 complex, suppressed invadopodia formation in v-Src-transformed 3Y1 rat fibroblasts (Mizutani *et al.*, 2002). Also, activation of N-WASP was detected at actively degrading invadopodia (Lorenz *et al.*, 2004). Furthermore, N-WASP depletion in metastatic rat adenocarcinoma MTLn3 cells reduced their ability to form invadopodia whereas WAVE1 and WAVE2 knockdown cells formed invadopodia as efficiently as control cells (Yamaguchi *et al.*, 2005). These results suggested that N-WASP in implicated in invadopodia biogenesis and as a consequence, ECM degradation.

Many upstream activators of N-WASP such as Grb2, Nck, WASP-interacting SH3 protein (WISH) and WASP-interacting protein (WIP) (Carlier *et al.*, 2000; Fukuoka *et al.*, 2001; Rohatgi *et al.*, 2001) have been identified. The contribution of many of these molecular components in

invadopodia biogenesis has also been investigated. Invadopodia formation and degradation activity was markedly suppressed in the same cells by depletion of Nck but not Grb2 (Yamaguchi *et al.*, 2005). WIP is thought to link Nck and N-WASP to induce actin polymerization (Benesch *et al.*, 2002). The ectopic expression of the wild-type form showed that this protein accumulated significantly at invadopodia. Additionally, transfection of the N-WASP binding domain (WBD) of WIP showed a marked reduction of invadopodia formation (Yamaguchi *et al.*, 2005). These data suggested that the interaction between N-WASP and WIP was needed for invadopodia formation. An additional note of interest is that similar to Arp2/3, N-WASP is overexpressed in several cancer types (Yamaguchi and Condeelis, 2007).

2.3.4. Cortactin

Cortactin is an actin-binding protein involved in the coordination of cell migration, cytoskeleton remodeling, and intracellular protein transport (Ammer and Weed, 2008; Olazabal and Machesky, 2001). Human cortactin is encoded by the CTTN gene on chromosome 11q13, frequently amplified in breast, head and neck squamous carcinoma and bladder cancer (Bringuier *et al.*, 1996; Schuuring, 1995). Cortactin features an N-terminal acidic domain containing a conserved DDW motif that binds and weakly activates the Arp2/3 complex (Uruno *et al.*, 2001). This is followed by a variable number of 37 amino acid repeats (depending on the splice variant; van Rossum *et al.*, 2003), constituting the actin-binding domain of which only the fourth is required for F-actin-binding activity, and has been suggested to stabilize the newly created branches between filaments (Weaver *et al.*, 2001, 2003). Cortactin was originally identified as a major substrate of Src (Wu *et al.*, 1991) and later found to be tyrosine-phosphorylated in response to stimuli that induce remodeling of the actin cytoskeleton, as for instance FGF, EGF, or integrins (Vuori and Ruoslahti, 1995; Zhan *et al.*, 1993). In particular, phosphorylation of Y421, 466, and 482 in the proline-rich domain (PRD) has been shown to be required for motility of endothelial cells (Huang *et al.*, 1998) and metastatic dissemination of breast carcinoma cells (Li *et al.*, 2001).

Cortactin has also been shown to play a fundamental role in invadopodia formation and function. After an earlier study showing that microinjection of antibodies against cortactin blocked matrix degradation at invadopodia (Bowden *et al.*, 1999), other reports have corroborated and extended this finding with diverse approaches including RNA interference and functional domain analysis. These results have highlighted the importance of cortactin phosphorylation by Src in invadopodia function (Artym *et al.*, 2006) and suggested that cortactin might function by regulating metalloprotease secretion at sites of ECM degradation (Clark *et al.*, 2007). One study investigated the role of various protein kinases in cortactin function at invadopodia,

showing that phosphorylation of S405 and S418 in the PRD by ERK1/2 as well as S113 in the first actin-binding domain by PAK, respectively, was crucial to invadopodia biogenesis (Ayala *et al.*, 2008). If one considers, however, that a mass spectrometric study mapped 17 new phosphorylated residues in cortactin in addition to those already known (Martin *et al.*, 2006), it is likely that the phosphorylation pattern of cortactin and its functional implications are even more complex.

2.3.5. Cofilin

Cofilin belongs to a family of related proteins with similar biochemical activities called the actin depolymerizing factor (ADF)/cofilin family, which are critical regulators of actin dynamics and protrusive activities in cells. The reorganization of the actin cytoskeleton at the leading edge of eukaryotic cells requires coordinated actin polymerization and depolymerization. To drive the cell front forward, branched actin filaments are generated at the leading edge through the action of the Arp2/3 complex. ADF/cofilin family of proteins disassembles F-actin from the rear of the actin network to recycle actin monomers to the leading edge for further rounds of polymerization (Orlichenko *et al.*, 2006). Recent data have demonstrated the involvement of cofilin in invadopodia function. In particular, its depletion resulted in the formation of small, short lived and, thus, poorly-degrading invadopodia, suggesting that cofilin plays a role in the stabilization and/or maturation process instead of initiation (Yamaguchi *et al.*, 2005). Cofilin and its regulatory proteins have been also implicated in tumor cell invasion and metastasis and in cancer (Wang *et al.*, 2007).

2.4. Signaling to the cytoskeleton

The characterization of the molecular effectors downstream of integrin engagement in invadopodia, while obviously of great importance, is still poorly advanced. Some general conclusions can be drawn, however, in terms of the protein kinases, small G-proteins, and signaling activators/effectors implicated in invadopodia biogenesis and function.

2.4.1. Tyrosine kinases

Invadopodia were originally discovered as specialized surface protrusions enriched in Src kinase (Chen, 1989); high levels of tyrosine phosphorylation and Src-dependence were subsequently and specifically associated to such structures (Mueller *et al.*, 1992). The non-receptor Src family of tyrosine kinases (SFK) consists of nine members. They all exhibit a well-conserved membrane-targeting SH4 region at the N-terminus and a "unique" domain, highly diverse among family members, followed by the SH3, SH2, and the tyrosine kinase domain, containing the activating phosphorylation site (Y416) of Src kinases. The C-terminal tail features an autoinhibitory phosphorylation

site (Y527 in Src) (Thomas and Brugge, 1997). Src-transformed cells are characterized by reduced cellular contacts, such as focal adhesions in mesenchymal cells and cell–cell adhesion in epithelial cells (Frame *et al.*, 2002).

Cells treated with SFK inhibitors failed to form invadopodia and consequently to degrade the ECM; also, transfection with kinase-active or kinase-inactive Src mutants, respectively, increased or decreased the levels of ECM degradation, as compared to wild-type Src-expressing cells (Bowden *et al.*, 2006; Hauck *et al.*, 2002). These findings establish that SFK activity is absolutely required for invadopodia formation and to sustain efficient degradation of the ECM. A number of substrates can be potentially associated with the role played by SFK in invadopodia formation and function. These include cortactin (Ayala *et al.*, 2008), Tks5 and 4 (see below, Buschman *et al.*, 2009; Seals *et al.*, 2005) and caveolin 1 (see below, Caldieri *et al.*, 2009).

2.4.2. Serine/threonine kinases

More recently, various serine/threonine kinases have been brought into the picture and shown to be implicated in invadopodia biogenesis. The well-known extracellular signal-regulated serine/threonine kinases ERK1/2 have been associated with invadopodia biogenesis (Ayala *et al.*, 2008; Tague *et al.*, 2004). The ERKs are part of the large modular network of the mitogen-activated protein kinase (MAPK) pathways that regulate a plethora of physiological processes including cell growth, differentiation, and apoptosis. The ERKs are activated by diverse stimuli including growth factors acting through receptor tyrosine kinases (RTKs), cytokines binding to receptors that consequently activate tyrosine kinases, or agonists of G protein-coupled receptors (Kolch, 2005). How and when ERKs contribute to invadopodia formation remains to be clarified, although some reports have implicated the small GTPase ARF6 as an activator (Tague *et al.*, 2004) and the multidomain protein cortactin as a main substrate (Ayala *et al.*, 2008).

The serine/threonine kinases of the PAK family are main downstream effectors of the small Rho family GTPases Rac1 and Cdc42 playing roles in the regulation of cell morphogenesis, motility, survival, mitosis, and angiogenesis. The family comprises conventional PAK1, PAK2, and PAK3 isoenzymes, each containing an autoinhibitory domain and a group of newly discovered members PAK4, PAK5, and PAK6 (Bokoch, 2003; Kumar *et al.*, 2006; Zhao and Manser, 2005). In general, the substrates of conventional PAKs are involved in the regulation of the cytoskeleton and adhesion. Interestingly enough, the PAK1 gene maps to the 11q13 amplicon, the same region that contains the gene that encodes cortactin (Bekri *et al.*, 1997). Hence, similar to cortactin, overexpression of PAK has been documented in a number of cancers and T-cell lymphoma (Balasenthil *et al.*, 2004; Carter *et al.*, 2004; Mao *et al.*, 2003; Schraml *et al.*, 2003). Specifically, PAK1 expression is upregulated in human hepatocellular carcinoma and its overexpression is associated with more aggressive tumor behavior (Ching

et al., 2007). PAK1 also enhanced the motility of these cells as well as the phosphorylation of the downstream effector JNK that in turn phosphorylates paxillin to induce cell migration (Huang *et al.*, 2003).

PAK1 has been shown to phosphorylate cortactin on S113 *in vitro*. As a consequence cortactin binding to F-actin was reduced, suggesting a role for PAK-dependent phosphorylation of cortactin in the regulation of branched actin filaments dynamics (Webb *et al.*, 2006). It has been recently found that transfection of the autoinhibitory domain of PAK1, known to inhibit the endogenous kinase (Zhao *et al.*, 1998) induced a significant decrease in ECM degradation, thus suggesting that PAK activity supports invadopodia formation (Ayala *et al.*, 2008). Furthermore, a nonphosphorylatable S113A cortactin mutant reduced invadopodia formation and ECM degradation, whereas the phosphorylation-mimicking S113D cortactin variant stimulated matrix degradation and localized to invadopodia (Ayala *et al.*, 2008). Other PAK substrates of relevance to invadopodia formation, if any, remain to be determined. To this effect it is worth mentioning that PAK1 is able to interact with, and phosphorylate the ARPC1B subunit of the Arp2/3 complex (Vadlamudi *et al.*, 2004), thus possibly facilitates assembly and maintenance of the complex (Gournier *et al.*, 2001) hence regulating cell motility and invasivity (Vadlamudi *et al.*, 2004).

2.4.3. Rho-family GTPases

The Rho family Rho, Rac, and Cdc42 small molecular weight GTPases of the Ras superfamily have been intensely studied within the context of cell motility, invasion, and polarity. These proteins cycle between a GDP-bound and a GTP-bound form. The exchange of GDP for GTP induces a conformational change that allows interaction with downstream effectors; this active state is terminated by hydrolysis of bound GTP to GDP. Through this cycle, Rho-GTPases control many cell functions by interacting with, and stimulating various effector targets including protein kinases, actin nucleators, and phospholipases and thus affecting fundamental physiological processes, such as cell shape, morphology, polarization, motility, and metastasis formation by acting on the actin cytoskeleton (Hall, 2005). In fibroblasts, Cdc42 controls microspike and filopodia formation and is a master regulator of cell polarity (Etienne-Manneville, 2004; Heasman and Ridley, 2008; Nobes and Hall, 1995); Rac plays a central role in lamellipodia and ruffling whereas Rho in stress fibers and focal adhesion formation. For example, at the molecular level, Rac and Cdc42 both activate Arp2/3 through the effectors Sra-1 and N-WASP, respectively. Rho affects myosin II activity and consequently cell contraction.

The most compelling evidence to date points to Cdc42 as acting upstream of invadopodia formation, depending on the cell model. Dominant-active mutants of Cdc42 or Rac enhanced dot-like and diffused fibronectin degradation respectively in RPMI17951 melanoma cells (Nakahara *et al.*, 2003).

Conversely, the expression of a dominant-active mutant of Rho did not affect degradation (Nakahara *et al.*, 2003). When Cdc42 is downregulated by RNA interference, or when a constitutively inactive mutant is transfected, invadopodia formation is inhibited in the metastatic MTLn3 rat mammary adenocarcinoma cell line (Yamaguchi *et al.*, 2005). In immunofluorescence studies, transfected Cdc42 but not Rho A or Rac1 was detected at invadopodia (Baldassarre *et al.*, 2006). In addition, expression of dominant positive or negative RhoA and Rac mutants did not affect invadopodia function (Baldassarre, Ayala, and Buccione, unpublished data). Nevertheless, a role of Rho at invadopodia is suggested by the fact that phosphorylation of p190RhoGAP was found to activate the membrane-protrusive activity required for invadopodia formation and cell invasion (Nakahara *et al.*, 1997b). Of interest in connection to the above is the finding that the interaction of the multidomain polarity protein IQGAP1 with proteins of the secretion machinery (to be discussed later) is triggered by active Cdc42 and RhoA and is essential for matrix degradation (Sakurai-Yageta *et al.*, 2008).

As mentioned above, the small GTPases have limited hydrolytic and exchange activity on their own and thus require accessory proteins to function efficiently and to ensure proper regulation. These are included in three main classes, the guanine nucleotide exchange factors (GEF), the GTPase activating proteins (GAP), and the guanine nucleotide dissociation inhibitors (GDI). The GEF proteins stimulate the exchange of GDP for GTP to generate the activated form whereas the GAP proteins accelerate the intrinsically low GTPase activity thus increasing deactivation rate. Finally, the GDI class of proteins interacts with the GDP-bound forms and act as negative regulators, controlling cycling between membranes and cytosol. The known GEFs are much more numerous (about 60 members) than the Rho family GTPases themselves (Schmidt and Hall, 2002), and it can therefore be hypothesized that the different GEFs hold the key in regulating the specificity of downstream signaling from Rho GTPases in diverse cellular functions and cell systems (Zhou *et al.*, 1998). There is evidence suggesting that GEFs might be important players in invasion and metastasis as many of them were originally identified as oncogenes after transfection of immortalized fibroblast cell lines (Olson, 1996). The functional consequence of GEF overexpression is to elevate cellular levels of activated Rho GTPases, and hence deregulated GEF expression might lead to aberrant growth, invasiveness, and/or increased metastatic potential. Although how GEFs are regulated is still unknown, they clearly represent powerful candidates as spatial and temporal Rho-GTPase regulators.

Only very recently has a GEF been brought into picture in invadopodia biology with a report showing that the Cdc42 GEF Fgd1 is required for invadopodia biogenesis and function in melanoma A375MM, breast MDA-MB-231, and prostate PC3 carcinoma cells. The same report also showed that Fgd1 is expressed in human prostate and breast cancer as

opposed to normal tissue. In addition, the expression levels correlated with the aggressiveness of the tumors (Ayala *et al.*, 2009). Fgd1 appeared to be a transient component of invadopodia. In particular, Fgd1-positive, F-actin (or cortactin)-negative puncta with no underlying matrix degradation observed at earlier times, might be nascent invadopodia where Fgd1 appeared ahead of actin. Structures where Fgd1 colocalized with F-actin and underlying degradation were hypothesized to be fully active ''early'' invadopodia. These observations are in accord with the proposed stepwise mechanism for invadopodia biogenesis (Artym *et al.*, 2006) and consistent with a role in triggering actin nucleation via Cdc42 activation.

Function-abrogating mutations in Fgd1 cause faciogenital dysplasia (FGDY) or Aarskog–Scott syndrome, a human X-linked developmental disorder that affects the size and shape of skeletal elements resulting in disproportionate short stature and facial, skeletal and urogenital anomalies (Pasteris *et al.*, 1994). Although the specific role played by Fgd1 in skeletal formation is still unclear, the etiopathology of FGDY indicates that Fgd1 is an important regulator of bone development. Indeed, Fgd1 expression initiates in the embryo with the onset of ossification whereas after birth, it is expressed more broadly in skeletal tissue (Gorski *et al.*, 2000). To reconcile the pathogenesis of FGDY with findings on invadopodia formation and tumor progression is the subject of pure speculation. ECM remodeling is critical not only in pathological processes such as atherosclerosis or cancer, but also in physiological events such as immune response and inflammation, wound healing, embryonic morphogenesis, and differentiation. Fgd1 might thus play a physiological role in matrix remodeling during development, and hence the severe clinical symptoms associated to Fgd1 loss. In the adult, instead, aberrant expression could lead to the pathological events associated with tumorigenesis.

2.4.4. ARF-family GTPases

The ADP-ribosylation factors (ARFs) belong to another class of Ras superfamily GTPases. Six mammalian isoforms are known and classified by sequence homology. They too cycle between inactive GDP-bound and active GTP-bound forms. Although the best characterized member of the family is ARF1, ARF6 has been intensely studied and is known to regulate endosomal membrane trafficking, exocytosis, and actin remodeling at the cell surface (Donaldson, 2003; Donaldson and Honda, 2005). ARF1 and ARF6 exhibit almost identical effector domain regions (Switch I and II) and consequently share many interactors, but the presence of glutamine and serine residues exclusive to ARF6 confers this protein with the ability to control actin rearrangement (Al-Awar *et al.*, 2000).

ARF6 has been shown to play a role in acquisition of an invasive phenotype downstream of v-Src activation, by promoting traffic-mediated adherens junction disassembly and epithelial cell migration (Palacios *et al.*, 2001).

Cell migration was also promoted by the overexpression of ARNO, an ARF6 GEF (Santy *et al.*, 2001). Further studies showed ARF6 to be localized at invadopodia in MDA-MB-231 breast cancer cells (Hashimoto *et al.*, 2004) and in LOX melanoma cells (Tague *et al.*, 2004). The relevance of ARF6 in ECM degradation is thus evident and further supported by the direct correlation between ARF6 expression levels and the invasive phenotype (Hashimoto *et al.*, 2004). The mechanism of action of ARF6 at invadopodia, however, is not completely defined but appears to be dependent on ERK activation, required for ARF6-dependent invadopodia formation and activity (Tague *et al.*, 2004).

Connected to the above is the finding that AMAP1, a GTP-ARF6 effector in invasion (Hashimoto *et al.*, 2005) and expressed at high levels in invasive breast cancer cells, has been found to be a component of invadopodia (Onodera *et al.*, 2005). AMAP1 may act as a linker between paxillin and cortactin, two players in the ECM degradation process. It has become apparent that the AMAP1-mediated invasion machinery requires its binding to the adaptor protein CIN85 to interact with Cbl, a positive regulator of tumor progression (see also below, Nam *et al.*, 2007). Finally, a study performed on ASAP1, the mouse ortholog of AMAP1, revealed that it is associated with and positively regulates invadopodia. Specifically, the Src phosphorylation site on ASAP1, as well as the SH3 and the BAR domains, would be required for invadopodia formation, suggesting that ASAP1 participates in the tyrosine signaling cascade and might be an important scaffold to spatially and temporally target the activity of diverse protein to sites of ECM degradation (Bharti *et al.*, 2007).

2.4.5. Adaptors and effectors

There are many potential effector proteins downstream of the signaling cascades regulating invadopodia formation, that can be extracted from a number of reviews (Buccione *et al.*, 2004; Gimona *et al.*, 2008; Linder, 2007; Stylli *et al.*, 2008). One of the most characterized, cortactin, has been presented in Section 2.3.4; a few further examples will be discussed with an emphasis on those that appear to be substrates of the protein kinases acting at invadopodia and/or that have the potential of being key linkers between the signaling apparatus and the molecular machinery driving membrane and cytoskeleton remodeling.

Proteins of the dynamin family are large GTPases with common structural features: an N-terminal GTPase domain, a middle domain, a PH domain involved in membrane binding, a GTPase effector domain (GED) that stimulates the GTPase activity and participates in self-assembly, and a C-terminal PRD that contains several SH3-binding sites for dynamin partners (Danino and Hinshaw, 2001; McNiven *et al.*, 2000a). The dynamins control a variety of events such as receptor-mediated endocytosis, caveolae internalization, phagocytosis, and transport from the trans-Golgi

network toward the plasma membrane (Hinshaw, 2000; McNiven *et al.*, 2000a; Schmid *et al.*, 1998). The ubiquitous member, dynamin 2, interacts with the actin cytoskeleton in the regulation of actin filament organization and subsequently cell shape via cortactin (McNiven *et al.*, 2000b), actin comet formation (Lee and De Camilli, 2002; Orth *et al.*, 2002), and formation of podosomes (Ochoa *et al.*, 2000). Dynamin 2 has been shown to localize at invadopodia. In addition, dominant inactive mutant overexpression significantly reduced invadopodia formation and ECM degradation in A375MM melanoma cells (Baldassarre *et al.*, 2003). More recently, these findings have been confirmed by RNA interference of dynamin 2 (Caldieri and Buccione, unpublished results). The mechanism of action of dynamin 2 at invadopodia remains elusive however, although the available evidence points to a function in actin modulation rather than in the control of membrane trafficking events (Baldassarre *et al.*, 2003, 2006). A possible indication could arise from the finding that SFK-dependent phosphorylation of dynamin 2 regulates caveolae and albumin transport. Specifically, albumin binding to its cell surface receptor induced Src-dependent phosphorylation of dynamin 2, which in turn increased its association with caveolin 1, the caveolae scaffold protein. Expression of non-Src-phosphorylatable dynamin 2 (Y231, 597F) resulted in reduced association with caveolin 1 (Shajahan *et al.*, 2004; Yao *et al.*, 2005), impaired albumin uptake, and reduced transendothelial albumin transport (Shajahan *et al.*, 2004). In view of the newly reported function of caveolin 1 in invadopodia biogenesis (see below and Caldieri *et al.*, 2009), this represents a potential inroad to a better understanding of dynamin 2 function in invadopodia biology.

The novel Src substrate Tks5, formerly known as FISH, was recently identified at invadopodia of Src-transformed fibroblasts and a series of cancer cell lines and found to be overexpressed in ductal and metastatic breast carcinoma and skin cancer (Abram *et al.*, 2003; Seals *et al.*, 2005). Tks5 is a scaffolding protein exhibiting five SH3 and one PX domains, essential for invadopodia formation and function and for protease-dependent invasion in Matrigel. Also, exogenous expression of Tks5 in cells with low endogenous levels (such as epithelial cells) provided these with the ability to form podosomes/invadopodia (Abram *et al.*, 2003; Seals *et al.*, 2005). Although further work is required to fully elucidate its role, the emerging protein interaction profile of Tks5 is revealing many potentially crucial aspects of invadopodia biogenesis. For example, Tks5 binds and modulates the actin polymerization regulator N-WASP (Oikawa *et al.*, 2008) and can interact with members of the disintegrin and metalloprotease (ADAM) family of proteins, namely ADAMs 12, 15, and 19, involved in processes such as cell adhesion and motility. In particular Tks5 colocalizes with ADAM 12 in podosomes/invadopodia of Src transformed fibroblasts (Abram *et al.*, 2003) and thus regulates protease activity at sites of ECM degradation. Tks5 has also been

proposed to contribute to tumor progression *in vivo* (Blouw *et al.*, 2008). More recently, Tks4, a novel protein that is closely related to Tks5, has been found to be tyrosine-phosphorylated and predominantly localized at invadopodia-like structures in Src-transformed fibroblasts. Also, Tks4 knockdown inhibited ECM degradation. Interestingly, Tks4 and 5 do not appear to have overlapping functions as ECM degradation was not rescued by overexpression of Tks5. A possible mechanism of action is related to the finding that MT1-MMP was not recruited to invadopodia in Tks4-depleted cells (Buschman *et al.*, 2009).

IQGAP is a family of conserved multidomain proteins that can mediate binding to diverse target proteins. Of the three isoforms described so far in mammals, IQGAP1 is ubiquitous (Brandt and Grosse, 2007). The main function of this protein is to modulate cytoskeletal architecture and polarity. In fact, IQGAP1 enhances actin polymerization *in vitro* (Bashour *et al.*, 1997) and localizes at lamellipodia (Hart *et al.*, 1996). It also modulates the cytoskeleton through regulation of Rho GTPases; specifically, IQGAPs act to stabilize the GTP-bound forms of Cdc42 and Rac1 (Brill *et al.*, 1996; Hart *et al.*, 1996; Noritake *et al.*, 2004). IQGAP1 also interacts with the microtubule protein CLIP170, thus capturing growing microtubules at the leading edge of migrating fibroblasts, resulting in cell polarization (Fukata *et al.*, 2002). IQGAP1 is upregulated by gene amplification in common types of gastric carcinoma (Sugimoto *et al.*, 2001) and its expression is increased at the invasion front of colorectal carcinoma (Nabeshima *et al.*, 2002). This pattern is characteristic of advanced carcinomas with high invasive potential. Recent exciting findings have shown that IQGAP1 localizes to invadopodia and its depletion leads to a considerable reduction of matrix degradation, while the transfection of a constitutively active mutant increased invadopodia-dependent proteolytic activity. IQGAP has been suggested to act through active Cdc42- and RhoA-triggered interactions with the exocyst subunits Sec3 and Sec8. This interaction is required for the accumulation of cell surface MT1-MMP at invadopodia (Sakurai-Yageta *et al.*, 2008). This intriguing mechanism of action opens up new avenues for research and highlights the tight relationship between the cytoskeleton remodeling and membrane trafficking machineries (see below).

Paxillin plays a central role in the coordination of the spatial and temporal action of the Rho GTPases, by recruiting an array of accessory proteins (see Section 2.4.3). Paxillin is a multidomain scaffold protein that localizes to the intracellular surface of sites of cell adhesion to the ECM. It features many protein-binding modules, mostly under the control of phosphorylation, and hence serves as a platform for the recruitment of numerous regulatory and structural proteins to control the cell adhesion, cytoskeletal remodeling, and gene expression in cell migration, invasion, and survival (Deakin and Turner, 2008; Turner *et al.*, 2001). Paxillin was found to be localized and tyrosine-phosphorylated at invadopodia and formed a

complex with PKD and cortactin. Interestingly, formation of this complex was found to correlate with the degree of invasivity of cell lines tested (Bowden *et al.*, 1999; Mueller *et al.*, 1992). The potential role of paxillin in invadopodia formation and/or function, however, is far from defined.

Also known as SETA, Ruk, or SH3KBP1, CIN85 belongs to a family of ubiquitously expressed adaptor molecules containing three SH3 domains, a proline-rich region, and a coiled-coil C-terminal domain (Dikic, 2002). These molecules selectively control the spatial and temporal assembly of multi-protein complexes that transmit intracellular signals involved in cell regulation, differentiation, migration, and survival. Of particular interest is the fact that CIN85 associates to other components involved in RTK signaling. The SH3 domains of CIN85 bind the E3 ligase Cbl that belongs to a family of proteins that associate with E2 ubiquitin-conjugating enzymes to direct protein ubiquitination (Schmidt and Dikic, 2005). Tyrosine-phosphorylated residues of RTKs bind the SH2 domain of Cbl, whose activation stimulates in turn its association to CIN85. Cbl in this complex mediates the ubiquitination of RTK and CIN85 (Haglund *et al.*, 2002). Cbl-mediated ubiquitination has been well documented to downregulate RTK signaling such as for EGFR, CSFR-1, and c-Met, which have been implicated in the malignancy of many tumors. Cbl and CIN85 are also known to regulate the actin cytoskeleton (Schmidt and Dikic, 2005). The relevance for invadopodia biology stems from the finding that CIN85 interacts with AMAP1, an ARF6 effector and cortactin partner and component of invadopodia (see Section 2.4.4). CIN85 was also found to be a component of invadopodia and binding to AMAP1 was required for matrix degradation. It has been hypothesized that mono-ubiquitination of AMAP1 is relevant for MDA-MB-231 cell invasion (Nam *et al.*, 2007).

2.5. Interaction with and degradation of the ECM

2.5.1. Proteases

Early studies found invadopodia to be membrane domains enriched in MMP2 and the surface expressed serine protease seprase, where these components directly correlated with the invasive potential of tumoral cells (Monsky, 1994). Later, the urokinase-type plasminogen activator (uPA) proteolytic system was also found to have a role at invadopodia (Artym *et al.*, 2002; Kindzelskii *et al.*, 2004). The uPA system consists of uPA, its receptor uPAR, and plasminogen; uPA is secreted as a zymogen whose activation is accelerated by its binding to uPAR. Active uPA catalyzes the conversion of plasminogen to plasmin, which in turn degrades a variety of matrix proteins because of its broad spectrum of substrate specificities (Dano *et al.*, 2005; Nozaki *et al.*, 2006). Over the years, it has clearly emerged that the proteolytic potential of invadopodia is mainly due to members of the MMP family. More than 25 members have been identified that, as a group,

degrade virtually all components of the ECM (Egeblad and Werb, 2002; Seiki, 2003). The MMP family can be broadly divided into the soluble and membrane types. The latter are the minority and are membrane-bound proteins featuring either a single type I transmembrane domain and a short cytoplasmic stretch (MMP14, 15, 16, and 24 or, alternatively, MT1-, MT2-, MT3-, and MT5-MMP), a glycosphingolipid anchor (MMP17 and 25 or MT4- and MT6-MMP), or a type II transmembrane domain (MMP23). MT1-MMP plays a pivotal role in a number of physiological and pathological processes via mechanisms which go beyond the degradation of ECM components (Seiki, 2003; Sounni *et al.*, 2003). MT1-MMP is considered as a master regulator of protease-mediated cell invasion in general (Holmbeck *et al.*, 2004; Seiki and Yana, 2003), and this has proved the case also in invadopodia activity in diverse cell models (Artym *et al.*, 2006; Clark *et al.*, 2007; Steffen *et al.*, 2008). MT1-MMP docking to invadopodia is required for ECM degradation in melanoma cells, and overexpression (Nakahara *et al.*, 1997a) or knockdown (Artym *et al.*, 2006; Steffen *et al.*, 2008) of MT1-MMP correlates with enhanced or depressed invadopodia formation and function, respectively.

The currently accepted model for invadopodia formation postulates that invadopodia initiate as a nucleation site by accumulation of actin and cortactin at areas of cell membrane adherence to ECM. This is followed by ongoing increases in the amounts of actin and cortactin along with the appearance and increase in MT1-MMP. The beginning of matrix degradation defines the formation of mature invadopodia, enriched in actin, cortactin, phosphotyrosine, and MT1-MMP. The dispersal of actin and cortactin results in the formation of MT1-MMP-enriched late invadopodia capable of continuing focal degradation (Artym *et al.*, 2006). Although the relationship between invadopodia, their protease complement and the ability to degrade the ECM is thus apparently quite straightforward with simple variations on the same theme depending on the cell type, the situation might be more complex. Indeed, when invadopodia-forming cells (e.g., A375MM melanoma, MDA-MB-231 breast carcinoma, or others) are cultured on an ECM layer in the presence of a reversible hydroxamate broad-spectrum metalloprotease inhibitor, invadopodia do not form and therefore no degradation of the ECM occurs. Upon inhibitor washout, the cells resume the ability to form invadopodia and hence degrade the matrix (Ayala *et al.*, 2008; Baldassarre *et al.*, 2006). This suggests that there might be a positive feed-forward loop triggering invadopodia initiation or maturation possibly mediated by ECM degradation products.

2.5.2. Membrane trafficking

An important aspect of invadopodia biology is to understand how metalloproteases are specifically targeted to invadopodia to execute focal degradation of the ECM. As mentioned in Section 2.2, integrins, for instance, may

function as docking sites to specifically recruit proteases (Baciu *et al.*, 2003; Galvez *et al.*, 2002). An important role, however, might be played by the secretory pathway as the prolonged and continued degradation of the ECM at invadopodia could require an active transport of protease-delivering carriers from the Golgi area to the designated sites of ECM degradation. It remains unclear, however, how and if this transport is organized, although light is now being shed on this process thanks to recent reports addressing the integration between trafficking and cytoskeleton machineries in invadopodia formation and function. In one, the transport vesicle-tethering exocyst complex was shown to be required for matrix proteolysis at invadopodia and breast carcinoma cell invasion (Sakurai-Yageta *et al.*, 2008). The exocyst complex consists of eight subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84) and mediates the tethering of post-Golgi and endocytic recycling intermediates at the plasma membrane (Hsu *et al.*, 2004). Genetic and cell biology studies have shown that in mammalian cells, the exocyst complex is necessary for cellular functions that require polarized exocytosis to confined regions of the plasma membrane, such as in cell adhesion and migration (Grindstaff *et al.*, 1998). At invadopodia, MT1-MMP targeting depends on the interaction of exocyst subunits Sec3 and Sec8 with IQGAP1 (Sakurai-Yageta *et al.*, 2008). In another report, it was suggested that the docking/fusion protein v-SNARE TI-VAMP/VAMP7 is also required to make MT1-MMP available at sites of degradation (Steffen *et al.*, 2008). This protein belongs to the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) class of proteins that mediate intracellular membrane fusion in endocytic and secretory pathways in eukaryotic cells (Jahn and Scheller, 2006). In particular, VAMP7 functions in the transport from the trans-Golgi network to late endosomes/lysosomes (Advani *et al.*, 1999) and apical transport in polarized cells (Galli *et al.*, 1998). Depletion of VAMP7 in MDA-MB-231 cells significantly inhibited ECM degradation and the amount of MT1-MMP at degradation sites. Furthermore, loss of VAMP7 caused a switch in mesenchymal to ameboid migration in a 3D environment (Steffen *et al.*, 2008), supporting the role of VAMP7 as essential for MT1-dependent ECM degradation and further increasing our understanding of the fusion machinery acting at invadopodia. Taken together these findings substantially broaden our knowledge in the role played by the intracellular trafficking machinery and open intriguing scenarios in terms of polarized sorting of components in invadopodia formation and function.

Polarized trafficking of proteins is part of the complex system that ensures that different proteins and lipids are targeted to the correct surface, preventing the mixing of membrane components that are physiologically distinct. This process was first characterized in epithelial cells where newly synthesized proteins in the trans-Golgi network, destined for the apical

membrane, are segregated into cholesterol and sphingolipid-enriched membrane microdomains (Keller and Simons, 1998). A recent study has shown that invadopodia biogenesis, functionality, and structural integrity rely on appropriate levels of plasma membrane cholesterol, and that invadopodia themselves display the properties of cholesterol-rich membranes (Caldieri *et al.*, 2009). Invadopodia were, in fact, found to be sensitive to plasma membrane cholesterol manipulation and typically resistant to mild nonionic detergent extraction. The study also suggested that caveolin 1 is a key regulator of the cholesterol homeostasis at the cell surface through modulation by SFK-mediated phosphorylation and is required for invadopodia formation and function. Interestingly, some cancer types have been found to be cholesterol-enriched (Freeman *et al.*, 2007; Zhuang *et al.*, 2005) and caveolin 1 plays a significant role in tumorigenesis (Kato *et al.*, 2002; Williams and Lisanti, 2005; Yang *et al.*, 1999). These results further our understanding of how signal transduction, local cytoskeletal remodeling events, and polarized trafficking are spatially and temporally confined and integrated to carry out the focalized degradation of ECM.

2.5.3. Role of the substrate

Despite the close contact with the substrate, it is still poorly understood if and how physical and chemical ECM cues regulate invadopodia formation and function. Recent reports have addressed this issue. In one, effects of varying levels of physical rigidity and protein concentration were tested on invadopodia formation finding that ECM rigidity directly increases both the number and activity of invadopodia. Furthermore, it was determined that ECM-rigidity signals lead to the increased degrading activity at invadopodia, via a myosin II-FAK/Cas pathway (Alexander *et al.*, 2008). These data provide an interpretive framework for the reported correlation of tissue density with cancer aggressiveness. A related study based on an integration between computational modeling and experimentation investigated invadopodia formation and function in relation to ECM cross-linking and fiber density. The results suggest that less dense matrices such as collagen gels have little impact on invadopodia penetration and activity. Denser substrates such as gelatin are effective obstacles to invadopodial protrusion and activity (Enderling *et al.*, 2008). These results provide a novel framework for further studies on ECM structure and modifications that affect invadopodia and tissue invasion by cells.

Additional studies are required to address these important issues and will take advantage of the emerging technologies in artificial culture substrates such as micro- and nano-patterned glass and silicon, elastomeric surfaces, and surfaces with varying levels of roughness.

3. Open Questions and Concluding Remarks

The field of invadopodia biology is relatively new and currently driven by a limited number of laboratories with a certain heterogeneity of cell models; hence, there are many more open questions compared to relatively mature areas of research. An international open network of laboratories has been established, the Invadosome Consortium (http://www.invadosomes.org), to provide a framework for research on cell adhesion and tissue invasion and to promote the exchange of knowledge in this field.

Among the open issues, a central one will be to understand whether invadopodia are just a paradigm, albeit powerful, to study the tight interrelationships between the signaling, trafficking and cytoskeleton remodeling machineries (which of course is quite important in itself), or whether they can be also promoted as a translational/applicative model to study the invasive process and consequently to test novel therapeutical approaches. These and other issues will be addressed below.

3.1. Podosomes versus invadopodia

Podosomes are highly dynamic, actin-rich adhesion structures of monocyte-derived cells. They are involved in the adhesion to, and perhaps movement on solid substrates, and consist of a densely packed actin core surrounded by a ring of components commonly found in focal adhesion structures (Gimona and Buccione, 2006; Gimona *et al.*, 2008; Linder, 2007; Linder and Aepfelbacher, 2003). A picture has now emerged suggesting that podosomes represent physiologically relevant organelles that contribute to the linking of the cell membrane to solid surfaces, and may be relevant for tissue invasion and matrix remodeling by controlling focal degradation of ECM and activation of MMPs (Linder, 2007). Regulators of podosome formation, maintenance, and turnover include non-RTKs like Src and focal adhesion kinase (FAK), Rho GTPases (mainly Rac1, RhoA, and Cdc42), a diverse set of actin cytoskeleton modulators, and also the microtubule and intermediate filament cytoskeletons. Of note, the role of the latter two is virtually unexplored for invadopodia. Recent work has demonstrated that epithelial cells, as well as vascular endothelial and smooth muscle cells can also form podosomes, and that they contain the same molecular complement as their monocytic counterparts (Hai *et al.*, 2002; Osiak *et al.*, 2005; Spinardi *et al.*, 2004).

It is thus evident that molecular, morphological, and functional parameters must be applied to distinguish podosomes from invadopodia. A clear, detailed framework has been provided to help make this distinction (Linder, 2007), which can be summarized in the following concepts. Podosomes are

dynamic, rapidly turning-over (2–12 min), small (1 × 0.4 mm), dot-like structures organized in rings and/or distributed at the leading edge as opposed to the persistent (up to 3 h), larger (up to 8 × 5 mm), irregularly shaped invadopodia, which tend to be confined to the central area of cells. The latter are typically formed by cancer-derived cells and are less numerous (1–10 per cell) as compared to podosomes (20–100 per cell), but more aggressive toward the substrate.

3.2. Invadopodia in three dimensions

Invadopodia are studied using cells on 2D substrata where they form below the cell body, extending a few micrometers into the ECM scaffold producing punctuate-like small lytic foci. Also the molecular mechanism underlying these structures has been mainly characterized only in breast and melanoma cell lines, although more recent data extend invadopodia formation to further tumor types (Ayala *et al.*, 2009; Clark *et al.*, 2007). Consequently, whether these contacts, which have been exclusively defined in some cell culture systems, could represent artifacts or have related counterparts in 3D settings either in reconstituted ECM models or *in vivo* (i.e., within tissues) is incompletely defined. Intriguingly, the fact that proteins known to play a crucial role in the actin meshwork of invadopodia, such as cortactin, WASP, and Arp2/3, are misregulated in some cancer types and connected to metastasis formation, suggests a correlation between the actin dynamics leading to ECM degradation *in vitro* and the invasive potential of cancer cells *in vivo* (Vignjevic and Montagnac, 2008). Nevertheless, strong evidence is lacking as to whether invadopodia have the potential to form clearings in the ECM through which the cell eventually migrates and whether the lytic zones known from 3D invasion (Wolf and Friedl, 2009; Wolf *et al.*, 2007) contain bona fide invadopodia. The identification of lytic protrusions in 3D tissue invasion, comparable to invadopodia, and the establishment of their relevance to invasive migration and ECM remodeling needs to be determined. Ultimately, one would want to know when, where, and with which consequences invadopodia contribute to 3D cell invasion and tissue remodeling. These goals will require sophisticated, state-of-the-art imaging technology including multiphoton-excited fluorescence and second harmonic wave generation (Wolf and Friedl, 2005) and are being currently pursued in a few laboratories.

3.3. Invadopodia as a model for drug discovery

Potentially, biological models based on invadopodia formation and function provide two possible avenues for exploitation in the drug discovery/testing process. On one side, ECM degradation at invadopodia might be considered an assay to test the effects of potential lead compounds or to screen RNAi and

drug libraries. This type of approach, currently in progress in some laboratories including the Authors', has the added advantage of potentially accelerating the biological discovery process. On the other, invadopodia could be considered as actual therapeutic targets for anti-invasion or anti-metastatic therapies, where the leading concept is that disruption of invadopodia should prove beneficial (Weaver, 2006). The latter approach, however, suffers somewhat for the lack of known invadopodia-specific components, but because invadopodia are not important for cell viability, could prove successful in the long run. Clearly, this approach would greatly benefit from the ongoing studies investigating invadopodia-specific components and targets and from the validation of invadopodia in 3D settings.

ACKNOWLEDGMENTS

The Authors' laboratory work was supported by grants to RB from the Italian Association for Cancer Research (AIRC, Milano, Italy), the European Commission (contract LSHC-CT-2004-503049), the Ministero della Salute (Ricerca finalizzata (Art. 12 bis D.Lvo 502/92), and the Fondazione Cassa di Risparmio della Provincia di Teramo. GC was supported by fellowships from the Italian Foundation for Cancer Research (FIRC) and the "Calogero Musarra" Foundation. IA was supported by a postdoctoral fellowship of the Alfredo Leonardi Fund and G.L. Pfeiffer Foundation.

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CHAPTER TWO

Viral Channel-Forming Proteins

Wolfgang B. Fischer *and* Jens Krüger

Contents

Abstract

Channel-forming proteins are found in a number of viral genomes. In some cases, their role in the viral life cycle is well understood, in some cases it needs still to be elucidated. A common theme is that their mode of action involves a change of electrochemical or proton gradient across the lipid membrane which

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International Review of Cell and Molecular Biology, Volume 275
ISSN 1937-6448, DOI: 10.1016/S1937-6448(09)75002-6

modulates the viral or cellular activity. Blocking these proteins can be a suitable therapeutic strategy as for some viruses this may be "lethal." Besides the many biological relevant questions still to be answered, there are also many open questions concerning the biophysical side as well as structural information and the mechanism of function on a molecular level. The immanent biophysical issues are addressed and the work in the field is summarized.

Key Words: Viruses, Viral channel proteins, Antiviral therapy, Protein assembly.

1. Introduction

Genomes of viruses encode a series of proteins which span the membrane and alter the permeability of viral and cellular membranes (Fischer, 2005; Gonzales and Carrasco, 2003). In combination with *in vitro* experiments, it has been proposed that these proteins need to form pores or some kind of channel since small molecules as well as ions cross a lipid bilayer in the presence of these proteins. Due to their size, it is evident that these proteins have to self-assemble to form a gateway across the membrane. Self-assembly and formation of homo- or hetero-oligomers is a strategy which is also commonly used by the host cells to manufacture functional units such as ion channels that are usually about five times larger (Karlin, 2002). And on an even larger scale, porins (e.g., from Rhodobacter) are found to assemble in trimeric units even though their activity as a pore is due to the individual protein subunit (Danese and Silhavy, 1998; Schirmer, 1998; Yoshihara *et al.*, 1991).

Today, it is unquestionably accepted that M2 from influenza A is a channel-forming protein enabling the flux of protons (Lin and Schroeder, 2001; Mould *et al.*, 2000). M2 has also been the first ion channel target in antiviral therapy (Davies *et al.*, 1964). For others, such as Vpu from HIV-1 and p7 from HCV, there is evidence that they form channels *in vitro*. Recently, more proteins have been suggested to act as channels or pores such as 3a from SARS-Co (Lu *et al.*, 2006), BM2 from influenza B (Mould *et al.*, 2003), 2B from polio virus (van Kuppeveld *et al.*, 1997a), and others.

This review presents an update of the research field of viral channel-forming proteins guided by biophysical questions along the lines of a putative mechanism of function: how we can identify a putative channel in the genome of the virus, how these proteins are assembled, what their structure is, and finally what their mode of action is in terms of gating and selectivity.

In asking for the role of these proteins within the cellular life cycle of the viruses, one might speculate that they modulate cellular environment (1) via inducing changes of electrochemical or proton gradients and/or (2) via direct interaction between viral and host membrane proteins.

2. Channel-Forming Proteins

2.1. M2 from influenza A

M2 (Allen *et al.*, 1980; Winter and Fields, 1980) consists of 97 amino acids (aa), has a single transmembrane (TM) domain (Fig. 2.1A), and forms homo-tetramers from dimers covalently linked via disulfide bridges (Sakaguchi *et al.*,

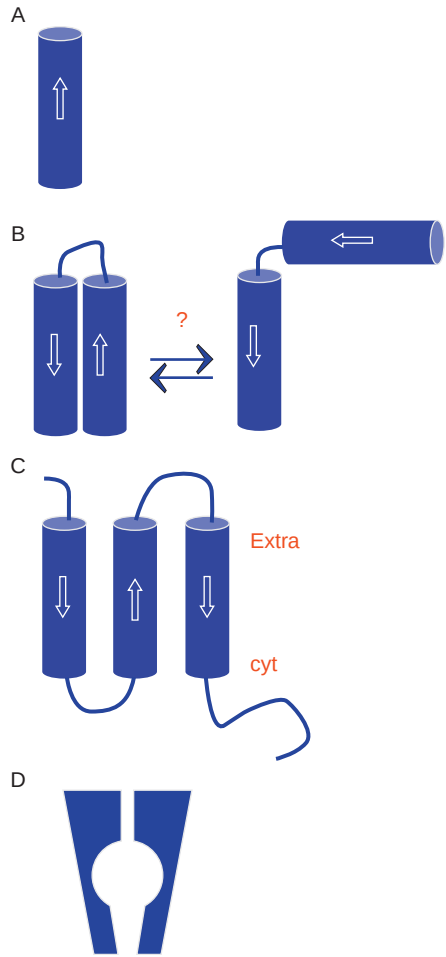

Figure 2.1 Topologies of the TM region of the to-date known viral channel-forming proteins. (A) A single TM domain [e.g., M2 (influenza A), Vpu (HIV-1), 6K (alphavirus)]. The proteins have a parallel alignment. (B) Two TM domains as proposed for p7 (HCV) or 2B (Polio). (C) Three TM domains proposed for 3a (SARS-CoV). (D) Kcv model based on sequence homology (Plugge *et al.*, 2000) to KcsA channel (Doyle *et al.*, 1998).

1997; Sugrue and Hay, 1991). It has a phosphorylation site in the cytoplasmic domain (Holsinger *et al.*, 1995) and is palmitoylated (Sugrue *et al.*, 1990b; Veit *et al.*, 1991). The protein is involved in the entry of the virus into the host cell via the endocytosis pathway which occurs by membrane fusion at a specific low pH (Ciampor *et al.*, 1992; Sugrue *et al.*, 1990a). This event takes place when the virion is entrapped in the endosome. Sensing the low pH in the endosome, M2 conducts protons into the interior of the virion thereby enabling conformational changes of viral proteins such as the matrix protein M1 and the fusion protein hemagglutinin (HA) eventually leading to fusion of the viral and endosomal membrane and uncoating of the viral genome. In addition, during the late stage of the infectious cycle of certain influenza A strains, M2 maintains a near-neutral pH in the Golgi, preventing HA from adopting the low-pH structure which would cause the assembly of nonfunctional HA in the virion. It seems also that M2 facilitates the budding process (McCown and Pekosz, 2006). For this role the cytoplasmic domain of M2 is responsible. This mode of action is not affected by the antiviral drug amantadine which otherwise blocks M2 channel activity.

A series of investigations have been done focusing on channel activity of M2 in dependence of lipid composition. It has been reported that the association of peptides corresponding to the TM domain of M2 is promoted in the presence of cholesterol as it is found in the Golgi (Cristian *et al.*, 2003). NMR spectroscopic investigations reveal that the tilt of such peptides varies according to membrane thickness (Nishimura *et al.*, 2002; Wang *et al.*, 2001).

M2 has little association with rafts (Leser and Lamb, 2005; Zhang *et al.*, 2000). Recently, a model for raft association has been suggested (Schroeder and Lin, 2005; Schroeder *et al.*, 2005). M2 from infected cells and eukaryotic expression systems copurifies with cholesterol (Schroeder *et al.*, 2005). However, channel activity in liposomes seems to be independent of cholesterol or raft lipids (Lin and Schroeder, 2001). Based on M2′ palmitoylation site Cys-50 and other motifs such as a cholesterol recognition/interaction amino consensus motif (CRAC) including a phosphatidylinositol phosphate (PIP) recognition site, it is therefore proposed that M2 can be raft anchored with its cytoplasmic domain. The actual TM domain, however, remains in a raft-free lipid environment. This mechanism may explain the incorporation of M2 into the rafts of the virion and also may be the reason for the low amount of M2 in the virion since it needs also raft-free areas in the membrane. In the light of the early discoveries, it has been speculated that other viruses, at least the enveloped viruses, should encode for proteins with similar properties.

2.2. Vpu from HIV-1

Vpu has been detected in the mid-1980s independently by two groups (Cohen *et al.*, 1988; Strebel *et al.*, 1988). Their results indicated a virus protein ''U'' of 81 aa with a single TM domain (Fig. 2.1A) and specific for

human immunodeficiency virus type-1 (HIV-1) and related simian immunodeficiency virus (SIV) isolates (Courgnaud *et al.*, 2002; Huet *et al.*, 1990). Its presence in T lymphocytes leads to a 5- to 10-fold increase in the release of progeny virions (Strebel *et al.*, 1988). However, in the absence of the Vpu open reading frame, viruses can still replicate and the protein then has been classified as an auxiliary protein (Cullen, 1998). Investigations using confocal microscopy have shown that Vpu is localized to the recycling endosome (Varthakavi *et al.*, 2006). With its retention signal to the Golgi (Pacyniak *et al.*, 2005), Stephens and coworkers have been able to visualize Vpu in the Golgi apparatus and the ER also using confocal microscopy (Hout *et al.*, 2006). A most recent study shows evidence of Vpu assembling as a pentamer (Hussain *et al.*, 2007).

Vpu is supposed to influence HIV egress from the host cell by three distinct mechanisms:

(1) Vpu is involved in the enhanced release of progeny virions (Klimkait *et al.*, 1990) by directing the HIV receptor CD4 to the ubiquitin-dependent proteasome degradation pathway in the ER (Bour *et al.*, 1995; Chen *et al.*, 1993; Friborg *et al.*, 1995; Margottin *et al.*, 1996; Tiganos *et al.*, 1997). The CD4–Vpu interaction is determined by the Vpu cytoplasmic domain (Chen *et al.*, 1993; Kimura *et al.*, 1994; Margottin *et al.*, 1996; Schubert *et al.*, 1996a; Willey *et al.*, 1992).

(2) It is assumed that Vpu forms ion-conducting channels with a positive effect on the budding process. This is supposed to be due to the oligomerization of the protein (Maldarelli *et al.*, 1993) and consequently the ability to form a channel through which ions can pass (Ewart *et al.*, 1996; Schubert *et al.*, 1996b). The finding that Vpu forms ion channels emerged from experiments with peptides corresponding to the TM domain and with full-length protein, both of which exhibit similar channel activity when reconstituted into artificial lipid bilayers. Oocytes expressed Vpu has shown channel activity in whole cell current recordings. Applying a range of −60 to −130 mV holding potentials generates currents in the nanoampere range (Schubert *et al.*, 1996b). In a similar experiment in which the sequence of amino acids of the TM domain has been randomly chosen, no significant current has been detected. Similarly, a 27-aa synthetic peptide corresponding with the same scrambled sequence TM domain of Vpu reconstituted into artificial bilayer does not generate any current (Schubert *et al.*, 1996b). The data can be taken as a proof that the wild type amino acid sequence of the TM domain is responsible for channel activity and that the TM domain and with it the channel activity may be functionally associated with enhanced particle release. The data above have been thoroughly discussed in the literature (Lamb and Pinto, 1997). At that state of investigations, it has been concluded that it still may be possible that Vpu

modulates another cation channel and that there is the need of a selective Vpu blocker to further investigate the issue while using whole cell recordings.

In a more recent bilayer study (peptide or protein reconstituted into a lipid bilayer according to Montal and Müller, 1972), it has been reported that a peptide corresponding to the first 32-aa of Vpu shows channel activity even at lowest or zero current (Mehnert *et al.*, 2007). Together with the finding of an asymmetric voltage-dependent open rate, it has been suggested that the closure of the bundle is voltage independent and due to other factors such as the lateral pressure profile of the bilayer. Mutant studies based on the synthetic peptides have shown that presumably pore-lining serines are essential for channel activity and that the tryptophan is involved in the gating kinetics of the bundle (Mehnert *et al.*, 2008).

(3) There is increasing evidence of Vpu interacting with other cellular factors. It has been reported that action of Vpu to enhance particle release is dependent on the cells which are invaded by HIV-1 (Varthakavi *et al.*, 2003). Most recently, it has been found that Vpu interacts with a potassium channel (TASK-1) of the host cell, thereby blocking its channel activity in a dose-dependent manner and thus indirectly affecting the release of progeny virions (Hsu *et al.*, 2004). Interesting to note is the sequence similarity between Vpu and the first TM domain (TM-1) of the TASK channel. Expressing just the TM-1 domain, a similar effect of lowering TASK channel activity occurs in the host cell. This has been taken as an indication that an alteration of channel activity may be important for viral release, but it may not necessarily be due to channel activity of Vpu itself. In addition, Vpu interacts with the cellular protein BST-2 which would otherwise prevent the pinching off of the virus particle (Neil *et al.*, 2008; van Damme *et al.*, 2008). For this mode of action, both the TM and the cytoplasmic domain of Vpu are necessary. BST-2 has a TM domain and a luminal GPI anchor and is colocalized with structural gag proteins of HIV-1 in the endosome and the plasma membrane. Vpu deficient HIV-1 will be retained at the plasma membrane. Unlike for M2, research on Vpu lacks still essential experiments like those involving escape mutants to assess the intracellular role of this protein.

2.3. p7 from HCV

The genome of HCV encodes approximately 10 proteins which are expressed as a large ''polyprotein'' and cleaved into single proteins by viral and host proteases and peptidases (Penin *et al.*, 2004). One of this protein is p7, a 63-aa TM protein (Lin *et al.*, 1994). It sites between E2 and NS2 and all three proteins represent the precursor before final cleavage.

Thus, occasionally E2-p7 remains uncleaved. Up to now it is not yet clear whether p7 belongs to the structural proteins, such as E1 and E2, or the nonstructural proteins, such as NS3, NS4A, NS4B, NS5A, and NS5B. It is also not yet confirmed whether p7 is part of the virion. Deletion of p7 leads to noninfectious viruses, suggesting that p7 is essential for the production of the virus (Harada *et al.*, 2000). The protein is detected on the cell surface and with a large portion retained in the endoplasmic reticulum (Carrère-Kremer *et al.*, 2002). It is a polytopic membrane protein with its N- and C-termini exposed to the extracellular environment (Carrère-Kremer *et al.*, 2002) (Fig. 2.1B). The translocation experiments have been done with CD4-p7 chimeras detected by immunofluorescence in nonpermeabilized cells while the studies on the topology have been based on Myc epitope mapping. Secondary structure prediction proposes two TM domains connected by a short basic segment. Both ends are found to face the interior of the endoplasmic reticulum. Protein p7 is suggested to be less immunogenic, since the generation of antisera has failed so far. The exact role of this protein remains to be elucidated. The protein seems to be important for infectivity of the virion (Sakai *et al.*, 2003; Steinmann *et al.*, 2007a) but not essential for the replication of viral RNA in hepatoma cell lines (Lohmann *et al.*, 1999). It is assumed that p7 has a role within the compartments of the secretory pathway (Carrère-Kremer *et al.*, 2002). Recent modeling analysis suggests that a histidine residue is pointing into a putative pore, formed by the assembly of the protein (Patargias *et al.*, 2006). Transmission electron microscopy (TEM) data of HIS-p7 reconstituted into unilamellar vesicles indicate that the protein assembly is a hexamer with a putative diameter of 3–5 nm (Griffin *et al.*, 2003). In a more recent study, it is suggested that p7 forms also homoheptamers when analyzed as a GST-FLAG-p7 protein using the same technique (Clarke *et al.*, 2006). Full-length p7 reconstituted into lipid bilayer shows channel activity which can be blocked by several drugs (Griffin *et al.*, 2003; Pavlovic *et al.*, 2003; Premkumar *et al.*, 2004). Structural data is lacking to date.

2.4. 2B from polio virus

2B is part of nonstructural proteins encoded by enteroviruses (e.g., poliovirus, coxsackie virus, and ECHO virus) (de Jong *et al.*, 2003; Nieva *et al.*, 2003). The protein (coxsackie) is localized in the Golgi to affect the permeability of secretory membranes and the plasma membrane (de Jong *et al.*, 2003) but is not transported outside the Golgi. The plasma membrane has been found in experiments to be more permeable to calcium, thereby modulating apoptosis (Campanella *et al.*, 2004), and to low molecular weight compounds (Aldabe *et al.*, 1996; Doedens and Kirkegaard, 1995; van Kuppeveld *et al.*, 1997a). How the retention in the Golgi affects the plasma membrane is unclear. Two proposals have been made to explain the retention of 2B in

the Golgi: (1) the oligomers are too large ("kin-recognition" model: Nilsson *et al.*, 1993, 1996) and (2) lack of specific signals (Munro, 1998). 2B is supposed to consist of two hydrophobic transmembrane stretches (van Kuppeveld *et al.*, 1995, 1997b) (Fig. 2.1B). Similar to the other channel-forming proteins, 2B forms multimers (Cuconati *et al.*, 1998; de Jong *et al.*, 2002; van Kuppeveld *et al.*, 2002). The roles of several amino acids on oligomerization, permeabilization of the membrane, and the localization within the cell have been identified (de Jong *et al.*, 2004). Mutations of hydrophilic residues within the short linker region between the two TM domains readily impair the multimerization and also decrease membrane permeability. Mutating residues (tryptophans) toward the C-terminus of the second TM domain also shows an abrogation of membrane permeability without affecting multimerization of the protein. The results are based on a hygromycin B assay. In this assay, protein activity is monitored under the suppressing affect of hygromycin B. Hygromycin B is supposed to enter the cell through leakages or pores formed in the cell membrane. Permeability tests have also been done with unilamellar vesicles in which a 2B maltose-binding protein (MBP) fusion construct has been reconstituted (Agirre *et al.*, 2002). With this test, membrane permeability was shown to be increased for molecules with a molecular weight of up to 660 kDa. It has been shown that the fusion construct forms SDS-resistant tetramers with an approximate pore size of 6 Å. The topology is suggested to consist of two TM domains, one of which is lysine rich, a short linker region followed by another TM domain which is folded back into the membrane. The suggestion is based on the defined cutoff in size for the permeating molecules. A carpet-like mechanism in which one of the TM domains may lie on the surface of the membrane while the other forms the pore, should lead to pores which allow the permeation of molecules of a large variation of size, which is in contrast to their findings. Channel conductance has not yet been reported for 2B.

2.5. 6K from alphavirus

Members of the alphavirus genus express 6K proteins with a sequence in the range of 58–61 aa (Welch and Sefton, 1980) (Fig. 2.1A). The role in the life cycle of the viruses is not yet known. Expression of 6K proteins in *Escherichia coli* increases membrane permeability fostering the budding process when expressed in eukaryotic cells (Liljestrom *et al.*, 1991; Loewy *et al.*, 1995; Sanz *et al.*, 1994). Its topology is assumed to be a single helix according to theoretical secondary structure prediction programs (Sonnhammer *et al.*, 1998). Using *in vitro* translation-translocation assays, it is suggested that 6K consists of two helices (Liljestrom and Garoff, 1991). Full-length protein expressed in *E. coli*, purified and reconstituted into lipid bilayers, has shown channel activity with a preference for cations (Melton *et al.*, 2002).

2.6. 3a and E proteins from SARS coronavirus

Recently, a 274-aa protein called 3a and located in an open reading frame between the S and E protein loci in the genome of the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) has been reported to modulate membrane currents when expressed in *Xenopus* oocytes (Lu *et al.*, 2006). Its topology has been identified to consist of three TM domains with the C-terminus in the cytoplasm (Fig. 2.1C). The protein forms homodimers and tetramers and is located in the plasma membrane of infected cells.

Another short 9–12 kDa protein (Siddell, 1995), called E protein, with a short 10-aa hydrophobic region (Shen *et al.*, 2003) is encoded by SARS-CoV. The protein is nonessential for the survival of the virus, and its exact function remains to be elucidated. A synthetic 76-aa construct of the full-length protein has been identified to show channel activity (Wilson *et al.*, 2004). Structural analysis using Fourier-transform infrared (FTIR) spectroscopy, X-ray scattering, and electron microscopy together with global search molecular dynamics (MD) simulations reveals a hairpin like motif for the proposed TM region of the protein (Arbely *et al.*, 2004).

Other proteins from the genome of SARS-CoV are currently under investigation and show promising results that they my form channels (C.-C. Chen, J. Krüger, P. Henklein, Y.-M. Chen, and W. B. Fischer, unpublished results).

2.7. Kcv from PBCV-1

Chlorella virus PBCV-1 (family Phycodnaviridae) encodes a 94-aa membrane protein (Plugge *et al.*, 2000). Hydropathy analysis reveals two putative hydrophobic stretches which are separated by about 44-aa. This part of the sequence follows the same sequence as found for the pore region of the KcsA channel (Doyle *et al.*, 1998). The protein when expressed in Xenopus oocytes exhibits K^+ selective channel activity (Plugge *et al.*, 2000) and is suggested to be active during viral entry (Mehmel *et al.*, 2003) (Fig. 2.1D). It is expected that the number of channels from plant viruses may increase in the near future, since their genomes are larger than those from animal viruses.

2.8. NB and BM2

Like influenza A, influenza B and C encode for small transmembrane proteins, NB (Fischer *et al.*, 2000b; Sunstrom *et al.*, 1996), BM2 (Mould *et al.*, 2003; Paterson *et al.*, 2003), and CM2 (Hongo *et al.*, 1997; Kukol and Arkin, 2000). NB and BM2 have been shown channel activity in reconstitution experiments. These proteins are type III integral membrane proteins

with an $N_{out}C_{in}$ motif which are expressed at the surface of virus-infected cells (Paterson *et al.*, 2003) and in the virion (NB: Betakova *et al.*, 1996; BM2: Odagiri *et al.*, 1999; CM2: Hongo *et al.*, 1997). In both cases, proton selectivity has been reported. BM2 is active in the Golgi (Mould *et al.*, 2003) equilibrating the pH between the Golgi and the cytoplasm, a function similar to M2 from influenza A. Also, the TM topology with a HxxxW motif is similar to M2 and strengthens the experimental findings. Influenza M2 protein appears to play an additional role during virus assembly, which is not dependent on the channel function (Schroeder and Lin, 2005; Schroeder *et al.*, 2005). Recent findings on BM2 indicate that the protein captures the M1–vRNP complex during the budding process (Imai *et al.*, 2004, 2008). Following the recognition that BM2 is the ion channel responsible for biological functions, the role of NB is now obscure (Hatta and Kawaoka, 2003).

3. Detecting a Channel-Forming Protein

3.1. Sequence identification

Discovery of a putative channel-forming protein is driven by the detection of hydrophobic stretches within the amino acid sequence of a viral protein. Specific amino acids have a certain probability to reside within the TM stretch. The probability is based on investigations of known membrane proteins, available in protein databases.

Using secondary structure prediction tools as well as tools for TM segment prediction, it is possible to identify suitable candidates. These tools usually assume an α-helical or β-barrel motif for the membrane spanning part. So far only helical motifs have been found for most of the membrane proteins (Doyle *et al.*, 1998; Kuo *et al.*, 2003; Miyazawa *et al.*, 2003; Stroud *et al.*, 2003). These predictions can, of cause, only suggest a certain idealized hydrophobic stretch; kinks or bends within the transmembrane domain which may be meaningful for the mechanism of function cannot be predicted.

An example of the prediction of the TM domain of 3a protein from SARS-CoV is shown in Fig. 2.2. A series of programs such as Membrane Protein Explorer (MPEx, http://blanco.biomol.uci.edu/mpex/), Dense Alignment Surface prediction of transmembrane regions in proteins (DAS, http://www.enzim.hu/DAS/DAS.html), TMpred (prediction of transmembrane regions and orientations, http://www.ch.embnet.org/software/TMPRED_form.html), TMHMM (prediction of transmembrane helices, http://www.cbs.dtu.dk/services/TMHMM/), and HMMTop (prediction of TM helices and topology of proteins, http://www.enzim.hu/hmmtop/index.html) are commonly used. The programs try to identify the membrane spanning part based on sequence homology with well

```
                40          50          60          70
           ATATIPLQAS  LPFGWLVIGV  AFLAVFQSAT  KIIALNKRWQ

MPEx          ******  **********  **********  ***
DAS                   **********  *******
TMpred                **********  ********
TMHMM        *******  **********  ******
HMMTop          ***   **********  ******
DASTM                 **********  *********

                80          90         100
           LALYKGFQFI  CNLLLLFVTI  YSHLLLVAAG

MPEx             **   **********  *******
DAS              ***  **********  *********
TMpred          *****  **********  *********
TMHMM            ****  **********  *********
HMMTop           ***   **********  **********
DASTM            ***   **********  **********

               110         120         130
           MEAQFLYLYA  LIYFLQCINA  CRIIMRCWLC

MPEx           *****  **********  ****
DAS            *****  **********  ****
TMpred         *****  **********  ****
TMHMM        ********  **********  *****
HMMTop     *
DASTM          ******  **********  *****
```

Figure 2.2 Prediction programs used to assess the topology of the channel-forming protein 3a from SARS-CoV. The asterisks indicate a presumably helical TM conformation. The consensus among the programs is highlighted in light grey, but would need experimental confirmation.

characterized proteins and on lipophilicity of individual amino acids derived from octanol/water distribution coefficients. The prediction from all of these programs does not always overlap perfectly well. Whilst the core region is usually well defined, the residues presumably located within the lipid head group region are harder to grasp. Therefore, it is recommended to use not only one prediction program but to work with a consensus based on different methods. Using multiple secondary structure prediction tools three TM domains of SARS-CoV 3a have been identified with a longer linker region between TM1 and TM2 and a shorter linker region between TM2 and TM3. Also for Vpu (Fischer *et al.*, 2000a) and p7 (Patargias *et al.*, 2006), the predictions reveal one and two TM domains, respectively.

The analysis mentioned is then the starting point for further prediction of the tertiary structure of the assembled proteins within the TM region. Sequence homology with other proteins may also give a clue about the structure and role of these proteins and consequently about their mechanism of function.

3.2. Detection of channel activity

Channel activity of all of these proteins has so far been verified experimentally with whole cell recordings in which the particular viral protein is overexpressed in either *Xenopus* oocytes or other cell lines (Fischer, 2005; Fischer and Sansom, 2002; Gonzales and Carrasco, 2003). Another source of evidence for channel-forming properties is derived from reconstitution of the proteins and parts of them in artificial membranes (bilayer technique: Montal and Müller, 1972). In this respect, data from Vpu from HIV-1 (Ewart *et al.*, 1996; Mehnert *et al.*, 2007; Schubert *et al.*, 1996a) (Fig. 2.3), p7 from HCV (Griffin *et al.*, 2003; Pavlovic *et al.*, 2003), and other proteins (Melton *et al.*, 2002; Piller *et al.*, 1996; Wilson *et al.*, 2004) have been accumulated. For Vpu, a scrambled sequence of the TM domain, which is

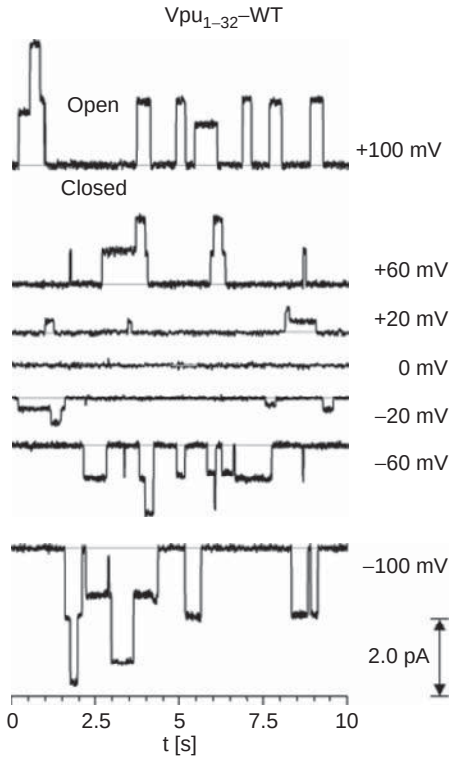

Figure 2.3 Typical channel recordings of a peptide corresponding to the first 32-aa of Vpu (HIV-1) reconstituted into lipid bilayers [POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine) and DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) 1:4] at various holding potentials. The system was hold in 300 mM KCl, 5 mM K-HEPES at pH 7.0.

thought to be solely responsible for channel function, has shown no channel activity and thus confirms the credibility of the technique (Schubert *et al.*, 1996b). Based on the use of this technique, the statement can be made that these proteins self-assemble in lipid membranes and display channel characteristics. Thus, within the cellular membranes they are expected to behave in a similar way.

However, there is increasing evidence at least for Vpu that the protein interferes with several host membrane proteins at various locations within the cell. Since no feedback mechanism regulating Vpu production is known to date, it is reasonable to assume that Vpu can engage in these protein–protein interactions (CD4: Bour *et al.*, 1995; Chen *et al.*, 1993; Friborg *et al.*, 1995; Kimura *et al.*, 1994; Margottin *et al.*, 1996; Schubert *et al.*, 1996a; Tiganos *et al.*, 1997; Willey *et al.*, 1992; TASK: Hsu *et al.*, 2004; BST-2: Neil *et al.*, 2008; van Damme *et al.*, 2008) but also can self-assemble forming channels or pores. The same situation counts for the other channel-forming proteins and needs to be elucidated in the future.

4. Functional and Structural Characterization

4.1. Biophysics of the channel-forming proteins and channel–pore dualism

In models of ion-conducting peptide assemblies hydrophilic residues are usually proposed to face the lumen of the pores (Dieckmann *et al.*, 1999; Tieleman *et al.*, 1999). In recent investigations on viral ion channels, the orientation of hydrophilic residues has been done accordingly (Fischer and Sansom, 2002). The requirement of an adequate counterplay between hydrophilic and hydrophobic residues for a TM peptide to show ion channel function is outlined by the synthetic peptides consisting of specific Leu-Ser repeats (Lear *et al.*, 1988). Modeling these peptides in a pore, the hydrophilic residues are oriented toward the lumen (Dieckmann *et al.*, 1999). The position of these hydrophilic residues in conjunction with their hydrophobic counterparts makes the channel selective for protons and other ions. It has been found that a serine side chains in 6K protein from alphavirus affects ion selectivity if mutated (Melton *et al.*, 2002). Computational studies on the assembly of two TM helices from p7 from HCV indicate that hydrophilic residues such as serines and threonines, but also histidine, align within a putative pore (Patargias *et al.*, 2006). For M2, histidines and tryptophans are lining the pore and are important for the proton-conducting properties of the channel (Hu *et al.*, 2006).

Thus, from the topological point of view, the viral channels fulfill the criteria of being able to show ion selectivity. They have a helical TM

motif with hydrophilic pore facing residues and there are indications that side chains create a selectivity filter (Wilson *et al.*, 2004) as well as hydrophic stretches (Mehnert *et al.*, 2008). Recent crystallographic data of a pentameric ligand-gated ion channel from bacteria belonging to the cys-loop family support considerations on the orientation of pore-lining residues (Hilf and Dutzler, 2008). With an increasing number of channel-forming viral proteins with several TM domains, it may be possible that also other channel architectures similar to those of voltage-gated channels are adopted, as in the case of the Kcv channel from chlorella virus (Plugge *et al.*, 2000).

Whilst M2 is held together at least partially by covalent links (Cys-Cys), the other channels are solely held together by noncovalent protein–protein interactions. Thus, in the latter case and in comparison of the pore-lining motifs of larger channels, the viral channel-forming proteins comprise a minimalist approach towards channel architecture. The hydrophilic residues may be just enough to attract and accumulate the ions to be conducted.

Any mechanism of gating may involve minimal conformational changes. The mechanism may also be driven by the lipid environment. Lipid composition and thickness in the Golgi network, for example, could induce altered conformations which allow an ion flux. For M2, a tendency for associating with rafts in the membrane is outlined (Schroeder *et al.*, 2005). For Vpu, a lipid dependency for closure is also suggested on the basis of channel recordings in artificial bilayers reflecting small conformational changes (Mehnert *et al.*, 2007, 2008). The lateral pressure profile is proposed to induce the closure. Consequently, channel activity may depend also on membrane thickness, another indication for activity which may depend on lipids in the Golgi and the cell membrane.

In general, evidence is emerging that for a gating mechanism only subtle conformational change are necessary, a suggestion based on computational studies on nanopores (Beckstein *et al.*, 2001, 2004) and the nAChR structure (Beckstein and Sansom, 2006). This suggestion is confirmed by experimental studies on the nAChR (Cymes and Grosman, 2008). However, these suggestions still remain controversial since large-scale conformational changes have also been proposed for the larger channels (Cymes and Grosman, 2008) and also M2 from influenza A based on X-ray (Stouffer *et al.*, 2008) and NMR studies (Schnell and Chou, 2008).

Experiments have shown that some of the channels, such as Vpu, alter membrane permeability for small molecules (Gonzales and Carrasco, 2003). In line with the finding that, apart from the influenza M2 family, most of the viral ion-conducting channels are only weakly ion selective it is suggested that these proteins may embody a ''channel–pore dualism''. The term is coined in analogy to the particle–wave dualism of light: depending on the experimental conditions they exhibit channel characteristics or pore (low or no ion selectivity) characteristics.

4.2. Structural information

There is a large amount of structural information available for M2 and Vpu (see Fischer, 2005, references therein). The information is based from early studies using CD spectroscopy (M2: Duff *et al.*, 1992; Vpu: Wray *et al.*, 1999), NMR spectroscopy (M2: Kovacs and Cross, 1997; Kovacs *et al.*, 2000; Vpu: Ma *et al.*, 2002; Park *et al.*, 2003), FTIR spectroscopy (M2: Kukol *et al.*, 1999; Vpu: Kukol and Arkin, 1999), and X-ray reflectivity (Zheng *et al.*, 2001). Most of the experiments have used parts of the TM domain or parts of the cytoplasmic domains in the case of Vpu. Recently for M2 the first crystal structure has been published showing a TM domain assembly in absence of a hydrophobic lipid environment in a state at low-pH conditions cocrystallized with amantadine (Stouffer *et al.*, 2008). Without the drug in the pore the conformation seems to represent a solvent accessible pore and therefore an open state of the bundle. This is in contrast of findings from solution NMR spectroscopic studies (Schnell and Chou, 2008). The data show the tetrameric assembly with the tryptophans (Trp-41) occluding the putative pore and interpreted this as the closed state of the channel. In the study, amantadine is found in binding pockets outside the pore.

Vpu is the only protein for which the cytoplasmic domain structure has been characterized by NMR spectroscopy. The spectra reveal either two (Federau *et al.*, 1996) or three helices (Willbold *et al.*, 1997) connected via short loops. In addition, interactions of the cytoplasmic domain with cellular proteins have also been reported for Vpu (Coadou *et al.*, 2003; Gharbi-Benarous *et al.*, 2004).

Depending on the experimental conditions, TEM data of p7 reveal a hexameric (Griffin *et al.*, 2003) or heptameric assembly (Clarke *et al.*, 2006).

4.3. Modeling the mechanism of function

Modeling the mechanism of function has two aspects: experimental data need to be derived and the data have to be converted into theoretical models which then have to be filled with atomistic details.

The working hypothesis is that the viral proteins are formed in the ER and exist as monomeric units prior to any assembly. Assembling is a common feature for membrane proteins in general (Engelman, 2005; Popot and Engelman, 2000) as well as for other channel or pore-forming proteins such as alamethicin and melittin (Bechinger, 1997; Tossi *et al.*, 2000). For these proteins, the topology of the pore once formed is also an assembly of helical TM domains (Boheim *et al.*, 1983). Up to now only gramicidin channel is known to consist of two β-barrel units forming hydrogen bonds in a head-to-head formation at their N-termini (Woolley and Wallace, 1992).

To model the pore or channel some care need to be taken to obtain structural details of the monomeric protein. After careful assessment of the

conformational space of the monomeric TM domain, the domain may then be replicated and reoriented to form the appropriate bundle. This has now been done for Vpu (Krüger and Fischer, 2008). At this stage also the effect of lipid composition and thickness on the structure of the TM domain of Vpu can be addressed. It has been reported that Vpu adopts to the environment via kinking rather than forcing the lipid environment to adjust (Krüger and Fischer, 2008). This is in contrast to experimental findings of X-ray reflectivity data but may be due to the high protein to lipid ratio (0.02) used in the experiments where already assemblies may have formed (Khattari *et al.*, 2006). None of the sequences of the putative channel-forming proteins contain a Leu heptad (Gurezka *et al.*, 1999) or GxxxG motives (Russ and Engelman, 2000) with the latter especially known to promote close helix–helix packing.

For a functional and selective pore, it is required to have an ingenious balance of hydrophilic and hydrophobic pore-lining residues. Purely hydrophobic pores would pose a barrier for the permeating ion similar to the lipid environment. At contrast a highly hydrophilic motif would trap the passing ion. Modeling a hydrophobic pore, Beckstein and Sansom (2003) have shown that the amount of water within the pore changes continuously between ''water filled'' and ''empty'' (termed by the authors as ''vapor'' phase) depending on the pore diameter. Therefore, the term ''oscillating liquid/vapor phase'' within the pore has been created. Conductance can only be observed when a sufficient amount of water molecules is present within the gorge portion of the pore. The observed oscillation between the liquid and the vapor state within the pore is just one aspect which addresses the role of a specific type of wall within a channel. The lumen of a biological ion channels consists of a complex alignment of polar and nonpolar residues. Many more aspects have to be considered for a proper description of the ion permeation such as its interaction with the residues at the entrance/lumen of the pore and the behavior of the hardly removable first tetrameric hydration shell within the pore.

Recently, the TM domain of Vpu (Ulmschneider and Ulmschneider, 2007) has been folded within a hydrophobic slab mimicking a lipid bilayer. In this approach, the slab shows a gradual change of the dielectric constant toward the center of the membranes. Monte Carlo simulations have been used in combination with replica exchange methods to assess the fold of the domains. This is another approach toward a fully *in silico* modeling of membrane protein structure.

Several approaches on the level of bundles have been tried for Vpu to elucidate the mechanism of function once the pore is formed. Molecular dynamics simulations in combination with physical models of ion permeation have been undertaken. In all of these studies solely the TM domain of Vpu has been used. The TM domain was oligomerized into tetrameric, pentameric, and hexameric bundles. In one of the studies, after using MD

simulations to achieve a reasonably stable structural model, the energy profiles for Na^+ and Cl^- along the pore have been calculated by placing them at various positions within the pore (Grice *et al.*, 1997). Recently, steered MD simulations were used to assess the potential of mean force of the ions across the lumen of the pore (Patargias *et al.*, 2009). Based on the energy profile the pentameric model supports the experimental findings of a weak cation-specific channel. In a combined computational and experimental approach, structural features of the Vpu bundle, such as the tilt of the helices in the mechanism of function and the role of a putative selectivity filter based on a EYR-motif at the C-terminus, has been demonstrated (Cordes *et al.*, 2002). Together with other MD simulation work in which the instability of the hexameric bundle has been explicitly shown (Moore *et al.*, 1998), the pentameric bundle model is now fairly established until further proof to the contrary. The essential question remains, what happens between the stage of the monomer and the functional bundle? Two kinetic pathways are possible, either the assembly happens in an all or nothing step with minor adjustments or more likely the monomers assemble sequentially.

For the influenza A M2 channel, proton conductance has been modeled using MD simulations (Chen *et al.*, 2007; Smondyrev and Voth, 2002). Based on theoretical considerations, there are two possible scenarios for the proton to pass the pore and specifically the histidines (His-37): either the protonated histidine flips and carries the proton to the other side, or all histidines undergo a ''swing motion'' due to electrostatic repulsion and thereby open the pore accordingly (Schweighofer and Pohorille, 2000).

5. Channel-Forming Proteins in Antiviral Therapy

Inhibition of proteins which are essential in the virus life cycle is a basic approach in antiviral therapy. In the case of channels and pores, there are in principle two strategies (1) to inhibit the channel itself by a suitable inhibitor, either small organic molecule or peptide drug and (2) to prevent the formation of the channel by hindering the assembly of the monomers, where custom designed ''anti-oligomerization'' peptides appear to be most promising.

5.1. Small molecule drugs

There are several small molecule drugs under investigation which inhibit some of the channel proteins. A derivative of amiloride such as hexamethylene amiloride has been shown to be effective against HIV-1 and in particular against Vpu (Ewart *et al.*, 2002). Experiments have been done

in HELA cells which coexpressed Vpu and Gag proteins as well as with pure Vpu reconstituted into artificial membranes from proteoliposomes. Also p7 from HCV, synthesized by solid phase peptide synthesis and reconstituted into lipid bilayers, is affected in the presence of hexamethylene amiloride (Ewart *et al.*, 2002; Premkumar *et al.*, 2004). M protein from Dengue virus-1 also forms channels when reconstituted into artificial membranes and can be blocked by hexamethylene amiloride and amantadine (Premkumar *et al.*, 2005).

Amantadine, known as one of the first drugs against influenza A, blocks the proton channel M2 in a concentration of up to 5 μM (Chizhmakov *et al.*, 1996; Hay *et al.*, 1985; Tu *et al.*, 1996) by changing M2 dynamics (Hu *et al.*, 2007). Depending on the technique used, the drug may either enter the pore (X-ray crystallography: Stouffer *et al.*, 2008) or diffuses into hydrophilic/hydrophobic pockets in the outer rim of the bundle (NMR spectroscopy: Schnell and Chou, 2008). Amantadine/rimantadine resistance is on the rise because most mutations determining drug resistance do not impair channel function. New derivatives of these drugs are being developed in the hope of overcoming drug resistance (Kolocouris *et al.*, 1996). Amantadine is also under investigation for treatment of chronic hepatitis C virus infection (Lim *et al.*, 2005) and seems to affect p7 when this protein is used within a GST-His-p7 or GST-p7 construct (Griffin *et al.*, 2003, 2004). In a recent study with full-length p7, amantadine shows no affect on the channel activity of p7 in a range of up to 10 μg/ml (Steinmann *et al.*, 2007b). In a computational study using global search algorithms, a hexameric model of p7 has been suggested (Patargias *et al.*, 2006). Amantadine has been docked into the pore and its residence is suggested to be in the vicinity of the histidine in the first TM domain at position 17. A clinical study has shown that amantadine does not lead to a mutation at the position of His-17 (Castelain *et al.*, 2007). The attribute affects the pH-dependent entry pathway of HCV. One still can argue that the residence of amantadine at the site of His-17 is possible, but as can be seen in the study of Patargias *et al.* (2006), it does not necessarily occlude the pore. As a consequence, it may not to be a potent candidate for blocking of p7. Kcv from PBCV-1 (Plugge *et al.*, 2000) and Vpu also respond on amantadine and derivatives (Kolocouris *et al.*, 1996), but only to fairly high doses.

It has been shown that iminosugar derivatives are affective against bovine viral diarrhea virus (Durantel *et al.*, 2001). In follow-up studies, it could be shown that p7 from HCV is a possible target of these small molecules as well (Pavlovic *et al.*, 2003). With a liposome assay for p7 activity, large-scale drug screening for p7 comes into reach (StGelais *et al.*, 2007). Investigations on the interaction of p7 with iminosugars indicate an effect in the range of up to 10 μg/ml (Steinmann *et al.*, 2007b). Especially derivatives such as *N*-nonyl-deoxynojirimycin (*N*N-DNJ) and *N*-nonyl-deoxygalactonojirimycin (*N*N-DGJ) are potential candidates.

5.2. Peptide drugs

Peptide drugs have several advantages over small molecule drugs (1) they are highly active and specific and (2) they accumulate in tissue to a lower extent and therefore exhibit lower toxicity. There is a major drawback such as the difficulty to deliver the drug to the target site. Usually peptide drugs have very low oral bioavailability and need to be injected (Hamman *et al.*, 2005). They are commonly used for severe chronic diseases. Peptide drugs have a low uptake by the gastrointestinal tract and experience rapid presystemic enzymatic degradation. However, advances in peptide manufacturing and improvements in peptide drug delivery systems have and will further rejuvenate this field.

The concept of interfering with the molecular action by using parts of protein itself has been shown in HIV research. It has been found that Vpu, a channel-forming protein of HIV-1, interferes with the function of the acid-sensitive leakage K^+ channel (TASK) (Hsu *et al.*, 2004). Vpu has high sequence homology with parts of the TASK protein. The authors have additionally shown that parts of TASK can also hamper channel activity of TASK. The mechanism of function is proposed to be a disturbed assembly of the protein in the presence of the protein fractions.

The concept of interfering with protein assembly within the aqueous phase has already been used to interrupt the fusion mechanism of gp41 from HIV-1 (Root *et al.*, 2001). The interaction of the trimeric gp41 with the cellular membrane induces conformational change into a bundle of six helices which is called the ''trimer-of-hairpins.'' This intermediate catalyzes the fusion of the viral with the cellular membrane. A protein which corresponds to only five out of these six helices has been synthesized, called ''five-helix'' protein. The gap generated by the missing sixth helix in this assembly acts as a high-affinity target site for gp41.

6. Concluding Remarks

After almost 20 years of research viral channel-forming proteins still leave a lot of open questions. Elucidation of their modes of action should be accelerated since for some viruses these proteins are essential and consequently a valuable drug target. Several questions remain to be answered: what is the relation of channel and pore functionality; how do they assemble and which oligomeric structure do they adopt; and finally, which sequence elements and interactions govern the fate of nascent viral channel monomers to form either homo-oligomeric channels or instead interact with host cell proteins?

For a full understanding of the mode of action, the full-length proteins need to be considered more thoroughly. Up to now all functional analysis is based on looking solely on the TM domains. For Vpu, bilayer experiments and assays indicate that the protein can be seen as a unification of two domains, the TM and the cytoplasmic domain, each of which can act independently from the other. It may be due to future research whether this is also the case for the other proteins.

ACKNOWLEDGMENTS

WBF thanks the NSC, NYMU, and the Government of Taiwan for financial support. JK acknowledges the receipt of an Alexander von Humboldt—NSC fellowship. We thank Cornelia Schroeder (Dresden) for valuable discussions and critically reading the manuscript.

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CHAPTER THREE

Notch Signaling in Chondrogenesis

Camilla Karlsson *and* Anders Lindahl

Contents

Abstract

The different stages of cartilage development are well described but no transcription factor capable of specifically inducing differentiation to articular cartilage has been identified and little is known about the molecular mechanisms regulating cartilage development. Notch signaling is an evolutionarily conserved pathway taking part in many developmental and cell type specification processes. It has been demonstrated that markers for Notch signaling are differentially expressed during cartilage development and there is evidence for their functional role during this process. Notch signaling has further been implicated in osteoarthritis and Notch1 has been suggested as a marker for chondrogenic progenitor cells. This review summarizes the current knowledge on the role of the Notch signaling pathway in cartilage development and osteoarthritis.

Key Words: Notch signaling, Chondrocytes, HES, Cartilage development, Osteoarthritis.

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International Review of Cell and Molecular Biology, Volume 275
ISSN 1937-6448, DOI: 10.1016/S1937-6448(09)75003-8

1. Introduction

Despite the fact that the different stages of cartilage development *in vivo* are rather well described, no transcription factor capable of specifically inducing differentiation to articular chondrocytes has been identified and little is known about the molecular mechanisms regulating cartilage development. The human genome projects revealed that we contain fewer genes for tissue development than expected. Sequence data also revealed that similar genes influence the development of comparable structures across the animal kingdom. The fact that the genes directing the formation of limbs are also involved in the formation of the nervous system confirms that one gene can be involved in the development of more than one tissue, that is, nature uses the same tool for many purposes.

2. Helix–Loop–Helix Proteins

Davis and colleagues first discovered the helix–loop–helix (HLH) family of genes in 1987. They found a gene, which they called MyoD1, whose expression in transfected fibroblast-like 10T1/2 cells converted them to stable myoblasts (Davis *et al.*, 1987). In the article, they also stated, ''It is striking that the gene products of the achaete–scute locus, which play a role in *Drosophila* neurogeneic cell determination, also share the same common region of myc homology with MyoD1. A common amino acid sequence in determination factors of at least two cell lineages (myogenic and neurogenic) raises the possibility that other cell lineages may use analogous determination factors that share this sequence.'' Two years later, the HLH motif was discovered by Murre *et al.* (1989a,b) in the murine transcription factors E12 and E47 and it was also noted that this motif was present in MyoD, three myc genes, and two genes of the achaete–scute complex. In the following years, differentiation of various types of tissues was demonstrated to be regulated by transcription factors containing the HLH motif (Bain *et al.*, 1994; Lee *et al.*, 1995; Porcher *et al.*, 1996; Zhuang *et al.*, 1994). Until today, this family of genes comprises more than 200 evolutionarily conserved members (Atchley and Fitch, 1997). These genes are characterized by two highly conserved and functionally distinct domains, a DNA-binding domain and a motif responsible for protein–protein interactions (Murre *et al.*, 1989a). A sequence of mainly basic amino acids at the amino-terminal end permits binding to a promoter sequence called the E-box (CANNTG), and hence these proteins are often referred to as basic HLH (bHLH) proteins (Davis *et al.*, 1990; Voronova and Baltimore, 1990). This domain is usually adjacent to a

domain consisting of hydrophobic residues, called the HLH domain due to its protein motif of two amphipatic α helices that are separated by a loop (Murre *et al.*, 1989b). This motif is responsible for homo-and/or heterodimerization with other HLH proteins (Murre *et al.*, 1989b).

As mentioned above, the discovery of the HLH motif by Murre *et al.* resulted in the identification of many other HLH proteins. They devised a classification of these proteins, dividing them into seven groups on the basis of tissue distribution, dimerization capabilities, and DNA-binding specificities (Massari and Murre, 2000). Class I, also known as the E-proteins, include E12, E47, E2-2, and Daughterless (Murre *et al.*, 1994). These proteins are abundantly expressed and capable of forming either homo- or heterodimers (Murre *et al.*, 1989b). Their DNA-binding specificity is limited to the E-box. Class II includes members such as MyoD, myogenin, Atonal, NeuroD, and the achaete–scute complex, which all show a tissue-restricted expression pattern (Massari and Murre, 2000). They are, with few exceptions, incapable of forming homodimers and preferentially heterodimerize with the E-proteins (Murre *et al.*, 1989b). The activities of the above classes of proteins are regulated by HLH proteins from class V to VI (Benezra *et al.*, 1990). Proteins lacking the basic region, comprising the inhibitor of differentiation (ID)/DNA binding and extra macrochaetae (*emc*) genes, define class V (Benezra *et al.*, 1990; Ellis *et al.*, 1990). Class VI proteins are characterized by a proline residue in their basic region. This group include the *Drosophila* proteins hairy and enhancer of split (HES) and the mammalian homolog HES (Klambt *et al.*, 1989; Rushlow *et al.*, 1989).

The developmental importance of HLH proteins has been studied in several different tissues, including myogenesis, hematopoiesis, and neurogenesis (Kageyama *et al.*, 1997; Porcher *et al.*, 1996; Weintraub *et al.*, 1991). For a cell that is specified to the neural fate, transition from proliferation to neurogenesis involves an increase in expression of proneural bHLH genes like the mouse achaete–scute complex homolog 1 (Mash1), the mouse atonal homolog Math1, and NeuroD (Akazawa *et al.*, 1995; Guillemot and Joyner, 1993). These proteins then form heterodimers with E-proteins, resulting in E-box binding and expression of genes specific for differentiated neurons. In addition to these bHLH proteins promoting differentiation, dominant-negative transcription factors are also important. They balance the action of positive transcription factors which, in turn, allows differentiation to be properly controlled (Ishibashi *et al.*, 1995; Tomita *et al.*, 1996). Absence of some of these negative HLH proteins accelerates neural differentiation, resulting in severe defects of the nervous system (Ishibashi *et al.*, 1995; Tomita *et al.*, 1996). Examples of negative bHLH proteins are, as briefly mentioned above, the HES and ID proteins. HES proteins repress transcription in two different ways (Fig. 3.1). One mechanism is suppression of formation of functional heterodimers at the

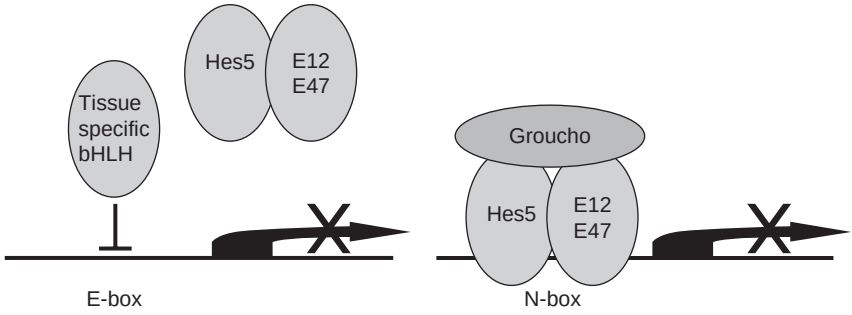

Figure 3.1 Two mechanisms demonstrating the suppressive function of HES proteins.

protein–protein interaction level. As described above, tissue-specific bHLH proteins dimerize with ubiquitously expressed E2A proteins, resulting in E-box binding (Murre *et al.*, 1989b). One mechanism for the HES proteins to inhibit differentiation is thus by depriving functional heterodimers (consisting of tissue-specific bHLH and E2A) of E2A by competitive binding (Hirata *et al.*, 2000; Sasai *et al.*, 1992). During neurogenesis, HES1 is expressed by neuronal precursor cells but cannot be detected in mature neurons; that is, the expression of HES1 significantly decreases as neuronal differentiation proceeds (Akazawa *et al.*, 1992). In these neural precursor cells, HES1 bind to E2A, thereby depriving Mash1 of its dimerization partner (Sasai *et al.*, 1992). When differentiation proceeds, the expression of HES1 is decreased, E2A binds therefore to Mash1 again resulting in expression of genes inducing neurogenesis. The second proposed mechanism for suppression is binding-site-dependent transcriptional repression. HES, but not ID, can bind directly to the so-called N-box sequence (CACNAG) of DNA and repress transcription by recruiting the corepressor complex TLE/Grg (Grbavec and Stifani, 1996; Sasai *et al.*, 1992). This promoter sequence regulates the expression of, for example, the achaete–scute genes (Chen *et al.*, 1997). Binding to the N-box thus represses expression of genes inducing differentiation. In neuronal precursor cells, HES1 binds to the N-box in the promoter region of Mash1, inhibiting Mash1 expression and therefore also inhibiting differentiation (Chen *et al.*, 1997; Ishibashi *et al.*, 1994). During development, HES1 expression decreases, releasing its inhibition on Mash1 transcription so that the neurogenesis can proceed (Akazawa *et al.*, 1992; Kageyama *et al.*, 1997). The higher binding affinity of HES genes to the N-box than to the E-box is the result of a proline residue in the middle of the basic region. The importance of HLH transcription factors and their dominant-negative counterparts for the development of several different tissues prompted us to investigate their importance for chondrogenesis.

3. Notch Genes

3.1. Regulation of HES gene transcription

During development, a limited number of signaling pathways specifies many different fates of the cell. One of these pathways is the Notch signaling pathway (Fig. 3.2). Mohr (1919) discovered the gene encoding the Notch protein in *Drosophila melanogaster*. He stated, ''A female, just hatched, having wings serrated or notched at the end, was found in the purple stock bottle, October 7, 1918.'' The gene was thus named Notch as partial loss of function resulted in notches at the wing margin. So far, four mammalian Notch genes (Notch1-4), encoding cell surface transmembrane receptors have been identified. The realization that the Notch signaling pathway regulates a broad spectrum of cell fates and developmental processes (in organisms ranging from *Drosophila* to humans) resulted in a veritable explosion of Notch-related studies in the past decade (Artavanis-Tsakonas *et al.*, 1999; Bray, 1998).

The Notch signaling pathway is deceptively simple. It is initiated through the binding of a membrane-tethered ligand (in mammals: Jagged1, 2 and Delta1, 2, 4) to the Notch receptor, triggering ligand endocytosis which induces a conformational change in the Notch receptor (Nichols *et al.*, 2007).

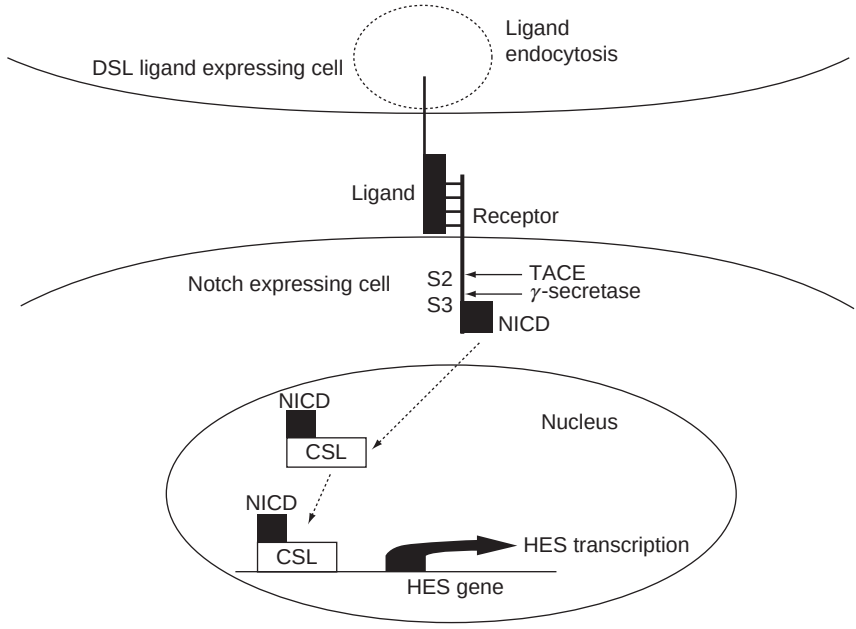

Figure 3.2 Schematic picture of the Notch signaling pathway.

The S2 site on the extracellular domain of Notch is then exposed for cleavage by a metalloproteinase (A disintegrin and metalloproteinase-17 (ADAM-17) or TNF-α-converting enzyme (TACE)) (Brou *et al.*, 2000). γ-Secretase can then cleave the Notch receptor at the S3 site, located within its intramembrane domain, releasing the Notch intracellular domain (NICD) (Schroeter *et al.*, 1998). The NICD then translocates into the nucleus, where it forms a complex with the DNA binding proteins CBF1(RBP-J)/Su(H)/LAG1 (CSL), converting them from transcriptional repressors to transcriptional activators, inducing transcription of HES genes (Honjo, 1996). Since Notch signaling leads to upregulation of HES expression, activation of Notch thus inhibits differentiation of several tissues and maintains progenitor cells.

3.2. Expression of Notch markers during chondrogenesis

Only a few articles, somewhat conflicting, have been published regarding the distribution of Notch markers during the development of articular cartilage. Watanabe *et al.* (2003) showed that Notch1 was expressed in mesenchymal condensations in the forelimb of E12.5 mouse embryos. At the maturation of cartilage in E16.5 embryos, Notch1 was expressed at the periphery of the developing cartilage and in the developing tendon; thus, a small population of the cells with sustained Notch1 expression become perichondrial cells (Watanabe *et al.*, 2003). Notch1-positive cells were also observed in prehypertrophic and hypertrophic zones of the embryos. The authors propose that this expression pattern of Notch1 suggests a dual role for Notch signaling in cartilage development; first, it controls condensation of mesenchymal stem cells (MSCs) and at later stages, it suppresses hypertrophy. They further suggest that Notch1 may have a role in the formation of stem cells in the perichondroium. Hayes *et al.* (2003) demonstrated that Notch1 was distributed at the surface layer of the mouse cartilage prior to joint cavitation (E15, E16) as well as after cavitation (E17). After birth and at 3 months of age, Notch1 was restricted to the deep zone (Hayes *et al.*, 2003). The authors discuss that the expression of Notch1 in the surface of the developing articular cartilage may be important in determining cell fate during chondrogenesis since appositional growth depends upon the presence of progenitor cells on the surface. They further showed that Notch2, Notch4, Delta, and Jagged2 were present throughout the cartilage during development (Hayes *et al.*, 2003). They detected this broad distribution of Notch markers in adult mice as well, except for the uppermost 1–2 cell layers, which were negative for these markers (Hayes *et al.*, 2003). In adult bovine cartilage and chicken cartilage, on the other hand, Dowthwaite *et al.* (2004) showed that Notch1 is expressed in chondrocytes located at the articular surface, and is not restricted to deeper zones of the cartilage after birth. These results are in accordance with the results from our

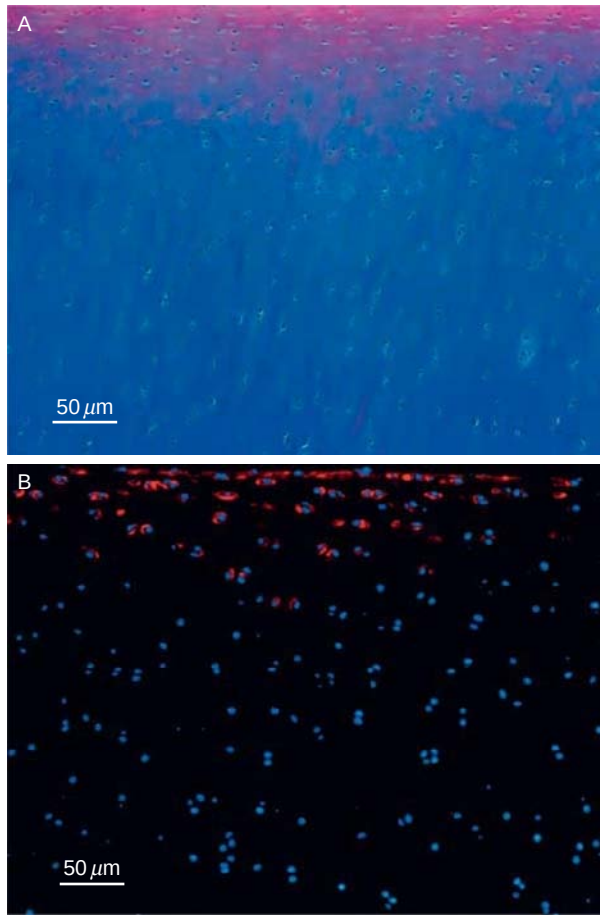

Figure 3.3 Alcian Blue van Gieson staining of the articular cartilage (A) and immunohistochemical localization of Notch1 (B).

group demonstrating expression of Notch1, HES5, and Jagged1 in the superficial zone of the human articular cartilage (Fig. 3.3) (Karlsson *et al.*, 2007b). This difference in expression may represent differences in the overall life spans of the species or it may simply be a species difference. *In vitro*, it has been demonstrated that the expression of human HES5 as well as most Notch receptors and their ligands, including Notch1, Notch3, Delta1, and Jagged1 decreased during chondrocyte differentiation (Karlsson *et al.*, 2007b). A thorough investigation of the expression of these markers during chondrogenesis *in vivo*, preferably on several different species, would be an important step in elucidating the function of Notch signaling for cartilage development.

4. Notch1 as a Marker for Progenitor Cells in Adult Cartilage

Progenitor cells are responsible for regeneration and repair of several tissues. Since Notch1 is a marker for a progenitor cell population in several different tissues, it has been investigated whether this is the case in cartilage as well (Chiba, 2006; Harada and Ohshima, 2004; Johansson *et al.*, 1999; Karanu *et al.*, 2003; Schwarting *et al.*, 2007). It has been demonstrated that articular cartilage grows appositionally, implicating the existence of a population of progenitor cells within the superficial layer of the cartilage. Dowthwaite *et al.* (2004) demonstrated in 7-day-old calves that 86% of the chondrocytes in the surface zone express Notch1 while a lower expression was detected in the middle and deep zones (10% and 34%, respectively). This is in line with our results showing that Notch1 positive cells localized to the superficial zone of the cartilage, thus suggesting that Notch1 might be a marker for progenitor cells in cartilage (Karlsson *et al.*, 2008b). Dowthwaite *et al.* (2004) further isolated bovine Notch1 positive cells from the superficial zone and demonstrated that these cells also had an increased colony-forming efficiency compared with unselected cells. They further stress that approximately 75% of surface zone cells express Notch 1 and only 1–2% of these selected cells form colonies, Notch 1 expression *per se* is not a specific marker of progenitor chondrocytes. Inhibition of Notch signaling further abolishes colony-forming ability whilst activated Notch rescues this inhibition. They also speculate that Notch 1 signaling may play one of the two roles for chondrocytes: it may function to maintain cells in a proliferative state, that is maintain clonality, or it may promote chondrocyte differentiation and hence cartilage growth. They further engrafted bovine surface zone-derived cells into the wing bud of stage 22 chick embryos and found tissue-specific matrix synthesis *in ovo* highlighting the plasticity of this cell population and supporting the notion that these cells represent a progenitor population as plasticity is a key marker of a stem cell population. The results obtained by Dowthwaite *et al.* are somewhat in contrast to data from our group. We isolated Notch1 positive chondrocytes from adult bovines but could not detect any increased colony-forming efficiency or plasticity to differentiate into chondrogenic, adipogenic, and osteogenic lineages compared to the Notch1 negative population of chondrocytes (Fig. 3.4) (Karlsson *et al.*, 2008b). This discrepancy might be because animals of different ages were used (7-day-old calves vs. 2–3-year-old bulls). The proliferative state of the chondrocytes in a 7-day-old calf might partly explain the higher expression of Notch1 in younger animals. Another possible explanation might be the different developmental stages of the cartilage. As discussed above, the expression of both Notch1 and HES5 significantly decreases during chondrogenesis. The large content of

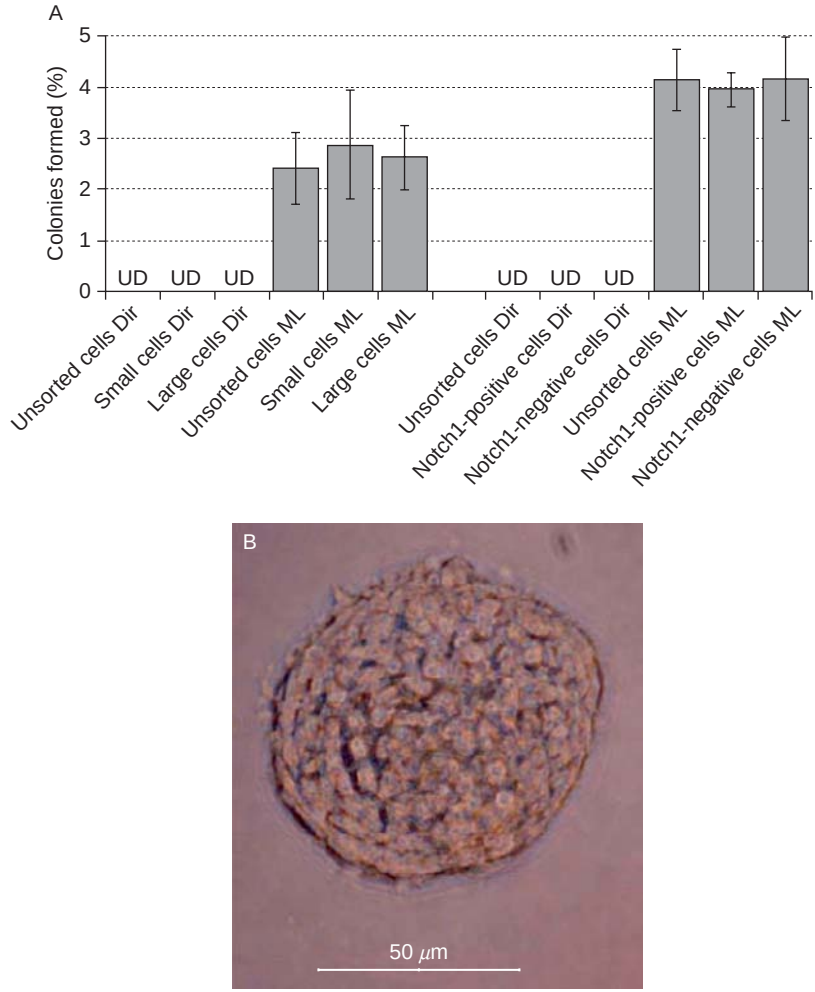

Figure 3.4 Colony-forming efficiency of different sizes of chondrocytes as well as Notch1 positive and negative cells both directly after isolation and after expansion.

terminally differentiated chondrocytes in adult cartilage might thus explain the less abundant expression of Notch1. Despite the lack of progenitor cell properties of the Notch1 positive chondrocyte population, the surface cells expressing Notch1 might still have another phenotype than the cells in deeper layers not expressing Notch1. Collagen type I is mainly expressed by chondrocytes located in the surface zone of the articular cartilage, as described above. In addition, less collagen type II and proteoglycans are accumulated in this area. As demonstrated by Watanabe *et al.* (2003), ATDC5 cells overexpressing Notch1 significantly decrease their expression of collagen type II

and aggrecan. The high expression of Notch1 detected in chondrocytes located in the surface zone of the articular cartilage might thus to some extent be responsible for the altered, more immature phenotype detected in these cells.

5. Function of Notch Signaling During Chondrogenesis

A few studies have been conducted regarding the function of Notch signaling for chondrogenesis. Watanabe *et al.* (2003) demonstrated that overexpression of Notch1 in ATDC5 cells allowed to differentiate in the monolayer resulted in the reduced expression of collagen type II, aggrecan, scleraxis, and SRY-box containing gene 9 (Sox9). They further demonstrated that coculture of ATDC5 cells with myeloma clone D10, which constitutively express Delta1, resulted in significant reduction of Alcian Blue positive nodule formation (Watanabe *et al.*, 2003). They thus demonstrated that activating either endogenous or exogenous Notch signaling results in inhibited chondrogenesis. Our results, demonstrating a significant decrease in the expression of Notch markers during differentiation of human chondrocytes, are in accordance with this notion, suggesting that Notch signaling retains chondrocytes in an immature state (Karlsson *et al.*, 2007b). The high expression of HES5 in dedifferentiated chondrocytes suggests that HES5 is important for early cartilage development, and that a downregulation is required for expression of genes inducing a hyaline phenotype. In accordance with our results, Akazawa *et al.* (1992) have shown that rat HES5 is expressed at a high level in the embryonic brain, whilst the level of HES5 mRNA is low in the adult brain. A similar expression pattern of HES5 in mice is also observed in the neural retina.

Fujimaki *et al.* (2006) studied the importance of Notch signaling for murine limb bud cells cultured in micromass cultures. They demonstrated that the expression of several Notch markers peaked between Day 3 and Day 5 of micromass culture, that is at the condensation phase. Blocked Notch signaling resulted in increased initiation of the condensation phase as well as induction of the collagen type II, Sox9, and GDF5, implying that Notch signaling may play an important role in chondrogenic differentiation by negatively regulating the initiation of prechondrogenic condensation (Fujimaki *et al.*, 2006). In contrast to the above discussed results, Vujovic *et al.* (2007) demonstrated that blocked Notch signaling reduced the rate of MSC proliferation. Blocked Notch signaling further reduced the pellet size by ~50% and Toludine Blue stainings demonstrated less accumulation of proteoglycans compared to control pellets. No differences in collagen type II expression were detected. With regard to the results from Fujimaki

et al. (2006), they suggest that a transient Notch signaling might be required for chondrogenesis, and that either blockage or activation of the Notch signaling pathway result in inhibited chondrogenesis.

The abundant but nonoverlapping expression of Delta1 and Delta4 in adult cartilage suggests that these two ligands might have different functions in mature cartilage. Notch signaling is known to create boundaries between two different cell types (Karlsson *et al.*, 2007b). The differential expression of Delta1 and Delta4 might distinguish the superficial zone of the cartilage from the transitional zone.

One interesting question to discuss is how this signaling system can work in chondrocytes *in vivo* that do not have any cytoplasmatic contact with other cells. One answer might be the use of soluble ligands. It has, for example, been demonstrated that the extracellular portion of the Delta can be cleaved off and function as a soluble ligand. It has also been shown that the Notch signaling can be activated even when the ligand and receptor are present on the same cell, which is another possibility for the chondrocytes to signal via Notch receptors.

5.1. Importance of HES1 and HES5 for cartilage formation *in vivo*

Rather unexpectedly, deletion of HES5 and one HES1 allele did not affect articular cartilage development (Fig. 3.6) (Karlsson *et al.*, 2007b). These results were explained either by the HES1 and HES5 genes not being involved in cartilage development or by functional redundancy between the genes belonging to the family of HES genes: that is, one could compensate the disruption of one gene: that is, disruption of one gene could be compansated for by the activity of another. This overlapping function of a family of genes is very common for developmentally important genes, including genes in the Notch signaling system (de la Pompa *et al.*, 1997; Kong *et al.*, 2004; Oates and Ho, 2002). During cartilage development, the expression of Notch2 and 4, Delta, and Jagged2 display a very similar distribution pattern, suggesting redundancy within the Notch signaling system in the cartilage (Hiraoka *et al.*, 2006). Other attempts have been made in elucidating the roles of Notch signaling for cartilage and bone formation. Francis *et al.* (2005) conditionally deleted the Notch1 gene in limb mesenchymal cells, but no altered phenotype was detected. They further suggested Notch2 to compensate for the loss of HES1. RBP-Jk, which forms a complex with NICD activating transcription of HES genes, is thus required for Notch signaling from several different Notch receptors. One way of finding out the importance of Notch signaling for *in vivo* chondrogenesis might thus be to construct mice lacking the RBP-Jk gene.

Overexpression of genes in the Notch signaling pathway as well as pharmacological blockage of Notch signaling from all Notch receptors

in vitro results in altered chondrogenesis, demonstrating the importance of Notch signaling for cartilage development (Fujimaki *et al.*, 2006; Vujovic *et al.*, 2007; Watanabe *et al.*, 2003). If Notch signaling is of importance for chondrogenesis *in vivo* as well, the Notch receptors must, according to our results, signal via genes other than HES1 and HES5. The known Notch transducers in mice of the HES gene family are HES1, HES5, and HES7, that is, not all HES genes are regulated by Notch signaling (Bessho *et al.*, 2001; Koyano-Nakagawa *et al.*, 2000; Nishimura *et al.*, 1998). HES7 is specifically expressed in the presomitic mesoderm in a dynamic manner, and thus not likely to affect joint development and endochondral bone formation (Bessho *et al.*, 2001). One possible pathway for Notch signaling is via the more recently discovered family of Hey genes encoding a new group of transcription factors related to the HES genes (Maier and Gessler, 2000). This far, their expression during cartilage development and endochondral bone formation is unknown.

5.2. Differential Notch signaling in MSCs and chondrocytes

The joint is formed by condensation of MSCs (DeLise *et al.*, 2000; Hall and Miyake, 2000). Despite this, chondrocytes and MSCs tend to differentiate into different cartilage phenotypes, articular and hypertrophic, respectively. To further elucidate the role of Notch signaling, it is therefore of interest to study the expression of Notch markers during chondrogenesis of these two cell types.

Crowe *et al.* (1999) had earlier shown that the Delta-Notch2 signaling pathway is important in regulating the progression of prehypertrophic chondrocytes to hypertrophic chondrocytes during chick growth plate elongation. In this system, Delta-Notch2 signaling that occurs downstream of the Indian hedgehog (IHH), bone morphogenetic protein (BMP), and parathyroid hormone-related protein (PTHrP) pathways inhibiting the differentiation of prehypertrophic to hypertrophic chondrocytes and over-expression of Delta results in stunted limbs with reduced ossification (Crowe *et al.*, 1999). The detected increase in expression of Notch2 and Delta4 during chondrogenesis *in vitro* might thus prevent the cells from differentiating into a hypertrophic phenotype (Fig. 3.5). The same might be true for the abundant expression of Delta1 and Delta4 detected in cartilage biopsies from healthy donors (Karlsson *et al.*, 2007b). This effect of Delta-Notch2 signaling might possibly go through HES1 since we have demonstrated that HES1, Delta4, and Notch2 have the same expression pattern during *in vitro* chondrogenesis. de Jong *et al.* (2004) demonstrated that HES1 is downregulated by the osteogenic factor BMP2, whereas it is induced by the inhibitory factor TGF-β, suggesting an inhibitory role of HES1 in osteoblast differentiation. We have earlier demonstrated that a high expression of collagen type X detected in MSCs correlate to a low expression of

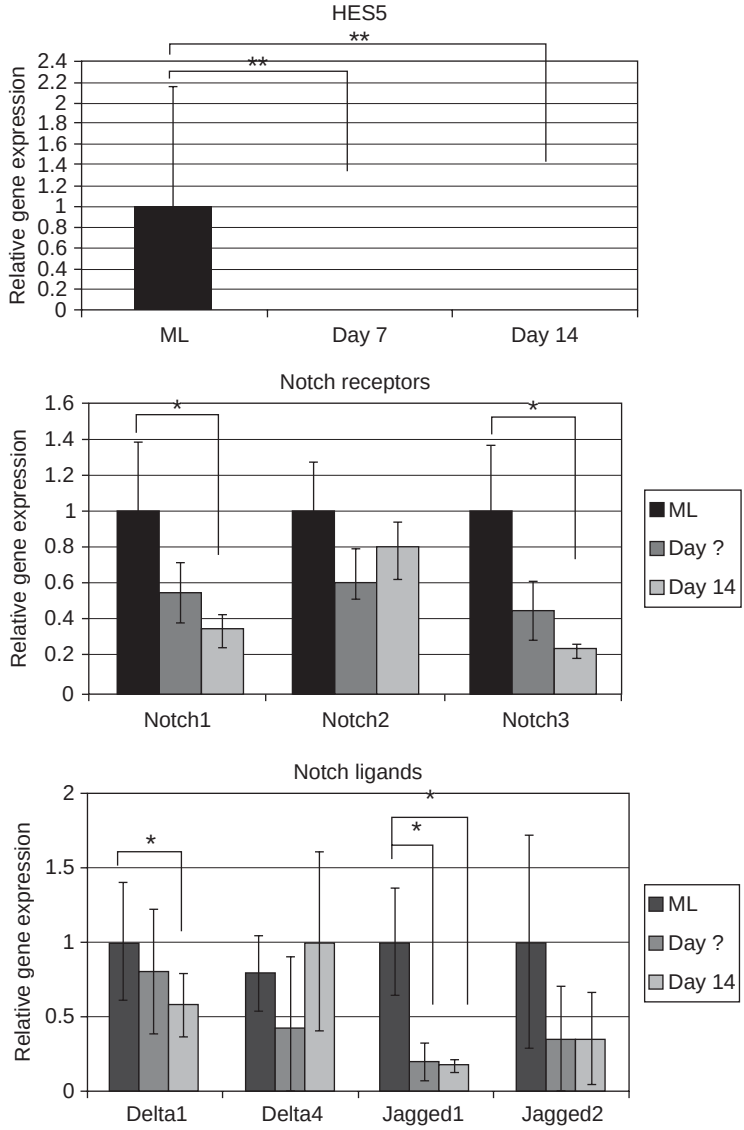

Figure 3.5 **Expression of HES5, Notch receptors, and ligands during chondrogenesis** ***in vitro.***

HES1 (Karlsson *et al.*, 2007a). It has actually been demonstrated that HES1 physically interacts with core binding factor alpha1 (Cbfa1), which is strongly expressed in terminally hypertrophic chondrocytes, and represses the expression of osteopontin (McLarren *et al.*, 2000). In accordance, transient transfection of NICD attenuates the promoter activities of Cbfa1

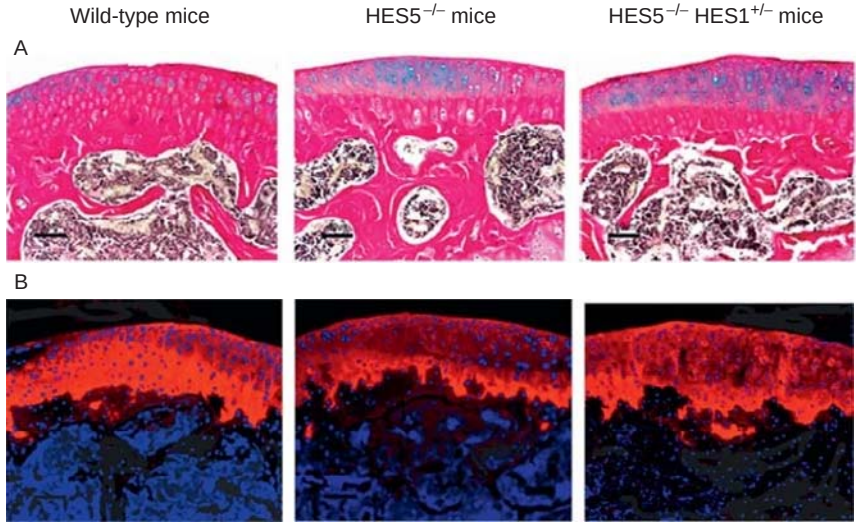

Figure 3.6 Alcian Blue van Gieson staining (A) and detection of collagen type II (B) in wild-type and transgenic mice lacking the HES5 genes and one HES1 allele.

(Shindo *et al.*, 2003). It has also been demonstrated that there are two N-box sequences in the osteocalcin promoter that can serve as HES1 binding sites and negatively regulate osteocalcin expression (Tezuka *et al.*, 2002). The low expression of HES1 detected in MSCs, especially in those with a high expression of collagen type X, thus release repression of osteopontin and possibly other genes involved in osteogenesis, including collagen type X, promoting osteogenesis and hypertrophy.

In some tissues, Notch1 and Notch2 have opposing effects on HES1 expression. Fan *et al.* (2004) detected a correlation between Notch2 and HES1 expression, whereas they could not detect any correlation between Notch1 and HES1. During chondrogenesis *in vitro*, we observed that while the expression of Notch1 was significantly decreased, the expression of HES1, Notch2, and Delta4 first decreased but then increased in expression from Day 7 to Day 14 of pellet mass culture (Karlsson *et al.*, 2007a,b). It thus seems as if transcription of HES1 is differentially regulated by Notch1 and Notch2 in chondrocytes as well, possibly resulting in different functions of these two receptors in chondrocytes. As described above, Notch2 and HES1 prevent hypertrophy. On the other hand, transfection of NICD1 to C3H10T1/2 cells stimulated osteoblastic differentiation (Tezuka *et al.*, 2002). NICD1 expression in primary human bone marrow MSCs also induced both spontaneous and stimulated osteoblastic cell differentiation, suggesting that osteoblast differentiation is regulated positively by Notch1 (Tezuka *et al.*, 2002). These results are in accordance with our data,

demonstrating a significant downregulation of Notch1 expression during chondrogenesis of chondrocytes. The differences detected in chondrogenic potential between articular chondrocytes and MSCs might thus to some extent be explained by their differential expression of several transcriptional factors belonging to the HLH family.

6. Notch Signaling in Osteoarthritis

The major degenerative disease associated with articular cartilage is arthritis (Felson *et al.*, 2000). It can be further subdivided into two major classes: rheumatoid arthritis (RA) and osteoarthritis (OA). OA is the most common form of arthritis and one of the most common forms of musculoskeletal diseases encountered in all countries of the globe. In Europe, a joint is replaced every 1.5 min due to OA. A study of Notch signaling has been conducted in RA, which like OA, is characterized by phenotypic alterations, increased proliferation, and overexpression of tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β). These studies have reported increased expression of Notch1, Notch4, and Jagged2 in RA synovium (Ando *et al.*, 2003). This is in accordance with our results demonstrating that Notch1, Jagged1, and HES5 are abundantly expressed in OA cartilage compared to healthy cartilage (Karlsson *et al.*, 2008a). The abundant expression of HES5 also suggested that there was increased signaling from the Notch receptors in OA chondrocytes. Our results are again in accordance with Hiraoka *et al.* (2006) demonstrating that normal articular cartilage contains Notch1 positive cells and that their frequency is increased in OA, especially in clusters of chondrocytes. Interestingly, Notch receptors and their ligands are also more strongly expressed in the synovium of patients with RA and OA compared to healthy controls (Ishii *et al.*, 2001; Yabe *et al.*, 2005).

Ando *et al.* (2003) also showed that TNF-α stimulation of synoviocytes from patients with RA induces expression of Notch1, 4, and Jagged2 as well as increased signaling from the Notch1 receptor shown by nuclear translocation of NICD1. This increased Notch signaling in the biopsies from RA synovium where the Notch1 expression is localized to the nuclei (Nakazawa *et al.*, 2001). The functional involvement of Notch signaling in RA has also been demonstrated. Nakazawa *et al.* (2001) have shown that inhibition of endogenous Notch1 expression led to suppression of TNF-α induced synovial cell proliferation, indicating that Notch1 signaling is involved in TNF-α induced abnormal growth of RA synoviocytes. The effect of Notch signaling for proliferation of chondrocytes and MSCs have also been studied by others, resulting in somewhat conflicting data, perhaps due to the use of different methods (Dowthwaite *et al.*, 2004; Vujovic *et al.*, 2007; Watanabe *et al.*, 2003). Our results demonstrating that Notch

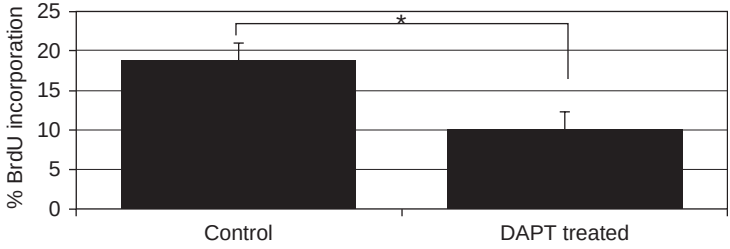

Figure 3.7 The effect of Notch signaling for proliferation.

signaling is associated with significantly increased proliferation is thus in accordance with Nakazawa *et al.* (2001) (Fig. 3.7) (Karlsson *et al.*, 2007b). We thus hypothesize that the increased expression of Notch markers detected in OA cartilage is due to either increased proliferation or phenotypic alterations (Karlsson *et al.*, 2008a). Several studies have shown that there is proliferative activity in OA chondrocytes compared to normal articular chondrocytes, which show very limited, if any, proliferative activity (Hulth *et al.*, 1972; Karlsson *et al.*, 2007a; Meachim and Collins, 1962; Rothwell and Bentley, 1973). These results explain the chondrocyte cloning observed in OA cartilage, which one found primarily in the upper cartilage zone, that is, in the same location as the Notch1 positive cells. The increased proliferative activity might be due to better access to proliferative factors from the synovial fluid due to fissures and degradation of the matrix (Lee *et al.*, 1993; Meachim and Collins, 1962). Another theory is that damage to the collagen matrix, which is particularly detected in the superficial zone, might contribute to the increased proliferation. This proliferation resulting in higher cellular content is unlikely to represent efficient tissue repair since chondrocyte clusters in the upper zone does not add significantly to matrix anabolism (Aigner *et al.*, 1997). The increased proliferation detected in OA might also be due to the increased expression of Notch markers.

Dowthwaite *et al.* (2004) have shown that the Notch signaling pathway might be involved in the degradation of matrix seen in OA, since blockage of Notch signaling in IL-1β treated bovine cartilage explants abolished the GAG loss associated with IL-1β treatment. The presence of inflammation in OA, to some extent resembling that found in RA, increased proliferation, and phenotypic alterations, prompted us to study Notch signaling in OA cartilage.

Other phenotypic alterations detected in OA cartilage are hypertrophy and dedifferentiation (Aigner *et al.*, 1993, 1999; Kirsch *et al.*, 2000; Sandell and Aigner, 2001). The abundant expression of Notch1, Jagged1, and HES5 detected in OA cartilage biopsies might thus also be due to, or result in, a more primitive phenotype characteristic for OA cartilage (Aigner *et al.*, 1993;

Sandell and Aigner, 2001). In healthy cartilage, a more primitive phenotype is detected in the surface zone, seen as a higher expression of collagen type I and decreased expression of collagen type II compared to the other zones. In OA cartilage, a more primitive gene expression is also detected in cells in the transitional zone. This extended area of primitive gene expression might explain the increased number of cells expressing Notch markers. Another possibility is that Notch signaling in these upper layers prevents hypertrophy of these cells, since expression of collagen type X is mainly detected in the radial zone (Girkontaite *et al.*, 1996). It has further been suggested by Watanabe *et al.* (2003) that Notch suppresses hypertrophy at later stages of chondrocyte differentiation. For a more detailed discussion regarding Notch signaling and hypertrophy, see the section above regarding chondrogenesis of MSCs.

In contrast to the data from Nakazawa *et al.* (2001), showing that TNF-α induces nuclear translocation of NICD1 in synoviocytes, we clearly showed that there is no TNF-α induced translocation of NICD1 in chondrocytes. Chondrocytes and synoviocytes thus respond differently to cytokine stimulation not only with regard to transcription of Notch markers but also to activation of the Notch signaling pathway. These results indicate that cleavage of the NICD1 is associated with control mechanisms regulating its cellular localization. One cannot explain the significantly cytokine-induced repression of HES5 transcription by the decreased cleavage of NICD1 or accumulation of NICD1 in the cytoplasm. Recently, it was demonstrated that the signaling cascades emanating from the IL-1β and TNF-α receptors converge, resulting in activation of nuclear factor kappa b (NFκB) (Chen *et al.*, 1997). The effect of cytokineinduced NFκB activation on HES5 repression was studied using pyrrolidine-dithiocarbamate (PDTC), a substance inducing a phenotype corresponding to genetic ablation of NFκB activation (Hu *et al.*, 2001). We have shown that the effect of TNF-α stimulation on HES5 transcription was, at least to some extent, transmitted via NFκB rather than the canonical NICD pathway (Karlsson *et al.*, 2008a). There are contradictory results regarding the effect of NFκB activation on HES1 transcription; both repressed and induced HES1 transcription have been demonstrated (Aguilera *et al.*, 2004; Espinosa *et al.*, 2003). Our results are thus in accordance with Espinosa *et al.* (2003), demonstrating that when NFκB is inhibited, p65 and several nuclear corepressors are retained in the cytoplasm bound to IkappaB (IκB). The reduced nuclear levels of these corepressors decrease the threshold for Notch-dependent gene activation, resulting in increased expression of HES1 (Espinosa *et al.*, 2003). Cytokine-induced NFκB might thus interfere with the transcriptional complex containing NICD resulting in decreased binding to the HES5 promoter. Another possibility is that NFκB binds to the HES5 promoter, interfering with the binding of the NICD complex, resulting in transcriptional repression of HES5.

The functional importance of the increased Notch signaling detected in OA biopsies was studied using microarray analyses of chondrocytes with pharmacologically blocked Notch signaling due to γ-secretase inhibition compared to untreated chondrocytes (Karlsson *et al.*, 2008a). Microarray analyses of cells treated with γ-secretase inhibitors have previously been used to reveal genes controlled by Notch signaling (Cheng *et al.*, 2003; Fujimaki *et al.*, 2006; Searfoss *et al.*, 2003). We demonstrated that genes known to inhibit differentiation and increase proliferation, including CD10, ID4, HES4, and HES5, were induced by Notch signaling (Diaz-Romero *et al.*, 2005; Hatakeyama *et al.*, 2004; Riechmann and Sablitzky, 1995). The cloning and dedifferentiation detected in OA might thus, to some extent, be a result of the increased Notch signaling resulting in increased expression of CD10, ID4, HES4, and HES5. This is also in accordance with previous results, demonstrating that blocked Notch signaling results in decreased chondrocyte proliferation. We have also shown that chondrocytes at low confluence, that is, in a highly proliferative state have a higher Notch1 expression compared to confluent cells (unpublished data).

Notch signaling further repressed transcription of lubricin. Normally, lubricin is expressed by chondrocytes in the superficial layer, imparting mechanical properties and lubrication to the articular surface-minimizing friction in synovial joints (Felson *et al.*, 2000). It has recently been demonstrated that there is a loss of lubricin transcription and immunostaining after the onset of early OA and it has been suggested that this loss might have a role in the pathogenesis of OA (Young *et al.*, 2006). The increased Notch signaling detected in OA might partly be responsible for the repressed transcription of lubricin detected in OA. Other genes whose expression was repressed by Notch signaling included BMP2, IL8, and MMP9. BMP2 is upregulated in OA and acts as an anabolic stimulus promoting synthesis of collagen type II while IL8 and MMP9, also upregulated in OA, are responsible for the catabolic responses (Fukui *et al.*, 2003; Murphy *et al.*, 2002). The abundant Notch signaling detected in OA cartilage thus tends to counteract the high expression of these genes seen in OA.

7. Concluding Remarks

Taken together, Notch signaling seems to be important for chondrocytes in several different aspects; it regulates chondrogenesis, it is a possible marker for cartilage progenitor cells, and it is involved in OA. Despite the recent interest in Notch signaling in chondrocyte research, more studies are needed to pinpoint its function, especially regarding redundancy within the Notch family and the control of the hypertrophic pathway of chondrocyte differentiation.

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CHAPTER FOUR

The Controversial Role of Mast Cells in Tumor Growth

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Contents

Abstract

Mast cells (MCs) were first described by Paul Ehrlich (Beiträge zur Theorie und Praxis der Histologischen Färbung, Thesis, Leipzig University, 1878). They have long been implicated in the pathogenesis of allergic reactions and protective responses to parasites. However, their functional role has been found to be complex and multifarious. MCs are also involved in various cell-mediated immune reactions and found in tissues from multiple disease sites, and as a component of the host reaction to bacteria, parasite, and even virus infections. They also participate in angiogenic and tissue repair processes after injury. The importance of a possible functional link between chronic inflammation and cancer has long been recognized. As most tumors contain inflammatory cell

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International Review of Cell and Molecular Biology, Volume 275
ISSN 1937-6448, DOI: 10.1016/S1937-6448(09)75004-X

infiltrates, which often include plentiful MCs, a possible contribution of these cells to tumor development has emerged. In this review, general biology of mast cells, their development, anatomical distribution, and phenotype as well as their secretory products will first be discussed. The specific involvement of MCs in tumor biology and tumor fate will then be considered, with particular emphasis on their capacity to stimulate tumor growth by promoting angiogenesis and lymphangiogenesis. Finally, it is suggested that mast cells may serve as a novel therapeutic target for cancer treatment.

Key Words: Angiogenesis, Mast cells, Tumor progression.

1. Introduction

Mast cells (MCs) are versatile, tissue-homing secretory cells, which were first described by Ehrlich (1878) in his doctoral thesis (Crivellato *et al.*, 2003a). He named these cells Mastzellen because he believed that the intracellular granules, which appeared purple in color when stained with aniline blue dyes, contained phagocytosed materials or nutrients. MCs have long been implicated in pathogenesis of allergic reactions and certain protective responses to parasites. Their functional role, however, has been discovered to be increasingly complex and multifarious. MCs have been implicated in various cell-mediated immune reactions and are found in tissues from multiple disease sites, and as a component of the host reaction to bacteria, parasite, and even virus infections. They have also been shown to participate in angiogenic and tissue repair processes after injury (Galli *et al.*, 2005a,b).

The importance of a possible functional link between chronic inflammation and cancer has long been recognized. As most tumors contain inflammatory cell infiltrates, which often include plentiful MCs, the question as to the possible contribution of MCs to tumor development has emerged progressively. A major point linking MCs to cancer is the well-recognized capacity of these cells to synthesize and release potent angiogenic cytokines. Although some evidence suggests that MCs can promote tumorigenesis and tumor progression, there are some clinical sets as well as experimental tumor models in which MCs seem to have functions that favor the host. Thus, the involvement of MCs in tumor development is complex and far from being settled.

In this review, general biology of these cells, their development, anatomical distribution and phenotype as well as their secretory products will first be discussed. The specific involvement of MCs in tumor biology and tumor fate will then be considered, with particular emphasis on the capacity of these cells to stimulate tumor growth by promoting angiogenesis and lymphangiogenesis. The last section describes drugs that affect the MC number.

2. Biological Functions of Mast Cells

MCs are multi-functional long-lived secretory cells, characterized by the content of numerous cytoplasmic granules storing histamine (also serotonin in rodents), proteoglycans, cytokines, and neutral serine proteases. MCs are phylogenetically old cells, which apparently occur in all species with blood circulation. In histological preparations, MCs usually appear as round or elongated cells with a diameter ranging between 8 and 20 μm, depending on the organ examined. The single nucleus of a MC shows round or oval shape and the cytoplasm contains numerous secretory granules that stain metachromatically with thiazine dyes such as toluidine blue. On electron microscopy, the MC exhibits a nonsegmented monolobed nucleus with peripherally condensed chromatin. The cytoplasm contains a few mitochondria, short profiles of the rough endoplasmic reticulum, free ribosomes, and numerous membrane-bound, moderately electron-dense secretory granules with an average diameter of 1.5 μm (Dvorak, 1991) (Fig. 4.1). Secretory granules may present different substructural patterns, such as

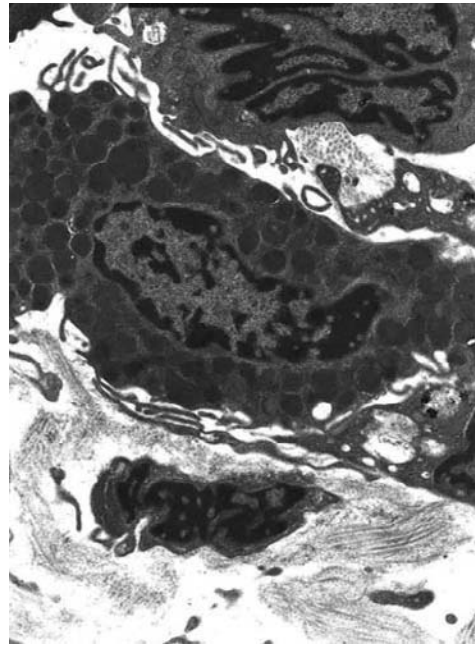

Figure 4.1 Electron micrograph of a human normal mast cell in the skin. This cell presents an elongated shape and a cytoplasm filled with plentiful electron-dense granules with homogeneous structure. Original magnification: ×6300.

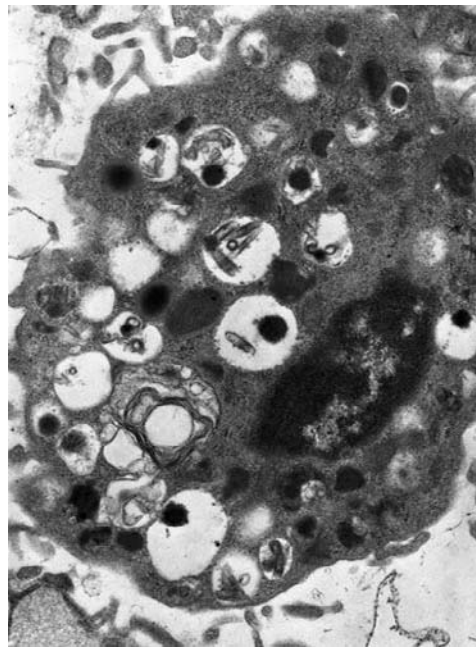

Figure 4.2 Electron micrograph of human mast cell from the colon mucosa in a patient with inflammatory bowel disease. The cytoplasm contains numerous, partially emptied granules with residues of scroll material. This particulate, nonexocytotic secretory pattern is called piecemeal degranulation. Original magnification: ×10,000.

homogeneous, crystalline, scroll, particle or thread-like pattern or a combination of them (Fig. 4.2). Granule ultrastructure has been partly related to their content of serine proteases. Indeed, granules with the chymase protease preferentially exhibit homogeneous or crystalline substructures, whereas granules lacking this protease show mainly a scroll pattern (Schwartz *et al.*, 1987). However, significant granule heterogeneity can be found in any particular tissue and even between granules of a single MC. Of note, MC populations show marked differences in their phenotypic expression in different species, as well as at distinct anatomical sites, a phenomenon called ''MC heterogeneity'' (Galli, 1990). In addition to the typical secretory granules, human and mouse MCs also contain nonmembrane-bound, highly osmiophilic granules, called lipid bodies (Dvorak, 1991). They are fewer in number and generally larger than secretory granules, and serve as a significant site for arachidonic acid storage and metabolism. Besides cytoplasmic granules, all mammalian MCs express common characteristics, including the stem cell factor (SCF) plasma membrane receptor KIT and the high affinity plasma membrane receptor (FcεRI) binding IgE antibodies.

Most of our knowledge on MCs is derived from MC ''knock-in'' mouse models which have allowed researchers for testing and verifying whether MCs contribute to specific functions. The *Kit*-mutant mice, which are deficient in MCs, have been used in a series of experimental settings (Galli *et al.*, 2005a). The *Kit* gene encodes for the KIT protein (CD117) or SCF receptor on the surface of MCs and other cell types, such as germ cells, melanocytes, and intestinal Cajal cells (Galli *et al.*, 1992; Metcalfe *et al.*, 1997; Nocka *et al.*, 1990). SCF, or KIT receptor ligand, represents the main survival and developmental factor for MCs. Lack of expression of a functional KIT receptor because of spontaneous mutation in both copies of *Kit*, as it occurs in genetically MC-deficient WBB6F1-Kit^{W}-$Kit^{W\text{-}v}$ mice (W/W^{v} mice), results in a virtual absence of tissue MCs (Kitamura *et al.*, 1978). Kit^{W} contains a point mutation that encodes a truncated KIT protein, which lacks the transmembrane domain and is therefore not expressed on the cell surface; $Kit^{W\text{-}v}$ encodes a mutation in the KIT tyrosine kinase domain that markedly decreases the kinase activity of the receptor. A *Kit*-mutant mouse has been characterized more recently, the C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mouse (Grimbaldeston *et al.*, 2005; Zhou *et al.*, 2007). $Kit^{W\text{-}sh}$ contains an inversion mutation of the transcriptional regulatory elements upstream of the *Kit* transcription start site on mouse chromosome 5 (Galli *et al.*, 2005b). Lack of MCs in *Kit*-mutant mice can be selectively repaired by the adoptive transfer of genetically compatible wild-type or mutant MCs derived from *in vitro* cultures to create the so called MC ''knock-in'' mice.

The cross-linking of immunoglobulin E (IgE) with bivalent or multivalent antigen results in the aggregation of FcεRI, which is sufficient for initiating down-stream signal transduction events that activate cell degranulation as well as the *de novo* synthesis and secretion of lipid mediators and cytokines (Blank and Rivera, 2004). Release of preformed and newly formed MC products from activated MCs leads to a series of profound biological effects. For the sake of clearness, the effects of MC activation may be conceptualized into two partly overlapping categories, that is immunological and nonimmunological. MCs may also be activated by ''alternative,'' IgE-independent pathways, such as aggregation of FcγRIII by IgG/antigen complexes, KIT and Toll-like receptor mechanisms, exposure to chemokines, anaphylatoxins C3a and C5a, fragments of fibrinogen, and fibronectin (Gommerman *et al.*, 2000; Johnson *et al.*, 1975; Marshall, 2004; Prodeus *et al.*, 1997; Wojtecka-Lukasik and Maslinski, 1992).

When MCs are activated, they immediately extrude granule-associated substances, such as histamine and proteases. Within minutes, they generate lipid-derived mediators (Brody and Metcalfe, 1998). Then, MC activation is followed also within hours, by the *de novo* synthesis of numerous cytokines and chemokines (Galli *et al.*, 2005a). MCs have long been regarded as key effector cells in IgE-associated immune responses, including allergic disorders and certain protective immune responses to parasites (Galli *et al.*, 2008a; Gurish and Austen, 2001). Consequently, MCs have mainly been considered

in the past for their detrimental role in type I allergic reactions, such as anaphylaxis, hay fever, eczema, or asthma. Several lines of evidence, however, indicate that MCs exert key immunological functions and are critical protagonists in host defence in the context of both innate and adaptive immune responses. In addition, there is growing evidence that MCs exert distinct nonimmunological activities, playing a relevant role in processes like wound healing after injury, tissue homeostasis and remodeling, fibrosis as well as tissue angiogenesis.

2.1. Immunological functions

MCs have increasingly been recognized as crucial effectors in both innate and adaptive immune responses. This concept is mostly derived from studies using MC-deficient mice. In these experimental settings, MCs have been shown to protect against bacteria, fungi, protozoa, and perhaps even viruses through the release of proinflammatory and chemotactic mediators (Féger *et al.*, 2002). Indeed, recent reports in the literature indicate that MCs can mediate a variety of antimicrobial activities. MCs, for instance, are able to recognize and kill opsonized bacteria. *Salmonella typhimurium* coated with the C3b fragment of complement is recognized through complement receptor 3 (CR3) on the MC membrane (Sher *et al.*, 1979). In addition, MCs express several IgG receptors that are involved in the binding of IgG-coated bacteria to the MC membrane (Talkington and Nickell, 2001). Following their binding to MCs, osponized bacteria are phagocytosed. MCs are a rich source of early-response cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-4, which are decisive in initiating the immune and inflammatory responses of the host to the invading pathogens (Galli *et al.*, 2005b). MCs release TNF-α stored in secretory granules after incubation with bacteria both *in vitro* and *in vivo* (Echternacher *et al.*, 1996; Malaviya *et al.*, 1996). In mutant W/W^v mice, the absence of MCs leads to a defective innate immune response against bacteria. In a model of acute septic peritonitis by cecal ligation and puncture, W/W^v mice exhibited a dramatically increased mortality compared with the wild-type mice (Echternacher *et al.*, 1996). Remarkably, the adoptive transfer of MCs to the peritoneum protected the MC-deficient mice from the lethal effects of cecal ligation and puncture. Similarly, MC-deficient W/W^v mice are less protected against experimentally induced lung enterobacterial infections than MC-sufficient or MC-reconstructed W/W^v mice (Malaviya *et al.*, 1996). The impaired killing of bacteria in MC-deficient mice was directly correlated with reduced neutrophil infiltration in lungs, partly as a result of lower levels of the MC-derived chemotactic TNF-α in these mice. Indeed, TNF-α-deficient mice have increased mortality in the ''cecal ligation and puncture'' model compared with wild-type mice (Maurer *et al.*, 1998). Recent evidence indicate that mMCP-2, a mouse MC serine protease of

the chymase type, can contribute to neutrophil recruitment and host survival during cecal ligation and punctute in mice (Orinska *et al.*, 2007). In a mouse model of sepsis, it has been shown that MC may decrease neurotensin-induced hypotension as well as sepsis-related mortality by degrading neurotensin through the protease neurolysin (Piliponsky *et al.*, 2008). Interestingly, other MC-derived products, such as leukotriene B_4 (LTB_4), human trypatse βI, macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β, MIP-2, monocyte chemoattractant protein-1, RANTES (regulated upon activation, normal T cell expressed and secreted) (CCL5), and IL-8 (CXCL8) also appear to contribute to the influx of neutrophils induced by activated MCs (Féger *et al.*, 2002).

By contrast, MCs may have deleterious effects during bacterial infections by excessive or inappropriate release of inflammatory mediators leading to detrimental effects to the host. For instance, there are indications that Shiga toxin produced by *Shigella dysenteriae* may stimulate intestinal MCs to release excessive amounts of inflammatory mediators derived from arachidonic acid metabolism, in particular leukotriene C_4 (LTC_4), leading to diarrhea and dysentery (Pulimood *et al.*, 1998). MCs may also trigger inflammation in *Helicobacter pylori* infection, and MC accumulation in the mucosa of patients with gastritis and MC degranulation by *H. pylori* products have been described (Masini *et al.*, 1994; Nakajima *et al.*, 1997; Plebani *et al.*, 1994). In addition, although MCs are capable of phagocytose and kill various opsonized bacteria, this capacity may be subverted by microbes endocytosed in nonopsonic conditions (Shin *et al.*, 2000). In these cases, the internalized pathogen is sequestered in a MC endosomal compartment that escapes acidification and entry of oxygen radicals. The net result is that the bacteria are not killed by MCs but remain in the MC cytoplasm as an intracellular reservoir (Féger *et al.*, 2002).

MCs may also contribute to optimal initiation of acquired immunity by orchestrating migration, maturation, and function of dendritic cells and by interacting with T and B cells (Nakae *et al.*, 2005). MCs promote dendritic cell migration and lymphocyte recruitment mainly through secretion of factors such as histamine, chemokines, LTB_4, and TNF (Demeure *et al.*, 2005; Jawdat *et al.*, 2006; Maurer *et al.*, 2006). MCs may contribute also to the processing and presentation of bacterial antigens to immune cells. Indeed, MCs express MHC class I and II molecules, and can process and present antigens *in vitro* (Kambayashi *et al.*, 2008). Furthermore, they represent sources of costimulatory activity by expressing molecules of the B7 family, members of the TNF and TNF receptor families, CD28 and CD40 ligand (Nakae *et al.*, 2006). In addition, they may modulate the amplitude of the inflammatory response by secreting anti-inflammatory products, which promote homeostasis, for instance, by limiting endothelin-1-induced toxicity (Maurer *et al.*, 2004). MCs can also limit cutaneous responses to chronic UVB irradiation via IL-10 secretion and enhance

resistance to snake or honeybee venoms possibly by protease degradation (Grimbaldeston *et al.*, 2007; Metz *et al.*, 2006). In humans, MCs have long been recognized as crucial effectors in T helper 2 (Th2) cell-dependent, IgE-associated allergic disorders, such as urticaria, angioedema, allergic rhinitis, atopic dermatitis, bronchospasm, and some food allergies. In this context, activated MCs release Th2 cytokines, namely IL-4, IL-5, IL-9, and IL-13 that polarize the immune reaction and lead to IgE production by B cells.

Several lines of evidence indicate that MCs may play a role in autoimmunity, affecting disorders like arthritis, multiple sclerosis, bullous pemphigoid, and Graves' ophthalmia. They help initiate rheumatoid arthritis (Lee *et al.*, 2002). W/W^v mice lacking MCs do not develop the rodent equivalent of this debilitating condition. In humans, an increased number of MCs are found in the synovial tissues and fluids of patients with rheumatoid arthritis and at the site of cartilage erosion, reflecting the presence of MC chemotactic or survival factors, such as SCF and transforming growth factor (TGF)-β, in the synovial fluid (Olsson *et al.*, 2001). The invading MCs show ultrastructural signs of cell degranulation and produce several inflammatory mediators; notably TNF-α, IL-1β, and vascular endothelial growth factor (VEGF). TNF-α reportedly plays a pivotal role in the pathogenesis of rheumatoid arthritis, especially in its ability to regulate IL-1β expression, this being important for the induction of prostanoid and matrix metalloproteinase (MMP) production by synovial fibroblasts and chondrocytes. In addition, MCs promote leakage of fluids into the joints, which in turn allows penetration of self-targeted antibodies that might lead to tissue damage by activating the complement cascade (Nigrovic and Lee, 2007). Growing evidence suggests that MCs play a crucial role in the inflammatory process and subsequent demyelinization observed in patients suffering from multiple sclerosis. Indeed, recent results from animal models with experimental autoimmune encephalomyelitis (EAE) clearly indicate that these cells act at multiple levels to influence both the induction and the severity of the disease, possibly by enhancing Th1 cell response through secretion of IL-4 (Christy and Brown, 2007; Gregory *et al.*, 2006). Bullous pemphigoid is another human disease, whereby MCs have been proposed to exert a relevant pathogenic role. This autoimmune skin disease is characterized by subepidermal blisters resulting from autoantibodies against two hemidesmosomal antigens, BP230 and BP180. Intradermal injection of antibodies against BP180 into neonatal mice causes a blistering disease mimicking bullous pemphigoid. Injection of antibodies against BP180 into MC-lacking W/W^v mice does not induce bullous pemphigoid, nor does the injection into wild-type mice pretreated with the MC stabilizer cromolyn sodium induce it (Chen *et al.*, 2002). Interstitial cystitis has gained increasing attention for an involvement of MC in its pathogenesis. Indeed, the presence of activated MCs in close proximity to suburothelial nerves is a consistent feature of this yet-to-be-clarified urological pathology (Elbadawi, 1997).

There are also indications that MCs may be implicated in immunological tolerance. MCs indeed serve as enforcers for regulatory T cells, turning down the immune system's reaction to skin allograft possibly by IL-10 secretion (Lu *et al.*, 2006).

2.2. Nonimmunological functions

There is now emerging evidence that MCs exert relevant functions in tissue homeostasis, remodeling, repair, and fibrosis (Artuc *et al.*, 1999, 2002; Dvorak and Kissel, 1991; Gruber *et al.*, 1997; Metcalfe *et al.*, 1997; Weller *et al.*, 2006). These functions are accomplished by a direct MC stimulation of specific connective tissue cell types, in particular fibroblasts, and by the release or activation of a series of extracellular matrix (ECM)-degrading enzymes. The presence of MCs in connective tissues has been linked to the development of fibrosis through the production of mediators, cytokines, proteases and growth factors, such as histamine, heparin, tryptase, fibroblast growth factor (FGF)-2, TNF-α, and TGF-β, which stimulate the proliferation of myofibroblasts and fibrosis. TGF-β, exerts a variety of effects on wound repair including the induction and/or facilitation of directed cell migration, angiogenesis, and granulation tissue formation. In addition, TGF-β exerts a potent chemotactic effect on MCs (Gruber *et al.*, 1994). MCs are capable of both responding to and producing TGF-β. Moreover, latent TGF-β bound to ECM can be released but not activated by MC-derived chymase (Taipale *et al.*, 1995). During the process of wounding, MC granules released into the tissue are phagocytozed by fibroblasts and endothelial cells and may thus contribute to persistently increased tissue histamine levels (Seibold *et al.*, 1990). MCs release platelet-derived growth factor (PDGF) into wounded tissue and thereby influence the healing process from very early stages onward. Tryptases and FGF-2 are potent activators of fibroblast migration and proliferation (Artuc *et al.*, 2002; Rouss *et al.*, 1991). In an *in vivo* model of wound healing, an increased number of MCs, positively stained for FGF-2, were detected during the fibroproliferative stage (Liebler *et al.*, 1997). Of particular interest is the observation that tryptases can stimulate the synthesis and release of collagen from fibroblasts in culture, as well as provoke secretion of collagenase (Cairns and Walls, 1997). Moreover, tryptases cleave fibronectin and type VI collagen. In addition, this class of molecules has the ability to activate the preenzyme forms of some MMP and urinary plasminogen activators (uPA). Indeed, these enzymes have been implied to have a major role in tissue degradation. The ability of tryptases to induce the proliferation of airway smooth muscle cells could be of relevance in conditions such as bronchial asthma, in which smooth-muscle cell hyperplasia is a feature (Thabrew *et al.*, 1996). Tryptases may also have a role in tissue repair processes as a growth factor for epithelial cells. Chymases may contribute to the role of MCs in tissue remodeling by

cleaving type IV collagen and by splitting the dermal–epidermal junction. The production of type VIII collagen by human MCs *in vivo* may influence repair processes as this collagen is believed to facilitate the assembly of endothelial cells and tubes, and its synthesis precedes that of procollagen type I. In addition, MCs contain nerve growth factor (NGF) which actively stimulates neurogenesis after injury. Indeed, in the rat intestinal mucosa, reconstitution of nerve fibers, after experimentally induced inflammation and nerve fiber degeneration, is accompanied by a significant increase in mucosal MC density (Stead *et al.*, 1991). In addition, MCs and MC-derived TNF can promote elongation of cutaneous nerves during contact hypersensitivity in mice (Kakurai *et al.*, 2006).

MC involvement in the pathogenesis of coronary spasm, cardiomyopathy, atherosclerosis, and myocardial ischemia has been suggested. It has been shown that chymase cleaves angiotensin I to angiotensin II more effectively than the angiotensin-converting enzyme (Church and Levi-Schaffer, 1997). Studies in the canine model of myocardial ischemia and reperfusion indicate a role for MC mediators in initiating the cytokine cascade which is ultimately responsible for neutrophil accumulation in the ischemic area. In addition, MCs have been claimed to play a crucial role in leading to the subsequent fibrotic process (Frangogiannis *et al.*, 1998). By using C57BL/6-*Kit* $^{W\text{-}sh/W\text{-}sh}$ mice crossed with atherosclerosis-prone mice deficient in low-density lipoprotein receptor, *in vivo* evidence has been obtained for the implication of MCs in the atherogenic process, as the absence of MCs causes smaller atherosclerotic lesions with fewer inflammatory cell infiltrates (Sun *et al.*, 2007a). MCs may also contribute to the pathogenesis of elastase-induced abdominal aortic aneurysms in mice, as C57BL/6-*Kit* $^{W\text{-}sh/W\text{-}sh}$ mice fail to develop such aneurysms (Sun *et al.*, 2007b).

3. Mast Cell Development, Anatomical Distribution, and Phenotype

MCs develop, like other leukocytes, from hematopoietic stem cells but do not mature before exiting the bone marrow and circulate as committed progenitors (Gurish and Austen, 2001). Kitamura *et al.* (1977) first showed that MCs are derived from bone marrow precursors. Using the abnormal giant cellular granules of beige mice (C57BL-Bg^{J}/Bg^{J}) as a traceable marker, they found that tissue MCs developed from grafted beige bone marrow in irradiated wild-type recipient mice. In humans, MCs are derived from CD34^{+}, CD13^{+}, FcεRI^{-}, KIT^{+} committed progenitors (Kirschenbaum *et al.*, 1991). Committed progenitors, circulating as mononuclear agranular-cells, traverse the vascular space and complete their maturation after moving into diverse peripheral tissues (Rodewald *et al.*, 1996). Here, they

acquire concomitant phenotypic diversity. Recently, a cell population ($Lin^{-}Kit^{+}Fc\gamma RII/III^{hi}\beta7^{hi}$) has been identified in the mouse spleen with the characteristics of a bipotent progenitor for the basophil and MC lineages (Arinobu *et al.*, 2005). This cell population, termed basophil/MC common progenitor (BMCP), can be generated mainly from granulocyte/macrophage progenitors in the bone marrow.

Tissue homing as well as local differentiation and maturation of MC precursors are critically regulated by tissue microenvironmental factors, in particular the SCF secreted by fibroblasts, stromal cells and endothelial cells, which represents the most important cytokine involved in human and rodent MC development (Ashman, 1999). Under various experimental conditions, SCF is chemotactic for MCs and their progenitors. For example, local treatment of mice with SCF can induce marked local increase in MC number, reflecting both enhanced recruitment/retention and/or maturation of MC precursors, and proliferation of more mature MCs (Tsai *et al.*, 1991). The importance of SCF as a MC growth factor is underlined by the fact that mice with certain loss-of-function mutations affecting either SCF or its receptor KIT are devoid of MCs. Indeed, lack of expression of a functional KIT receptor because of spontaneous mutation in both copies of *Kit*, as it occurs in genetically MC-deficient WBB6F1-Kit^{W}-$Kit^{W\text{-}v}$ mice (W/W^{v} mice), results in a virtual absence of tissue MCs (Kitamura *et al.*, 1978). Remarkably, MCs develop in W/W^{v} mice if these mice receive bone marrow cells from normal littermates. Integrins are also involved in the process of recruitment of MC precursors into inflamed mucosal tissues. Human MC progenitors express $\alpha4\beta1$ integrin, which regulates their adhesion to activated endothelial cells (Boyce *et al.*, 2002). Inflammation-induced recruitment of human MC progenitors to the lungs requires both $\alpha4\beta7$ and $\alpha4\beta1$ binding to ''vascular cell adhesion molecule-1'' (VCAM-1), implicating organ-specific control of MC progenitor influx (Gurish and Boyce, 2006). In the mouse, large numbers of MC-committed progenitors are constitutively recruited in the small intestine by a mechanism involving the $\alpha4\beta7$ integrin (Gurish and Austen, 2001). This integrin expressed on the surface of MC progenitors binds to the ''mucosal address in cell adhesion molecule-1'' (MAdCAM-1) and to VCAM-1 as endothelial counterligands for this integrin. In addition, chemokine receptors expressed by MC progenitors are most likely involved in directing the progenitors from the circulation into the tissues where they mature. Human MC progenitors derived *in vitro* from cord blood express a set of chemokine receptors including CXCR2, CCR3, CXCR4, and CCR5, and respond to the corresponding ligands *in vitro* (Ochi *et al.*, 1999).

SCF not only drives MC homing, proliferation and differentiation but also MC survival, migration and functional activation. In rodents, IL-3 plays an additional fundamental role in MC development (Lantz *et al.*, 1998). By contrast, the role of IL-3 in the development of human MC is controversial

(Saito, 2005). Other cytokines and growth factors which regulate MC development and differentiation include IL-4, IL-9, IL-10, TGF-β, and NGF (Okayama and Kawakami, 2006). Committed progenitors are supposed to populate peripheral tissues, functioning as a local reservoir. These undifferentiated but committed progenitors do not develop into mature MCs unless adequate inflammatory stimuli ensue. In the adult mouse, for instance, it has been shown that the mucosa of the intestine contains the largest peripheral pool of these committed progenitors (Guy-Grand *et al.*, 1984). Mature MCs can be very long-lived cells, surviving in some cases for years, and can retain their ability to proliferate under certain conditions (Galli and Lantz, 1999; Féger *et al.*, 2002).

Thus, MCs acquire their mature phenotype locally in the tissues where they ultimately reside. MCs are found in almost all the major organs and tissues of the body, particularly in association with connective tissue structures such as blood vessels, lymphatic vessels, and nerves, and in proximity to surfaces that interface the external environment such as those of the respiratory, gastrointestinal, and urogenital system, as well as the skin. This selective accumulation at tissue sites where foreign material attempts to invade the host suggests that MCs are among the first cells to initiate defence mechanisms. MCs are not found in avascular tissues such as mineralized bone, cartilage and the cornea. In the human body, MCs collectively comprise a substantial cell population. It has been estimated that if all human tissue MCs were amassed together in a single organ, it would equal the size of a normal spleen (Sayed *et al.*, 2008). This indicates a strong selective pressure in maintaining this cell type throughout the evolutionary scale.

On the basis of their specific staining characteristics, preferential tissue homing, contents of proteases, and reactivity, two morphologically distinct subpopulations of rodent MCs were initially identified as connective tissue MCs CTMCs and mucosal MCs MMCs (Table 4.1) (Enerbäck, 1986). CTMCs can be distinguished from MMCs by staining with safranin because of the presence of large amounts of heparin in the secretory granules. Morphological, biochemical, and functional heterogeneity has been found in human MCs also. Human MCs are conventionally divided into two types depending on the expression of different proteases in their granules and other functional features (Irani *et al.*, 1986). MCs, which contain tryptase only, are designated as MC_T or ''immune cell associated'' MCs. They are predominantly located in the respiratory and intestinal mucosa, where they colocalize around T lymphocytes. MCs that contain both tryptase and chymase, along with other proteases such as carboxypeptidase A and cathepsin G, are referred to as MC_{TC}. They are predominantly found in connective tissue areas, such as skin, submucosa of stomach and intestine, breast parenchyma, myocardium, lymph nodes, conjunctiva, and synovium. These two subsets of human MCs differ also in terms of their mediator

Table 4.1 Characteristics of the two major human mast cell phenotypes

Mast cells	Location	Major enzymes	Major arachidonic acid metabolites	Major cytokines
Connective tissue mast cells (CTMC)				
	Submucosa of gastrointestinal tract and skin	Tryptase, chymase, carboxypeptidase	LTC_4 (↓), PG-D2	
	IgE-dependent and independent activation			IL-5 (↓), IL-6 (↓), IL-3, IL-4, IL-7, IL-8, IL10, IL-13, IL-16, TNF-α, SCF
Mucosal mast cells (MMC)				
	Mucosa of gastrointestinal tract, respiratory tract		LTC_4, PG-D2 (↓)	
	IgE-dependent and independent activation	Tryptase		IL-4(↓), IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-16, TNF-α, GM-CSF, SCF, TGF-β

content and reactivity. A third type of MC, called MC_C has been identified. This MC expresses chymase without tryptase and resides mainly in the submucosa and mucosa of the stomach, small intestinal submucosa, and colonic mucosa (Irani and Schwartz, 1994). Interestingly, human MC_T most closely correspond to murine MMCs, whereas MC_{TC} resemble murine CTMCs. MCs from different anatomical sites are able to generate distinct profiles of cytokine expression and different responses to secretagogues. Indeed, MCs from skin seem to be very sensitive to stimulation with substance P, compound 48/80, and morphine, all leading to histamine release, whereas heart and lung MCs only react to compound 48/80 (Fureder *et al.*, 1995). Intestinal MCs do not react to compound 48/80, whereas uterine MCs fail to respond to substance P (Shanahan *et al.*, 1985).

4. Mast Cell-Derived Secretory Products

When MCs are activated, they extrude granule-associated substances, such as histamine, immediately and within minutes, generate lipid-derived mediators (Table 4.2) (Galli *et al.*, 2005a). MC activation is followed also, within hours, by the *de novo* synthesis of numerous cytokines and chemokines (Table 4.2).

Secretory granules of MCs contain crystalline complexes of preformed mediators ionically bound to a matrix of proteoglycans. The metachromatic staining of MC granules probably reflects their content of proteoglycans, such as chondroitin sulfates and heparin. In the mouse, the proteoglycan content of MC granules varies in the different MC subtypes. CTMCs contain heparin that lacks in MMCs. Conversely, MMCs express chondroitin sulfates A and B, which are not found in CTMCs, whereas both MC subtypes store chondroitin sulfate E in their granules (Féger *et al.*, 2002). The dominant proteoglycan in human MCs is heparin, which constitutes

Table 4.2 Major human mast cell-derived mediators

Preformed mediators	Histamine, serotonin, heparin, neutral proteases (tryptase and chymase, carboxypeptidase, cathepsin G), major basic protein, acid hydrolases, peroxidase, phospholypases
Lipid mediators	LTB_4, LTC_4, PGE_2, PGD_2, PAF
Cytokines	TNF-α, TGF-β, IFN-α, IL-1α, IL-1β, IL-5, IL-6, IL-13, IL-16, IL-18
Chemokines	IL-8, I-309, MCP-1, MIP-1αS, MIP1β, MCP-3, RANTES, eotaxin
Growth factors	SCF, M-CSF, GM-CSF, FGF-2, VEGF, NGF, PDGF

some 75% of the total, with a mixture of chondroitin sulfates making up the remainder (Church and Levi-Schaffer, 1997). In humans, the heparin content in MC_T and MC_{TC} is roughly the same. Chondroitin sulfate and heparin proteoglycans are thought to bind histamine, neutral proteases, and carboxypeptidases primarily by ionic interactions and therefore contribute to the packaging and storage of these molecules in the granules. Mice that lack the enzyme *N*-deacetylase/*N*-sulfotransferase-2 (NDST-2) and are unable to produce fully sulfated heparin, exhibit severe defects in the granule structure of MCs, with impaired storage of certain proteases and reduced content of histamine (Forsberg *et al.*, 1999; Humphries *et al.*, 1999). During degranulation, the various mediators packaged with proteoglycans dissociate at different rates, histamine very rapidly but tryptase and chymase much more slowly.

Histamine, the first discovered mediator in MCs, is present at a concentration of 1–4 pg/cell in human MCs. Histamine exerts many effects pertinent to the immediate phase of allergic response, including vasodilation, increased vasopermeability, contraction of bronchial and intestinal smooth muscle cells, and increased mucous production. Histamine mediates its action via the histamine receptors 1–3, which can transduce signals leading to the variety of symptoms associated with acute allergic reactions (Galli *et al.*, 2008a,b).

MCs are a rich reservoir of neutral proteases. The major MC protease is tryptase, a 130 kd serine protease, which is stored in a fully active form in the granule. It represents the most abundant constituent of human MCs. Some 10 pg/cell has been detected in MCs in the lung and up to 35 pg/cell in skin MCs (Schwartz *et al.*, 1987). Tryptase cleaves various bronchial and intestinal neuropeptides and matrix components, as well as IgE molecules, thereby possibly down regulating the allergic response (Rauter *et al.*, 2008). In addition, it is emerging as a potent growth factor for fibroblasts, endothelial cells, and muscle cells (Blair *et al.*, 1997; Gruber *et al.*, 1997). Chymase, a 30 kD protease, is present within the granules of the MC_{TC} subset of MCs, in an estimated concentration of 4.5 pg/cell (Metcalfe *et al.*, 1997; Schwartz *et al.*, 1987). Like tryptase, it degrades some neuropeptides and interleukins, and cleaves collagen and other ECM components (Huang *et al.*, 1998). In the mouse, at least five different granule-associated chymases (mMCP-1, mMCP-2, MMCP-3, MMCP-4, MMCP-5) and three different granule-associated tryptases (mMCP-6, mMCP-7, mMMP-11/transmembrane tryptase [mTMT]) have been described at the protein level (Huang *et al.*, 1998). There appear to be multiple forms of human tryptases as well (tryptases α, I, II/β, III) (Miller *et al.*, 1989, 1990; Vanderslice *et al.*, 1990). Two other proteases, carboxypeptidase and cathepsin G, have been associated with the MC_{TC} subset of human MCs. Interestingly, the specific protease content of individual MCs can vary depending on the MC microenvironment. For example, MMCs in mice express mMCP-1 and mMCP-2, whereas CTMCs express a different pattern of proteases, namely mMCP-3, mMCP-4,

mMCP-5, mMCP-6, mMCP-7, and carboxypeptidase (Miller and Pemberton, 2002; Stevens *et al.*, 1993). MC proteases play an important role in innate host defence. MC tryptase mMCP-6, for instance, has a critical protective function in bacterial and parasite infection. mMCP-6-deficient mice are less able to clear *Klebsiella pneumoniae* injected into their peritoneal cavities, probably because of less recruitment of neutrophils (Thakurdas *et al.*, 2007). Delayed expulsion of the adult helminth and increased deposition of larvae in muscles occur in mMCP-1-deficient mice infected with *Trichinella spiralis* (Knight *et al.*, 2000). mMCP-6 as well is important for the clearance of the chronic *T. spiralis* infection (Shin *et al.*, 2008).

Preformed substances stored in the secretory granules can be released by two morphologically distinct secretory pathways, referred to as exocytosis (also called ''anaphylactic degranulation'') and piecemeal degranulation (or intragranular activation) (Fig. 4.2) (Dvorak, 2005). Exocytosis consists of a rapid and massive secretory process, which characteristically occurs during IgE-dependent hypersensitivity reactions. In exocytosis, cytoplasmic granule membranes fuse with each other and with the plasma membrane, giving rise to open secretory channels, which allow the release of granule contents into the local extracellular environment. Piecemeal degranulation, conversely, represents a particulate mode of MC secretion, characterized by a slow discharge of granule contents in a ''piecemeal'' fashion, without membrane fusion events and granule opening to the cell exterior. This degranulation pattern has frequently been observed in MCs infiltrating areas of chronic inflammation or tumors (Dvorak, 1991).

The most important MC-derived lipid mediators are cyclooxygenase and lipoxygenase metabolites of arachidonic acid (Galli *et al.*, 2005a). All these products have potent inflammatory activity and can also modulate the release process. The major cyclooxygenase product of MCs is prostanglandin D_2 (PGD_2), and the major lipooxygenase products derived from them are the sulfiodopeptide LTC_4: LTC_4 and its peptidolytic derivates LTD_4 and LTE_3. Human MCs can also produce LTB_4, although in much smaller quantities than PGD_2 or LCT_4, and some MC populations represent a source of platelet activating factor (PAF).

More than thirty different cytokines and chemokines have been shown to be produced by human and mouse MCs. MC secretory granules contain pools of stored TNF-α which has pleiotropic proinflammatory effects (Gordon and Galli, 1990, 1991). TNF-α has been implicated in neutrophil recruitment, inducing upregulation of the endothelial-leukocyte adhesion molecule (ELAM-1) (Walsh *et al.*, 1991). TNF-α has also been known to enhance the bactericidal activities of neutrophils (Kenny *et al.*, 1993). Certain MC populations may also have preformed as stores of VEGF (Boesiger *et al.*, 1998). In addition, human MCs have the capacity to generate IL-8, thereby contributing to neutrophil recruitment (Moller *et al.*, 1993). Under allergic conditions MCs produce significant amounts of IL-1 that

may contribute to lymphatic infiltration (Bochner *et al.*, 1990) and IL-4, essential for the triggering of Th2 lymphocytes that themselves produce IL-4 to initiate inflammatory cell accumulation and B lymphocyte immunoglobulin class switching to IgE (Bradding *et al.*, 1993). Other cytokines involved in MCs found in normal and in asthmatic airways are IL-5 and IL-6 which, together with IL-4 and IL-13, would enhance Th2-type immune response and eosinophil chemotaxis, thereby indicating that MCs may play an important role in initiating and maintaining the inflammatory response in asthma (Bradding *et al.*, 1995). Interestingly, a unique profile of cytokines is induced depending upon the nature of the stimulus or type of infection. Human intestinal MCs have been shown to spontaneously produce proinflammatory cytokines such as TNF-α, IL-6, and IL-8 at low levels without stimulation of the cell (Lorentz and Bischoff, 2001). Stimulation by IgE receptor cross-linking leads to an enhanced production of proinflammatory cytokines and *de novo* production of Th2 cytokines such as IL-3, IL-5, IL-10, and IL-13. Gram-negative bacteria, in contrast to IgE receptor cross-linking, do not induce the release of Th2 cytokines but enhance that of proinflammatory cytokines. Interestingly, MCs from different anatomical sites are able to generate distinct profiles of cytokine expression. In the tissues of bronchial and nasal mucosae from normal, asthmatic, and allergic rhinitis patients, MC_T release IL-5, IL-6, and some IL-4, whereas MC_{TC} preferentially express IL-4 but little IL-5 and IL-6. A similar predominant IL-4 pattern is recognizable in skin MCs which contain both tryptase and chymase (MC_{TC}). Such differences in the distribution of cytokine expression between subsets of MCs suggest a difference in the capacity of MC subsets to produce various cytokines and therefore a difference in their specific roles in allergic inflammation.

MCs have been shown to express an important set of chemokines, which influence recruitment of dendritic cells, lymphocytes, other inflammatory cells, and tissue resident cells at sites of tissue inflammation, as well as migration of dendritic cells to lymph nodes. Cross-linking of FcεRI on MCs induces the release of the CCL1 chemokine, which acts to recruit Langerhans-type dendritic cells to sites of atopic skin inflammation (Gombert *et al.*, 2005). MCs also express CCL19, the ligand for CCR7, a chemokine receptor required for dendritic cell migration (Humrich *et al.*, 2006). Other products of MC activation, including CCL5 (RANTES) and TNF-α, can promote dendritic cell (DC) migration (Yamazaki *et al.*, 1998). MCs also appear to orchestrate the migration of T cells. Upon activation, MCs express chemoattractants such as LTB_4, IL-16, XCL1 (also called lymphotactin), CCL3 (also called MIP-1α), CCL2 (mMCP-1), CCL5, CCXCL10, CCL19, and CCL21 (Galli *et al.*, 2005b; Sayed *et al.*, 2008). MC-derived LTB_4 is essential for the recruitment of both $CD4^+$ and $CD8^+$ effector cells to sites of inflammation (Ott *et al.*, 2003). CXCL8 (IL-8) is another MC-derived chemokine which exerts basic functions on inflammatory cell recruitment and endothelial cell activation.

5. Mast Cells in Experimental Carcinogenesis

The introduction of novel experimental procedures to induce carcinogenesis in laboratory animals has contributed to increase our understanding of the role of MCs in early tumor growth and angiogenesis. Development of squamous cell carcinoma in a HPV (human papilloma virus) 16-infected transgenic mouse model of epithelial carcinogenesis provided experimental evidence in favor of an early participation of MCs in tumor growth and angiogenesis (Coussens *et al.*, 1999). Accumulation of MCs increased at the hyperplastic and dysplastic stages and in the invasive front of tumor growth, with a typical localization around capillaries and epithelial basement membrane, where MCs degranulated releasing MC-specific proteases tryptase and chymase. On the contrary, MCs were absent within the tumor mass itself. Infiltration of MCs and activation of MMP-9 coincided with the angiogenic switch in premalignant lesions and addition of chymase alone was sufficient to stimulate an angiogenic phenotype when coincubated with a hyperplastic skin sample *in vitro*, while tryptase plays a role in tissue remodeling.

Gounaris *et al.* (2007) developed a transgenic mouse model overexpressing an inducible, stabilized β-catenin in intestinal enterocytes, as well as the $APC^{\Delta 468}$, as an independent hereditary model of polyposis. In both mice, developing polyps were rapidly infiltrated by $CD34^{+}$ MCs and staining confirmed the presence of both connective and mucosal types of MCs in developing tumors. The latter type was more prevalent at the site of newly developing polyps, while the former was more prevalent in the stroma at later stages of tumor development. Treatment with anti-TNF-α antibodies resulted in fewer polyps, reduced polyp growth, and decreased MC infiltration. Reconstitution of W^{sh}/W^{sh} MC-deficient mice with $CD34^{-/-}$ $CD43^{-/-}$ bone marrow also resulted in a decrease in polyp growth, as compared to that in mice reconstituted with wild-type bone marrow. Overall, these data suggest that MCs are an essential component for preneoplastic polyp development, as well as a possible target for therapeutic intervention.

In a pancreatic β-cell tumor model, activation of Myc *in vivo* has been shown to induce several chemokines in developing tumors, including CCL2 and CCL5. The latter triggers rapid recruitment of MCs to the tumor site, but not into the islets themselves, required for macroscopic tumor expansion (Soucek *et al.*, 2007). MCs were the only inflammatory cells increased in the vicinity of tumor cells at the early stage of tumor growth and their infiltration correlated with the expansion of islet tumors. Macrophages and neutrophils are also recruited after the onset of tumor angiogenesis. Treatment of established β-cell tumors with disodium cromoglycate (cromolyn), an inhibitor of MC degranulation, rapidly triggers hypoxia, and cell death of

tumor and endothelial cells. The authors conclude that MCs are necessary for tumor angiogenesis and growth and suggest that inhibition of MC function may prove to be therapeutically useful in restraining the growth of pancreatic cancer.

Angiopoietin-1 (Ang-1) secreted by primary murine MCs promotes marked neovascularization in an *in vivo* transplantation assay in nude mice (Nakayama *et al.*, 2004). The MCs studied were bone marrow mast cells (BMMCs) from c57BL/6 mice and two mouse plasmocytoma cell lines. A greater angiogenesis was demonstrated when BMMCs were transplanted into mice together with plasmocytoma cells than when each of the cell types was transplanted alone. The use of Ang-1-neutralizing antibodies reduced significantly the growth of plasmocytoma containing MCs. Overall, these data demonstrated that MC-derived Ang-1 promotes growth of plasmocytomas by stimulating neovascularization.

Huang *et al.* (2008) demonstrated that tumor-derived SCF recruits MCs to the tumor environment and also activates them. Using a SCF-knock-down H22 tumor model, tumor growth was reduced and this correlated with a reduction in MC accumulation in the developing tumor. SCF-stimulated MCs released active MMP-9 into the local environment, disrupting extracellular matrix and releasing further matrix-bound SCF, acting as a positive feed-back loop on MC activation within the tumor. Activation by high levels of SCF *in vitro* led to upregulation of proinflammatory genes, such as IL-6, TNF-α, and VEGF, which could be inhibited by antibodies against the SCF receptor, KIT.

Hart *et al.* (2002), using the W/W^v mice, demonstrated a direct correlation between MC density in the dermis and susceptibility to ultraviolet-B-induced systemic immunosuppression. These mice which are homozygous for the W (white spotting) mutation, and therefore severely MC deficient, are unresponsive to ultraviolet-induced immunosuppression unless first injected with MC precursors at the irradiated site (Hart *et al.*, 2002). The W locus encoded the KIT tyrosine kinase receptor that binds SCF (Church and Levi-Schaffer, 1997). The MC products involved in ultraviolet-induced immunosuppression are believed to be TNF-α and histamine (Hart *et al.*, 2001).

6. Mast Cells and Tumor Growth in Humans: Pro and Contra

MCs accumulate at sites of tumor growth in response to numerous chemoattractants (Conti *et al.*, 1997). MCs have a vast array of mediators, some of which have promoting, and others inhibitory, effects on malignancies (Fig. 4.3) (Theoharides and Conti, 2004). The phenotypic expression of MCs is not static, its secretory pattern varies according to the

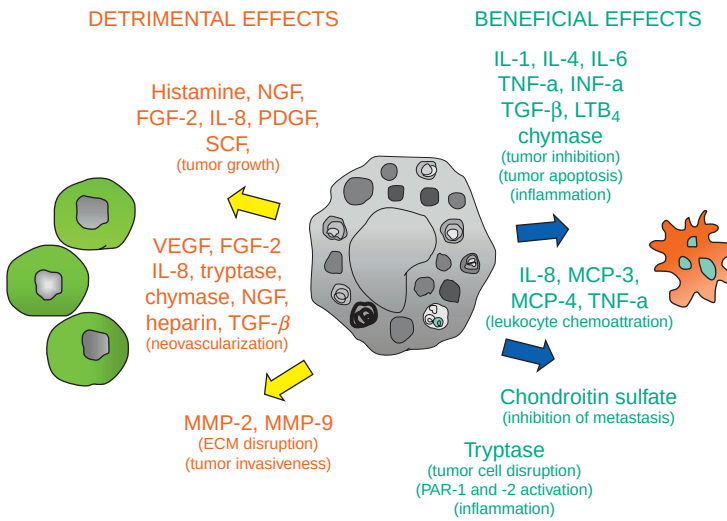

Figure 4.3 The potential dual role of mast cells in tumor fate. Mast cells may exert detrimental effects to the host by releasing cytokines and growth factors, such as FGF-2, NGF, PDGF, SCF, VEGF and IL-8, which stimulate tumor cell expansion. Mast cells are a major source of histamine, which can induce tumor cell proliferation through H1 receptors, while suppressing the immune system through H2 receptors. Mast cells produce several angiogenic factors, as well as matrix metalloproteinases, which promote tumor vascularization and tumor invasiveness, respectively. By contrast, mast cells may promote inhibition of tumor cell growth, tumor cell apoptosis and inflammation by releasing cytokines such as IL-1, IL-4, IL-6, and TNF-α. TNF-α, in particular, is very effective in leukocyte chemoattraction. Chondroitin sulfate may inhibit tumor cell diffusion and tryptase causes both tumor cell disruption and inflammation through activation of protease-activated receptors (PAR-1 and -2). Abbreviations: FGF-2, fibroblast growth factor-2; NGF, nerve growth factor; PDGF, platelet-derived growth factor; SCF, stem cell factor; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; MMP-2, MMP-9, matrix metalloproteinase-2, -9; IL-1, IL-2, IL-6; IL-8, interleukin-1, -2, -6, -8; ECM, extracellular matrix INF-α, interferon-α; LTB_4, leukotriene B4; MCP-3, MCP-4, mast cell protease-3, -4.

microenvironment, and MCs have the ability to secrete individual granules or distinct mediators selectively. Secretion of cytokines from MCs could occur without degranulation. This has been termed "differential release," "intragranular activation," or "piecemeal degranulation," and may be associated with the ability of MCs to release some mediators selectively without degranulation (Theoharides *et al.*, 2007).

Dabbous *et al.* (1986) showed that MC degranulation is associated with disruption and lysis of the tumor extracellular matrix, either directly through the action of their enzymes or indirectly through modulation of the collagenolytic activity of fibroblasts, macrophages, and tumor cells (Barom *et al.*, 2001; Stack and Johnson, 1994). Tryptase activates latent MMPs and plasminogen activator, which, in turn, degrade the extracellular matrix (Stack and

Johnson, 1994). MC chymase was demonstrated to cause apoptosis in different target cells (Hara *et al.*, 1999) and to induce the accumulation of tumor associated macrophages, neutrophils, and other inflammatory cells *in vivo* (He and Walls, 1998).

Heparin enhances both the activity and production of collagenase *in vitro* and releases plasminogen activator from endothelial cells (Markwardt and Klocking, 1977; Sakamoto *et al.*, 1973). MCs are the major source of histamine, which mediates many symptoms of allergic reactions. Histamine can induce tumor cell proliferation through H1 receptors identified in human malignant carcinoma, while suppressing the immune system through H2 receptors (Niwa *et al.*, 1991). High histamine concentration inhibits human primary melanoma-cell proliferation, presumably by acting through H1 receptors, an action enhanced by IL-2, whereas low amounts of it through H2 receptors increase proliferation (Lazar-Molnar *et al.*, 2002). It has been reported that the incidence of metastases, as well as the appearance of tumors, correlates inversely with tissue histamine level (Burtin *et al.*, 1985).

MCs exert direct mitogenic effect on tumor cells. For example, FGF-2 and IL-8 are directly mitogenic to melanocytes and melanoma cells (Halaban *et al.*, 1988; Schadendorf *et al.*, 1993). Recently, we have investigated the pattern of distribution of MCs in biopsy samples obtained from four different human tumors, utilizing an image analysis system and a mathematical model to make a quantitative approach to characterizing their spatial distribution (Guidolin *et al.*, 2008). In all tumors, MCs demonstrated a virtually random spatial distribution, albeit with varying densities, suggesting that MC–MC interactions could play a minor role in the formation of the MC pattern in neoplastic tissues. The random distribution of the cells in the tissues could be accounted for by a random walk migration under the influence of cell–matrix interactions or chemotactic fields potentially generated by tumor or endothelial cells.

Tumors of MCs are extremely rare, but certainly not as rare as commonly thought. The most common tumors of MCs are those confined to the skin and manifest in childhood (Lennert and Parwaresch, 1979). The group of the cutaneous mastocytosis consists of two types. The most common is designated urticaria pigmentosa and represents a disseminated involvement of the skin, while the localyzed type is made of solitary or multiple nodules and is called benign mastocytoma (Lennert and Parwaresch, 1979). The clinical course in cutaneous mastocytosis is benign, in many cases skin lesions disappear during puberty (Caplan, 1963).

Systemic mastocytosis is usually diagnosed in adulthood and is characterized by multiorgan involvement (with or without skin lesions) and disease persistence (Lennert and Parwaresh, 1979). Indolent variants as well as aggressive variants of systemic mastocytosis have been reported (Lennert and Parwaresh, 1979). Patients with systemic mastocytosis may be diagnosed with an associated clonal hematologic non-MC lineage disease

(Travis *et al.*, 1988) and MC-leukemia is a rare subtype of systemic mastocytosis defined by circulating MCs and a rapidly deteriorating clinical course in most cases (Travis *et al.*, 1988).

Increased MC number has been correlated with a poor prognosis in several human tumors such as human melanoma (Ribatti *et al.*, 2003b), oral squamous carcinoma (Iamaroon *et al.*, 2003; Wanachantarak, 2003), and squamous cell carcinoma of the lip (Rojas *et al.*, 2005). Human tumors in which MC infiltration correlates with a poor prognosis include: melanoma, oral squamous carcinoma, Hodgkin's lymphoma, diffuse large B cell lymphoma, angioimmunoblastic T cell lymphoma, Waldenstrom's macroglobulinemia, and prostate cancer.

Sharma *et al.* (1992) reported a higher number of MCs in nodular sclerotic-type Hodgkin's lymphoma (HL) than in other types of HL and the number of MCs was higher in fibrotic areas than in cellular areas. Molin *et al.* (2002, 2004) observed a worse prognosis for a nodular sclerosing HL exhibiting a high MC number. Fukushima *et al.* (2006) demonstrated an increased number of tryptase-positive and chymase-positive MCs in fibrotic areas in diffuse large B cell lymphoma (DLBCL) lymph nodes. The greatest number of MCs among T cell lymphomas was observed in angioimmunoblastic T cell lymphoma (Fukushima *et al.*, 2001). Dave *et al.* (2004) utilized gene array to study the relationship between prognosis and a specific gene expression profile and concluded that the length of survival among patients with follicular lymphoma correlates with the molecular features of nonmalignant immune cells present within the tumor at the time of diagnosis. One of the genes observed to correlate most negatively with survival was that of microphthalmia-associated transcription factor (MIFT), a transcription factor which has been found to be highly expressed in MCs and to play a critical role in the regulation of several key MC-specific genes (Nechushtan and Razin, 2002). Tournilhac *et al.* (2006) demonstrated that MCs may support tumor cell expansion in Waldenstrom's macroglobulinemia through constitutive CD154-CD40 signaling. In detail, MCs expressed CD154, a potent inducer of malignant B cell proliferation, while bone marrow lymphoplasmacytic cells functionally expressed the CD154 receptor, CD40. Moreover, the use of CD154-CD40 signal inhibitor partially inhibited MC-mediated bone marrow lymphoplasmacytic proliferation and/or tumor colony formation.

Nonomura *et al.* (2007) demonstrated that in prostate cancer MC counts were higher around cancer foci in patients with higher Gleason scores than in those with low Gleason scores. The MC number correlated with clinical stage and multivariate analysis revealed that MC count was a significant prognostic factor.

Increased MC number has been correlated also with a good prognosis in several human tumors. Human tumors in which MC infiltration correlates with a good prognosis include: breast cancer, nonsmall-cell lung carcinoma,

and ovarian cancer. Aaltomaa *et al.* (1993) found a positive correlation between survival and increased MC number in a study of 187 breast cancer biopsies. Dabiri *et al.* (2004) analyzed the correlation between MC number in breast cancer and patients' prognosis in a study of 438 patients. They found a strong correlation between the presence of MCs and a favorable prognosis. Perivascular tumor-associated MCs in mammary adenocarcinoma could secrete several cytokines and proteolytic enzymes that could be detrimental to the tumor cells. For instance, IL-4, which binds to IL-4 receptors expressed by human breast carcinoma cells, could lead to apoptosis in breast cancer (Gooch *et al.*, 1998). The histamine content of human breast cancer tissue is much higher than adjacent normal tissue and act as a local immunosuppressant (Ohno *et al.*, 2002). Moreover, the mean level of serum tryptase in women with breast cancer is three times higher than that in healthy women (Samoszuck and Corwin, 2003). Kankkunen *et al.* (1997) observed that significant increase in MC counts in breast carcinoma versus benign lesions is due to tryptase-containing MCs. It was found that in benign lesions, the number of MCs exhibiting tryptase activity was similar to that of chymase-active MCs. However, malignant tumors had two to three times more tryptase-containing than chymase-containing MCs, while the tryptase activity was significantly higher than that in benign lesions. Moreover, in malignant lesions, tryptase-containing MCs were concentrated at the tumor edge, whereas chymase-containing MCs were not increased in this area. Breast cancer patients with metastases in the axillary nodes reveal greater numbers of MCs in all nodes examined compared with patients without metastasis (Thorense *et al.*, 1982).

Welsh *et al.* (2005) analyzed the presence of MCs in the tumor stroma of 175 patients with surgically resected nonsmall-cell lung carcinoma and demonstrated that both macrophage and MC infiltration of the tumor islets was associated with a marked increase in 5-year survival, independently of other favorable prognostic factors including stage. Chan *et al.* (2005) studied samples of ovarian cancer from 44 patients and demonstrated that tumors with higher microvascular density had a higher mean survival compared with low MC density or low microvessel density.

7. Mast Cells and Tumor Angiogenesis and Lymphangiogenesis

Inflammatory cells regulate endothelial cell functions related to physiological angiogenesis, as well as inflammatory and tumor-associated angiogenesis. It was Rudolf Virchow in 1863, who critically recognized the presence of inflammatory cells infiltrating neoplastic tissues and first

established a causative connection between the ''lymphoreticular infiltrate'' at sites of chronic inflammation and cancer.

In neoplastic tissues, inflammatory cells act in concert with tumor cells, stromal cells and endothelial cells to create a microenvironment, which is critical for the survival, development and diffusion of the neoplastic mass. These synergies may represent important mechanisms for tumor development and metastasis by providing efficient vascular supply and easy pathway to escape. Indeed, the most aggressive human cancers, such as malignant melanoma, breast carcinoma, and colorectal adenocarcinoma, are associated with a dramatic host response composed of various inflammatory cells, especially macrophages and MCs.

MCs also promote lymphangiogenesis. Endostatin, a proteolytic fragment of collagen XVIII, is a potent inhibitor of angiogenesis and tumor growth. In transgenic J4 mice, which overexpress endostatin in their keratinocytes, carcinogen-induced skin squamous cell carcinomas were less aggressive and more often well differentiated than those in the control mice, indicating that endostatin regulates the terminal differentiation of keratinocytes (Brideau *et al.*, 2007). Tumor angiogenesis was inhibited by endostatin at an early stage in skin tumor development. Inhibition of angiogenesis was accompanied by significant reduction of lymphatic vessels and lymph node metastasis. Remarkably, accumulation of tumor-infiltrating VEGF-C-positive MCs was markedly decreased in the tumors of the J4 mice. In addition, endostatin inhibited the adhesion and migration of murine MC/9 MCs on fibronectin *in vitro*. These data suggest that endostatin can inhibit tumor lymphangiogenesis by decreasing the VEGF-C levels in the tumors via inhibition of MC migration and adhesion.

7.1. Angiogenenic factors stored in mast cells

Angiogenic factors stored in MCs include: heparin, histamine, MMP-2, MMP-9, tryptase, chymase, FGF-2, VEGF, TGF-β, TNF-α, IL-8, and NGF.

Heparin, a granule-associated mediator, stimulates endothelial cell proliferation and migration *in vitro* (Alessandri *et al.*, 1984; Thorton *et al.*, 1983). *In vivo*, however, it stimulates (Norrby and Sorbo, 1992; Norrby, 1993; Ribatti *et al.*, 1987), inhibits (Jakobson and Hahnenberger, 1991; Norrby, 1993; Wilks *et al.*, 1991) or has no effect (Castellot *et al.*, 1982; Taylor and Folkman, 1982). These differences seem to be related to its molecular size (high-molecular-weight heparin has been shown to stimulate angiogenesis, whereas low-molecular-weight inhibits it) and degree of sulfation. Heparin acts as a soluble form of the low-affinity FGF-2 receptor (Folkman and Shing, 1992), which displaces FGF-2 in the biologically active-form and allows its rapid interaction with endothelial cells (Yayou *et al.*, 1991).

Histamine has an angiogenic effect through both H1 and H2 receptors (Sorbo *et al.*, 1994). It may also increase the permeability of newly formed

microvessels during tumor angiogenesis, and hence the leakage of plasma proteins and deposition of fibrin. Degradation products of fibrin are angiogenic *in vivo* (Thompson *et al.*, 1995).

Microvascular leakiness in tumors is also a consequence of a defective organization of microvascular endothelium. In fact, tumor endothelial cells do not form a normal monolayer and, therefore, do not have a normal barrier function (McDonald and Boluk, 2002). The cells are disorganized and irregularly shaped, overlap one another, or have luminal projections, and give rise to abluminal sprouts.

MCs synthesize and store large amounts of MMP-2, MMP-9, and serine-proteinases of two subclasses: tryptase and chymase (Di Girolamo and Wakefiled, 2000; Fang *et al.*, 1999; Vincent *et al.*, 2000). Given the ability of MMP-2 and MMP-9 to degrade type IV, V, VII, and X collagens, as well as fibronectin, (Raza and Cornelius, 2000), the major components of the interstitial stroma and subendothelial basement membrane, the findings suggest that MCs may contribute to the progression from *in situ* to invasive and metastatic solid tumors, characterized by an enhanced angiogenesis and secretion of proteolytic enzymes (Raza and Cornelius, 2000).

Tryptase and chymase are involved in angiogenesis after their release from activated MC granules (Fig. 4.4). Their proteolytic activities degrade extracellular matrix components or release matrix-associated growth factors (Taiplae *et al.*, 1995), and they also act indirectly by activating latent MMP (Gruber *et al.*, 1989) and plasminogen activators (Stack and Johnson, 1994). Blair *et al.* (1997) have demonstrated the angiogenic potential of tryptase *in vitro* and its important role in neovascularization. Tryptase added to microvascular endothelial cells cultured on Matrigel caused a pronounced increase in capillary growth, and this was suppressed by specific tryptase inhibitors. Moreover, tryptase directly induced endothelial cell proliferation in a dose-dependent fashion.

Muramatsu *et al.* (2000a,b) used the hamster sponge-implant model to show that angiogenesis is induced by angiotensin II and inhibited by

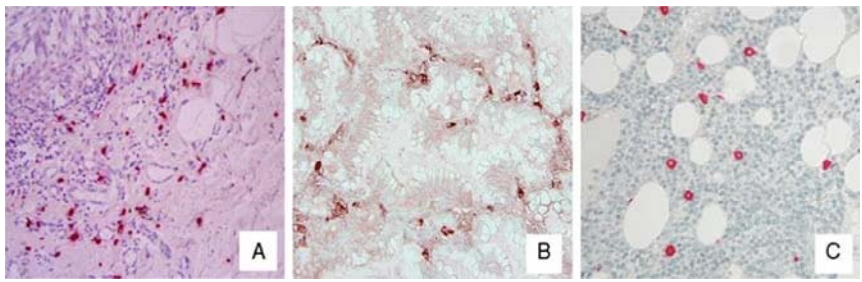

Figure 4.4 **Tryptase-positive mast cells in bioptic specimens of human melanoma (A), nonsmall-cell lung cancer (B) and B cell chronic lymphocytic leukemia (C). Original magnification: (A)–(C), ×250.**

chymase inhibitors, suggesting that MC-derived chymase is an important mediator of MC-dependent angiogenesis. MCs release several polypeptide growth factors, including FGF-2, VEGF, TGF-β, TNF-α, and IL-8. These cytokines are involved both in normal as well as tumor-associated angiogenesis. The spectrum of cytokines expressed appears to vary depending on the maturity state of the MCs and of the tissue of residence. Qu *et al.* (1998) demonstrated that FGF-2 is localized in the cytoplasmic and extruded granules of MCs in several human tissues. Grutzkau *et al.* (1998) demonstrated the expression of VEGF in the human MC line HMC-1 and in human skin MCs. NGF, also contained in MC secretory granules, induces endothelial cell proliferation *in vitro* and angiogenesis *in vivo* in the chick embryo chorioallantoic membrane (CAM) assay (Cantarella *et al.*, 2002; Emanueli *et al.*, 2002).

7.2. Mast cells in experimental tumor angiogenesis

Kessler *et al.* (1976) demonstrated that tumor angiogenesis factor (TAF) elicited a vasoproliferative response when implanted upon the CAM of the chick embryo. This response was first observed stereomicroscopically 2–3 days after implantation and a 40-fold increase in MC density was observed in the vicinity of the implants in 24 h. Poole and Zetter (1983) demonstrated that rat peritoneal MCs migrate in response to conditioned medium from several tumor cell lines. The active chemoattractant(s) in this conditioned medium appeared to be peptide(s) with a molecular weight of 300–1000. They proposed that the chemoattraction of MCs by tumor-derived peptides may be an important, early event in tumor neovascularization.

Starkey *et al.* (1988) investigated the role of host MCs in tumor-associated angiogenesis by comparing the angiogenic response of genetically MC-deficient W/W^v mice and MC-sufficient +/+ littermates to subcutaneously growing B16-BL6 tumors. The response was slower and initially less intense in W/W^v. Fewer W/W^v than +/+ mice developed spontaneous lung metastases. Bone marrow repair of the MC deficiency restored the incidence of hematogeneous metastases to approach that of +/+ mice. These results demonstrate a role for MCs *in vivo* during tumor angiogenesis and also suggest a role for host MCs in hematogeneous metastases.

We have demonstrated that isolated MCs and their secretory granules, but not degranulated MCs, induce an angiogenic response in the CAM assay (Fig. 4.5) (Ribatti *et al.*, 2001). Addition of anti-FGF-2 or anti-VEGF antibodies reduced the angiogenic response of both MCs and their secretory granules by 50% and 30%, respectively. These data support the evidence that the angiogenic properties of MCs depend on the angiogenic molecules contained in their secretory granules, and indicate that FGF-2 and VEGF are the angiogenic cytokines primarily and perhaps synergistically responsible for this vasoproliferative activity.

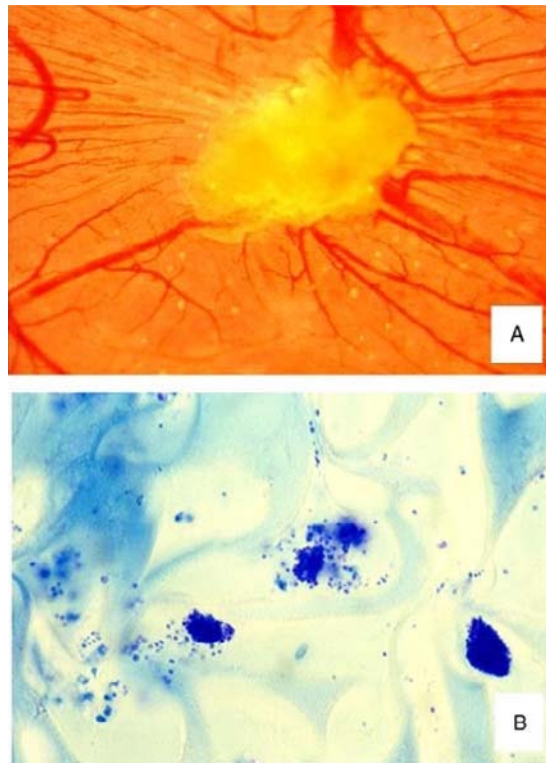

Figure 4.5 A mast cell suspension has been delivered on the top of the chick embryo chorioallantoic membrane on Day 8 of incubation using a gelatin sponge implant. Macroscopic observation on Day 12 shows the gelatin sponge surrounded by numerous allantoic vessels that develop radially towards the implant in a "spoked-wheel" pattern (A). The histological analysis shows among the sponge trabeculae metachromatic mast cells and their secretory granules (B). Original magnification: (A) ×50, (B) ×250 (adapted with modifications from Ribatti *et al.*, 2001).

7.3. Mast cells in human solid and hematological tumor angiogenesis

Solid human tumors, in which MC density correlates with microvascular density, include: human endometrial carcinoma, nonsmall-cell lung cancer, melanoma, squamous cell cancer of the oesophagus, and lymph nodes from patients with breast cancer.

Meanwhile, the following are hematological human tumors in which MC density correlates with microvascular density: B cell non-Hodgkin's lymphoma, Hodgkin's lymphoma, multiple myeloma, myelodysplastic syndromes and B cell chronic lymphocytic leukemia.

Tryptase-positive MCs increased in number and vascularization increased in a linear fashion from dysplasia to invasive cancer of the uterine cervix (Benitez-Bribiesca *et al.*, 2001). We have demonstrated that angiogenesis in human endometrial carcinoma is highly correlated with MC tryptase-positive cell counts and that these parameters increase in agreement with tumor progression (Ribatti *et al.*, 2005).

An association of VEGF and MCs with angiogenesis has been demonstrated in laryngeal carcinoma (Sawatsubashi *et al.*, 2000), in nonsmall-cell lung carcinoma, where most intratumoral MCs express VEGF (Imada *et al.*, 2000; Takanami *et al.*, 2000; Tomita *et al.*, 2000), and in melanoma, where MCs express both VEGF (Toth *et al.*, 2000) and FGF-2 (Ribatti *et al.*, 2003a). Lastly, a prognostic significance has been attributed to MCs and microvascular density in squamous cell cancer of the oesophagus (Elpek *et al.*, 2001) and in melanoma (Ribatti *et al.*, 2003b). Imada *et al.* (2000) studied 85 cases of stage I nonsmall-cell lung carcinoma and demonstrated a higher number of tryptase-positive MCs compared to cases of squamous cell carcinoma. A high correlation was observed between intratumoral MC counts and microvessel counts and double staining showed that most intratumoral MCs expressed VEGF.

In melanoma, MC accumulation around the margin of tumors has been observed to peak just as the tumor acquire the angiogenic phenotype and peritumoral MC counts correlated strongly with microvascular density, melanoma progression, and prognosis (Toth *et al.*, 2003a,b; Ribatti *et al.*, 2003a). Furthermore, in melanoma, MCs were closer to one another and to the vessels (Guidolin *et al.*, 2006). This close association between MCs and the endothelium might indicate that MCs are involved in the maintenance reaction necessary for the long lasting functional integrity of the endothelium. We have demonstrated that angiogenesis increases in parallel to the number of tryptase-positive MCs in lymph nodes from patients with breast cancer and that their values are significantly higher in lymph nodes with micrometastases as compared with those without metastasis (Ribatti *et al.*, 2007).

Angiogenesis in benign lymphadenopathies and B cell non-Hodgkin's lymphomas (B-NHL), measured as microvessel counts, is correlated with the total and MC tryptase-positive counts, and both increase in step with the increase in Working Formulation malignancy grades (Ribatti *et al.*, 1998, 2000). In NHL, the cellular expressions of VEGF and FGF-2, as well as MC and vessel counts, were assessed (Fukushima *et al.*, 2001). The number of MCs was greater in T cell lymphomas than in B cell lymphomas and in all NHL a significant correlation was found between vessel count and number of MCs, and between vessel count and number of VEGF-expressing cells. Double fluorescence staining of VEGF mRNA and MC tryptase revealed that MCs expressed VEGF mRNA. These data suggest that MCs intervene in angiogenesis in these lesions by expressing VEGF.

Glimelius *et al.* (2005) evaluated the relationship between the number of MCs and the microvessel count in tissue samples from HL-involved lymph nodes by immunohistochemistry and did not demonstrate any correlation between high microvessel count and the number of MCs.

Bone marrow angiogenesis, evaluated as microvessel area, and MC counts are highly correlated in patients with inactive and active multiple myeloma (MM) and in those with monoclonal gammopathies of undetermined significance (MGUS). Both parameters increase simultaneously in active MM (Ribatti *et al.*, 1999).

In both B-NHL and MM, MCs rest near or around blood or lymphatic capillaries. Their ultrastructural picture includes a typical morphological semilunar feature, or piecemeal partial degranulation of their secretory granules, unlike the IgE-mediated massive degranulation that occurs during immediate hypersensitivity reactions (Crivellato *et al.*, 2002, 2003b; Ribatti *et al.*, 1998, 1999) (Fig. 4.6). This morphology is typical of the slow degranulation that takes place in delayed hypersensitivity reactions and chronic inflammation (Kops *et al.*, 1984). Semilunar appearance may reflect slow but progressive release of angiogenic factors favouring chronic and progressive stimulation of MC degranulation.

Bone marrow samples of patients with myelodysplastic syndromes display a high correlation between microvessel counts and both total and tryptase-positive MCs, and both parameters increase simultaneously with tumor progression (Ribatti *et al.*, 2002). There is also a correlation between

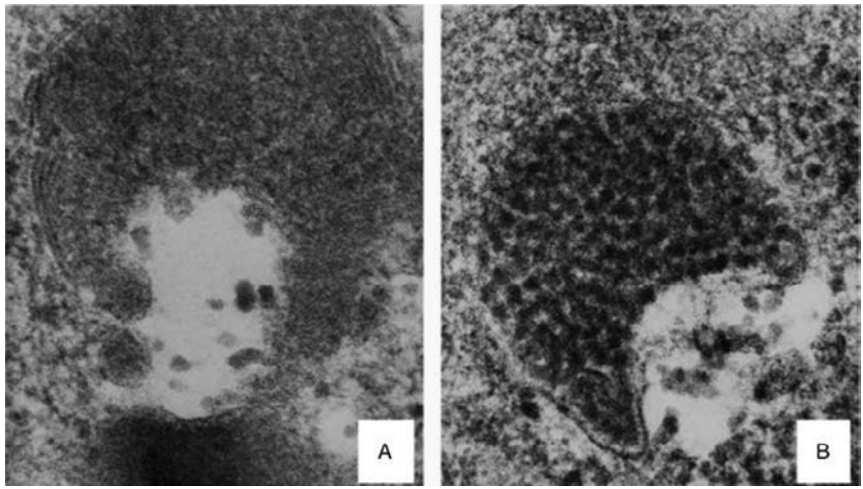

Figure 4.6 Ultrastructural findings of mast cell granules from high-grade B cell non-Hodgkin's lymphoma. Note in (A) semilunar granular aspect and in (B) a full complement of dense particles recognized in this type of granule. Original magnification: (A, B), ×25,000 (adapted with modifications from Crivellato *et al.*, 2002).

the extent of angiogenesis and the number of tryptase-positive MCs in patients with early B cell chronic lymphocytic leukemia, and tryptase-postive MCs predict their clinical outcome (Molica *et al.*, 2003; Ribatti *et al.*, 2003c).

8. Concluding Remarks

The roles of MCs in normal physiology and in the pathogenesis of several human diseases in addition to allergic reactions have been a subject of increasing interest. MCs are found in multiple tissues and their presence in human cancer has been established for many years. However, the literature data have described both a positive and a negative correlation between their number and prognosis in various human tumor types. These discrepancies recognized also in experimental studies could be due to differences in the animal model used and the stage of carcinogenesis investigated.

MCs are recruited in tumor development and they play a key role in both angiogenesis and tissue remodeling, promoting both tumor initiation and growth. In addition, as tumor growth progresses, MCs recruit immune cells or alternatively suppress antitumoral responses. Reports addressing MCs specifically as mediators in tumor immunosuppression come from Huang *et al.* (2008), where they mobilize MCs to infiltrate tumors by means of SCF. Tumor-derived, SCF dependent MC infiltration and activation resulted in remodeling of the tumor microenvironment and tumor growth.

MCs may act as a new target for the adjuvant treatment of tumors through the selective inhibition of angiogenesis, tissue remodeling and tumor-promoting molecules, permitting the secretion of cytotoxic cytokines and preventing MC-mediated immune suppression. Moreover, some of the new targeted anticancer therapies have pronounced effects on MCs; in fact, it may be possible that some of their antitumor effect is closely related to their effect on MCs.

Preliminary studies using anti-KIT antibodies (Huang *et al.*, 2008), anti-TNF-α antibodies (Gounaris *et al.*, 2007), or the MC stabilizer disodium cromoglycate (cromolyn) (Soucek *et al.*, 2007) in mouse models demonstrated promising results even if administered after the initiation of tumor development. Treatment of mice bearing mammary adenocarinoma and pancreatic cancer with cromolyn led to clotting of blood vessels, hypoxia, and tumor cell apopstosis (Samoszuk and Corwin, 2003; Soucek *et al.*, 2007). Unfortunately, cromolyn is a weak inhibitor of human MC secretion and is poorly adsorbed, so it is unlikely to be effective in treating cancer in humans.

The first tyrosine kinase inhibitor introduced into the clinic, STI571 (Imatinib mesylate, Gleevec) has inhibitory activity against the signaling cascade activated by c-kit receptor (CD117) (Heinrich *et al.*, 2000). This inhibitory activity is the basis of the use of this drug against gastrointestinal stromal tumors (GIST), most of which harbor a *kit* mutation (Kitamura and Hirotab, 2004). STI571 is also in use for some varieties of mastocytosis, although some c-kit activating mutations involved in mastocytosis are resistant to its inhibitory activity (Akin and Metcalfe, 2004) and cromolyn has been administered with very limited side effects for various allergic inflammatory conditions (Edwards, 1995; Norris, 1996; Pardanani *et al.*, 2003). Ranitidine, a H2 receptor antagonist that is used as adjuvant therapy, prolonged survival of colorectal cancer patients (Nielsen *et al.*, 2002). Another H2 receptor antagonist, famotidine, given preoperatively for 14 days, enhanced tumor infiltrating lymphocytes and increased metastatic lymph node reactive changes in breast cancer in humans (Parshad *et al.*, 2002). Molica *et al.* (2007) demonstrated that the reduction of the extent of bone marrow angiogenesis observed after sequential therapy with low doses of subcutaneous alentzumab after a clinical response to fludarabile induction therapy was associated to a reduction in the number of MCs.

ACKNOWLEDGMENTS

This work was supported in part by MIUR (PRIN 2007), Rome, and Fondazione Cassa di Risparmio di Puglia, Bari, Italy.

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CHAPTER FIVE

Limbal Stem Cells: Application in Ocular Biomedicine

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International Review of Cell and Molecular Biology, Volume 275
ISSN 1937-6448, DOI: 10.1016/S1937-6448(09)75005-1

Abstract

Corneal opacification due to limbal stem cell deficiency (LSCD) is an important cause for ocular morbidity, resulting from a number of intrinsic and extrinsic factors. While the extrinsic factors include conditions such as chemical or thermal injuries, intrinsic include dysfunction, or reduction in the number of stem cells either due to pathological changes in autoimmune diseases or secondary to certain clinical conditions such as diabetes, dry eye disorders, or multiple previous eye surgeries. LSCD is characterized by a classic triad of signs—conjunctivalization, neovascularization and decrease in vision. With the increasing knowledge of limbal stem cells, the treatment of this condition has evolved from simple debridement to use of biological materials, direct transplantation of the healthy limbal tissue from the contralateral eye, or allogenic source to the use of cultivated limbal epithelial sheets. This chapter provides an update on the disease pathology, various treatment methodologies, with specific emphasis on the fast developing field of cell therapy and tissue engineering.

Key Words: Limbal stem cells, Limbal stem cell deficiency, Tissue engineering, Microarrays, Proteomics, Cryopreservation, Clinical application.

1. Introduction

The eye is a highly specialized organ of photoreception, a process that involves the conversion of different quanta of light energy into nerve action potentials. This intricate function is achieved by the coordination and interdependence of ocular surface elements (epithelia and adnexa of the eye) and the tear film. Similar to the other epithelium in the body, ocular surface epithelia are renewed constantly, by the adult stem cells (SCs) located in the limbus (Thoft and Friend, 1983). Any damage to this functional and physical interdependent structures leads to vision-threatening conditions ranging from decreased to complete loss of vision posing a challenge to the clinicians. The most important of such conditions is the limbal stem cell deficiency (LSCD) caused by a number of individual or environmental or incident specific factors (Dua and Azuara-Blanco, 2000). For a long time the treatment for this condition has been transplantation of healthy tissue from autologous, allogenic, or cadaveric source (Pan *et al.*, 2000; Pellegrini *et al.*, 1997; Tsai and Tseng, 1994; Tsai *et al.*, 2000a,b; Tseng *et al.*, 1995; Ti *et al.*, 2002). With the introduction of cell therapy and tissue engineering, transplantation of cultivated limbal stem cells (LSCs) has been the choice by many groups across world, leading to a large number of

case series and cohorts with a long follow-up (Sangwan *et al.*, 2005a,b, 2006; Ti *et al.*, 2004). Though the principle of treatment remains the same, there is a variation in the procedures involved. With the availability of new molecular tools and increasing information obtained from contemporary fields of stem cell biology, the armamentarium of characteristics that identify the native and cultured limbal cells has evolved over the last few years. This chapter attempts to provide an overall update on the disease pathology, its treatment methodologies experimented with a specific note on the fast developing field of cell therapy and tissue engineering.

2. Ocular Surface and Limbal Stem Cells

2.1. Anatomy and physiology of ocular surface

The ocular surface is anatomically composed of the entire epithelial surface of the cornea, limbus and conjunctiva and is physically continuous with the eyelids and adenexa. Functionally, ocular surface is a unit comprising the tear film, surface epithelium of the cornea, limbus and conjunctiva as well as the lacrimal glands and eyelids. The ocular surface epithelium is a stratified, non-keratinizing epithelium that is connected to the underlying connective tissue stroma through adhesion complexes. Figure 5.1 represents the histology of these ocular surface epithelia. From a functional standpoint, the ocular surface has critical protective and refractive roles. The blinking mechanism of the eyelids protects the eye from unwanted stimuli and serves to spread the tear meniscus over the ocular surface and debrides foreign matter from the ocular surface. The tear film provides lubrication to the ocular surface, permits proper focusing of light by providing a smooth refractive surface, and has enzymes and peptides which have important antimicrobial properties. The anterior surface of the cornea contributes to more than two-thirds of the total refractive power of the eye and hence is critical for clear vision. The tight junctions between adjacent epithelial cells provide a nonporous barrier that prevents the tear film as well as microbes from gaining entry into the epithelium.

2.1.1. Preocular tear film

The preocular tear film lubricates the ocular surface and is composed of three layers—the innermost mucin layer secreted by the goblet cells and bonded to the glycocalyx of the surface epithelial cells; the middle aqueous layer secreted by the lacrimal gland and the accessory tear glands of Krause and Wolf ring, composed of dissolved salts, proteins, enzymes, and antimicrobial substances and the superficial oily layer derived from secretions largely of the meibomian glands and a small contribution from the glands of Zeiss, composed of wax and cholesterol esters with some phospholipids and hydrocarbons. This layer retards evaporation of the tear film.

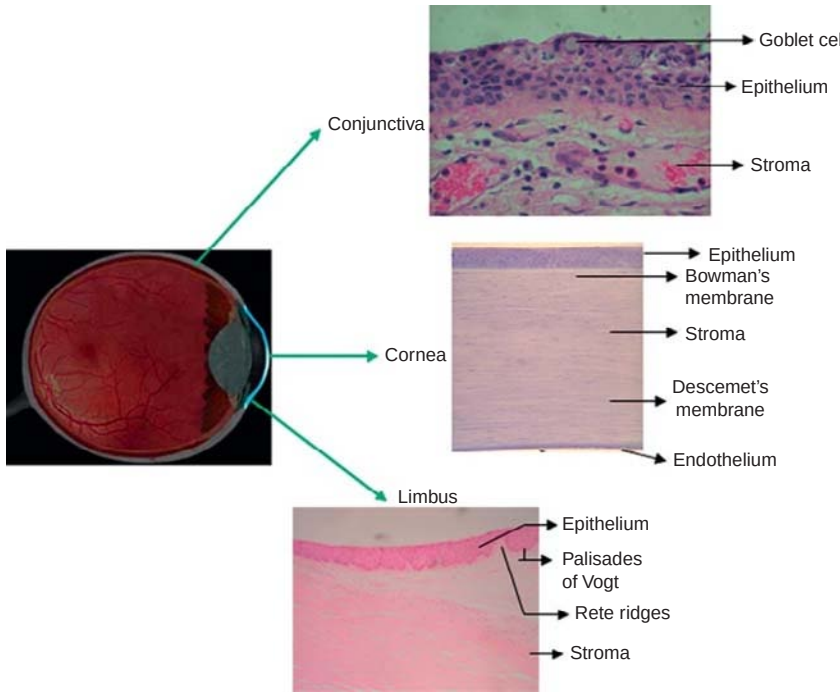

Figure 5.1 **Anatomy of ocular epithelia. Location and histology of ocular surface epithelium conjunctival, corneal, and limbal epithelia. Note the nonkeratinized, stratified epithelium with underlying connective tissue stroma in all the three epithelia. Also note the presence of goblet cells with a vascular stroma in conjunctiva, and palisades of Vogt, which are absent toward the corneal epithelium, an avascular stromal bed with distinct basement membranes and a single layer of endothelium of the cornea.**

2.1.2. Conjunctival epithelium

Conjunctival epithelium is the predominant epithelium of the ocular surface extending from the posterior margin of the eyelids, covering the posterior surface to the peripheral edge of the tarsal plate, then folds back (the fold being referred to as the fornix) to drape the sclera and then continues as the limbal epithelium. Thus the conjunctiva is typically described as comprising of three regions namely palpebral, bulbar, and fornical conjunctiva. The cellular morphology of the conjunctival epithelium differs in these three regions. It is stratified cuboidal over the tarsus, columnar in the fornices, and squamous over the globe. Mucus secreting goblet cells account for up to 10% of the basal epithelial cells of the conjunctiva. They are most numerous over the tarsal and inferonasal bulbar conjunctiva. Unlike the normal cornea, the conjunctival epithelium lacks an organized basement membrane (BM) and rests loosely on the fibrovascular tissue of the substantia propria.

Slow cycling label-retaining cells have been identified in the bulbar, fornical and palpebral conjunctival epithelium, but a stem cell enrichment is seen in the fornicial conjuntciva (Wei *et al.*, 1995). These SCs are the source of epithelial cells that replenish and replace conjunctival epithelium.

2.1.3. Corneal epithelium

Mature corneal epithelium is five or six layers of squamous epithelial cells, about 0.05 mm in thickness. The cells in the basal layer are columnar, in the middle layers are winged, and in apical layer are relatively flattened. Individual cells are connected to each other and to BM by desmosomes and hemidesmosomes respectively. The surface epithelial cells are uniform and smooth acting as a convex lens when covered with tears. Electron microscopy of the corneal epithelium shows numerous finger-like projections or microvilli that emanate from the apical cells, which project into the tear film, trapping tear fluid and thus preventing desiccation of the ocular surface.

2.1.4. Limbal epithelium

Limbal epithelium is located between the corneal and conjunctival epithelia and is about the thickness of the corneal epithelium. The basal epithelium of the limbus has an undulated appearance called the palisades of Vogt—a distinctive feature of the human corneoscleral limbus. These undulations cause tongue like epithelial projections (termed as rete ridges) into the underlying stroma wherein vasculature from the episcleral arteries provides the blood supply to it (Van Buskrik, 1989). This rich vascular supply is thought to be critical in providing nutrients and oxygen to these mitotically quiescent SCs.

2.2. Concept of limbal stem cells

The ocular surface cells constantly renew. Early studies documented a centripetal movement of the corneal epithelial cells from the limbus to the central cornea, suggesting that proliferating precursor cells were likely located at the limbus (Hanna, 1966). Indirect initial clinical evidence of the possible source of epithelial replenishment at the limbus was provided by Davanger and Evensen (1971). The concept of limbal-based SCs was further supported by the observation that it was impossible to create permanent corneal epithelial defects in laboratory animals without damaging the limbus (Srinivasan *et al.*, 1979). Schermer *et al.* (1986) demonstrated the differentiation marker a 64-kDa corneal keratin at the limbus, strongly suggesting a limbal location of corneal SCs. Additional cytological proof was provided by Cotsarelis *et al.* (1989) who demonstrated slow cycling limbal epithelial basal cells that could preferentially be stimulated to proliferate. Thoft (1989) was the first to hypothesize that the epithelial rete ridges

possibly serve as a repository for corneal epithelial precursor cells and that these cells are destined to differentiate into corneal cells. This hypothesis was subsequently proved to be accurate, confirming that cells in the basal epithelium of the palisades of Vogt indeed are the cells that differentiate into corneal epithelial cells and are important in both aging and diseases of the cornea.

SCs have an unlimited capacity for self-renewal, are slow cycling and exhibit low mitotic activity. Once SC differentiation begins, it is irreversible and occurs by transient amplification. Transient amplifying cells (TACs) are capable of a finite number of cell divisions, migrate centripetally and can be found in the limbal and peripheral corneal basal epithelium. The TACs differentiate into postmitotic cells (PMCs) of the suprabasal corneal epithelium. Further differentiation of PMCs gives rise to the terminal differentiated cells (TDCs) of the superficial corneal epithelium (Figure 5.2). It is now clear that healthy SCs are necessary to maintain a normal corneal epithelium, to participate in wound healing and also in barrier function so as to prevent overgrowth of conjunctival epithelium on the corneal epithelium (Cotsarelis *et al.*, 1989; Ebato *et al.*, 1987).

2.3. Diseases of ocular surface

Because all elements of the ocular surface are integrated and work in concert ocular surface disease of any etiology has the common elements of an unstable tear film, vision change due to irregular refraction and surface epithelial changes that could progress to defective wound healing, surface destruction, and profound vision loss. The primary pathogenesis may have to do with dysfunction of one or more of the elements of the ocular surface such as the tear film and/or lacrimal gland, corneal and/or conjunctival epithelium (traumatic/infectious/allergic/inflammatory or congenital conditions) or eyelids (malposition, infections, inflammation). However, the other elements of the ocular surface are affected subsequently. For example, primary deficiency or alteration of the tear film (tear film deficiency or dysfunction) can lead to an unstable tear film, a hallmark of various dry eye disorders. The latter could then cause secondary changes of the epithelial phenotype (Lemp, 1995). Conversely, primary or secondary changes of the ocular surface epithelial phenotype can also lead to a secondary dry eye disorder. Similarly, eyelid malposition or disease can cause secondary changes in the tear film and the ocular surface epithelium.

2.4. Limbal stem cell deficiency

LSCD has been defined as the destruction or dysfunction of the SC containing limbal epithelium, leading to failure of corneal epithelial regeneration, with resultant re-epithelialization by the conjunctival epithelial cells,

accompanied by chronic inflammation, stromal scarring, neovascularization, and persistent epithelial defects (PEDs) (Chen and Tseng, 1991; Kruse *et al.*, 1990).

2.4.1. Etiology and classification

LSCD may be primary or secondary (Kruse, 1994; Puangsricharern and Tseng, 1995). Primary LSCD is characterized by the absence of identifiable external factors and an insufficient microenvironment to support the LSCs as seen in hereditary aniridia, congenital erythrokeratoderma, keratitis with multiple endocrine deficiency and poor nutritional or cytokine supply, neurotrophic keratopathy, peripheral inflammation (Puangsricharern and Tseng, 1995) and sclerocornea. Here, the dysfunction/poor regulation of stromal microenvironment of limbal epithelial stem cells results in gradual loss of SC population or TACs generation and amplification.

Secondary LSCD occurs due to the destruction of LSCs by external factors as seen in trauma [chemical–acid or alkali or thermal injuries (Tseng, 1985), or ultraviolet and ionizing radiation (Fujishima *et al.*, 1996)], systemic conditions [Steven Johnson Syndrome (SJS), ocular cicatricial pemphigoid (OCP) (Pfister, 1994), multiple endocrine disorders and Vitamin A deficiency], iatrogenic [multiple ocular surgeries, excision of pterygia, limbal neoplasm, cyclocryotherapy, antimetabolites—topical mitomycin C (MMC), systemic chemotherapy or asymptomatic but presenting with LSCD (Holland and Schwartz, 1997)], contact lens wear (Lajtha, 1979; Leblond, 1981) and severe ocular diseases (keratoconjunctivitis sicca, postinfectious keratitis, neurotrophic keratitis, vernal/atopic keratoconjunctivitis, tumors, and pterygium), and multiple ocular surgeries (Dua and Azuara-Blanco, 2000). Table 5.1 summarizes the corneal diseases arising out of dysfunction of the limbal niche or deficiency of LSCs.

LSCD may be classified as partial or total (Dua and Azuara-Blanco, 2000). In partial deficiency there is localized deficiency of LSCs in a region of limbus

Table 5.1 Pathological classification of corneal diseases

S. No	Destructive loss of stem cells	Dysfunction of limbal niche
1	Chemical or thermal burns	Aniridia/Iris coloboma
2	Steven Johnson syndrome/TEN	Multiple endocrine deficiency
3	multiple surgeries or Cryotherapies to limbus (Iatrogenic)	Chronic limbitis or peripheral Inflammatory/ulcerative disorders
4	Contact lens-induced keratopathy	Neuronal or ischemic neurotrophic keratopathy
5	Severe microbial keratitis	Pterygium or pseudopterygium
6	Anti-metabolite (5FU, MMC)	Chronic bullous keratopathy
7	Radiation	Idiopathic

but an intact population of SCs in other areas. This results in sectoral conjunctivalization in the areas of SCD (Chen and Tseng, 1990). In total LSCD there is dysfunction or destruction of the entire LSC population resulting in conjunctivalization of the entire cornea (Holland and Schwartz, 1996).

2.4.2. Clinical features

Clinically, LSCD presents with decreased vision, photophobia, tearing, blepharospasm, and recurrent episodes of pain (epithelial breakdown), as well as a history of chronic inflammation with redness (Dua and Azuara-Blanco, 2000; Puangsricharern and Tseng, 1995; Tseng, 1985). The hallmark of LSCD is a triad of signs noted in the cornea: conjunctivalization, neovascularization and chronic inflammation (Chen and Tseng, 1990, 1991; Kruse *et al.*, 1990). Also there could be a variation in the thickness and transparency of the corneal epithelium, seen as a dull and irregular reflex of the corneal epithelium on slit lamp examination. Tiny "bud-like projections" of normal corneal epithelium extending into the conjunctivalized area may be seen. There is an ingrowth of thickened fibrovascular pannus, chronic keratitis, scarring and calcification, abnormal (stippled) fluorescein staining due to increased permeability of the conjunctivalized corneal epithelium (Coster *et al.*, 1995; Dua and Forrester, 1990; Dua *et al.*, 1994), loss of palisades of Vogt (Kinoshita *et al.*, 1986), scarring, persistent and/or recurrent epithelial defects, stromal inflammation, sterile infiltrates, ulceration, melting and perforation (Dua and Azuara-Blanco, 2000). Figure 5.2 depicts the regeneration of corneal epithelium from LSCs and clinical features of LSCD at presentation. The clinical diagnosis may be confirmed using impression cytology, by staining the specimen with periodic acid Schiff stain to identify goblet cells, or monoclonal antibodies to cytokeratin CK3 and CK19 to confirm a conjunctival phenotype (Calonge *et al.*, 2004).

3. Conventional Treatment Modalities of Limbal Stem Cell Deficiency

3.1. Conventional

Prior to the recognition of the location and importance of SCs, the standard treatment for corneal opacification secondary to ocular surface failure was corneal transplantation. This restored ocular surface health was because of the presence of TACs in the corneal epithelium of the transplant. However, there was invariably a breakdown of the ocular surface within a few months and recurrence of epithelial problems, conjunctivalization and secondary corneal opacification. The choice of treatment depends on whether the condition is unilateral or bilateral and involves some or all of the LSCs. Figure 5.3 depicts an algorithmic approach to managing LSCD.

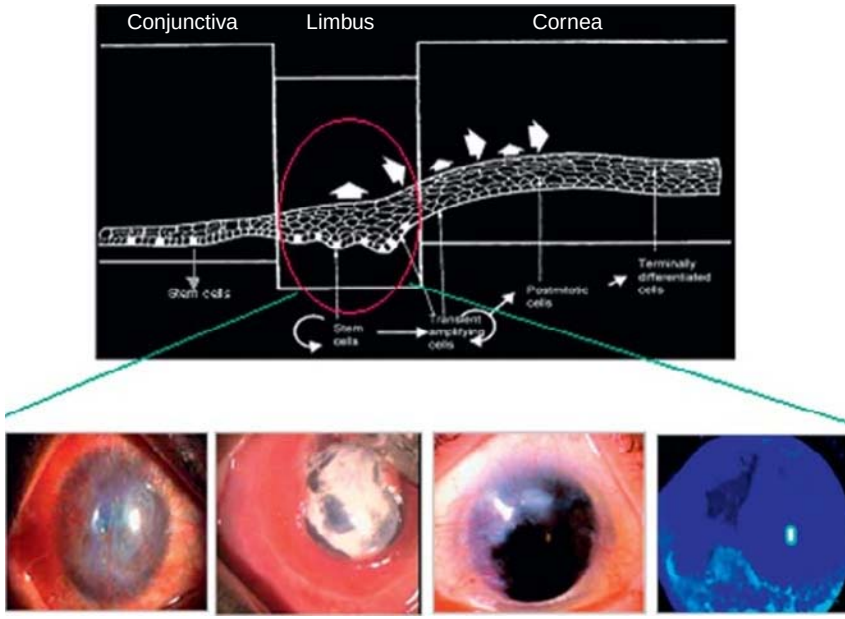

Figure 5.2 Regeneration of corneal epithelium and clinical signs of LSCD. Regeneration of corneal epithelium is by migration of stem cells (differentiation) from the basal layer of limbal epithelium toward the cornea. An insult to this process (marked in red) leads to limbal stem cell deficiency presented clinically with vascularized cornea, persistent epithelial defect, conjunctivalization, and stippled flourescein staining of the conjunctivalized cornea (from let to right).

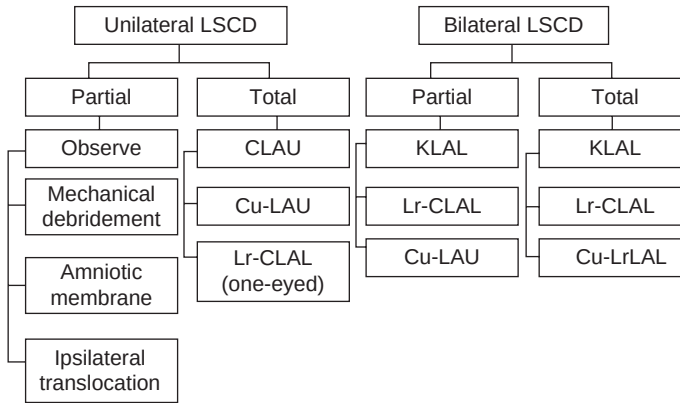

Figure 5.3 Algorithamic approach to treatment of LSCD. Surgical management of limbal stem cell deficiency based on the severity. LSCD, limbal stem cell deficiency; CLAU, conjunctival limbal autograft; KLAL, keratolimbal allograft; Lr-CLAL, living related conjunctival allograft; Cu-LAU, cultured limbal autograft; Cu-LAL, cultured limbal allograft.

3.2. Amniotic membrane transplantation

An alternative approach in eyes with partial LSCD is amniotic membrane transplantation (AMT) in the area where unhealthy epithelium has been denuded (Tseng *et al.*, 1998). The biological properties of amniotic membrane (AM) such as a thick BM, avascular stromal matrix, potential to promote epithelialization and epithelial differentiation, prevention of apoptosis and nonimmunogenicity are responsible for the successful transplantations (Lee and Tseng, 1997). These biological properties are also responsible for AMT retarding conjunctivalization in eyes with focal limbal deficiency while permitting adjacent healthy epithelium to repopulate and cover the AM, thus restoring ocular surface integrity and health. Figure 5.4 depicts clinical follow-up in a patient who had undergone AMT in the acute phase of LSCD

3.3. Limbal transplantation

The definitive treatment for LSCD is limbal transplantation using auto or allograft limbal tissues each of which have their associated risks and benefits. The success of LSC transplantation is determined by a variety of factors and may be adversely affected by concomitant lid pathology, dry eye, and uncontrolled systemic disorders. Hence, the management of associated adenexal conditions such as eyelid/eyelash malposition and management of dry eye is a prerequisite for successful ocular surface reconstruction and needs to precede stem cell transplantation.

Unilateral cases with total LSCD may benefit from either a conjunctival limbal autograft (CLAU) or transplantation of *ex vivo* cultured LSCs from the fellow eye. In bilateral cases with total LSCD or in a one-eyed patient who develops total LSCD in the seeing eye, an allograft limbal transplant utilizing donor tissue from a cadaver or a living relative is the only option. If donor tissue is being harvested from a living relative, the preferred approach is to culture and expand the donor tissue in the laboratory and to transplant the expanded tissue on the patient. If an allograft is being obtained from eye bank tissues, most surgeons prefer to harvest almost 10 h from the donor eye and

Figure 5.4 Amniotic membrane transplantation in LSCD. The clinical follow-up of a patient who underwent an amniotic membrane transplantation in the acute phase of chemical injury showing eye with AMT (A); 4 years posttransplant showing a clear cornea (B), and limbal pigmentation (C).

suture this to the limbal area of the recipient. In partial LSCD, a piece of healthy limbal tissue from the same eye may be cultured and subsequently transplanted to the area of diseased epithelium. Another technique described for focal LSCD is ipsilateral translocation of healthy limbal tissue to an area of partial LSCD. In a small series of patients, this has been shown to provide good outcomes (Nishiwaki-Dantas *et al.*, 2001). The relative paucity of additional studies with this approach makes this a less, well-studied and understood approach for management of partial LSCD. The visual acuity of a patient with ocular surface disease who has undergone LSC transplantation may improve without any further surgical intervention. However, in cases where the corneal stromal opacification hampers visual recovery, a penetrating keratoplasty (PK) may be necessary. While some authors suggest PK and LSC transplantation to be done at one sitting (Rao *et al.*, 1999; Theng and Tan, 1997). Croasdale *et al.* (1999) recommend an interval of 3 months between the two and some others (Ilari and Daya, 2002) recommend a deep lamellar keratoplasty 1 year post-LSCs transplantation, if the endothelium is healthy. In our experience, the latter approach of staged surface reconstruction followed by subsequent corneal transplantation (lamellar in any situation where the endothelium is deemed healthy and penetrating in eyes with endothelial injury) is the preferred approach to visual rehabilitation of such eyes.

Postoperative treatment typically consists of preservative-free topical antibiotic and prednisolone 0.5% four times daily for the first 2 weeks. Frequent application of preservative-free artificial tears and highly viscous methylcellulose four times daily is also used with long-term low-dose topical steroids. Autologous serum eye drops (20% solution of serum with sterile saline, aliquoted and stored at $-20\ ^{\circ}C$) can help promote epithelial healing and are hence used by some groups hourly until the epithelialization is completed. During the early postoperative period the limbal explant is carefully monitored for any areas of epithelial loss as conjunctival epithelium could cross the explant at these sites and gain access to the corneal surface (particularly when a stromal spacer is used, which acts as a limited barrier to conjunctival encroachment). If conjunctival encroachment is observed, mechanical debridement of conjunctival cells from the explant, spacer or even the corneal surface should be promptly carried out. It is important to mention that the cornerstone to success of allograft transplants is aggressive systemic immunosuppression which is mandatory for survival of allografts.

In limbal allografts the surface disorder can recur if there is immunological destruction of the transplanted LSCs. A high rate of immune reactions can be expected because of the vascularity of the limbus, the high immunogenic stimulus of the limbal transplant related to the relative abundance of Langerhan's cells and HLA-DR antigens. Hence, an effective immunosuppression is considered essential indefinitely or until graft is working. The immunosuppressive dosage can be increased or decreased if it proves ineffective or causes adverse effects, respectively. We recommend full dose triple drug regimen consisting systemic steroids, azathioprine and cyclosporine A.

Allograft rejection of transplanted limbus can threaten the viability of donor stem cells and needs to be recognized early. Clinical signs of rejection include the development of limbal injection and inflammation and/or acute or chronic severe surface abnormalities. Daya *et al.* (2000) have reported the clinical features of limbal allograft rejection varying according to the presentation as acute rejection when associated with intense sector injection at the limbus, edema and infiltration of the lenticule, punctate keratopathy, and epithelial defects; low-grade rejection when there is mild diffuse or perilimbal injection, elevated perilimbal area, punctate epithelial keratopathy and epithelial irregularity. Rejections are treated aggressively with systemic prednisone as well as topical hourly prednisolone acetate 1%.

3.4. Cultivated limbal stem or epithelial cell treatment—Autologous, allogenic (living related, cadaveric)

The concept that the LSCs are critical for ocular surface health (Thoft and Friend, 1983) leads to the subsequent development of stem cell transplantation as the treatment of choice for LSCD (Chen and Tseng, 1991; Shaptron *et al.*, 1981). The LSCs may be drawn either from the fellow eye (autograft), a cadaver (allograft) or a living relative (allograft) and harvested with a carrier, which may be either the conjunctiva (conjunctival limbal auto- or allograft) or cornea (keratolimbal allograft). While stem cell transplantation represents the definitive treatment for LSCD, this may not be necessary in some situations. For example, asymptomatic patients with quadrantic, peripheral, or partial conjunctivalization secondary to focal (less than 3 h) stem cell deficiency may be kept under periodic close observation. If a tendency for surface compromise or breakdown central to the conjunctivalization is observed, such patients may have repeated mechanical debridement also known as sequential sector conjunctival epitheliectomy (Dua, 1998). The rationale for this treatment is to remove the area of diseased epithelium and to let this repopulated with epithelial cells from the adjacent healthy limbus in this eye. Figure 5.5 depicts the successful transplantation of cultivated limbal epithelial transplantation (CLET) in a patient with LSCD. The technique of cultivation and clinical efficacy of transplantations is described in the sections below.

4. Concept of Cultivated Limbal Stem Cell Transplantation

4.1. Tissue source

Animals specifically rabbits have been the choice of a limbal epithelial cell source for *in vitro* cultures for a long time. In humans the richest source has been the discarded corneoscleral rims (CSR) following PK, or CSR rim of

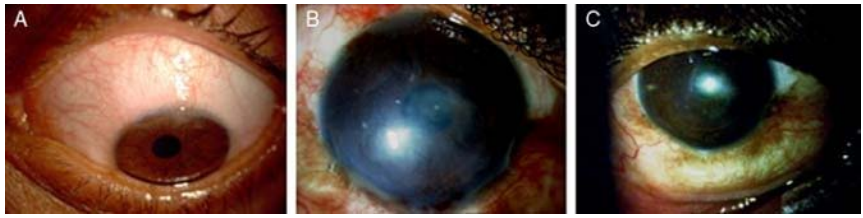

Figure 5.5 Cultivated limbal epithelial transplantation in LSCD. The clinical outcome in a patient with LSCD, who has undergone a CLET for visual rehabilitation showing the site of biopsy (arrow) (A); stable ocular surface with residual scarring 7 weeks postcultivated limbal autograft transplantation (B), reduction in density of scar and a stable ocular surface 10 months postoperatively (C).

rejected corneas from cadaveric eyes obtained from the eye bank. Another important source is from the patients suffering from LSCD. We initiated the standardization of our technique with limbal tissue from corneoscleral rim obtained from our eye bank and later with patient's samples for purposes of transplantation.

4.1.1. Limbal tissue

Various groups have used limbal tissue as the chief source of limbal epithelial cells from among ocular surface epithelia (conjunctiva, limbus, and cornea) for CLET (Lindberg *et al.*, 1993). The prime factor of debate still remains to be the choice between fresh and cadaveric limbal tissues as a tissue source.

4.1.2. Cadaveric versus fresh limbal tissue

The potential of cadaveric tissues for use in *ex vivo* expansion of limbal epithelial cells is governed by the age of the donor, duration of preservation in medium, culture technique and so on. However, the *in vivo* behavior of these cultured limbal epithelial cells is further influenced by survival of the transplanted cultured epithelium, percentage of LSCs in the cultured epithelium, persistence of these cells in the new environment, homing of donor cells into the recipient limbal niche, establishment of communication between the LSCs (internal factors) and the surrounding stroma (niche or external factors).

Fresh limbal biopsies are the best choice for cultivation of limbal epithelial cells because of the high potential for active growth of all tissue bits. Fresh biopsies collected in the requisite culture medium (HCE or DMEM) could be cultured either immediately or stored at room temperature till cultured. Our experience with stored fresh biopsies has been very encouraging in getting good quality cells even with tissues stored for about 48–72 h post-harvesting. The fact that there could be an initial delay in the initiation of growth in the stored biopsies cannot be ruled out.

Studies by Vemuganti *et al.* (2004) have shown that fresh limbal tissue with 100% viability is preferable to a cadaveric limbal tissue with 51% viability for cultivation. James *et al.* (2001) showed a decreasing trend in growth potential of limbal tissues with increasing age. The growth potential of limbal tissue from discarded corneoscleral rims obtained from the operating room was 66.6%, as compared to 100% in tissues obtained from freshly enucleated eyeballs. Tungsiripat *et al.* (2004) determined the viability of different layers of central and limbal corneal epithelium on days 0, 3, 6, and 9 after harvest (microkeratome dissection) stored at 4 °C in corneal storage medium in eye bank. Their results showed that the basal epithelial layers of all tissues and time points displayed greater mean viability than the overlying middle epithelial layers, and among the basal epithelia, viability of the limbus was significantly greater than that of the central epithelium with 100–95% in the limbus versus 98.4-68.6% in the central epithelial during the 9-day period confirming the hardiness of the stem cell region. Cryopreservation of the limbal tissue or cultured limbal epithelial cells is an alternative to reduce the risks of donor site compromise in repeat CLETs and would also become an important source of cells for allogenic transplantations (application based on HLA matching). A study by Yeh *et al.* (2008) showed that 60% DMEM, 30% FBS, and 10% DMSO were an optimal cryopreservation medium for *ex vivo* expanded LSCs, while Mi *et al.* (2008) and Qu *et al.* (2009) used liquid nitrogen storage for cryopreservation and the cryopreserved cells were further evaluated by transplantation into goat and rabbit LSCD models, respectively.

4.1.3. Alternative sources for LECs being explored

The difficulties associated with allogenic transplantation of cultivated limbal epithelial cells have been the guiding factor for exploring autologous tissue sources for ocular surface reconstruction. *Oral mucosal epithelial cells* (Ang *et al.*, 2006; Hayashida *et al.*, 2005; Madhira *et al.*, 2008; Nishida *et al.*, 2004b), epidermal adult stem cells derived from ear skin (Yang *et al.*, 2008), conjunctival epithelial cells (Ono *et al.*, 2007; Tanioka *et al.*, 2006), and embryonic stem cells (Homma *et al.*, 2004) are already being explored for ocular surface reconstruction in animal (adult epidermal and embryonic stem cells) and human models (oral mucosal and conjunctival epithelial cells).

4.2. Nature of scaffolds

4.2.1. Choice of substrate

A corneal tissue replacement or a substrate for cultivation of limbal epithelial cells should have high optical clarity, appropriate refractive index, dimensions as that of cornea, toughness to withstand surgical procedure, that is, adequately robust for implantation, nontoxic, nonimmunogenic, noninflammatory and most importantly promote regeneration of corneal cells and

nerves. The literature reveals the application of several biological or biosynthetic substrates for ophthalmic applications some of which are described below.

4.2.2. Human amniotic membrane

Human amniotic membrane (HAM), the innermost layer of placenta is the most common substrate used for both *in vitro* cultures and as a vehicle for transfer of cells during transplantation, because of its structural similarities to the ocular surface, its biological and functional properties. HAM is also used as substrate to culture conjunctival epithelial cells. However, there are conflicting reports of HAM supporting goblet cell differentiation (Meller *et al.*, 2002) and HAM cultured conjunctival cells showing largely a non-goblet cell phenotype (Meller and Tseng, 1999).

Histologically HAM is comprised of a monolayer of cuboidal epithelial cells with large number of apical microvilli, a thick BM composed of a network of reticular fibers (closely resembling that of conjunctiva) and an avascular hypocellular stromal matrix comprising a loose fibroblast network. This structural integrity, transparency and elasticity of BM of HAM make it the most widely accepted substrate for ocular surface reconstruction.

Functionally HAM promotes epithelial cell adhesion, migration, and differentiation supporting growth of epithelial progenitor cells by prolonging their lifespan, maintaining their clonogenicity and preventing epithelial cell apoptosis. Factors such as *bFGF, HGF, and TGF* produced by HAM stimulate *epithelialization* and modulate *proliferation and differentiation* of stromal fibroblasts. The stromal matrix rich in *fetal hyaluronic acid* (suppresses TGF-γ signaling) not only aids in *reduction of scarring* postocular surface reconstruction surgeries but also suppresses expression of inflammatory cytokines such as IL-1a, IL-2, IL-8, INF-γ, TNF-γ, bFGF, and PDGF originating from the ocular surface epithelia. The *anti-inflammatory role* of HAM is by *attracting and sequestering inflammatory cells* infiltrating the ocular surface and by the action of various forms of *protease inhibitors*. Type IV collagen and laminin in the BM play a role in cell adhesion, acting as a natural substrate for the cell growth, integrates onto corneal surface on transplantation and enables easier handling during transplantation. Figure 5.6 represents the difference in HAM with and without native epithelium as observed under a phase contrast microscope.

Touhami *et al.* (2007) elucidated the mechanism of action of HAM in epithelialization by investigating the expression of ligands and receptors of the neurotrophin family such as nerve growth factor (NGF), neurotrophins (NT3 and NT4), brain-derived neurotrophic factor (BDNF), tyrosine kinase-transducing receptors TrkA, TrkB, and TrkC, and pan-NT low-affinity receptor (p75 NTR) by human limbal epithelial cells expanded on HAM, supporting the importance of NGF in expansion of limbal epithelial progenitor cells.

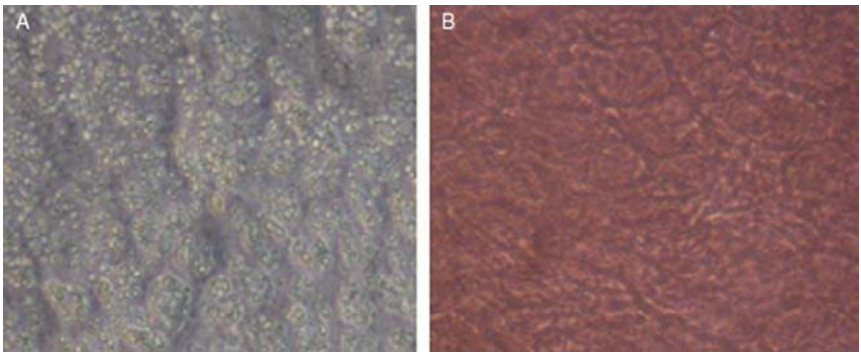

Figure 5.6 HAM with intact and devitalized epithelium. The morphology of HAM with the intact epithelium (A) and a devitalized epithelium (B) (devitalization by trypsin digestion and mechanical scrapping of the epithelium) as seen under a phase contrast microscope at a magnification of 100×.

Both fresh and preserved HAM have been found to function equally well on transplantation to ocular surface. The use of fresh HAM is subject to concerns such as serological testing of the donor both at the time of procurement and about 6 months later to eliminate the slightest risk of disease transmission, which may not be possible for a short-time interval between procurement and transplantations; wastage of unused tissue with nonpreserved as opposed to frozen HAM (up to 30 grafts could be prepared from one placenta); epithelial cells in fresh or preserved AMG are nonviable (probably associated with low-grade inflammatory response) obviating the need for a −70 °C refrigerator, which precludes its use outside big institutions. On the other hand, frozen HAM as per some groups is not that user-friendly because of its brittle nature and handling difficulties.

The standard protocol proposed by Kim and Tseng (1995) is used by various laboratories including ours for preparation of HAM. Briefly, placenta obtained from caesarian deliveries is washed with Ringer solution containing antibiotics and the amnion and chorion are separated. The separated amnion is spread on top of a nitrocellulose paper with epithelial side up, cut into required dimensions and stored in DMEM-glycerol at −70 °C. Just before use HAM is thawed at 37 °C. Rama *et al.* (2001b) also supported use of HAM stored at −140 °C, thawed and stored at 4 °C before grafting in RPMI medium and showed results similar to that thawed immediately before grafting.

Many groups have shown the role of HAM in maintaining the stemness of stem cells probably by providing the right kind of "niche" as is present *in vivo* in the stroma (Wang *et al.*, 2003). Shortt *et al.* (2009) evaluated the ability of different preparations of stored HAM to serve as substrates for limbal epithelial cell expansion, such as decellularization of HAM prior to seeding limbal epithelial cells, glycerol cryopreservation and peracetic acid

(PAA) sterilization and antibiotic disinfection and showed that (i) decellularization facilitates migration of limbal epithelial cells but not increased proliferation resulting in larger cells and does not affect the percentage of cells expressing ΔNp63α and ABCG2; (ii) glycerol cryopreservation resulted in poor morphology and a low proportion of cells expressing ΔNp63α and ABCG2; (iii) HAM frozen at −80 °C in HBSS was superior; (iv) decontamination by PAA or antibiotics does not appear to affect its function; and (v) use of glycerol as a cryoprotectant impairs this ability whereas simple frozen HAM appears to work extremely well for this purpose.

Grueterich *et al.* (2002) proposed that culturing explants on an intact HAM with devitalized epithelium favors expansion of an epithelial phenotype that closely resembles LSCs. Koizumi *et al.* (2007) compared the morphology and adhesion of the suspension cultured limbal epithelial cells with air lifting on intact and denuded HAM, cocultured with a mitomycin-C (MMC)-treated 3T3 fibroblast feeder layer. Their study showed that while cells on both the substrates were well attached to the HAM stroma, morphologically superior, well-stratified and/or -differentiated limbal cells could be cultured on denuded HAM compared to that on intact HAM hypothesizing that denuded HAM is probably more practical carrier for human limbal epithelial cell cultures in cell-suspension culture system. Baharvand *et al.* (2007) studied the proteome of the epithelium-denuded HAM to understand the mechanisms by which HAM (in comparison to matrigel and collagen) may confer its effects on the expansion of LSCs and showed that epithelium-denuded HAM (enriched in proteins such as lumican, osteoglycin/memican, collagen alpha type IV and fibrinogen) provides a superior niche for LSC proliferation and phenotype maintenance *in vitro*. Kolli *et al.* (2008) analyzed the outgrowths from human cadaveric limbal explants cultured on HAM by dividing the explant outgrowths into three zones—inner, middle, and outer depending on proximity to the explant and showed a successive decline in colony forming efficiency (CFE) and expression of ΔNp63 and ABCG2, an increase in expression of CK3 in zones further away from the explants supporting the importance of proximity of stem cells to their niche environment in maintaining their undifferentiated state.

4.2.3. Fibrin

Fibrin gels are prepared by diluting stock solutions to obtain a final 3 IU/ml thrombin, 35–55 mg/ml fibrinogen, 1.1% NaCl, and 1 mM $CaCl_2$. Aprotinin (25 μg/ml) is added to each culture on fibrin gel to prevent fibrinolysis and at the same time not affecting cell migration.

Rama *et al.* (2001a) evaluated the preservation of limbal features in LSCs cultivated on a fibrin substrate. On grafting, damaged corneas showed re-epithelialization within first week, inflammation and vascularization regressed within 3–4 weeks and by end of first month corneal surface was

covered by a transparent, normal-looking epithelium. This observation was later replicated by Talbot *et al.* (2006), who showed that cultures of dispase treated rabbit limbal epithelial cells cocultured with 3T3 fibroblast feeder layer on fibrin gel or on plastic culture dishes showed characteristics similar to *in situ* basal limbal cells (small cells with regular shapes, a low C/N ratio, CK3, $K4^-$). On transplantation onto damaged corneas, the fibrin gel disappears after grafting regenerating an epithelium of normal thickness from the cultured cells within a month, consistent with the results using HAM (degradation of HAM is slower than that of fibrin gels). Higa *et al.* (2007) used stratified corneal epithelial sheets prepared in culture dishes coated with biodegradable fibrin glue in comparison to denuded HAM and showed that carrier-free sheets were more differentiated compared with AM sheets, while retaining similar levels of colony-forming progenitor cells.

4.2.4. Lens capsule

Human anterior lens capsules (Galal *et al.*, 2007) obtained from patients undergoing cataract surgery were used as a scaffold/substrate for cultivation of limbal epithelial cells. Limbal biopsies plated epithelial-side down on the capsular specimen (crystalline epithelium is removed) in a 35-mm culture dish cultured for 2 weeks, showed a superior cell viability (95%) and desmosomes between growing cells, in both autologous and allogenic combinations.

4.2.5. Corneal or limbal stroma

Espana *et al.* (2003) grew limbal epithelial cells on limbal/corneal stroma and provided the evidence of role of stromal niche in controlling the plasticity of limbal and corneal epithelial differentiation in rabbits.

4.2.6. Therapeutic contact lens

Di Girolamo *et al.* (2007) showed that among siloxane-hydrogel contact lenses (lotrafilcon A and balafilcon A) used therapeutic contact lenses, Lotrafilcon A contact lenses sustained proliferation and migration of limbal epithelial cells cultured in autologous serum, with a corneal phenotype showing microvilli on the apical surface, while no growth was observed on balafilcon A contact lenses.

4.2.7. Tissue engineered substrates

Use of allogenic biological material is associated with the risks of disease transmission (e.g., HIV, hepatitis B and C, and bacterial and fungal infections) and allograft rejection, in addition to lack of mechanical toughness which allows for easy handling and suturing and prolonged endurance after transplantation, raising a need to develop new methods of cell carriers for ocular surface epithelial cell replacement. These disadvantages have necessitated formulation of biosynthetic materials custom fabricated as stromal substitutes to support epithelial cell growth and provide a ready supply of material for clinical use.

4.2.8. Collagen scaffolds

Reconstituted collagen membrane from HAM has been investigated as a source of collagen matrix, for culturing human fibroblasts based on the measurement of thermodynamic behavior by differential scanning calorimetric and thermogravimetric analysis, and tensile strength (Doillon *et al.*, 2003; Dravida *et al.*, 2008; Griffith *et al.*, 1999; Li *et al.*, 2003). Fibroblasts cultured on the chitosan cross-linked collagen membrane had good adherence and retained their morphology as indicated by microscopic analysis, supporting nontoxic nature of the membrane and application of reconstituted human amniotic collagen membrane as collagen scaffolds to culture fibroblasts *in vitro*.

4.2.9. Temperature-responsive gels

Temperature-responsive polymers chemically immobilized in thin films on cell-culture surfaces, facilitate cell adhesion and growth of cells in normal culture conditions at 37 °C, and can reversibly alter their hydration properties with temperature (hydrate and swell below 30 °C) prompting complete detachment of adherent cells without the use of proteolytic enzymes or treatment with EDTA (Schmaljohann *et al.*, 2003). Nishida *et al.* (2004a) have developed human or rabbit corneal epithelial cell sheets by using novel temperature-responsive culture surfaces.

Mebiol gel, a copolymer composed of thermoresponsive polymer poly(*N*-isopropylacrylamide-*co*-*n*-butyl methacrylate) (poly-NIPAAm-*co*-BMA) and hydrophilic polymer polyethylene glycol (PEG), is hydrophilic below 20 °C and hydrophobic above 20 °C forming cross-linking points and a homogenous three-dimensional network in water. The sol–gel transition temperature can be controlled by altering chemical composition of thermoreversible gelation polymer (TGP). Mebiol gel has been used as wound dressing, microcapsule for islets, electrophoretic gels for DNA separation, and 3D culture matrix for various cells. Cells or tissues can be embedded in a liquid Mebiol gel solution at lower than 20 °C and cultured three dimensionally in a hydrogel state at 37 °C. This hydrogel is far more transparent than HAM. Madhavan *et al.* (2004) showed rapid expansion of animal cells in Mebiol gel without signs of cytotoxicity. LSCs cultured by explant culture method on Mebiol gel by Sudha *et al.* (2006) showed good proliferation exhibiting a limbal phenotype of ABCG2^{+}/p63^{+}/integrin $\beta1^{+}$ and corneal differentiation phenotype of K3/K12^{+}/Cx43^{-} in absence of 3T3 feeder layers. Sitalakshmi *et al.* (2008) evaluated the efficacy of transplantation of LSCs cultured on Mebiol gel and showed that these cells may restore a nearly normal ocular epithelial surface in rabbit eyes with unilateral LSCD.

4.2.10. Myogel

Francis *et al.* (2009) showed that Myogel derived from autologous muscle biopsy and stromal extract supported growth, confluence and ABCG2 expression of *ex vivo* expanded LSCs from cadaveric limbal cells, in

comparison to Matrigel and denuded HAM, thus providing an alternative cell carrier.

4.2.11. Chitin

Glycosaminoglycans (GAGs) are major components of skin and cornea, and its role in wound healing has been studied both *in vitro* and *in vivo*. Chitosan, a member of the family of GAGs has been used as a scaffold for tissue engineering of skin and in ophthalmology for contact lens fabrication and ocular bandage lenses. The stiffness of a pure chitosan substrate makes it unfit for application on the curved ocular surface and hence combinations of chitosan with compounds such as gelatin (as denatured collagen) are being developed to improve the physical, chemical and biological properties. Thus a gelatin–chitosan (GC) composite biomembrane could be used for ocular surface tissue engineering (Zhu *et al.*, 2006).

4.2.12. Other scaffolds

Apart from the above substrates, various BM proteins such as laminin, fibronectin, and collagen IV have been extensively used as surface coating agents to grow epithelial cells (Nakagawa *et al.*, 1990). Schwab *et al.* (2000) used a bioengineered corneal surface replacement using cultured corneal epithelial stem cells seeded on a matrix derived from HAM. The resulting composite cultured tissue was transplanted to the ocular surface of the diseased eye. Li *et al.* (2005) attempted to isolate enriched stem cell population from human limbal epithelial cells by their rapid adherence on collagen IV coated dishes. Table 5.2 summarizes various substrates that have been used by various groups for cultivation of LSCs.

4.3. Media constitution

Composition of the medium used for the cultivation of LSCs plays a very critical role in the type and quality of the cells obtained as the cells take molecular cues for growth and differentiation from the components of the medium similar to those (molecular cues) *in vivo*. Thus cell-culture media are combination of growth factors and cytokines, salts, amino acids, electrolytes, hormones, or peptide growth factors (Boyce and Ham, 1983) and carbohydrates maintained at a specific pH used with or without a serum supplement.

For epidermal keratinocytes a series of media supplemented with growth promoting agents were formulated in Ham's laboratory. As described previously (Fatima *et al.*, 2006; Sangwan *et al.*, 2003; Vemuganti *et al.*, 2004) we use human corneal epithelial medium (HCEM) which is a recipe of minimal essential medium (MEM) and Ham's F12 (in 3:1 ration) supplemented with growth factors EGF (0.01 mg/l), Cholera Toxin (0.1 mg/l) and insulin (2.5–5 mg), and antibiotics penicillin, streptomycin, amphotericin and

Table 5.2 Summary of the substrates used by various groups for cultivation of LECs

S. No	Substrate used	Group	Year	Application
1	HAM	Schwab *et al.*	1999	Clinical
		Tsai *et al.*	2000	Clinical
		Sangwan *et al.*	2003	Clinical
		Wang *et al.*	2003	Research
		Sun *et al.*	2006	Research
		Li *et al.*	2006	Research
		Sangwan *et al.*	2006	Clinical
		He *et al.*	2006	Research
		Inatomi *et al.* (Oral mucosa)	2006	Clinical
2	Fibrin	Pelligrini *et al.*	1999	Clinical
		Talbot M *et al.*	2006	Research
3	Corneal stroma	Espana *et al.*	2003	Research
4	Soft contact lens	Pelligrini *et al.*	1997	Clinical
5	Culture inserts	Koizumi *et al.*	2002	Research
6	Mixed coated plates (laminin, fibronectin, collagen IV)	Nakagawa *et al.*	1990	Research
		Schwab *et al.*	1999	Clinical
		Li *et al.*	2004	Research
7	Temperature-responsive gels—poly (*N*-isopropylacrylamide)	Nishida *et al.*	2004	Research
		Sitalakshmi *et al.*	2008	Research
		Sudha *et al.*	2006	Research
8	Therapeutic contact lens	Di Girolamo *et al.*	2007	Research
9	Myogel	Francis *et al.*	2009	Research
10	Collagen scaffolds	Dravida *et al.*	2008	Research

gentamicin (at 75, 50, 1.25, and 4 mg/l, respectively). Hydrocortisone and cholera toxin were used initially but were discontinued later. Alternatively Dulbecco's minimal essential medium (DMEM) can also be used (Argüeso *et al.*, 2006; Pan *et al.*, 1999, 2000; Wylegala *et al.*, 2004). Both these media are supplemented with 10% serum (FBS or autologous) in serum supplemented cultures.

Few groups have also explored the possibilities of serum-free media for cultivation of limbal epithelial cells. Figueira *et al.* (2007) used TMEM (a modified Eagles medium, hormone and growth factor-free) a controlled, enriched medium for a wide range of cell cultures, supplemented with L-glutamine (292 μg/ml), ITS mixture (insulin, 10 μg/ml; transferrin,

5.5 μg/ml; and selenium, 5 ng/ml) and penicillin G (streptomycin sulfate, (10,000 U/ml each); amphotericin B, 25 μg/ml), as culture medium for limbal epithelial cells. Explant cultures in this medium were confluent in 2 weeks, with an immunophenotype of $\Delta Np63^+$, $CK3^-$ (similar to limbal basal cells) and $CK3^+$ on serial passaging (corneal epithelial phenotype). Aniscough and coworkers proposed the use of VitroGro™ complex, containing vitronectin and members of IGF family as a viable alternative to serum-containing media for proliferation of cell lines and keratinocytes derived from the skin and also *ex vivo* expansion of keratinocytes derived from the cornea. Although the medium significantly enhances proliferation of primary limbal cell cultures above serum-containing media, cultures grow to confluence only when supplemented with EGF and growth arrested 3T3 feeder cells, supporting establishment and cultivation of limbal cells to the same extent as the traditional Green's method (Green's media with serum and growth arrested 3T3 feeder cells). A new media (unknown composition) labeled as CnT20 from Millipore were used to grow limbal epithelial cells up to seven passages (unpublished data).

4.4. Techniques of cultivation

Different methods have been used to culture limbal epithelial cells *in vitro* since the first demonstration by Sun and Green (1977) using different principles of tissue culture, that is, anchorage dependant or anchorage independent cultures, described in the following sections. Most of the adult stem cell cultures have necessitated the use of feeder cells, which are mitotically inactive and metabolically active. We discuss here the role of feeder cells and the various culture techniques that have been used to culture the limbal–corneal epithelium from native limbal tissues.

4.4.1. Role of feeder layers

Feeder layers are mitotically inactivated normal cells that support the growth of a variety of fastidious cultured cell types including stem cells, by serving as basal layer cells supplying important metabolites without further growth or division of their own. In LSC cultures using feeder layers—these cells are known to simulate the stem cell microenvironment by secreting extracellular metabolites that maintain the stemness of stem cells.

3T3 Cells from mouse embryonic fibroblasts are the most common feeder layers used (Rheinwald and Green, 1975). These cells are inactivated by γ-irradiations (6000R), (Pellegrini *et al.*, 1997) or by MMC (4 μg/ml for 2 h at 37 °C under 5% CO_2) treatment. MMC-treated 3T3 fibroblast feeder layers have been used to cultivate epithelial cells from human limbus (Lindberg *et al.*, 1993), conjunctiva (Kawasaki *et al.*, 2006) and oral mucosa (Nishida *et al.*, 2004a,b). These are then trypsinized and plated onto plastic

dishes with a density of 2×10^4 cells/cm^2 and allowed to adhere. Limbal epithelial cells (seeded on substrate such as HAM) are now seeded onto these feeder layers (Grueterich *et al.* (2003).

Alternatively human sources for application as feeder cells are also being explored. A study by Lee *et al.* (2003) showed that human adult uterine endometrial cells (hUECs), human adult breast parenchymal cells (hBPCs), and human embryonic fibroblasts (hEFs) could be used as feeder cells for supporting the growth of human embryonic stem cells but their application for cultivation of limbal epithelial cells is yet to be evaluated. Chen *et al.* (2007) showed that the clonal growth of limbal epithelial cells was promoted by human amniotic epithelial cells compared to 3T3 feeder cells. Notara *et al.* (2007) used MRC-5, a hEFs cell line, and J2 3T3 mouse fibroblasts as feeder layers for expansion of human limbal epithelium under serum-free conditions.

In our experience, use of explant cultures obviates the need for feeder cells. This was a puzzling observation which we partly understood by identification of the stromal cells which emerge from delayed explant limbal cultures (Polisetty *et al.*, 2008). We speculate that these spindle cells act as ''intrinsic feeder cells'' in the explant culture system thus maintaining the ''stemness'' of cultures without the fibroblastic feeder cells of animal origin.

4.4.2. Explant culture technique

In this technique limbal tissues, from corneoscleral rims/limbal biopsies/animal source, are dissected to obtain limbal epithelium without the underlying stroma. Bits of limbal tissue (explants) are inoculated onto the BM side of HAM as shown in the schematic representation (Tsai *et al.*, 2000a,b), allowed to adhere and cultured to obtain limbal epithelial cultures. Joseph *et al.* (2004) chopped the epithelium into 3-mm tiny bits, placed onto 35 mm plastic culture plates, allowed to adhere for 10 min with the epithelial side of the explant facing upward. Figure 5.7 depicts the feeder layer- free explants culture technique on HAM (limbal alone or limbal and conjunctival coculture).

4.4.3. Suspension culture technique

In this technique single cell suspension of dispase II digested limbal epithelial cells is seeded onto either HAM (Koizumi *et al.*, 2002) or plastic tissue culture dishes (Lindberg *et al.*, 1993). Koizumi *et al.* (2002) hypothesized that suspension-cultured limbal epithelium was morphologically superior to the explant cultures and should ideally include stem cells, which was later disproved by Kim *et al.* (2004) who showed no significant difference in cultured limbal epithelia by both the methods. Table 5.3 summarizes the features of these two techniques of cultivation.

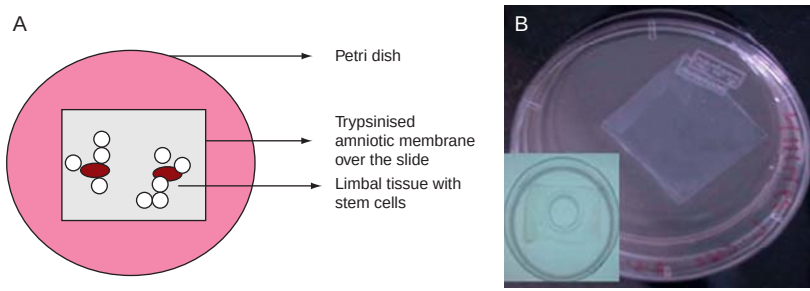

Figure 5.7 Explant culture technique. The submerged, feeder-free explant culture technique of culturing limbal explants. (A) shows a glass slide used to hold the amniotic membrane in place so as to provide a uniform surface for the cells growing from the explant, which is in turn placed in a petri plate, with medium to provide a submerged state. When cultured, small cells are seen arising from these tissue bits as shown. (B) is representation of the same showing HAM on glass slide for limbal cultures and with a ring barrier (inset) for cocultivation of limbal (inside the barrier) and conjunctival (outside the barrier) cells.

4.4.4. Organ culture technique

Increasing importance of role of *in vitro* models in the field of ophthalmology and an insufficiency of primary and immortalized cell lines to mimic the *in vivo* interactions between different cell types lead to organ culture models for the whole cornea or anterior chamber. Corneal organ culture originally was established for long-term *in vitro* study of corneal wound healing (Foreman *et al.*, 1996). Ideally, an *in vitro* corneal model should, (i) include an epithelium exposed to the air, (ii) have an automatic irrigation system replicating tear film and assisting epithelial desquamation, (iii) the anterior chamber should be perfused separately to allow maintenance at normal IOP, (iv) support long-term maintenance in culture for epithelial and endothelial wound healing studies retaining healthy and transparent stroma and keratocytes during the period, (v) be a cheaply and readily available corneal (species) source. Corneal epithelial proliferative potential (CEPP) depends on the age of the donor, time of death to enucleation, time of enucleation to organ culture and time in organ culture in the eye bank. Shanmuganathan *et al.* (2006) determined the epithelial proliferative capacity of organ cultured limbal tissue, correlated with various donor and eye banking factors and found that death to enucleation time was the only statistically significant factor affecting CEPP.

In this technique, approximately 4 mm of the limbal conjunctiva is excised from corneoscleral rims, rinsed in sterile PBS, placed epithelial-side down into a sterile cup containing MEM. Endothelial corneal concavity is filled with MEM containing 1% agarose and 1 mg/ml rat tail tendon collagen maintained at 42 °C, allowed to gel and cornea along with its supporting gel, is inverted and then transferred to a 35-mm dish, about 2 ml

Table 5.3 Summary of experimental results for explant versus suspension culture techniques

S. No	Feature	Explant culture technique	Suspension culture technique
1	Confluent cultures obtained at	14–21 days	10–14 days
2	Cellular morphology	Combination of smaller and larger cells	Smaller, more compact and uniform
3	Immunostaining	Smaller cells—p63, EGFR, K19, and integrin β1positive Larger cells—K3, involucrin, and connexin 43 positive	Smaller cells—p63, EGFR, K19, and integrin β1positive Larger cells—K3, involucrin, and connexin 43 positive
4	BrdU-label retaining cells (21 days chasing)	2.3 ± 0.7%	3.73 ± 1.5%
5	Phenotype of cell differentiation	Maintained	Maintained
Animal studies (Rabbit)			
6	Vimentin positivity (FACS)		<5%
7	Histological examination	Cultured epithelial cells poorly differentiated	Cultured epithelial cells could be better differentiated
8	ΔNp63 expression	Cells around the explants were positive while peripheral cells were negative	More number of cells showing positivity than in explant cultures

of culture medium is added drop wise to the surface of central cornea until limbal conjunctiva is covered, leaving the epithelium exposed to air. This model experimented in bovine corneas has been shown to have *in vivo* properties of maintenance of air–liquid interface, support epithelial stem cells, IOP of 18 mm Hg, posterior chamber perfusion rate of 2.5 μl/min and the cornea could be maintained long enough for epithelial and endothelial wound healing studies.

4.4.5. Airlift technique

The air–liquid interface system popularly known as ''Airlift'' technique is used for simulation of *in vivo* conditions, in many epithelial cell systems, including limbal epithelial cells for stratification (Xia, 1989). The mechanisms behind such effects are still unknown. Briefly the technique involves growing of cells for 12–14 days during which a monolayer of cells is formed, followed by lowering the level of medium so as to provide an air–liquid (water) (Koizumi *et al.*, 2001; Pellegrini *et al.*, 1997; Tsai *et al.*, 2000b) interface. The minimum culture duration with this technique is 30 days. Commercially available culture inserts (Fig. 5.8) made from polycarbonate membrane were used by Koizumi *et al.* (2002) to grow the limbal epithelial cells (Espana *et al.*, 2003).

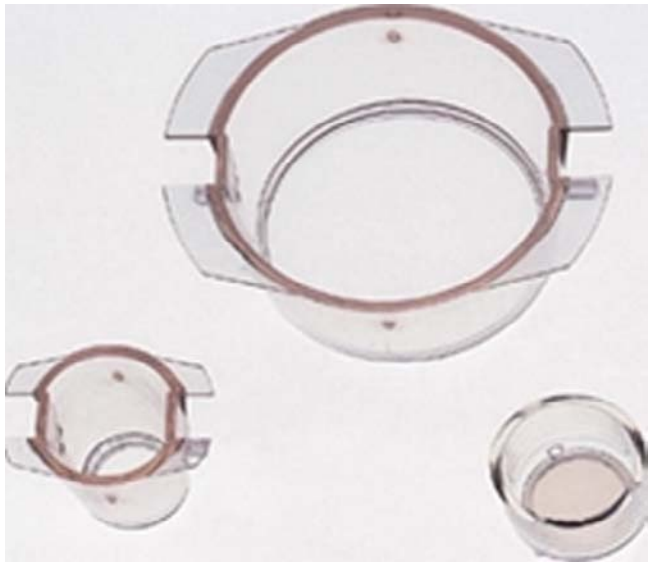

Figure 5.8 Culture inserts. The different models of culture inserts used for cultivation of limbal epithelial cells with feeder layers, acting as a barrier between the feeder layers and the growing limbal epithelial cells, and provide an air–liquid interface when the level of the culture medium is lowered.

4.5. Characterization

Characterization of the cultivated limbal epithelial cells is aimed to answer the most important aspects of extent of similarity between the cultivated cells and native corneal epithelium and percentage of stem cells in cultured epithelium.

4.5.1. Clonal assays/colony forming assays

Studies by Barrandon and Green (1987) reveal three clonal types of multiplying epidermal keratinocytes distinguishable by the marked difference in the frequency with which they give rise to terminal progeny as (i) Holoclones (greatest growth potential, likely to be stem cells) (Lajtha, 1979); (ii) Paraclones (grow rapidly at first, the total lifetime of no more than 15 cell generations, followed by growth arrest and terminal differentiation), and (iii) Meroclones (clone of mixed composition, gives rise to paraclones with appreciable frequency). The transitions from holoclone to meroclone to paraclone are unidirectional resulting in progressively restricted growth potential. These clonal keratinocyte are analogous to other differentiated cell types such as the hematopoietic system, the nervous system and muscle.

Clonal relationships between LSCs, the stage of development at which they are determined, the number of cells that are specified to become stem-like, and the lineage relationship between them still remain unknown due to the lack of definitive LSC markers. Two extreme hypotheses have been proposed for the clonal derivation of LSCs: (i) all LSCs descend from one cell that is specified during late embryogenesis and (ii) each LSC in the postnatal eye has been specified individually from a huge pool of randomly arranged cells that are only distantly related to each other. Studies on cornea in chimeras and mosaic mice infer that the real situation lies between these two extremes. On the basis of previous reports, it is possible to assign two stages in the development of the corneal epithelium (i) an embryonic and early postnatal phase in which cells throughout the epithelium proliferate by randomly orientated cell division and (ii) subsequently a more ordered maintenance phase of directional clonal growth, in which proliferative stem-like cells in the limbal region give rise to migrating TACs (Cotsarelis *et al.*, 1989; Lehrer *et al.*, 1998; Yew *et al.*, 2001). A study by Collinson *et al.* (2002) suggests a duration of 3 weeks for complete turnover in the uninjured corneal epithelium, inferring an alternative source of residual dividing cells.

4.5.2. Morphology

Explant cultures of the limbal epithelium when observed under a phase contrast microscope show clear spherical refractile cells emerging from the explants by about day 1, which adhere to the substrate as cuboidal cells by days 2–3 and extend circumferentially around the explant with characteristic

pushy margins. When confronted with another margin, these margins disappear due to contact inhibition forming a uniform monolayer of closely packed cuboidal cells in 12–15 days time in our culture system. Stratification of these cells could be seen when retained for longer durations of 3–4 weeks in culture or promoted by providing an air–liquid interface to growing cells.

Histology of the cultured limbal epithelium in whole mount preparation shows a monolayer of polygonal cells with vesicular nuclei, originating from the edge of the explanted tissue, while the paraffin embedded sections shows a multilayer (2–4 layers) of cuboidal epithelial cells on HAM, mimicking the *in situ* milieu. Ultrastructural study of the cultivated LSCs shows numerous microvilli on the apical cells, torturous cuboidal cells arranged as layers with distinct intercellular desmosomes, and hemidesmosomes with the underlying substrate. Figure 5.9 is a representation of morphological features of limbal epithelium cultured on denuded HAM.

4.5.3. Immunophenotyping

Stem cells and TACs make up the proliferative pool of the epidermis. Keratinocytes presumed to be stem cells have been enriched based on the expression of cell surface molecules (Bickenbach and Chism, 1998; Jones and Watt, 1993; Tani *et al.*, 2000); but no definitive differentiative marker has been identified for stem cells and TACs (Cotsarelis *et al.*, 1999; Lavker and Sun, 2000). Keratinocyte stem cells are believed to possess a large nucleus to cytoplasm ratio and have been described as small and relatively undifferentiated, both biochemically and ultrastructurally (Bickenbach, 1981; Holbrook and Odland, 1975; Lavker and Sun, 1982; Lavker *et al.*, 1991; Morris *et al.*, 1990; Pavlovitch *et al.*, 1991). The low side scatter (SSC) has been one of the parameters used for isolating hematopoietic stem cells (Johnsen and Knudsen, 1996). Recently, low SSC was also used to enrich spermatogonial stem cells in mouse testis (Shinohara *et al.*, 2000). A report by Epstein *et al.* (2005) has shown that the LSCs have a low granularity and high N/C ratio confirming their stem cell nature and this was enriched in the p63 expressing cell population.

Immunophenotyping of the cultivated cells for specific surface marker molecules by immunohisto/cytochemistry, FACS or MACS sorting to obtain cells of the required phenotype, traditionally using cornea specific cytokeratin pair K3/12, CK19, integrin $\beta 1$, ΔNp63 isoforms (specifically ΔNp63α) (Vascotto and Griffith, 2006), connexin 43, EGFR, Ki-67 (Joseph *et al.*, 2004) with recent inclusions of low affinity NGF receptor 75 (p75) (Di Girolamo *et al.*, 2009; Qi *et al.*, 2007, 2008; Touhami *et al.*, 2002), pan-cytokeratin and vimentin, ABCG2, gap junction intercellular communication (GJIC) (Grueterich and Tseng, 2002), c-kit (Vascotto and Griffith, 2006) aldose dehydrogenase 1 (ALDH1), and RHAMM/HMMR expression (Ahmad *et al.*, 2008), and so on. Definitive phenotype of the cultured limbal epithelial cells is not yet known but is a combinatorial

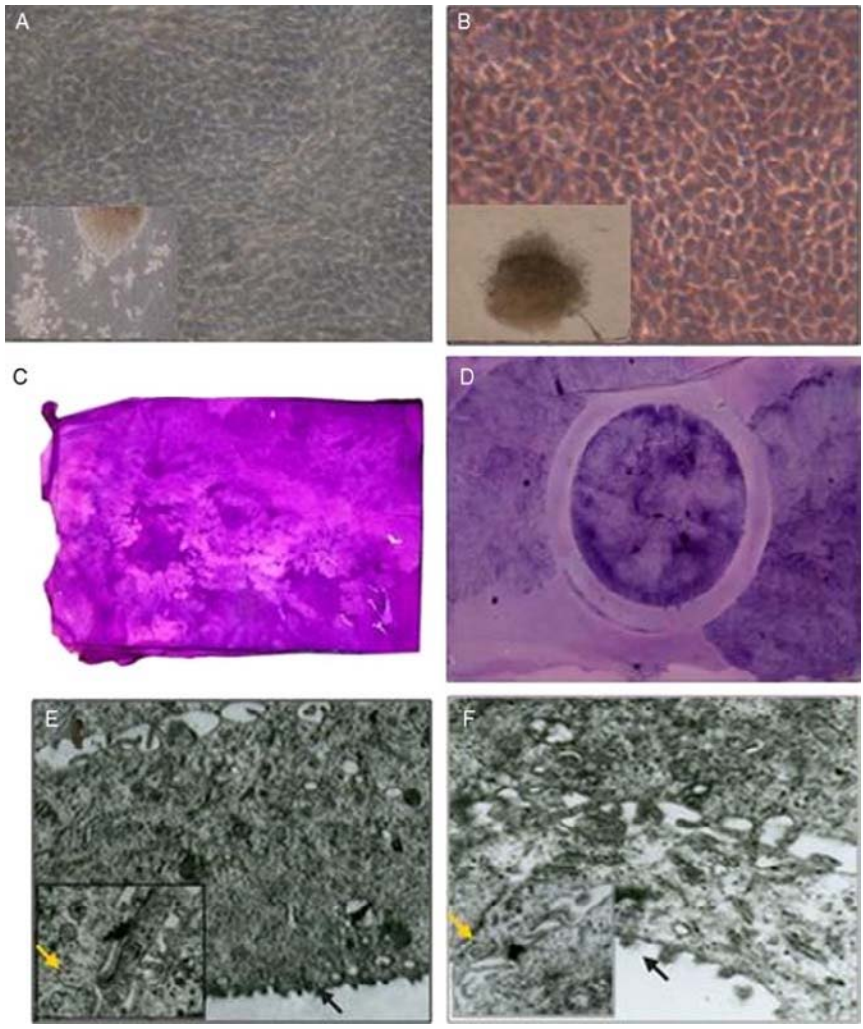

Figure 5.9 Morphological features of epithelium cultured on denuded HAM. The morphological features of limbal and conjunctival epithelial cultures grown on devitalized HAM, by feeder-free submerged explant culture technique showing the morphology of compactly packed, cuboidal limbal cells (A) and conjunctival cell (B) (cells growing from the explants could be appreciated in the inset) as observed under a phase contrast microscope by about 12–14 days of culture; extent of expanded limbal epithelial monolayer on HAM (C) and co-cultured limbal and conjunctival epithelium (D) as seen by whole mount preparations stained with H&E. The figure also shows the microvilli (black arrows) and desmosomes (yellow arrows) in cultivated limbal (E) and conjunctival (F) epithelia by about 3–4 weeks in culture.

profile of these markers. Immunophenotype results are to be supported by side population studies, BrdU incorporation studies (LRCs) or CFU assays to determine the percentage of stem cells in the population being transplanted. Table 5.4 summarizes the immunophenotype of the limbal and corneal epithelial cells.

4.5.4. Label retaining cell studies

Tissue stem cells are quiescent and retain a DNA synthesis label, while the label is diluted out in more mature dividing cells during a chase period. Retention of the label may result from segregation and selective retention of template DNA strands by stem cells undergoing asymmetric division rather than from cell quiescence in adult murine intestinal crypt cells, muscle satellite cells and mammary epithelial stem cells Capuco (2007). Those cells retaining the label over long period of time are the label retaining cells (LRCs) and have been identified as stem cells (retain template strands of DNA and to pass the newly synthesized strands or chromatids to their daughter cell during asymmetric division). It has generally been thought that, once cells begin DNA replication, they progress without interruption through S-phase and incorporate added DNA label (Tritiated thymidine [$^3H_1$] or bromo-, chloro-, or iodouridine (Conboy *et al.*, 2007; Fatima *et al.*, 2006) into their newly synthesized DNA. Labeling index (LI) is thus defined as the fraction of labeled cells present upon autoradiography of DNA labeled cells, for estimates of the size of the S-phase compartment in various tumors. Because stem cells are slow cycling, they rarely incorporate DNA labels after single pulse administration explaining why stem cell-rich regions (e.g., limbal epithelium, bulge region of the hair follicle, tips of deep rete ridges of palmar epithelium) usually do not incorporate large amounts of pulse-administered labels. BrdU has been the most widely accepted label for these studies because of the associated radioactive risks with other compounds ($^3H_1$). The only disadvantage of this study is that S-phase cells that do not synthesize DNA via enzymatic pathways (such as thymidylate synthetase pathway) do not utilize exogenous thymidine and hence do not incorporate thymidine analogues complicating the assay and inaccurate results on the fraction of S-phase cells. An example of such instance was reported by Hamilton and Dobbin (1982) in adenocarcinoma cells.

Study design for the incorporation of a thymidine analogue into the S-phase cells would depend on the need of the kind of information from the experiment. If the percentage of cells in S-phase at a given time point is the data of interest, a *pulse labeling experiment* is performed where in the cells in culture are labeled with a known concentration of the thymidine analogue at that particular time point for a period of 24 h, terminated after labeling and assayed for cells incorporating the compound using immunohistochemistry or immunofluorescence or FACS. If the data of interest are the percentage of cells retaining the label over a defined period of time, then

Table 5.4 Phenotypic markers for limbal and corneal epithelium

Markers	Limbal epithelium	Corneal epithelium
Surface markers		
CD34	−	−
Transferrin receptor CD71	Basal cell +	+
ABCG2	Basal cell+	−
Proliferation markers		
Ki67	−	+++
Cyclin D	Basal cell +	
Cyclin E	Basal cell +	
Intermediate filaments		
Cytokeratin K3	−	+++
Cytokeratin K19	Basal cell +	−
Cytokeratin K12	−	+++
Cytokeratin K14	Basal cell +	−
Vimentin	+', Co-expression with K19	−
Adhesion molecules		
Integrin beta1	+', Higher expression in limbal basal cells	+
Integrin α 6	− in basal cells, ++ in suprabasal cells	+
Connexin 43	−	+
Connexin 50	−	+
E-Cadherin	− in basal cells, + in suprabasal cells	+
Growth factor receptors		
EGF receptor	+, Higher expression in basal cells	++
Keratinocyte growth factor receptor (KGFR)		++
Miscellaneous		
α-Enolase	+	−
p63	+	+
Nestin	+ in suprabasal cells	+

a *pulse chase experiment* is performed where the cells are incubated with the labeling compound for a duration of 24 h in the initial dividing phase of culture, the point termed p0 and the labeling medium is substituted with

nonlabeling growth medium for the rest of the culture duration. Cultures are terminated at different time points (starting with p0) later, detected for label positive cells using any of the above techniques and the result compared against p0. To a large extent estimation of LRCs in the culture is confirmative of the percentage of stem cell population and is of great importance where the cells would be further used for transplantations. *In vivo* experiments are also designed in a similar way and the results are assayed using *in vivo* confocal microscopy or autoradiography techniques to trace the percentage of label positive cells.

4.5.5. Side population studies

Recent studies have shown the presence of side population (SP) cells, immature cells in the limbus and smaller in size compared to normal cells, similar to those from other tissues (Espana *et al.*, 2002). SP phenotype of these stem cells is indicated by expression of multiple drug resistance marker ABCG2, higher expression of the polycomb gene family member Bmi-1 and increased nestin expression, which may allow proper maintenance and self-renewal of limbal epithelial SP cells. Most of the limbal epithelial SP cells were found to be growth arrested in G0/G1, compared to those isolated from the epithelium explaining decreasing CFE with increasing purity of the SP fraction. SP cells also show a lower expression of Integrins $\alpha 6$ and $\beta 1$ highly expressed in cells with strong proliferative capabilities. Limbal epithelial SP cells are cell cycle arrested, exhibit no *in vitro* proliferative capabilities and thus defining epithelial stem cells by CFAs or detection of LRCs may not represent true stem cells, but rather immature progenitor cells. In fact, Oshima *et al.* (2001) discussing their results, on adult multipotent stem cells from hair follicles state that, these cells with high proliferative potential, are likely generated from more immature stem cells or progenitor cells comprising the portion of the non-SP (NSP) fraction with active telomerase, demonstrate a slow cell cycle, have CFU abilities and can produce a more differentiated phenotype. TA cells in the NSP cell fraction of the limbus can also migrate toward the central cornea to produce the corneal epithelial cells that express CK3 and CK12. (Umemoto *et al.*, 2006)

Park *et al.* (2006) showed that $0.73 \pm 0.14\%$ of the rabbit limbal epithelium were SP cells, with smaller cell size than the major population (MP) cells, quiescent and in undifferentiated state. These SP cells were able to regenerate cornea-like structure with basal enrichment of p63-positive cells by *in vitro* 3D culture and *in vivo* transplantation, all of which were best achieved by the whole population (WP) of cells comprising SP and MP cells. Following a central cornea wounding, a transient increase in the SP cells, followed by increase in CFE in the limbal MP cells (day 2) and then in the corneal MP cells (day 5) was observed. Colony-forming assay of the limbal SP cells gave approximately eightfold higher CFU than the limbal MP cells.

4.5.6. Gene expression profiles

Analysis of RNA by semiquantitative RT-PCR, quantitative RT-PCR, subtractive hybridization PCR, quantitative real-time PCR has been performed either singly or in combination for gene profiling or transcriptional profiling of the stem cell-enriched limbal basal cell population in their ''natural'' quiescent state with respect to its neighboring corneal basal epithelium. Other techniques such as SAGE, microarrays, and microRNA have also been employed for these studies. Laser capture microdissection has added value to these techniques by allowing the isolation of resting limbal and corneal basal cells from frozen sections with minimal tissue processing, thereby improving the yield and quality of RNA.

Analyses of RNA isolated from limbal and corneal basal cells by Zohu *et al.* (2006) using microarrays techniques assisted by laser capture microdissection, revealed a set of 100 genes that were differentially expressed in limbal cells versus corneal epithelial basal cells, confirmed by semiquantitative RT-PCR showed an upregulation of three limbal (epiregulin, basic keratin complex 2, fibroblast growth factor receptor 1 (FGFr1), and the adaptor molecule disabled homolog 2 (Dab2)) and three corneal genes contributing to the unique heterogeneity of these two closely related basal cell populations similar to the stem cell enriched populations of hair follicles, suggesting a signature set of genes as in neural, hematopoietic, and embryonic stem cells.

Serial analysis of gene expression (SAGE) of rat limbal and corneal epithelium by Adachi *et al.* (2006) showed that limbal-enriched transcripts such as WDNM1-like protein, mesothelin, marapsin, cytokeratins K4 and K15 and membrane spanning four-domain subfamily A member 8B were also expressed in the conjunctival epithelium, suggesting an association between several genes preferentially expressed in the limbal epithelium with cellular proliferation and migration.

4.5.7. Proteomics

The lacunas, in gene expression studies such as inability to correlate the changes in mRNA levels to changes in encoded protein levels, posttranslational modifications (phosphorylation, glycosylation, etc.) affecting cell function, have led to protein microarrays. In recent years proteomic approaches have added a wealth of data to gene expression analyses for studying signaling pathways and molecular mechanisms involved in self-renewal, pluripotency, and/or multipotency of stem cells and their differentiation. Technical improvements in peptide and protein separation, mass spectrometry, have still to answer issues of sample heterogeneity, posttranslational modifications, protein–protein interaction, and high throughput quantification of hydrophobic and low abundant proteins (Baharvand *et al.*, 2007). Different techniques such as 2D gel

electrophoresis, Western blots, mass spectrometry have been applied by different groups to study the proteome profile of LSCs.

Proteomic studies by Ma *et al.* (2006) showed an enhanced secretion of anti–angiogenic factors endostatin and restin, stratifin (which act as cell cycle controller, inducing MMPs 1 and 3 involved in the generation of restin and endostatin respectively). Studies by Shimmura *et al.* (2006) revealed proteins selectively secreted by limbal fibroblasts such as SPARC, vimentin, serine protease, collagen alpha 2 precursor, tissue inhibitor of metalloproteinase 2 (TIMP-2), and 5,10-methylenetetrahydrofolate reductase (FADH2). The study has also shown that most of the corneal anti–angiogenic factors belonging to ECM proteins acquire angio-inhibitory activity only after proper proteolytic processing. *Peptide arrays* containing peptides have been used for profiling p53 and kinase activities (Houseman *et al.*, 2002; Pellois *et al.*, 2002).

4.6. Quality assurance

As per regulatory guidelines, cultivated or *ex vivo* expanded cells for clinical transplantations have to be screened for absence of any bacterial, fungal, or microbial infections; any cryptic infections such as mycoplasma, toxoplasma; and any harmful byproducts of such organisms such as bacterial endotoxins, fungal mycotoxins and aflatoxins either by direct culture or using ELISA, RIA, direct staining or PCR amplification-based commercially available kits.

All filter sterilized media and chemicals are to be checked for sterility against aerobic and anaerobic bacterial and fungal agents. Biological substrates such as HAM are to be evaluated for HIV, HBs antigen and VDRL before processing and the processed HAM is evaluated for bacterial or fungal contaminations. Media (supplemented with serum—autologous or FBS) used for culturing cells are also evaluated for cryptic infections such as mycoplasma and toxoplasma. The cultivated cells are also similarly evaluated for sterility before being transplanted onto the patient.

4.6.1. Ideal cells for clinical application

Based on the characterization of cultivated cells on different substrates by different groups, compactly packed cuboidal cells (monolayer or stratified) with typical epithelial morphology; healthy cells as evident from morphological observation, viable with good number of mitotic figures, no apoptotic or dying cells, retaining the profile of limbal or corneal progeny, free of any exogenous bacterial, viral or fungal contaminants or their products such as endotoxins, mycotoxins and so on, cryptic infectious agents are the ideal cells for clinical application of *ex vivo* expanded cells. These criteria are in line with the regulations given by the US-FDA (USA) and Indian Council for Medical Research (ICMR) for cell therapy.

5. Review of Literature on Clinical Trials

5.1. General overview

While the literature has consistently seen reports on the short- and intermediate-term efficacy of stem cell transplantation for ocular surface reconstruction, it must be mentioned that all of these studies are limited in that they are retrospective, nonrandomized, the number of eyes in the cohorts being small and relatively short durations of follow-up.

It is clear from the literature that autograft transplantation is efficacious for both transplanted limbal tissue (Kenyon and Tseng, 1989; Rao *et al.*, 1999; Shimazaki *et al.*, 2006; Tseng *et al.*, 1995) as well as for cultivated epithelial transplants (Espana *et al.*, 2002; Rama *et al.*, 2001a; Sangwan, 2006; Sangwan *et al.*, 2005a,b; Tsai *et al.*, 2000a,b), but is limited by the relatively short follow-up. Although there are no known cases of limbal dysfunction after removal of donor tissue from a healthy eye, caution is required in cases with chemical burns because the apparently healthy eye may have been involved during the initial trauma. Removal of limbal tissue from a partially stem cell–deficient eye may cause irreversible damage. Cultivated epithelial sheets using autologous cells can reduce the trauma to the healthy eye by limiting the amount of excised tissue. However, techniques for cultivating epithelial sheets vary greatly among the reports of clinically successful patients.

Ang *et al.* (2007) reported less stromal scarring and better vision in the eye with CLET compared to fellow eye with a limbal allotransplant providing evidence on a documented benefit from cultivated epithelium in the same patient. Meta-analysis of more than 2374 articles of LSC transplants (limbal transplant versus cultivated transplants, with/without concurrent PK/Deep Anterior Lamellar Keratoplasty (DALK), etc.) by Cauchi *et al.* (2008) did not show a strong evidence of any advantage of one procedure over another for a specific type of LSCD. Long-term results demonstrating continued efficacy of cultivated epithelium are awaited.

While allografts are initially efficacious, it is clear their survival over time is critically dependant on various factors. Univariate analysis by Santos *et al.* (2005) on 33 eyes of 31 patients with total LSCD secondary to SJS and severe chemical burns reported a decreased allograft survival rate with time and also demonstrated that dry eye, keratinization, SJS, eyelid abnormalities and allogenic transplantation have a significant negative impact on the clinical outcome, is supported by evidences from Tsubota *et al.* (1996, 1999).

Based on the literature available at this time, there is a generalized consensus among corneal surgeons as to the indications and contraindications for attempting surgery (Shimmura and Tsubota, 2008). In eyes with a compromised ocular surface and stromal opacification, PK alone has a poor prognosis

Table 5.5 Summary of reports on clinical outcome of CLET

S. No.	Group	Year	Number of eyes	Follow-up months	Success (%)
1	Schwab *et al.*	2000	14	13	71.4
2	Rama *et al.*	2001	18	12–27	77
3	Shimazki *et al.*	2002	13	10	46.2
4	Daya *et al.*	2005	10	28	70
5	Sangwan *et al.*	2005	15	8.3	93
6	Sangwan *et al.*	2006	88	18.3	73.1

since the transplanted corneal button is inevitably replaced by invading vascularized tissue, further complicated by immunologic rejection and secondary glaucoma. While the initial results of PK combined with keratolimbal allograft (KLAL) to replace both the opaque stroma and LSCs seemed promising, the long-term results were not encouraging (Tsubota *et al.*, 1999). Report by Yao and associates (2002) combining DALK (intact endothelium and no immunologic rejection) with CLAU showed promising results in an otherwise complicated group of patients and since an autologous tissue was used, the use of systemic and topical immunosuppressants could be tapered much earlier compared with allografts, reducing the number of cases with secondary infections and glaucoma. Shimazaki *et al.* (2002) found that DALK is a safer procedure and is just as effective as PK in a randomized clinical trial. Table 5.5 summarizes reports on clinical outcome of CLET.

5.2. Proof of concept

Ocular surface reconstruction by cell therapy is a valuable model of cell therapy. This is because unlike other systems, it provides a unique opportunity to document the survival, networking, and integration of transplanted cells, through various techniques: (a) The corneal impression cytology (CIC) done to confirm the diagnosis is a proof that the ocular surface was covered by the conjunctival cells before the cell therapy. Subsequent to cell therapy, the re-epithelialization of the surface is provided by (b) the study of the pannus removed during the surgical debridement procedure prior to transplantation of cultivated limbal epithelium, (c) intact epithelium post-transplantation as evident by Fluorescence staining, (d) the absence of vasuclarization/pannus/conjucntivalization, (e) clinical confocal microscopy which documents the multilayering of corneal epithelial cells, (f) histologic proof of corneal epithelium in the corneal specimen received from patients undergoing PK following CLET for visual rehabilitation, (g) proof of surviving cells through DNA finger printing in allogenic CLET.

Traditionally, the diagnosis of conjunctivalization of the corneal surface relies on the detection of conjunctival epithelial goblet cells on impression

cytology specimens or conjunctival phenotype in corneal pannus specimens. Cross-sectional studies on corneal pannus showed presence of goblet cells, in addition to stromal vascularization and inflammatory cell infiltration (Espana *et al.*, 2004).

Conjunctival phenotype of the pannus was also shown by absence of CK3 (Fig. 5.10) and a strong CK19 expression by immunohistochemistry (Elder *et al.*, 1997) and Western blot, though a weak expression of CK3 transcripts, but not CK12 suggesting a noncorneal origin of cells, but some expressed both K3 and K19 (probably partial LSCD) as in aniridia. Western blot analysis of ΔNp63 in murine and rat models with LSCD was also consistent with the conjunctivalized pannus (Moore *et al.*, 2002). The inability of corneal impression cytology is to obtain a full thickness epithelial sample, even if conjunctivalization is detected, necessitate the studies of corneal cross sections as reported by Fatima *et al.* (2008).

In vivo confocal analysis of the transplanted eyes during the regular follow-up shows the homing and integration of cells onto the host environment, remnants of the degraded HAM (if any) (a sign of successful transplantation), *in vivo* stratification of the transplanted monolayer of cells and the corneal epithelial phenotype along with vision improvement (Fatima *et al.*, 2007).

Significant stromal scarring in chemical burns necessitates a PK for visual rehabilitation. Earlier results of PK in these eyes were not very encouraging probably because the limited life span and limited proliferative potential of the TACs from PK specimen were insufficient to restore the ocular surface epithelium on a long-term basis. CLET in these cases maintains the reservoir of corneal epithelial cells required for a stable and healthy corneal

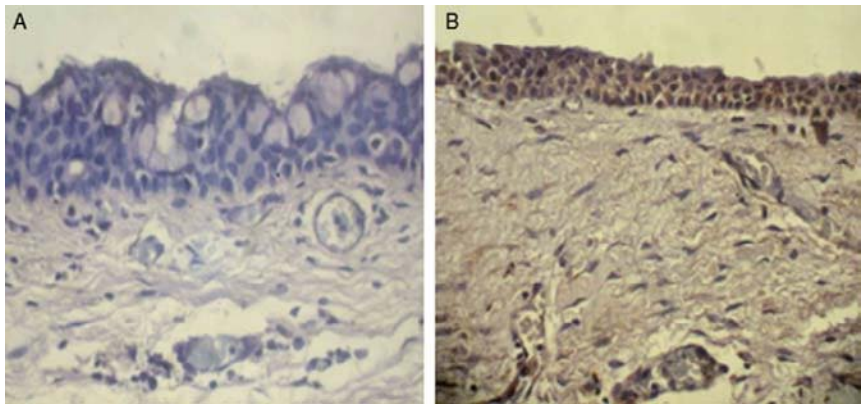

Figure 5.10 Immunophenotype of conjunctivalized pannus. The conjunctivalized pannus which is negative for the corneal phenotype specific CK3 before cultivated limbal epithelial transplantation (CLET) (A), and the corneal pannus which is positive for the corneal phenotype specific CK3 after CLET (B).

epithelium. Sangwan *et al.* (2005a) hypothesize that a transplanted monolayer ultimately proliferates *in vivo* to produce stratified (multilayered) epithelium following transplantation, supported by results from corneal buttons (Sangwan *et al.*, unpublished data, 2001), which showed a stratified corneal phenotype epithelium and a (collagen IV) staining of the basement membrane (Figure 5.11). This proves the concept that the transplanted LSCs home the host damaged cornea and become functional corneal epithelial cells as evident by the formation of the cells own basement membrane.

DNA analysis allows studies of graft survival, more so in the allogenic conditions where the damaged eye receives a donor cornea or LSC transplantation. Studies have shown that the surviving donor limbal epithelial cells in allogenic transplantations are responsible for the long-term graft survival, in contrast to some previous reports and that clinical efficacy of limbal transplantation does not necessarily correlate with the survival of donor cells on the ocular surface. Some instances find the presence of a chimera of two different donor cells along with recipient cells, probably indicative of the number of donors in that particular case. The technique also discriminates the presence of conjunctival cells when used in combination with phenotypic or histological evaluation. Although the information obtained by this assay is valuable, detection of donor cells may not be accurate in conditions of inadequate immunosuppression, or recurrent LSCD or partial LSCD; in cases of rejected or failed transplantations, detection of donor cells could be either for a short follow-up or may not be possible. The use of laser capture microdissection and CIC (Nelson,

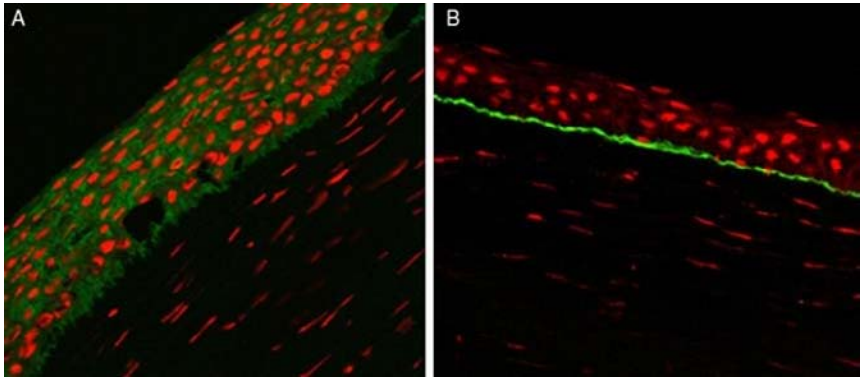

Figure 5.11 Immunophenotype of penetrating keratoplasty (PK) specimen. Immunophenotype of the PK after CLET shows stratified epithelium of corneal phenotype as stained by CK3 (green) (A) and the basement membrane secreted by these cells as stained by collagen IV staining (green) (B). Nuclei in both the specimens have been counter stained by Propidium iodide (red).

1988; Tseng, 1985; Williams *et al.*, 1995) allows for a clean dissection without involving the stroma or other contaminants. This is particularly important *in vivo* survival analysis which is inherently limited by not having the donor genotype available, and the nonrecipient polymorphisms are presumed to be of the donor type (Djalilian *et al.*, 2005).

Detection of DNA polymorphisms amplified by the polymerase chain reaction is a simple, rapid, and noninvasive method of following the course of transplanted cells at the ocular surface. These DNA fingerprinting methods allow assessment of quality and quantity of donor cell survival, as well as survival time. Williams *et al.* (1995) investigated the survival of donor-derived epithelial cells harvested by impression cytology after LSC transplantation (allo) using short tandem-repeat DNA polymorphisms (microsatellites) to distinguish donor and recipient cells and showed that cells of donor genotype were present over the grafted areas at the time of surgery but were not detected in the central cornea until 12 weeks postoperatively, indicating that repopulation of the epithelial surface from transplanted LSCs took considerable time while only recipient-type cells were detected in the grafted eye by 20th week. Henderson *et al.* (1997) attempted to investigate donor cell survival following corneal LSC grafting using amelogenin gene probe (a Y-specific DNA probe) with PCR. The same group later (Henderson *et al.*, 2001) demonstrated that a combination of impression cytology and single cell DNA fingerprinting is suitable for detecting transplanted cells after corneal limbal allografting.

6. Concluding Remarks

Although a rare disease, LSCD has become a unique disease which has unraveled the mysteries of stem cell concept in limbus, sequelae of depleted stores of LSCs, and has paved way for newer modalities of treatment using the principles of cell therapy. This has also helped the clinicians and scientists to explore different methods of cell cultures, scaffolds, and ways of characterizing these cells using emerging tools of cell biology. A meta-analysis of the published series from different parts of the world has thus provided adequate evidence of successful translational research from bench to bedside. It also came out as a unique model where objective proof of surviving cells could be obtained from the tissues removed during two staged surgeries: pannus removal during ocular surface reconstruction and followed by corneal transplantation as to restore vision. Even though the clinical outcome appears promising, a few areas that require further attention include quantification of transplanted cells, tissue and cell banking facilities, *in vivo* cell tracking so as to understand the homing and survival of transplanted cells into the limbal niche.

ACKNOWLEDGMENTS

Financial support was provided by Department of Biotechnology, Hyderabad Eye Research Foundation, C-Tracer. Dr Basti's work was supported in part by a grant from Research to Prevent Blindness, NY, to the Department of Ophthalmology.

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CHAPTER SIX

Cell and Molecular Biology of the Novel Protein Tyrosine-Phosphatase-Interacting Protein 51[1]

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Contents

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[1] This chapter is dedicated to Professor Andreas Oksche.

International Review of Cell and Molecular Biology, Volume 275
ISSN 1937-6448, DOI: 10.1016/S1937-6448(09)75006-3

Abstract

This chapter examines the current state of knowledge about the expression profile, as well as biochemical properties and biological functions of the evolutionarily conserved protein PTPIP51. PTPIP51 is apparently expressed in splice variants and shows a particularly high expression in epithelia, skeletal muscle, placenta, and germ cells, as well as during mammalian development and in cancer. PTPIP51 is an *in vitro* substrate of Src- and protein kinase A, the PTP1B/TCPTP protein tyrosine phosphatases and interacts with 14-3-3 proteins, the Nuf2 kinetochore protein, the ninein-interacting CGI-99 protein, diacylglycerol kinase alpha, and also with itself forming dimers and trimers.

Although the precise cellular function remains to be elucidated, the current data implicate PTPIP51 in signaling cascades mediating proliferation, differentiation, apoptosis, and motility.

Key Words: PTPIP51, PTP1B, TCPTP, Src-kinase, Raf-Erk signaling, 14-3-3, Nuf2 kinetochore protein, Cell cycle, Cell motility, Apoptosis, Differentiation.

1. Introduction

The human protein-tyrosine-phosphatase-interacting-protein 51 (PTPIP51) was detected in yeast two-hybrid screens aimed to identify potential substrates of the non-receptor protein tyrosine phosphatase 1B (PTP1B) and T-cell protein tyrosine phosphatase (TCPTP) and proteins being phosphorylated on tyrosine residues (Porsche, 2001). The protein was found in a number of tissues (Stenzinger *et al.*, 2005), with specific expression patterns. Although the structural characteristics revealed protein–protein interaction domains and several interacting proteins were identified, these features did not show the protein's function. Subcellular localization of the protein is partly mitochondrial and this may be related to a function in apoptosis. Here, we will review the available molecular and functional data, tissue distribution and discuss potential functional implications.

2. Molecular Biology of PTPIP51

2.1. Structure

The complete human PTPIP51 protein consists of 470 amino acids (aa) (Swiss-Prot Q96TC7, corresponding to 2251 nucleotides) with a calculated molecular mass of 52,118 kDa. The sequence is highly conserved in mammals and

chicken. The region 1–31 is predicted as a transmembrane helix-like structure and is required for mitochondrial localization and by analogy probably also for localization in other membranes (cf. Fig. 6.1). Amino acids 91–115 are likely to form a coiled-coil domain, one of the principal subunit oligomerization motifs. Another typical protein–protein interaction domain is present in the C-terminal half of PTPIP51 formed by tetratricopeptide (TPR) repeats. TPR modules are found in a number of proteins widely varying in cellular functions. Among these are proteins that control cell cycle and transcription, protein transport, or function as co-chaperones, protein phosphatases, modifiers of receptors, or enzymes. A TPR motif contains two antiparallel α-helices and tandem arrays of TPR motifs generate a right-hand helical structure with an amphipathic channel that may bind a target protein (Fig. 6.1) (Blatch and Lässle, 1999).

2.2. Phylogeny

The copy DNA (cDNA) of homologues of PTPIP51 was cloned from several mammalian tissues. The amino acid sequence of the mouse protein exhibits 84.9% identity with the human sequence. On the basis of sequence similarities in the TPR region, PTPIP51 was aligned with two other proteins into the family of sequence similarity 82 (FAM82) and received the designation FAM82C. An alignment of the sequences of PTPIP51 (FAM82C), FAM82A, and FAM82B is shown in Fig. 6.2. Sequence homologies are high in the C-terminal TPR-like region, but there are also similarities between FAM82A and PTIPIP51 in the N-terminal half. While homologies of PTPIP51 to invertebrate proteins appear to be low, FAM82B seems to be an ancestral protein and to be involved in chromosome segregation in *C. elegans* (Oishi *et al.*, 2007).

2.3. Genomic structure

The genomic information for PTPIP51 is located on human chromosome 15 (15q15.1) and is encoded by 12 exons. The first three exons comprise ATG codons at their beginning (exon 1) or close to the beginnings (exons 2 and 3) which are potentially used as alternative initiation codons of splice variants.

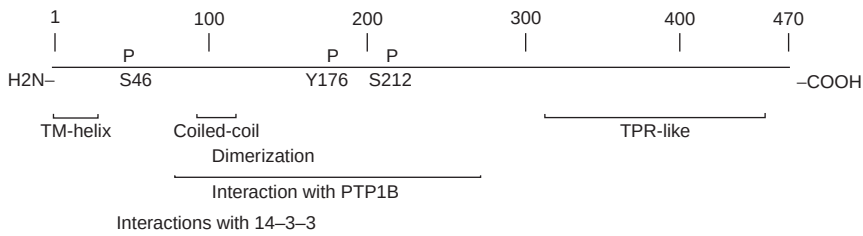

Figure 6.1 Schematic structure of human PTPIP51.

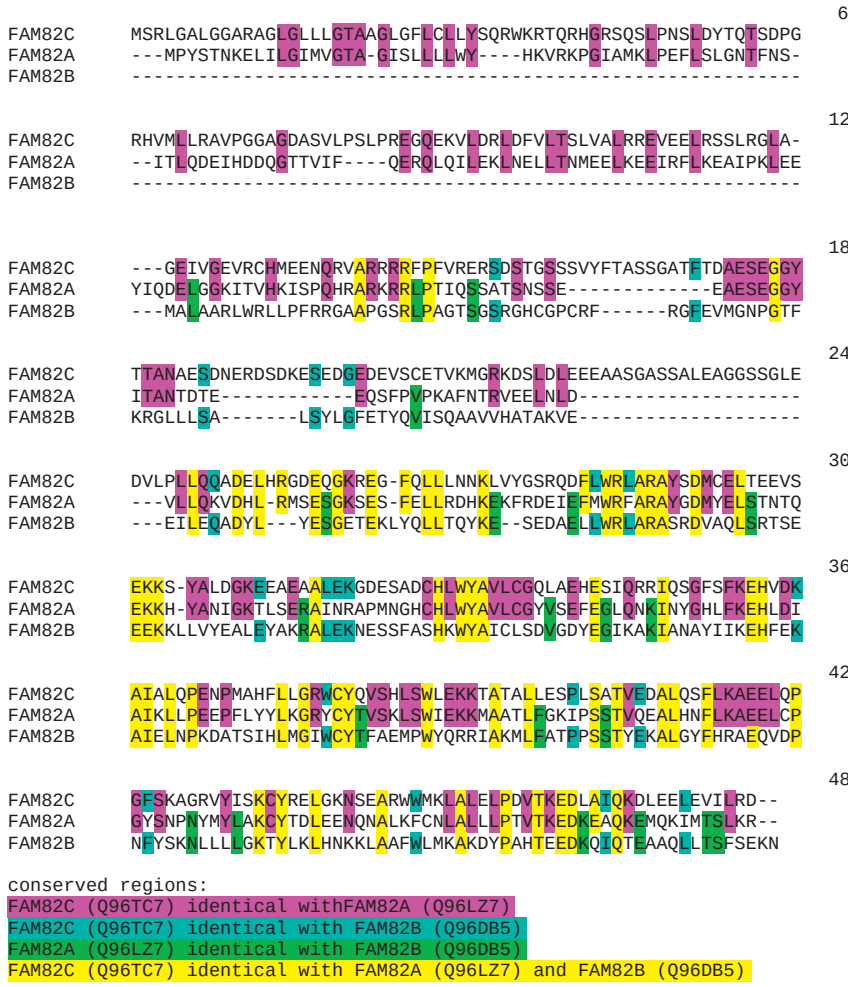

Figure 6.2 **Alignment of human FAM82 proteins (Kalign (2.0) alignment in ClustalW format). Most exchanges within conserved regions are conservative.**

The theoretical molecular masses of the resulting proteins are in fact close to the observed experimental values (see below). An additional ATG codon is located in exon 4. Human FAM82A is encoded on chromosome 2 (2p22.2) and human FAM82B on chromosome 8 (8q21.3) (Fig. 6.3).

2.4. Subcellular localization

Localization of PTPIP51 on the surface of mitochondria has been described and requires the N-terminal putative transmembrane motif (Lv *et al.*, 2006). Full-length PTPIP51 expressed in HEK293 cells as an epidermal growth

Exon	sequence	Expected Mr
1	**M**SRLGALGGARAGLGLLLGTAAGLGFLCLLYSQRWKRTQRHGRSQSLPNSLDYTQTSDPGRH	52118
2	V**M**LLRAVPGGAGDASVLPSLPREGQEKVLDRLDFVLTSLVALRREVEELRSSLRGLAGEIVGEV	45368
3	RCH**M**EENQRVARRRRFPFVRERSDSTGSSSVYFTASSGATFTDAESEG	38247
4	GYTTANAESDNERDSDKESEDGEDEVSCETVK**M**GRKDSLDLEEEAASGASSALEAGGSSGLEDV LPLLQQADELHRGDEQGKREGFQLLLNNKLV	29709
5	YGSRQDFLWRLARAYSDMCELTEEVSEKKSYALD	
6	GKEEAEAALEKGDESADCHL	
7	WYAVLCGQLAEHESIQRRIQSGFSFK	
8	EHVDKAIALQPENPMAHFLLGRWCYQ	
9	VSHLSWLEKKTATALLESPLSATVEDALQSFLK	
10	AEELQPGFSKAGRVYISK	
11	CYRELGKNSEARWWMKLALELPDVTKE	
12	DLAIQKDLEELEVILRD	

Figure 6.3 **Exons encoding PTPIP51 (FAM82C) and the proposed alternative structure of splice variants. The methionine encoded by putative alternative initiation points are underlined and printed in bold. The calculated M_r of the proteins derived from the corresponding initiation sites are given at the right hand. Gray underlay: proposed amino acids not expressed in splice mutants.**

factor receptor (EGFP) fusion protein also shows this localization (Fig. 6.4). However, the localization is not exclusively on mitochondria in tissues. PTPIP51 was also observed in other cytoplasmic compartments, at the endoplasmic reticulum(ER), and in the nucleus. Since the N-terminal transmembrane motif appears to be required for the localization at membranes, isoforms devoid of this motif may exhibit localizations different from the full-length form. Moreover, intracellular localization of PTPIP51 may be directed by phosphorylation (see Section 2.7) whose extent is influenced by the cell cycle (Daub *et al.*, 2008; Dephoure *et al.*, 2008).

2.5. Regulation of PTPIP51 expression

Roger and colleagues (2007) reported the negative control of PTPIP51 expression by ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) in differentiating rat retinal explant cultures and developing rat retina. PTPIP51 mRNA expression in retinal explants grown with medium alone increased from day 0 to day 6 and remained on a plateau for the consecutive days. CNTF and LIF treatment resulted in a marked inhibition of PTPIP51 expression observed *in vitro*. Upon addition of LIF and CNTF, inhibition peaked at day 6 by 52 and 45%, respectively. Notably, investigation of the *in vivo* PTPIP51 expression pattern observed for developing retina also revealed a continuous increase of PTPIP51 mRNA from day P0 to P14 (Roger *et al.*, 2007).

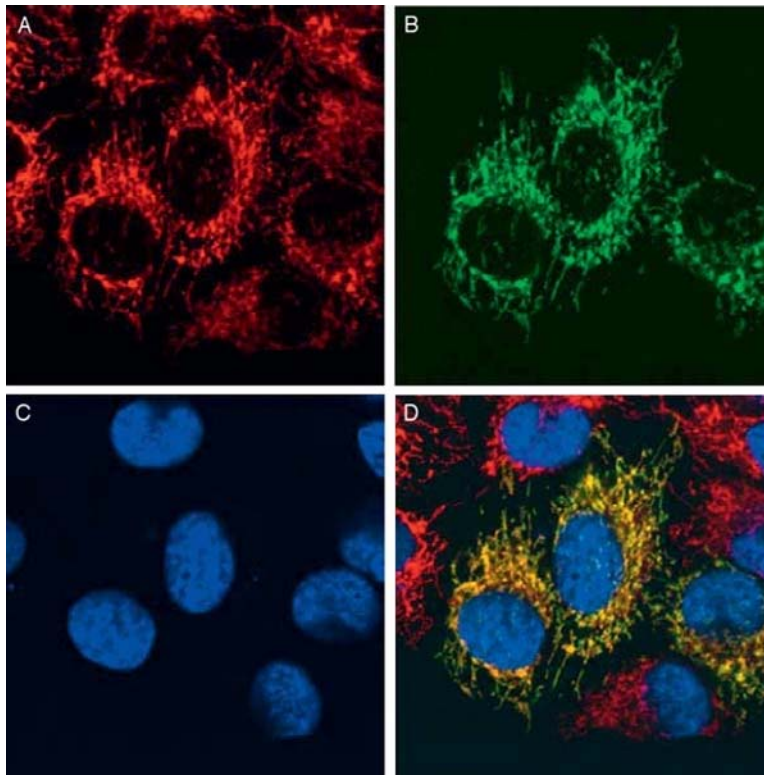

Figure 6.4 Localization of a PTPIP51–EGFP fusion protein expressed in HEK293 cells. HEK293 cells expressing full-length PTPIP51 fused to EGFP (B) were stained with MitoTracker (A) and Hoechst 33342 (C). The merged images show co-localization of mitochondria and PTPIP51–EGFP in the transfected cells (D).

Since CNTF and LIF belong to a family of structurally related cytokines, that is, the interleukin-6 family, it is tempting to speculate that a tissue-specific PTPIP51 expression is also influenced by other members of this cytokine family. For details of the biological function of the interleukin-6 family see Section 3.7.

2.6. Interaction of PTPIP51 with other proteins

2.6.1. Protein tyrosine phosphatase 1B and T-cell tyrosine phosphatase

To identify candidate proteins for reversible phosphorylation on tyrosine residues, we used PTP1B and the 48-kDa form of T-cell tyrosine phosphatase (TCPTP, Cool *et al.*, 1990) as bait proteins for the search of potential substrates in yeast two-hybrid screens (Porsche, 2001). The bait plasmids

contained cDNA sequences encoding either full-length PTP1B or a truncated (Δ1–11, Δ414–416) form of TCPTP which did not lead to intrinsic activation of the reporter genes in the absence of an interacting partner. Eleven cDNA clones encoding the sequence of PTPIP51 were isolated during a screen of a Jurkat cDNA library out of 21 clones fully characterized in the study. The sequences of the clones were incomplete, since they did not include an initiation codon close to their 5′-end. The reading frame was extended by rapid amplification of 5′ cDNA ends (5′-RACE) and corresponded with the cDNA sequence reported for a cDNA clone from brain (Genbank Accession No. AK001441, SwissProt Q9NVQ6). The full-length sequence for PTPIP51 encodes a 470-aa protein of 52.1 kDa molecular mass. But there is also evidence that smaller ~45, ~38, and ~30 kDa splice variants of the protein are expressed in a tissue-specific manner (Stenzinger *et al.*, 2005).

The interaction between PTPIP51 and the nuclear isoform of TCPTP was confirmed *in vitro*. A truncated form of PTPIP51 comprising aa 78 to 470, expressed in *Escherichia coli* in fusion with an N-terminal six histidines used as a tag on proteins (His_6-tag) and immobilized on a Ni^{2+}-nitrilotriacetic acid (Ni-NTA) column, was treated with an extract from nuclei of lymphocytes containing high concentrations of the nuclear isoform of TCPTP. After extensive washing, PTP activity was almost completely eluted together with PTPIP51(Δ1-77) whereas only a very small amount of PTP was nonspecifically bound to the Ni-agarose column when it was not preloaded with His_6-PTPIP51 (Fig. 6.5).

The interaction of PTP1B with PTPIP51 led to distinctly stronger signals compared to TCPTP in yeast two-hybrid assays. Interaction took place with the N-terminal region between aa 78 and 274 with the same intensity as in wild-type PTPIP51(Δ1–77) (T. Bürklen, personal communication). Yet, minor interactions were also observed with the C-terminal part of the protein (aa 255–470). The major interactions between phosphotyrosine phosphatases and PTPIP51, therefore, are not mediated by the TPR region but are closer located to the region serving as substrate of the phosphatase (see below). The same region was responsible for the interactions with TCPTP which were distinctly weaker as indicated by lower induction of β-galactosidase activity in the two-hybrid assay.

2.6.2. Homologous interactions of PTPIP51

PTPIP51(Δ1-77) was used as bait for a yeast two-hybrid screen of the Jurkat cell cDNA library. One of the interacting proteins was identified as PTPIP51 suggesting that the protein may dimerize or oligomerize (R. Büttel, personal communication). The localization of the interacting region was studied by two-hybrid assays testing the interactions of the LexA repressor (*E. coli*) DNA binding domain (LexA)-PTPIP51(Δ1–77) fusion protein with partial sequences of PTPIP51 fused to the B41 activation domain. The localization

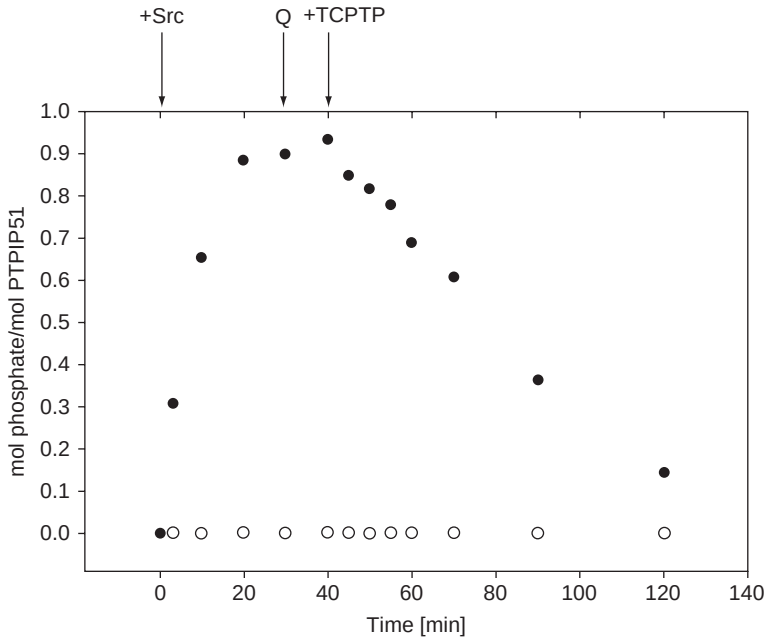

Figure 6.5 *In vitro* phosphorylation of PTPIP51 by Src protein tyrosine kinase and dephosphorylation by nuclear TCPTP. His_6-tagged PTPIP51 (aa 78–470) (filled circles) or PTPIP51 (aa 182–470) (open circles) was first incubated with Src in the presence of [γ-^{32}P]ATP (1 mM). ATP was degraded by the addition of glucose, $NADP^+$, hexokinase, and glucose 6-phosphate dehydrogenase after 40 min (indicated by Q). Partially purified TCPTP (nuclear isoform) was added after 50 min. The content of radioactive phosphate in PTPIP51 was determined at the indicated time.

of the dimerization region was located between aa 78 and 175 by these assays which includes the coiled-coil domain. The region between aa 104 and 118 comprises a short leucine zipper motif (L^{104}, L^{111}, L^{118}) and is, therefore, a candidate for subunit-subunit interactions. These lysines are also conserved in FAM82A. Approximate molecular masses of 170 $\pm$ 10 and 80 $\pm$ 10 kDa were estimated for the recombinant 44 kDa PTPIP(Δ1–77) by f ast protein liquid chromatography (FPLC) on Superose 6. These data support the presence of the protein as dimers and tetramers.

2.6.3. Heterologous interactions of PTPIP51

A two-hybrid screen performed with PTPIP51(Δ1–77) as a bait (R. Büttel, personal communication) also provided 17 cDNA clones (out of 28 fully characterized clones) encoding the kinetochore complex component (Nuf2) kinetochore protein that localizes to the centromere and is essential for

mitotic progression in vertebrate cells (Hori *et al.*, 2003). The microtubule-associated protein regulator of microtubule dynamics 1 (RMD-1) from *C. elegans*, RMD-1, which exhibits some sequence homology with human PTPIP51 in the TPR region was recently shown to function in chromosome segregation of *C. elegans* (Oishi *et al.*, 2007). Another interaction partner of PTPIP51 was C14ORF166 (also called CGI-99), a protein that interacts with Ninein and prevents its phosphorylation by glycogen synthase kinase 3 (GSK3) (Howng *et al.*, 2004). While the physical interaction of PTPIP51(Δ1–77) needs to be confirmed by independent methods, the interaction of PTPIP51(Δ1–77) with diacylglycerol kinase alpha (DGKα) was substantiated by pull-down of the enzyme with His_6-PTPIP51(Δ1–77) and by co-immunoprecipitation with polyclonal anti-PTPIP51(Δ1–77) (S. Becker-Weimann, personal communication). DGKα is also a substrate of Src and phosphorylation of tyrosine is required for its activation and its role in growth factor signaling inducing cell motility and angiogenesis (Chianale *et al.*, 2007; Merino *et al.*, 2007). DGKα suppresses tumor necrosis factor (TNF)-alpha-induced apoptosis of human melanoma cells through nuclear factor of kappa light chain enhancer in B-cell activation (NF-kappaB) (Yanagisawa *et al.*, 2007). On the other hand, DGKα also supports the secretion of exosomes carrying the pro-apoptotic Fas ligand (tumor necrosis factor ligand superfamily member 6), and the subsequent activation-induced cell death by apoptotic processes on T-cells lines and primary T-lymphoblasts (Alonso *et al.*, 2007). A pro-apoptotic role of PTPIP51 bound to mitochondria has been also postulated (Lv *et al.*, 2006). Further characterization of interactions of PTPIP51 with these proteins may shed light on the function of the protein in normal and tumor cells.

PTPIP51 also interacts with 14-3-3 proteins via two short motifs (aa 43–48 and 146–154) (Ewing *et al.*, 2007; Jin *et al.*, 2004) and activates the mitogen-activated protein kinase (MAPK)/extracellular regulated MAP kinase (ERK) pathway, potentially by forming a ternary complex with rapidly growing fibrosarcoma or rat fibrosarcoma (Raf). PTPIP51 also appears to regulate cell motility of HepG2 cells (Yu *et al.*, 2008).

2.7. Phosphorylation of PTPIP51

2.7.1. Phosphorylation of PTPIP51 by Src kinase

The interaction of PTPIP51 with tyrosine phosphatases of the PTP1B/TCPTP subfamily raised the hypothesis that it might reflect an enzyme-substrate relation. Incubation of PTPIP51 (Δ1–77) with Src tyrosine kinase led to the rapid incorporation of about 0.9 mol phosphate/mol protein. The phosphorylation took place on tyrosine as confirmed by immunoblots. Dephosphorylation was exerted by TCPTP as well as by PTP1B.

Since Src kinase did not phosphorylate PTPIP51(Δ1–181), the phosphorylation was expected to occur at one or both of the two tyrosine

residues present in the sequence region aa 78–180, that is, at Y158 and/or Y176. Since Y176 is located within a typical recognition site for Src family protein kinase, a Y176F mutation was introduced into the His_6-tagged wild-type and the mutated proteins were expressed in *E. coli* and purified. The Y176F mutation completely suppressed phosphorylation of PTPIP51(Δ1–77) while the non-mutated protein was strongly phosphorylated on tyrosine.

2.7.2. Phosphorylation on tyrosine *in situ*

The nucleotide sequences encoding wild-type and the Y176F mutant of full size PTPIP51 were cloned into the pEGFP-N1 plasmid and expressed in HEK293 cells. Due to the presence of high activities of endogenous tyrosine phosphatases, the phosphorylation of wild-type PTPIP51 only became detectable either if tyrosine dephosphorylation was blocked by treatment of the cells with pervanadate or Src was ectopically co-expressed. When cells were treated with 1 mM pervanadate, the wild-type PTPIP51 was strongly phosphorylated on tyrosine after 10 min. Phosphorylation of the Y176F mutant was distinctly weaker compared to the wild-type protein, but was nevertheless evident under prolonged exposure of the cells to pervanadate suggesting that additional tyrosines became phosphorylated in the full-length protein under *in situ* conditions. Taking into account that the PTPIP51(Δ1–77)Y176F mutant was resistant to phosphorylation by Src *in vitro,* the phosphorylation site may either be present in the 1–77 region of the protein or phosphorylation is exerted in the aa 78–470 region by a protein tyrosine kinase different from Src. Since Y53 was a candidate phosphorylation site for Src family protein tyrosine kinases, a Y53F mutation was introduced into full-length PTPIP51. The phosphorylation on tyrosine of PTPIP51(Y53F) was not distinctly different from the wild-type protein. The effect of this mutation was distinctly weaker compared to the Y176F mutation suggesting that Y176 was the main site of tyrosine phosphorylation of PTPIP51 but weak phosphorylation of other tyrosine residues may occur. This tyrosine is also conserved in human FAM82A but not in FAM82B.

Both PTP1B and its interaction partner PTPIP51 have been shown to support induction of apoptosis (Gonzalez-Rodriguez *et al.*, 2007; Lv *et al.*, 2006). Phosphorylation of mitochondrial components on tyrosines including cytochrome *c* and cytochrome *c* oxidase (Lee *et al.*, 2006) and translocation of modifying enzymes to mitochondria has received attention recently (Hüttemann *et al.*, 2007). The structure of the N-terminus of PTPIP51 resembles that of transmembrane domains and is crucial for mitochondrial localization of the full-length form. Recruitment of phosphotyrosine phosphatases of the PTP1B subfamily by full-length PTPIP51 would provide a plausible mechanism for the counteraction of antiapoptotic growth factor tyrosine kinase signaling.

2.7.3. Phosphorylation of PTPIP51 by protein kinase A

PTPIP51 and PTPIP51(Δ1–181) were phosphorylated by cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) *in vitro* as demonstrated by the incorporation of radioactive phosphate by incubation with PKA in the presence of [γ-^{32}P]ATP. A peptide of *m/z* 4210 (corresponding to residues 211-250) was present in the non-phosphorylated protein but disappeared in the phosphorylated sample, where it was replaced by a peptide of *m/z* 4290 corresponding to the same peptide containing a single phosphate (AbdelZaher Mostafa, 2004). The phosphorylated peptide contains five serine residues (no threonine), but the sequence context RKD*S*^{212}L is the only canonical phosphorylation site of PKA while the other serine residues lack basic aa at the –2 and –3 positions and a hydrophobic aa at the +1 position. The data, therefore, suggest that S^{212} serves as a phosphorylation site of PKA at least *in vitro*. This site is not conserved in FAM82A and FAM82B. PTPIP51 was recently found among proteins which undergo phosphorylation in cell cycle-dependent manner. S^{212} was phosphorylated in G1 and M phase (Daub *et al.*, 2008; Dephoure *et al.*, 2008). Other serine residues phosphorylated in G1 and M phase were S^{44}, S^{46}, and S^{225}, whereas S^{50} was only phosphorylated in G1. S^{46} is located in a potential, but minor, phosphorylation motif of PKA. The other serine residues appear to be substrates of different protein kinases (Figs. 6.6 and 6.7).

3. Signaling Partners of PTPIP51

The following section briefly summarizes the biochemical and biological properties and functions of the aforementioned *in vitro* interacting partners of PTPIP51.

As some of these signaling partners and their role in cellular processes, such as proliferation, differentiation, cell survival, apoptosis, and cell motility, as well as migration have been investigated rather extensively during the recent years, this section hopefully contributes to a better understanding of putative tissue-specific *in vivo* functions of PTPIP51, which will be discussed below.

3.1. PTP1B and TC-PTP

The protein tyrosine phosphatase (PTP)-superfamily is structurally diverse and encoded by 107 genes in humans, whose gene products tightly control phosphorylation dependent signaling pathways (Alonso *et al.*, 2004). PTP1B (Charbonneau *et al.*, 1989) and TC-PTP (Cool *et al.*, 1989), sharing over 70% aa sequence identity in their PTP domain, belong to the subclass of classical PTPs, comprising 38 tyrosine-specific enzymes (Andersen *et al.*,

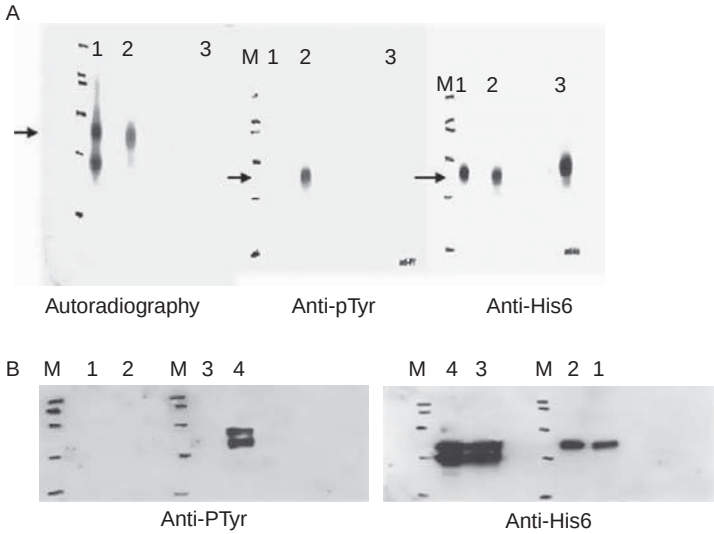

Figure 6.6 *In vitro* phosphorylation of PTPIP51 by Src protein tyrosine kinase and cAMP-dependent protein kinase (PKA). (A) Autoradiography (left) and immunoblots probed with anti-phosphothyrosine (PTyr) (middle) or anti-His_6 (right) for recombinant His_6-PTPIP51 (aa 78–470) (right) after treatment with PKA (lane 1), Src (lane 2) in the presence of [γ-^{32}P]ATP. Lane 3: Control in the absence of protein kinase. (B) Phosphorylation of wild-type (lanes 3 and 4) and Y176F mutant (lanes 1 and 2) forms of PTPIP51 (Δ1–77) by Src. Left: immunoblot with α-PTyr; right: immunoblot with α-His_6. Lanes 1 and 3: without Src; lanes 2 and 4 with Src.

2001). The most extensively studied prototype of the PTP superfamily is the widely expressed PTP1B, which is implicated in numerous intracellular cascades, thereby influencing the cellular outcome of metabolic, oncogenic, and cytokine signaling (Tonks, 2003).

The full-length form PTP1B predominantly localizes to the endoplasmic reticulum (ER) by its hydrophobic C-terminus (Frangioni *et al.*, 1992), but cell-specific variants with a distinct subcellular localization generated either by insulin- (Shifrin and Neel, 1993) and growth factor (Sell and Reese, 1999)-induced mRNA splicing or by calpain-mediated proteolytic cleavage (Akasaki *et al.*, 2006; Gulati *et al.*, 2004) have been reported. For TC-PTP, two splicing variants exist. While, the 48-kDa form is targeted to the ER (Ibarra-Sanchez *et al.*, 2000), the predominant 45-kDa form lacking the hydrophobic C-terminus is nuclear (Lorenzen *et al.*, 1995; Tiganis *et al.*, 1997).

PTP1B overexpression results in reduced phosphorylation of many receptor tyrosine kinases (RTKs) (e.g., for platelet derived growth factor (PDGF) and epidermal growth factor (EGF)) governing cellular growth, differentiation, survival and migration by many signaling pathways including the rat sarcoma proto-oncogene (Ras)-ERK sequence.

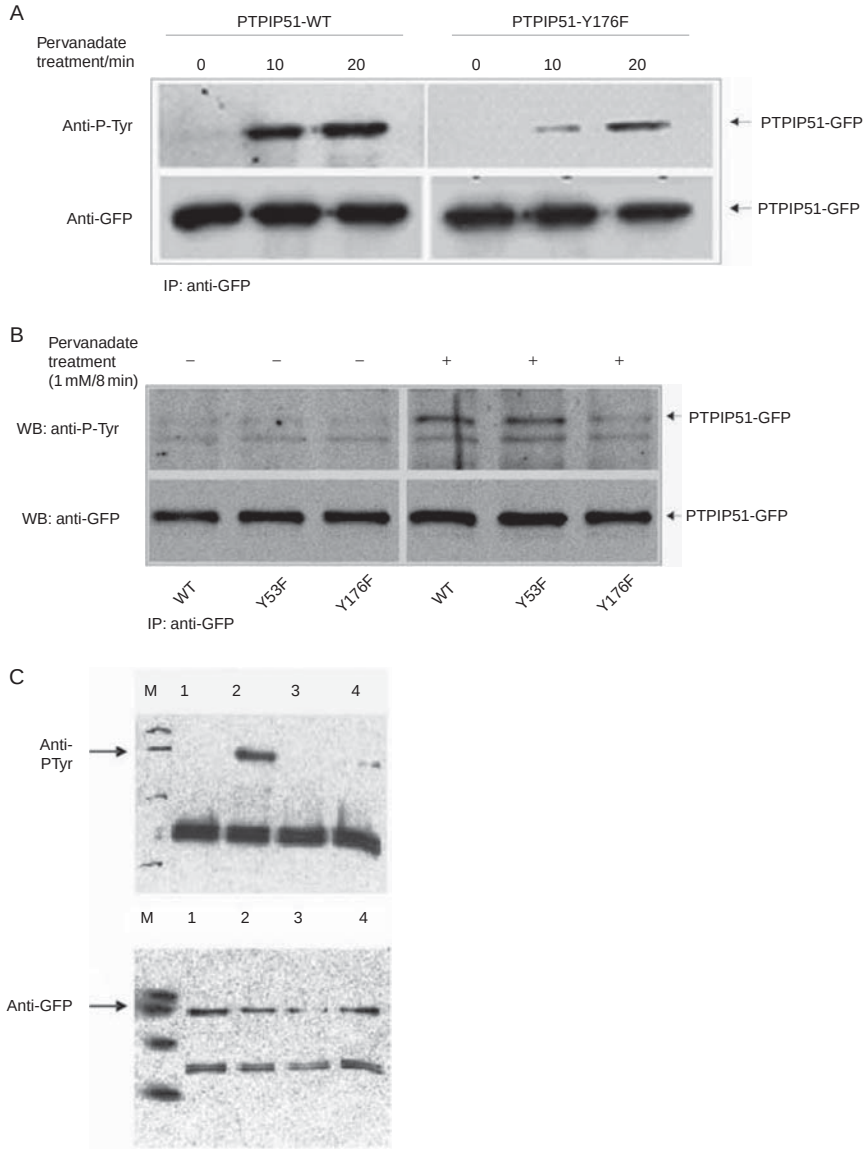

Figure 6.7 *In situ* phosphorylation of wild-type and mutant full-length PTPIP51 (fused to EGFP) in HEK293 cells. (A) *In situ* phosphorylation of wild-type (left) and Y176F mutant (right) PTPIP51 fused to EGFP and expressed in HEK293 cells. The cells were treated with 1 mM pervanadate for the indicated periods of time. Cell extracts were immunoprecipitated with anti-GFP. Upper panel: Immunoblot with anti-PTyr. Lower panel: Immunoblot with anti-GFP. The positions of PTPIP51 are indicated by arrows. (B) *In situ* phosphorylation of wild-type (1–2) and mutant (3–4) PTPIP51 fused to EGFP and expressed in HEK293 cells in the absence (left) and presence (right) of

Pioneering work regarding the role of PTP1B in metabolic signaling demonstrated that the purified enzyme is able to antagonize insulin signaling when injected into Xenopus oocytes (Tonks *et al.*, 1990). Consistent with this finding, two independent studies (Elchebly *et al.,* 1999; Klaman *et al.,* 2000) could show that PTP1B knockout mice displayed enhanced sensitivity to insulin and, moreover, seem to have a certain resistance to obesity induced by high fat diet. Remarkably, in contrast to what one could expect from theoretical considerations regarding the loss of PTP1B-mediated counterbalance of mitogenic and growth factor stimuli, the mice did not show an elevated predisposition to cancer compared to their wild-type littermates. As in Src knockout mice, complex compensatory mechanisms preventing hyperactivation of certain receptor protein tyrosine kinase (RTK) activated signaling cascades are likely to be responsible for this observation (Haj *et al.,* 2003).

With respect to oncogenesis, PTP1B is implicated in the regulation of cell adhesion and migration by modulating downstream signaling partners of the integrin cascade (Liu *et al.,* 1998). This is particularly mediated by dephosphorylation of the inhibitory tyrosine residue of c-Src (Y-530 human, Y527 mouse), a kinase, whose activity- and expression-alterations have been shown for many types of human cancer. A more detailed explanation on this subject is given in Section 3.2 dealing with the Src kinase family.

Moreover, PTP1B also promotes adhesive properties of the cadherin-catenin complex by dephosphorylation of β-catenin leading to N-cadherin-β-catenin assembly, which is crucial for tissue integrity (Xu *et al.*, 2002). A lack of PTP1B activity, leading to disruption of this molecular complex, may support cell motility and possibly tumor invasion and metastasis (Östman *et al.*, 2006). Notably, expression of PTP1B is induced by the breakpoint cluster region-Abl tyrosine kinase (Bcr-Abl) oncoprotein (LaMontagne *et al.*, 1998a) on the transcriptional level via the PTP1B promoter harboring a Bcr-Abl responsive sequence (Fukada and Tonks, 2003). Consistent with these results, PTP1B is able to antagonize Bcr-Abl induced transformation of cells (LaMontagne *et al.*, 1998b), which was found to be directly involved in the onset and progression of chronic myeloid leukemia.

pervanadate (1 mM, treatment for 8 min). Cell extracts were immunoprecipitated with anti-GFP. Upper panel: Immunoblot with anti-PTyr. Lower panel: Immunoblot with anti-GFP. The positions of PTPIP51 are indicated by arrows. (C) *In situ* phosphorylation of wild-type (1–2) and mutant (3–4) PTPIP51 fused to EGFP and expressed in HEK293 cells in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of co-expressed Src protein tyrosine kinase. Cell extracts were immunoprecipitated with anti-GFP. Upper panel: Immunoblot with anti-PTyr. Lower panel: Immunoblot with anti-GFP. The position of PTPIP51 is indicated by the arrow.

PTP1B upregulation also seems to be an early event in mammary tumorigenesis and early studies could demonstrate an association between overexpression of v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ErbB2) and PTP1B in human breast cancer samples (Kidd *et al.*, 1992; Slamon *et al.*, 1989; Wiener *et al.*, 1994). These data suggested that both proteins might be functionally linked in breast tumorigenesis. Indeed, two groups recently reported (Bentires-Alj and Neel, 2007; Julien *et al.*, 2007) that the absence of PTP1B in mice markedly delays breast cancer induced by activated ErbB2. Also, NDL2 mice showed a decreased incidence of lung metastasis, suggesting that PTP1B activity may influence tumor cell migration and invasion (Julien *et al.*, 2007). ErbB2-induced signaling functions by many cascades including c-Src, phosphatidylinositol 3-kinase (PI3K)/Akt, and Ras/Erk (Dankort and Muller, 2000) and at least in NDL2 mice, PTP1B deficiency leads to decreased Akt and Erk phosphorylation and increased Ras protein levels. This observation, however, seems—at least partly—due to the mouse model itself. The data, regarding PTP1B null mice expressing the polyoma middle T antigen (mouse mammary tumor virus-polyoma middle T antigen, MMTV-PyMT) presented by Bentires-Alj and Neel (2007), suggest that PTP1B also modulates an ErbB2-triggered process distinct from the pathways implicated in mammary tumorigenesis (Stuible *et al.*, 2008).

Interestingly, in the study conducted by Julien and coworkers (2007), MMTV-mediated overexpression of PTP1B alone was observed to induce the transformation of glandular mammary tissue toward a malignant phenotype—at least in mice. There is also a growing body of evidence that both PTP1B and TC-PTP contribute to apoptosis signaling. PTP1B was found to play a role in apoptotic cell death of human glioma cells (Akasaki *et al.*, 2006). In this scenario, the peroxisome proliferator-activated receptor y (PPARy) induces processing and subsequent activation of PTP1B, which in turn dephosphorylates signal transducer and activator of transcription 3 (STAT3), thereby priming glioma cells to TRAIL (TNF-related apoptosis-inducing ligand)-mediated apoptosis. Interestingly, phosphorylation of STAT3 requires cleavage of PTP1B into a soluble 42 kDa form, most likely by calpain (Kuchay *et al.*, 2007). Another study demonstrated that cells lacking PTP1B have a decreased susceptibility toward ER-stress-induced apoptosis (Gu *et al.*, 2004) and, moreover, PTP1B knockout mice are resistant to Fas-induced liver damage. The reason for the latter seems to be the inefficient suppression of ERK and NFkB signaling (Sangwan *et al.*, 2006).

TC-PTP is also involved in signaling cascades mediating apoptosis. There is evidence that TC-PTP overexpression leads to accumulating p53, which in turn triggers apoptosis of MCF-7 cells (breast epithelial adenocarcinoma) (Gupta *et al.*, 2002; Radha *et al.*, 1999). Generation of knockout mice provided insight in the pivotal role of TC-PTP and PTP1B in immune-cell signaling and its functional contributions to inflammation,

hematopoiesis and lymphopoiesis. TC-PTP deficient mice die between day 21 and day 35 after birth and a detailed examination revealed severely impaired lymphopoiesis and haematopoiesis corresponding with a decreased cellularity of the bone marrow, as well as splenomegaly, partly due to extramedullary erythropoiesis (You-Ten *et al.*, 1997).

In contrast to the decreased numbers of immature B-cells observed in TC-PTP double null mice, PTP1B double null mice displayed an increase in B-cells and total B-cell numbers and additional ablation of p53 resulted in an increasing incidence of B-cell lymphomas (Dubé *et al.*, 2005).

Most strikingly, TC-PTP ablated mice displayed a systemic inflammatory disease due to mononuclear cell infiltration of many tissues manifesting itself in gastritis, nephritis, sialadenitis, and myocarditis (Heinonen *et al.*, 2004). To some extent, the resulting phenotype can be explained by elevated interferon-γ (IFN-γ) levels, the increased production of TNF-α, IL-2 (IL, interleukin) and inducible nitric oxide synthase (iNOS) by mononuclear cells, as well as by the absent negative regulation through TC-PTP-mediated dephosphorylation of many cytokine-governed downstream signaling molecules (e.g., JAK1/2 (Janus kinase, JAK), STAT1, STAT3, STAT 5a/5b, STAT6) (Simoncic *et al.*, 2006a).

Both TC-PTP (Simoncic *et al.*, 2006b) and PTP1B (Heinonen *et al.*, 2006) control murine myelopoiesis and more specifically the monocyte/macrophage differentiation by negatively regulating the phosphorylation of the colony-stimulating factor-1 receptor (CSF-1R), which promotes proliferation, differentiation, and survival of macrophages and their precursor cells by Erk signaling (Pixley and Stanley, 2004).

3.2. Src-kinase

The Src family comprises nine members, some of which show a more restricted expression (FYN oncogene related to SRC, FGR, YES, Fyn; v-yes-1 Yamaguchi sarcoma viral related oncogene homolog, Lyn; lymphocyte-specific protein tyrosine kinase, Lck; hemopoietic cell kinase, Hck; Blk; Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog, Fgr; yes-related proto-oncogene tyrosine-protein kinase, Yrk), and some of which display a ubiquitous expression profile (Src, Fyn, and Yes) (Brown and Cooper, 1996). The highest expression of Src could be traced in the nervous system and in platelets and to a somewhat lower extent during myeloid differentiation (Barnekow and Gessler, 1986; Gee *et al.*, 1986). Homozygotes of the c-Src proto-oncogene in mice showed rather mild impairment of the phenotype including short stature and low birth weight compared to their littermates. Surprisingly, a detailed gross and histological examination revealed no impairment of the hematopoietic system and brain tissue, but one could observe osteopetrosis and failure of the incisor teeth to erupt through the gum. Most homozygotes died at 3–4

weeks, if weaned. The cause of death, however, was not clear (Soriano *et al.*, 1991). In contrast, as demonstrated by Klinghoffer and colleagues (1999), mouse embryos harboring functional null mutations of the three Src family kinases Src, Fyn, and proto-oncogene tyrosine-protein kinase YES (Yes) exhibited severe developmental defects and died perinatally. The data obtained from these studies indicated that there exists a considerable overlap regarding the function of Src, Fyn, and Yes, partially compensating the loss of function.

The main cellular functions of viral-Src and its cellular counterpart c-Src comprise proliferation, cellular motility, resistance to apoptosis and angiogenesis, all playing a role in developmental processes and cell homeostasis (Brown and Cooper, 1996). However, there is also evidence that Src plays a role not only in the onset, but also in the progression and metastatic spread of many tumors (Summy and Gallick, 2003). Although contribution of Src to tumorigenesis has been demonstrated for many malignant tumors, including breast (Muthuswamy and Muller, 1994; Verbeek *et al.*, 1996), ovarian (Wiener *et al.*, 1999), lung (Mazurenko *et al.*, 1992), and pancreatic cancer (Lutz *et al.*, 1998), the association between Src and neoplastic progression (Talamonti *et al.*, 1993) including metastatic spread (Mao *et al.*, 1997; Termuhlen *et al.*, 1993) has been studied most extensively in colon cancer and its precursor lesions (Cartwright *et al.,* 1994).

It seems to be the case that generally upregulated gene expression and protein activity contribute to cancer development and not mutations in the Src gene per se. There is, however, some evidence that a subset of metastatic colon cancers harbor an activating mutation leading to a transforming gene product (Irby *et al.*, 1999). Besides autophosphorylation (Superti-Furga, 1995), activity of Src is tightly regulated on tyrosine residue-527 by kinases, such as Csk and its homologue c-terminal Src kinase homologous kinase (Chk) (Nada *et al.*, 1993) and phosphatases, including PTP1B (Bjorge *et al.*, 2000) and TCPTP (van Vliet *et al.*, 2005). Dephosphorylation by PTP1B leads to activation and phosphorylation by Csk to inactivation of Src. The catalytic activity is also regulated by the Src homology 2 and 3 domain (SH2 and SH3 domain) of Src kinase. The SH3 domain in particular represses kinase activity by interaction with the catalytic, as well as the linker region between the SH2 and catalytic domains. Hence, interruption of the auto-interaction of SH2 or SH3 domains by specific ligands leads to activation of Src. Src family kinases communicate with numerous receptor pathways, including integrins, RTKs, EGFR, and Neu (ErbB2), as well as IL-6-R (Thomas and Brugge, 1997).

Focal adhesion formation and disassembly are important for cellular morphology, adhesion, and migration (Frame *et al.*, 2002). It has been shown that activated sarcoma viral oncogen homologue (v-Src) and also structurally related cellular variant of v-Src (c-Src) promote actin filament disassembly and also negatively modulate cellular adhesion by disrupting

cadherin/catenin association. Moreover, several groups also reported a regulation of matrix metalloproteases by c-Src, as well as v-Src and its effector kinase, focal adhesion kinase (FAK) thereby possibly contributing to the metastatic spread of cancer cells (Frame, 2002).

Src activity and expression were also shown to promote survival of cells detaching from the matrix, thereby conferring resistance to anoikis (Frisch and Francis, 1994). This effect seems to be mediated by the PI3-k/Akt pathway and MAPK cascade, thereby inducing B-cell CLL/lymphoma 2 protein (Bcl2)-family members (Coll *et al.*, 2002; Webb *et al.*, 2000). This mechanism may also contribute to cancer progression (Frame, 2004).

Deregulation of epithelial cadherin (E-cadherin) is crucial for the transition of epithelial cells to a mesenchymal-like phenotype (EMT, epithelial-to-mesenchymal transition). This transition is believed to increase the ability of epithelial cancer cells to migrate promoting tissue invasion, but also plays a role during development (Thiery, 2003). Experiments of several groups could show that Src expression interferes with proper E-cadherin translocation leading to acquisition of an EMT phenotype that is dependent on integrin-mediated signaling (Avizienyte *et al.*, 2002) and may also involve the MAPK cascade. Another Src signaling partner contributing to EMT formation is Hakai, which binds to E-cadherin causing disruption of epithelial cell contacts (Fujita *et al.*, 2002).

3.3. Protein kinase A

Since its discovery in the 1960s (Walsh *et al.*, 1968), cAMP-mediated PKA activity has been implicated in numerous cellular processes (Shabb, 2001). Of the two main classes of PKA isoenzymes, PKA type I is mainly confined to the cytoplasm, whereas PKA type II is bound to specific A-kinase anchoring protein (AKAP) scaffolds via the R II regulatory subunit tethering PKA to defined intracellular compartments, which allows signaling in a very small and defined domain (Baillie *et al.*, 2005).

Short-term effects of PKA activation comprise the phosphorylation dependent regulation of calcium signaling and interaction with many other proteins involved in Rho-, T-cell receptor- (Vang *et al.*, 2001) and MAPK signaling (Wu *et al.*, 1993), including Raf-1 (Schramm *et al.*, 1994) and v-mos Moloney murine sarcoma viral oncogene homolog (Mos), an oocyte-specific MAPK/ERK kinase (MEK) kinase required for oocyte maturation (Yang *et al.*, 1996). PKA also phosphorylates Bcl2-antagonist of cell death (BAD) suppressing its activity in the complex signaling network that impacts apoptosis (Lizcano *et al.*, 2000).

Mediated by a multitude of AKAP A-kinase anchoring peptides, PKA also plays a role in cytoskeletal organization, phosphorylating microtubules (Diviani and Scott, 2001), actin (Howe, 2004), and the centrosome, thereby

contributing to migration, cell cycle progression, and chromosomal stability (Matyakhina *et al.*, 2002).

Lüscher *et al.* (1999, 2000) could also show that PKA activity contributes to *N*-methyl-D-aspartic acid receptor (NMDAR)-dependent synaptic plasticity. PKA phosphorylation is also involved in the initiation and maintenance of sperm motility (Brokaw, 1987; Tash and Means, 1983). Again, several AKAPs, which are localized in the fibrous sheath of the head, mid, and principal piece of mammalian sperm cells (Carrera *et al.*, 1994; Miki *et al.*, 2002), seem to mediate these PKA effects.

Long-term effects on different tissues are mainly mediated by regulating the activity of members of the cAMP response element (CRE)-cAMP response element-binding protein (CREB) family. Targets of the PKA-CREB pathway are numerous (Mayr and Montminy, 2001) and include genes involved in metabolism, immune regulation cell cycle and survival, transport, reproduction, and development. Other transcription factor substrates of PKA are NF-κB (Zhong *et al.*, 1997) and nuclear factor of activated T-cells (NFAT) (Chow and Davis, 2000), as well as the retinoic acid receptors (Harish *et al.*, 2000) thereby modulating transcriptional activity. The latter are important regulators of development and differentiation in vertebrates (Duester, 2008).

3.4. Diacylglycerol kinase α

Diacylglycerol kinase α (DGKα) along with nine other isoforms comprises the mammalian DGK enzyme family (Merida *et al.*, 2008). Alternative splicing creates an even larger amount of distinct DGK subtypes. According to their structural features and specific functional domain, DGKs are divided into five different classes with DGKα belonging to the first one together with DGKβ and γ (Sakane *et al.*, 2007). Subcellular localization is cytosolic and sometimes nuclear, dependent on the isoform investigated (Martelli *et al.*, 2002). Activation is accompanied by a translocation to the cellular membrane, where diacylglycerol (DAG) is generated.

All these enzymes catalyze the phosphorylation of DAG into phosphatidic acid (PA), thereby interacting with a host of signaling partners, including RasGAP (Tsai *et al.*, 1990), Raf-1 (Ghosh *et al.*, 1996), mammalian target of rapamycin (mTOR) (Fang *et al.*, 2001), and protein-tyrosine phosphatase-1 (Jones and Hannun, 2002). The substrate DAG being released from certain phospholipids by the action of phospholipase C also has many target proteins, such as the conventional and novel forms of protein kinase C (cPKC and nPKC) (Ohno and Nishizuka, 2002) and Ras-guanyl nucleotide-releasing protein (GRP). Hence, DGK activation turns on and off DGK- and PA-mediated signaling pathways.

Since only a few DGK isoforms have been identified in *C. elegans* or *D. melanogaster*, the variety of mammalian isoforms seems to be pivotal

for cellular processes found in higher vertebrates. DGKs are important regulators during development, cytoskeleton formation and motility, immune-cell function, and also carcinogenesis and metastatic spread (van Blitterswijk and Houssa, 2000).

DGKα in particular displays high expression in peripheral T-cells, kidney tissue and in oligodendrocytes of the brain. Nonetheless, DGKα was also traced in several other cell lines of different origin and has been implicated in many cellular processes including secretory vesicular trafficking, proliferation, cell survival, as well as T-cell activation and tolerance (Zhong *et al.*, 2008).

In T-cells, IL-2 dependent DGKα activation stimulates cell proliferation. This effect seems to be mediated by Src-family kinase-dependent PI3 kinase (PI3K) (Cipres *et al.*, 2003). VEGF-A vascular endothelial growth factor–dependent activation of DGKα is mediated by Src kinase, thereby triggering proliferation and increased cell motility (Baldanzi *et al.*, 2004). Hepatocyte growth factor (Hgf), which is well known to promote proliferation, migration, and invasion of tumor cells (Danilkovitch-Miagkova and Zbar, 2002), also modulates, among other downstream effectors, Src kinase activity, which leads to DGKα induced enhanced cellular motility in endothelial, and epithelial cells, as well as COS cells (Cutrupi *et al.*, 2000). In this scenario, Src and DGKα seem to co-control actin filament and focal adhesion remodeling, thereby allowing cells to migrate (Chianale *et al.*, 2007). The exact mechanism by which DGKα is being activated by Src kinase has been unraveled recently and requires phosphorylation on the Y335 residue (Baldanzi *et al.*, 2008).

DGKα also promotes cell survival by counterbalancing the extrinsic pathway of apoptosis in T-cells (Alonso *et al.*, 2005) and several human melanoma cell lines (Yanagisawa *et al.*, 2007). Several studies elucidated the role of DGKα for T-cell function. T-cell receptor (TCR) signaling is attenuated by a DGKα mutant, which lacks kinase activity, thereby inhibiting RasGRP1 activity in T-cells (Olenchock *et al.*, 2006). This in turn leads to reduced proliferation and holds T-cells in an anergic state. It seems that at least in T-cells DGKα acts as a negative regulator of the Ras signaling pathway (Zha *et al.*, 2006).

3.5. Ras–Raf–Erk signaling

A-Raf, B-Raf, and C-Raf/Raf-1 comprising the mammalian Raf kinase family play a pivotal role in the MAPK cascade (Ras/Raf/MEK/ERK) that regulates diverse cellular functions, including gene transcription, apoptosis, and cell cycling (McCubrey *et al.*, 2007). Several effects of Raf, however, are not solely mediated by the MAPK pathway and some, including cell death control and migration were even found to be independent of MEK and ERK signaling (Baccarini, 2005). For example, Raf-1 can directly

interact with apoptosis signal-regulated kinase-1 (Akt-1) and mammalian sterile 20-like kinase 2 (MST-2) to inhibit apoptosis (Du *et al.*, 2004; O'Neill *et al.*, 2004). Moreover, Ehrenreiter and colleagues (2005) could show that Raf-1 knockout in fibroblasts and keratinocytes led to a defective cytoskeleton and migration failure. Mislocalized and hyperactivated Rok-α seems to be responsible for these malfunctions.

While A-Raf expression is restricted to urogenital and intestine cells and B-Raf is localized in neuronal and hormonal responsive cells, as well as hematopoietic tissue, Raf-1 is ubiquitously expressed in almost all types of embryonic and adult tissue (Storm *et al.*, 1990; Wojnowski *et al.*, 2000). *Raf-1* deficient embryonic knockout mice showing increased apoptosis particularly of the fetal liver die around midgestation (Mikula *et al.*, 2001).

Following ligand binding to a RTK, for example, EGFR, vascular endothelial growth factor receptor (VEGFR), PDGFR, cytokine receptors or integrins, classical activation of the MAPK cascade occurs through the adaptor proteins Shc (Src homology 2 domain containing protein) and Grb2 (GTP-exchange complex growth factor receptor bound-2) that recruite guanine nucleotide exchange factors like SOS-1 (son of sevenless) to the cellular membrane (Inhorn *et al.*, 1995; Matsuguchi *et al.*, 1995). This coupling complex promotes a conformational change of inactive Ras, thereby exchanging guanosine diphosphate (GDP) for guanosintriphosphate (GTP). This activation in turn, recruits Raf to the cellular membrane and leads to dimerization of Raf proteins and subsequent phosphorylation by Ras (Lanfrancone *et al.*, 1995; Marais *et al.*, 1995; Tauchi *et al.*, 1994). For full activation, however, Raf-1 needs to be additionally activated by a Src kinase (Marais *et al.*, 1997).

The fine-tuning of Raf-1 activity occurs—among many scaffolding proteins (Roy and Therrien, 2002; Sasaki *et al.*, 2003; Trakul and Rosner, 2005), 14-3-3 (Fantl *et al.*, 1994) and heat shock protein 90 (Hsp90) (Blagosklonny, 2002)—by its multiple regulatory phosphorylation sites (Chong *et al.*, 2003; Steelman *et al.*, 2008). Molecular autoinhibition is mediated by PKA (target residues: S43, S233, S259), Akt (S259), and protein kinase B (PKB) (S295), thereby allowing 14-3-3 binding, which renders Raf-1 inactive. Dephosporylation of S259 by the phosphatase protein phosphatase 2A (PP2A) in contrast leads to 14-3-3 dissociation and subsequently permits Ras–Raf–1 interaction (Dhillon and Kolch, 2002; Dhillon *et al.*, 2002). Interestingly, Raf can also be directly activated by protein kinase C (PKC) (Kolch *et al.*, 1993) and indirectly via its signaling partner phospholipase C (Buhl *et al.*, 1995).

Raf proteins directly activate the S/T dual specificity kinase MEK1 and MEK2 (MAPK) by phosphorylation of multiple serine residues (Marais *et al.*, 1997; Xu *et al.*, 1995). MEK1/2 in turn phosphorylate extracellular-signal-regulated kinases 1 and 2 (ERK), which, once activated, phosphorylate multiple transcription factors, such as v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets-1), cellular version of the v-jun sarcoma virus 17

oncogene homolog (c-Jun), and cellular myelocytomatosis oncogene (c-Myc). ERK also leads to indirect activation of NF-κB and can enter the nucleus to activate additional transcription factors, such as Elk1 and Fos by phosphorylation (Chen *et al.,* 2001). The effects of ERK activation are diverse and include cell cycle control, proliferation and migration, as well as modulation of apoptosis. The latter is achieved by phosphorylation of a variety of targets, including BAD (Zha *et al.,* 1996) and caspase 9 (Allan *et al.,* 2003), leading to inactivation, as well as the antiapoptotic protein Bcl2, leading to activation (Deng *et al.*, 2000, 2001). Also, Raf/MEK/ERK-mediated phosphorylation of Mcl-1 (myeloid cell leukemia sequence 1) and Bim (Bcl2-interacting mediator of cell death) protein ultimately prevents Bcl2-associated X protein (Bax) activation thereby inhibiting apoptosis (Harada *et al.*, 2004).

The *ras* proto-oncogene is frequently amplificated or mutated in human cancer (~30%) leading to constitutive activation of Ras and the MAPK-cascade particularly in pancreatic cancer (90%) and colon cancer (50%) (Friday and Adjei, 2008), but seems also to be involved in testicular germ cell tumor formation (McIntyre *et al.*, 2008). Aberrant Ras-signaling also plays a role in certain human developmental disorders (Aoki *et al.*, 2008).

While B-Raf mutations were mainly detected in melanoma (Brose *et al.*, 2002), thyroid cancer (Xing, 2007) and low-grade ovarian cancer (Sieben *et al.*, 2004), there is evidence that mutated *Raf-1* is present in therapy-induced acute myeloid leukemia (t-AML) (Zebisch *et al.*, 2006). Not surprisingly, many research groups work on small molecule inhibitors that aim to suppress MAPK activity (Downward 2003; Sebolt-Leopold and Herrera, 2004). Among those, MEK inhibitors seem to have the biggest potential with a certain clinical activity and low side effects as determined by phase I trials (Friday and Adjei, 2008). However, more effort and further trials are needed to fully evaluate the therapeutic potential of these biologicals.

3.6. 14-3-3 Proteins

14-3-3 signaling molecules constitute a family of evolutionary conserved eukaryotic proteins that regulate the activity of various enzymes, control the subcellular localization of proteins and function as adaptor molecules, thereby stimulating protein–protein interactions. The various functions enable 14-3-3 proteins to intervene in cell-cycle control, cell survival, apoptosis, transcription, protein trafficking, cytoskeleton formation, and metabolism (Ford *et al.*, 1994; Masters and Fu, 2001; van Hemert *et al.*, 2001). Initially thought to be restricted to neuronal tissue (Moore and Perez, 1967), members of this protein class have been found in all eukaryotic cell types and in all eukaryotic organisms studied so far (Fu *et al.*, 2000). Until now, more than 150 isoforms in 48 different species have been reported, in mammalians at least seven isotypes (β, γ, ε, ζ, δ, τ, and η) are known (Rosenquist *et al.*, 2000).

The pleiotropic effects of the acidic 14-3-3 isoforms are mediated by the formation of homo- and heterodimers and their specific binding activity to phosphoserine motifs of various proteins (Aitken *et al.*, 2002). Although some interactions are independent of phosphorylation, 14-3-3 target binding is mainly regulated by kinases creating phosphoserine-containing binding sites, such as PKA and PKC and—equally important—phosphatases (Aitken *et al.*, 2002; Dougherty and Morrison, 2004; Jaumot and Hancock, 2001).

The molecular anvil hypothesis proposes that 14-3-3 proteins cause conformational changes of the interacting partner, which mask or reveal functional motifs and alter the activity of 14-3-3 partner proteins (Yaffe, 2002). A well-investigated example is the interaction between the Raf-1/C-Raf kinase and 14-3-3 in the cytoplasm facilitating Raf-1 activation by ras and other proteins, thereby modulating the MAPK cascade (Fu *et al.*, 2000; Xing *et al.*, 2000).

Besides the activation state of an enzyme, the subcellular localization is of equal importance for the regulation of cellular processes. As reviewed by Muslin and Xing (2000), binding to 14-3-3 proteins often leads to an altered subcellular localization impairing the functional properties of proteins. The promotion of a cytoplasmic localization of many proteins, for example, the Akt substrate BAD (del Peso *et al.*, 1997), Cdc25, and cyclinB1/Cdc2, has been elucidated during the recent years. In the case of BAD, for example, 14-3-3 binding retains BAD in the cytoplasm and prevents the interaction with the antiapoptotic proteins Bcl2 and Bcl-XL (basal cell lymphoma-extra large protein) localized at mitochondria (Datta *et al.*, 2000; Yaffe *et al.*, 1997; Zha *et al.*, 1996). However, 14-3-3 also mediates the nuclear localization of some binding partners (Brunet *et al.*, 2002) and has been implicated in the endoplasmic reticulum-to-plasma membrane trafficking of certain multimeric complexes (Nufer and Hauri, 2003).

14-3-3 is pivotally required during eye and brain development and also maintains brain function (Chang and Rubin, 1997; Skoulakis and Davis, 1998). Hence, alterations in the 14-3-3 gene or gene product play a role in the onset and maintenance of many neurological disorders (Berg *et al.*, 2003), including Creutzfeldt-Jakob disease, Alzheimer's, and Huntington's disease. The δ 14-3-3 isoform is primarily restricted to epithelia and alterations of the protein level and methylation status of the gene were detected in various cancers of epithelial origin (Ferguson *et al.*, 2000; Iwata *et al.*, 2000).

3.7. CNTF and interleukin signaling

CNTF is a neuropoetic cytokine and belongs to the interleukin-6 family of structurally related hemato- and neuropoietic cytokines: IL-6, IL-11, LIF, oncostatin M (OSM), cardiotrophin-1 (CT-1), and cardiotrophin-like cytokine (CLC) (Heinrich *et al.*, 2003; Senaldi *et al.*, 1999; Shi *et al.*, 1999). Binding of these secreted glycoproteins to its multiunit receptor complex (Grötzinger

et al., 1999; Heinrich *et al.*, 2003) rapidly triggers not only the activation of the signal transducers and activators of transcription (STAT), but also the MAPK cascade through SHP-2 mediated linkage of the Grb2–SOS complex to glycoprotein 130 (gp130) (Holgado-Madruga *et al.*, 1996; Schiemann *et al.*, 1997).

By doing so, members of the interleukin family regulate an impressive array of cellular functions, including proliferation, differentiation, survival and apoptosis in the immune, hematopoietic, and neural system. CNTF in particular is involved in neuronal differentiation *in vitro* (Hughes *et al.*, 1988) and enhances survival of hippocampal (Ip *et al.*, 1991), cerebellar (Lärkfors *et al.*, 1994), sensory (Simon *et al.*, 1995), and motor neurons (Oppenheim *et al.*, 1991), as well as of retinal photoreceptors (LaVail *et al.*, 1998). Its expression is restricted to Schwann cells of the peripheral and astrocytes of the central nervous system (Stöckli *et al.*, 1991). While CNTF knockout mice displayed a rather mild phenotype basically resulting from the progressive loss of motor neurons (Masu *et al.*, 1993), CNTF-receptor (CNTFR) knockout mice died within 24 h postnatally because of more severe motor neuron deficits resulting in a suckling defect (DeChiara *et al.*, 1995). Recent studies also suggest that CNTF exerts an anti-inflammatory effect in the CNS (Meazza *et al.*, 1997) and, moreover, has a protective effect in patients suffering from multiple sclerosis (Giess *et al.*, 2002; Linker *et al.*, 2002). CNTF also plays a role in the weight regulation and diabetes onset by leptin-independent activation of hypothalamic satiety centers of mice (Lambert *et al.*, 2001).

As the MAPK cascade and its implications on PTPIP51 function have already been discussed in this chapter, this section will focus on the JAK–STAT pathway, which has been demonstrated to play the central role in cytokine signaling (Levy and Darnell, 2002). Activated Janus tyrosine kinases (JAK: JAK1, JAK2, and tyrosine kinase 2 (TYK2)) phosphorylate the cytoplasmic domain of the membrane receptor, thereby recruiting STATs to the cellular membrane, which, once activated by JAK, translocate to the nucleus and regulate gene transcription.

As shown by several groups JAK1 seems to be the most essential part in IL-6–type signal transduction (O'Shea *et al.*, 2002). Phosphorylation of signaling molecules is a key event in the IL-6-triggered cascade and negatively regulated by several phosphatases including PTP1B (Gu *et al.*, 2003; Myers *et al.*, 2001) and TC-PTP (Yamamoto *et al.*, 2002; Zhu *et al.*, 2002). While PTP1B interaction affects JAK-2 and TYK2 activity, TC-PTP promotes the inactivation of JAK1 and JAK3.

3.8. Apoptosis

Apoptotic cell death, first described by Kerr and colleagues (1972), is morphologically defined by specific cellular changes, namely nuclear shrinkage (pyknosis), chromatin condensation, and nuclear fragmentation (karyorrhexsis)

and is pivotally required for embryonic development (Penaloza *et al.*, 2006), the preservation of tissue homeostasis (Majno and Joris, 1995) and the onset and progression of various diseases (Fadee and Orrenius, 2005).

Besides their role in cytokine processing, inflammation (Martinon and Tschopp, 2007), terminal differentiation, and proliferation (Launay *et al.*, 2005), caspases are important players in the execution of apoptosis (Hengartner, 2000). Residing in the cytosol, these cysteine proteases are produced as inactive zymogens and cleave aspartic acid rich motifs upon activation (Thornberry and Lazebnik, 1998). The human genome encodes 12–13 distinct caspases. At least seven of them contribute to cell death and can be divided in initiator caspases (e.g., caspase-2, -8, -9, and -10) and executioner caspases (e.g., caspases-3, -6, and -7) (Cohen, 1997). Caspase, particularly caspase-3-mediated cleavage of proteins, including apoptosis inhibitors and promotion of DNA fragmentation (Ferri and Kroemer, 2000) by caspase-activated DNAse (CAD) (Enari *et al.*, 1998) leads to the classic morphological features mentioned above. Hence, successful prevention of apoptotic cell death by the inhibition of, for example, caspases provides the information that classic apoptosis has actually been executed.

Two major pathways are associated with caspase activation: the mitochondrial-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway (Danial and Korsmeyer, 2004).

The intrinsic pathway works by mitochondrial membrane permeabilization leading to liberalization of cytochrome *c* from the mitochondrial intermembrane space into the cytosol and subsequent formation of the apoptosome. This catalytic complex comprising cytochrome *c*, apoptotic peptidase activating factor 1 (Apaf-1), and caspase-9 activates the effector caspases-3 and -7, ultimately leading to cell death (Cain *et al.*, 2002). Among other players, the quantity and activity of both, Pro-(Bax, Bad, Bak) and antiapoptotic (e.g., Bcl2, BcL-XL) members of the Bcl2 family adjust the threshold of apoptosis susceptibility for the intrinsic pathway (Reed *et al.*, 1998).

Ligand binding (e.g., Fas and TNF) to death receptors initiates the intrinsic pathway, which relies on caspase-8 activation upon recruitment of adaptor proteins to the receptor (Ashkenazi and Dixit, 1998). Subsequently, this caspase triggers activation of the effector caspase-3. However, there is also evidence that the activation of cell death receptors is capable of and mandatory for inducing the downstream intrinsic pathway, at least in some cell types (Yin *et al.*, 1999).

Nevertheless, caspase activation does not necessarily lead to apoptosis (Garrido and Kroemer, 2004) and inhibition of apoptosis, for example, by pan-caspase inhibitors or staurosporine does not necessarily prevent cell death (Assefa *et al.*, 2000; Carter *et al.*, 2003; Maianski *et al.*, 2003; Zhang *et al.*, 2004), although classical hallmarks of apoptosis, for example, chromatin condensation and DNA fragmentation are absent. These findings

strongly suggest alternative, non-caspase dependent cell death programs (Kroemer and Martin, 2005), which, indeed, were found to shape the cell's fate *in vivo*—alone or in combination with caspases (Doonan *et al.*, 2003; Leist and Jäättelä, 2001; Lorenzo *et al.*, 1999). Phylogenetically older mechanisms of apoptosis-like cell death execution rely on several aspartate-, cysteine-, and serine-proteases, such as cathepsins, calpains, and granzymes (Johnson, 2000). Other downstream effectors include reactive oxygen species (ROS) (Fleury *et al.*, 2002), Ca^{2+}, and the mitochondrial flavoprotein apoptosis-inducing factor (AIF), which translocates to the nucleus and stimulates the formation of a degradosome leading to chromosome condensation and large-scale fragmentation of DNA (Susin *et al.*, 1999). As in caspase-mediated apoptosis, activation of these proteins can be governed by mitochondrial alterations or death receptor ligation and upstream signals can be blocked by antiapoptotic Bcl2-family members, as well as survival kinases (e.g., Bcr-Abl, AKT) (Hail *et al.*, 2006). Activated vitamin D3 is also reported to induce apoptosis-like programmed cell death in several cell lines (Mathiasen *et al.,* 1999; Naveilhan *et al.*, 1994). Apoptosis-like programmed cell death is usually accompanied by a cellular morphology that differs somewhat from the shape of real apoptotic cells. Chromatin condensation is less geometrical and phagocytosis recognition molecules are displayed on the cellular membrane prior to lysis (Leist and Jäättelä, 2001).

4. Expression Profile of PTPIP51

This section reviews the data available on the expression profile of PTPIP51 mRNA and its encoded protein in various human cell lines, as well as in developing and adult mammalian tissue and human cancer. Based on what is currently known about the tissue-specific roles of its interacting partners, putative functional implications of PTPIP51 are discussed.

4.1. Epithelia

4.1.1. Epidermis

In interfollicular epidermis, PTPIP51 mRNA is expressed in both, the basal and suprabasal layers, whereas PTPIP51 protein was only detectable in the suprabasal layers (Stenzinger *et al.*, 2005). The basal layer of the epidermis is composed of slow cycling epidermal stem cells mainly derived from the bulge of the hair follicle and their daughter cells generated by asymmetric cell division. Upon withdrawal from the cell cycle, these basal keratinocytes detach from the basement membrane, migrate upward, and execute a layer-specific program of terminal differentiation ultimately leading to a cornified envelope at the skin surface (Houben *et al.*, 2007). This process is accompanied by a

switch in keratin expression from keratins 5/14 to 1/10 (K5/K14 to K1/K10) (Fuchs and Green, 1980). Successful epidermal replenishment requires a carefully balanced interplay of proliferation, differentiation, and epidermal programmed cell death, a special form of apoptosis. Perturbations of this process lead to diseases, including psoriasis and cancer (Fuchs, 2007).

PTPIP51 is also expressed in follicular epidermis, more specifically in the inner root sheath (IRS), which forms the surrounding channel of the central hair shaft. Continuous cellular replenishment of the IRS is maintained by stem cell progeny forming the hair matrix that surrounds the dermal papilla. Comparable to interfollicular epidermis these matrix cells exit the cell cycle and differentiate while moving upward forming the hair shaft and IRS. This complex process is mainly driven by the WNT- and sonic hedgehog-pathway (Millar, 2002).

Sebaceous glands, appendages of the hair follicle, were also found to express PTPIP51. More specifically, proliferating basal sebocytes attached to the inner surface of the basement membrane showed a cytoplasmic PTPIP51-immunostaining. Disintegrated sebocytes execute a program of terminal differentiation, which ultimately leads to holocrine secretion upon cell death (Zouboulis *et al.*, 2008). In some of these cells, we observed a nuclear form of PTPIP51. As in epidermis, sebocyte development requires a finely tuned interplay to sustain a dynamic balance of growth and terminal differentiation. Additionally, PTPIP51 expression was also found in epithelial cells comprising the apocrine and eccrine glands (Stenzinger *et al.*, 2005).

In vitro experiments with human keratinocytes (HaCaT cells) revealed EGF, as well as 1,25 dihydroxyvitamin D3 ($1,25OH_2D_3$) and retinoic acid to influence the expression of PTPIP51 (Stenzinger *et al.*, 2006). Moreover, immunoblotting experiments suggest tyrosine phosphorylation of PTPIP51 is influenced by these agents, which are well known to play an important role in the regulation of epidermal homeostasis *in vivo*.

More specifically, while ~35% of untreated HaCaT cells express PTPIP51, application of nanomolar EGF concentrations led to a significant downregulation of PTPIP51-positive cells (~20%). Cell treated with higher concentrations (up to 10^{-6} M) of EGF showed a stepwise numerical increase of PTPIP51-positive cells to control levels. This effect is possibly mediated by downregulation of the EGFReceptor. EGF stimulates proliferation of epidermal keratinocytes (Cohen, 1983) and also appears to promote the expression of markers of late terminal differentiation (Wakita and Takigawa, 1999). Furthermore, EGF enhances the migration of keratinocytes *in vitro* (Barrandon and Green, 1987) and may therefore also play a role during development, wound healing, and metastatic spread *in vivo*. Interestingly, Src kinase, which has been shown to phosphorylate PTPIP51 *in vitro* (for details see Section 2.7.1), plays a major role in the promotion of cellular migration (for details see Section 3.2). Upon ligand binding, EGFReceptor-mediated intracellular signaling also abrogates apoptosis thereby promoting cell survival (Rodeck *et al.*, 1997).

The EGFReceptor exerts its function among other signaling cascades by the Ras-Raf-Mek-Erk cascade, known to be indirectly modulated by PTPIP51 at the Raf level possibly by the formation of a ternary complex with 14-3-3 isoforms (Yu *et al.*, 2008).

Treatment of HaCaT cells with increasing concentrations of $1,25OH_2D_3$ from 10^{-9} to 10^{-5} M was accompanied by a stepwise numerical increase of PTPIP51-positive cells up to ~58%. $1,25OH_2D_3$ is well known to regulate the differentiation of keratinocytes and exerts a concentration dependent antiproliferative (10^{-9} M and higher concentrations) effect (Bikle *et al.*, 2003).

The cellular response on retinoic acid observed in cultured keratinocytes is distinct from the *in vivo* effects on epidermal keratinocytes. While retinoids are known to suppress terminal differentiation under *in vitro* conditions, retinoids stimulate division of basal keratinocytes and also modulate cellular differentiation *in vivo* (Fisher and Voorhees, 1996). These different responses are most likely due to the distinct crosstalk between retinoids and other signaling pathways in cultured cells and under *in vivo* conditions. Human keratinocytes exposed to increasing concentrations of retinoic acid did not show an increase in PTPIP51-positive cells, except for supraphysiological concentrations (10^{-5} M), which led to the expression of PTPIP51 in 65% of keratinocytes. Since there is evidence that these concentrations of retinoids sensitize cultured keratinocytes to doxorubicin- and UVB-induced apoptosis (Mrass *et al.*, 2004) and, moreover, promote apoptotic cell death in differentiating keratinocytes (Rendl *et al.*, 2002), we assumed that PTPIP51 may also play a role in apoptotic cell death in HaCaT cells. To address this issue further, HaCaT cells were submitted to apoptosis-inducing concentrations of nocodazole (0.04 and 0.1 μM for 24 and 48 h, respectively), chelerythrine (10 and 20 μM for 24 h), and staurosporine (1 μM for 3 and 6 h, respectively). The reagent nocodazole acting by microtubular disruption induces mitotic arrest at the metaphase and anaphase transition followed by apoptotic cell death (Kitagawa and Niikura, 2008). This phenomenon is dependent on the concentration applied. The benzophenanthridine alkaloid chelerythrine is a well-known inhibitor of PKC, the latter playing an important role in terminal differentiation of mammalian epidermis. However, in dependence on the concentration applied, chelerythrine is also capable of triggering apoptosis in a variety of tumor cells (Malikova *et al.*, 2006), possibly mediated by direct targeting of Bcl2/Bcl-XL (Chan *et al.*, 2003). Staurosporine, a so-called nonspecific PKC inhibitor (Ruegg and Burgess, 1989; Tamaoki *et al.*, 1986), is reported to promote expression of proteins associated with terminal differentiation of epidermal keratinocytes (Honma *et al.*, 2006), but also induces apoptosis in cultured cells (Aho, 2004; Cirillo *et al.*, 2008).

All three agents, nocodazole, chelerythrine, and staurosporine were found to induce apoptosis in HaCaT cells determined by morphological

analysis and *in situ* terminal transferase dUTP nick end labeling assay (TUNEL assay) (unpublished data). Double immunostainings showed only apoptotic keratinocytes to display a very strong PTPIP51-immunosignal suggesting an upregulation of PTPIP51 in this cellular process. Moreover, the fraction of PTPIP51-positive cells (~35%) observed in controls raised significantly from ~35% to ~70% under staurosporine and ~90% under chelerythrine treatment. In accordance with the results described above, the latter finding may be interpreted as a differentiation-dependent induction of PTPIP51 in human keratinocytes.

Application of Z-Val-Ala-Asp (OMe)-fluoromethyl ketone (zVAD-FMK), a specific pan-caspase inhibitor, was not found to completely abrogate programmed cell death. Even more interesting, apoptosis in keratinocytes showing high PTPIP51 expression was only prevented in a subset of these cells. These data indicate that PTPIP51 may also play a role in phylogenetically older mechanisms of programmed cell death and is in accordance with the observations reported by Lv *et al.* (2006), who reported the full-length form of PTPIP51 to enhance apoptosis. For a detailed explanation of this issue see Section 3.8.

Merging the *in viv*o and *in vitro* data, it is tempting to speculate that PTPIP51 is not only associated with differentiation and apoptosis of cells but also serves as a signaling partner, which promotes these cellular processes in epidermis. Based on the recent findings of Yu *et al.* (2008), it is not unlikely that PTPIP51 also influences the motility of keratinocytes, which is essentially required for epidermal cell turnover. Investigating the exact function of PTPIP51 in the complex process of tissue homeostasis is certainly of interest and further research is needed to evaluate whether the *in vitro* data on PTPIP51 available so far can be directly applied to epidermal homeostasis *in vivo*.

4.1.2. Mesothelium

Mesothelial cells are derived from mesoderm and form a monolayer that lines the body cavities and internal organs. PTPIP51 is continuously expressed in all mesothelial cells from day E12 (Fig. 6.9A) onto the fully developed tissue (Stenzinger *et al.*, 2005 and unpublished results). Mesothelial cells are slowly renewing and express mesenchymal and epithelial cell intermediate filaments (Mutsaers, 2004). Mesothelial regeneration, for example, upon injury is believed to be mediated by two distinct mechanisms and seems to depend on the integrity of the basement membrane. The main regeneratory pathway relies on surface epithelial cells, which have retained their proliferative capacity. Upon exfoliation, these cells transform into a spindle-shaped fibroblastic morphology (EMT: epithelial-to-mesenchymal transition) and start to migrate to the defect, where they become flattened to cover the surface (Mutsaers and Wilkosz, 2007).

Destruction of the basement membrane activates a second pathway, which allows fibroblast like subserosal cells to migrate to the injured epithelial surface, where they acquire an epithelial phenotype. These processes are initiated by the para- and autocrine release of various growth factors including EGF, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF).

PTPIP51 was found to act as a positive regulator of MAPK-driven cell motility and its expression is influenced by EGF—at least in keratinocytes *in vitro*. Thus, PTPIP51 may play a role for tissue integrity and possibly repair mechanisms of the mesothelium. Interestingly, there is some evidence, that the activity of the Ras–Raf–Erk pathway plays a role in mesothelioma pathogenesis (Mukohara *et al.*, 2005), the latter being associated with the exposure to asbestos. Inhibition of MAPK *in vitro* results in a decrease in mesothelioma cell proliferation but has little impact on the prevention of apoptosis (Patel *et al.*, 2007). As discussed Section 3, PTPIP51 may also play a role in EMT, which is known to be co-controlled by Src-kinase activity.

4.1.3. Urothelium

The urothelium lining the surface of the urinary tract consists of three distinct cell layers with the terminally differentiated umbrella cells constituting the most superficial cell layer. The urothelium is involved in the control of permeability, immune response, and cell–cell communication. PTPIP51 expression was observed for all three layers and PTPIP51 antigen is particularly concentrated at the basal cell pole of the basal cell layer. As has been shown by Swiatkowski and colleagues (2003), the activity of the MAPK pathway particularly stimulated by EGF family members is essential for normal urothelial cell proliferation. Interestingly, papillary urothelial carcinoma are typically characterized by MAPK activation, which may lead to a self-limiting tumor growth making this cancer type less malignant than its invasive counterpart being less sensitive to MAPK inhibition (Schulz, 2006). With respect to the findings by Yu *et al.* (2008), PTPIP51 may also serve as a MAPK-signaling partner in urothelial cells. A more detailed explanation of the MAPK-pathway and its functional implications is given in Section 3.5. Since PTPIP51 is also expressed in the basal layer harboring cycling cells, PTPIP51 may be implicated in cellular proliferation as suggested by the work of Daub *et al.* (2008) and Dephoure *et al.* (2008), whose findings are discussed in Section 2.7.3.

4.1.4. Endothelium

PTPIP51 protein and mRNA were found in the majority of endothelial cells of both, venous and arterial vessels, as well as capillaries in all organs investigated. In placental tissue (Fig. 6.8F) and peritumoral stroma (Fig. 6.10D), however, all endothelial cells of the vasculature were found

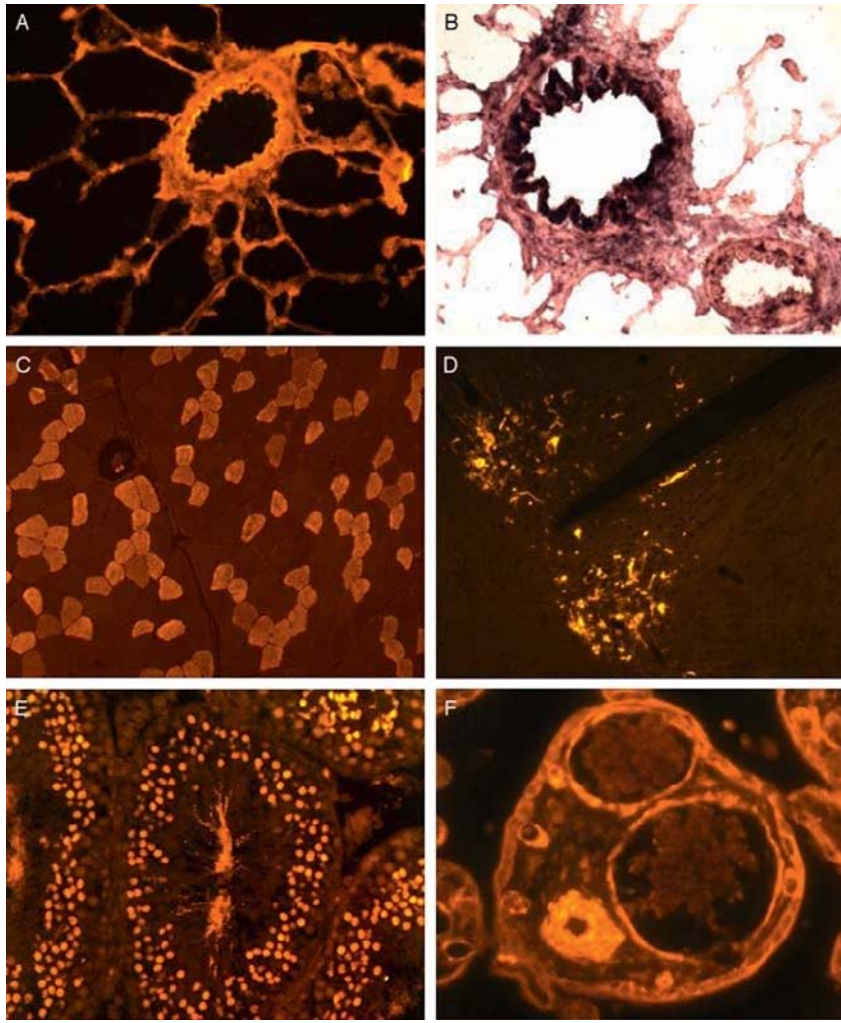

Figure 6.8 Localization of PTPIP51 in tissues from adult human, rat, and murine origin. (A) PTPIP51-immunostaining of rat bronchus (magnification: 20×). (B) *In situ* hybridization with anti-sense probe to PTPIP51 of rat bronchus (magnification: 40×). (C) PTPIP51-immunostaining of rat skeletal muscle (magnification: 20×). (D) PTPIP51-immunostaining of mouse paraventricular nucleus (magnification: 10×). (E) PTPIP51-immunostaining of rat seminiferous epithelium (magnification: 20×). (F) PTPIP51-immunostaining of human placental villi from term placenta (magnification: 40×).

to express PTPIP51. Besides neurons, endothelial cells are among the longest-living cells in mammalians. The vasculature is usually quiescent in the adult and only few adult tissues require ongoing angiogenesis, including placenta, ovary, and physiological repair processes following injury (Papetti

and Herman, 2002). Angiogenesis is also pivotally required for tumor development (Bergers and Benjamin, 2003).

Angiogenesis is a complex, multistep process that relies on the tightly regulated exertion of inhibiting and activating mediators, the latter group comprises several growth factors, including FGF, PDGF, EGF, and VEGF. VEGF is reported to stimulate endothelial cell proliferation and also enhances cell migration *in vitro*. Moreover, VEGF increases endothelial permeability by phosphorylation (Esser *et al.*, 1998) and subsequent disruption of the constitutively expressed vascular endothelial (VE)–cadherin complex located at the adherens junctions. The mechanism of vascular endothelial–cadherin (VE–cadherin) phosphorylation is not entirely clear but seems to rely on the PTPIP51-signaling partner Src kinase, which is reported to directly associate to VE–cadherin and VEGF-mediated phosphorylation is abrogated in Src deficient mice (Weis and Cheresh, 2005). A detailed explanation of the various cellular functions mediated by Src kinase is given in Section 3.2. Interestingly, Kevil and colleagues (1998) reported the MAPK pathway also to be involved in VEGF-mediated permeability *in vitro*. Activation of the MAP kinase cascade leads to a disorganization of the cellular localization of VE–cadherin and occludin at the adherens junctions.

4.1.5. Ciliated epithelia

Epithelia bearing motile cilia, such as the ependyma lining the brain vesicles, the respiratory epithelium (Fig. 6.8A and B), epithelium lining the uterine tubes, as well as efferent ductules of the testes express PTPIP51 mRNA and display a strong immune reaction on the apical cell membrane. Electron microscopy revealed PTPIP51 protein to be localized within the motile cilium. The latter consists of nine peripheral microtubule doublets arranged around a central core harboring two central microtubules. This structure, called axonema extends from the nine triplet microtubules of the basal body, a modified centriole, which acts as microtubule organizing center (Satir and Christensen, 2007).

Ciliary motility is mediated by axonemal dyneins causing the relative sliding of the nine outer axonemal doublets. Interestingly, cAMP-activated PKA, which has been shown to phosphorylate PTPIP51 (for details see Section 2.7.3.) is localized to the axoneme and well known to positively regulate ciliary beat frequency (Salathe, 2007). As reviewed in Section 2.6., PTPIP51 (FAM82C) harbors a TPR domain, which was shown to mediate microtubular association of RMD-1 (FAM82B), another member of the FAM82 family (Oishi *et al.*, 2007). Hence, PTPIP51 may act as an anchoring site for proteins, which directly exert ciliary motility and/or facilitates protein transport along the microtubules. Dependent on the tissue affected, defects in ciliary motility and protein transport may

result in respiratory tract infection, chronic rhinitis and sinusitis, infertility, and hydrocephalus (Afzelius, 2004).

4.2. Skeletal muscle

In adult skeletal muscle of various mammalian species, including human, the expression of PTPIP51 protein and mRNA is restricted to specific muscle fibers (Stenzinger *et al.*, 2005). Immunostaining for PTPIP51 of rat skeletal muscle sections (Fig. 6.8C) revealed a close correlation between PTPIP51 expressing fibers and the type IIa fiber type determined by ATPase-histochemistry (>90% match). Only ~10% of type I and IIb fibers were also found to express PTPIP51. These results suggest that PTPIP51 is not a housekeeping protein involved in the basic functioning of muscle cells (e.g., enzymes involved in synthesis and processing of DNA, RNA, proteins, or the major metabolic pathways), because its expression is mainly restricted to a specific fiber type.

Chronic low frequency stimulation of adult fast twitch skeletal muscle in rat leads to a sequential switch from fast type IIb to type IIa fibers and, to a much smaller extent, to slow type I fibers (Pette and Staron, 2001). Bridging the gap between these pure fiber types characterized by the expression of a single myosin heavy chain (MHC) isoform, the transitional process also generates hybrid fibers expressing two or more MHC isoforms. This process of transformation allows muscle fibers to adapt their contractility and metabolism to functional requirements (Pette, 2002).

As observed in serial histological sections of transformed muscle, PTPIP51 expression steadily increases during the fiber type shift from the fast glycolytic type IIb to the slow oxidative type I fibers leading to a higher proportion of type IIa fibers. We also investigated PTPIP51 expression of developing skeletal muscle in mouse embryos. From day 12 pc onward, all myoblasts were PTPIP51-positive (e.g., in the diaphragm, intercostals, tongue, and external eye muscles). It is tempting to speculate that PTPIP51 serves as an intracellular signaling partner during development of higher vertebrates. To further address this issue, we investigated the role of PTPIP51 in an *in vitro* cell culture system and found a differentiation-dependent increase in PTPIP51 mRNA and protein in myotubes and evolving myofibers (Barop *et al.*, 2009).

Cultured human myoblasts permanently express PTPIP51 protein at low level. Induction of myoblast fusion as triggered by withdrawal of EGF and basic fibroblast growth factor (bFGF) results in a continuous increase in the amount of PTPIP51. The process of myoblast alignment, elongation, and fusion is a pivotal step in the formation of myotubes and evolution of myofibers, respectively.

Further increase of PTPIP51 mRNA and protein is observed during differentiation of myofibers as determined by MHC and sarcoplasmic/

endoplasmic reticulum calcium ATPase (SERCA) expression. In accordance with these findings, a resumption of the proliferation process after 14 days of myofiber-differentiation mediated by proliferation medium containing EGF and FGF results in a time-dependent reduction of PTPIP51 mRNA in myotubes. The attenuation in expression, however, did not drop back to the basal expression of PTPIP51 observed in untreated cells.

Among other mitogenic stimuli both FGF and EGF stimulate cell proliferation (Doumit *et al.*, 1993) and inhibit myogenic differentiation (Yablonka-Reuveni, 1995) through activation of the MAPK-pathway (Böttcher and Niehrs, 2005; Milasincic *et al.*, 1996), thereby repressing the transcription factors myogenin and Myo D, both being essential for muscle differentiation (Tortorella *et al.*, 2001). Promotion and inhibition of myogenic differentiation seem to be carefully modulated on the level of Raf activity. DeChant *et al.* (2002) reported a low Raf signaling level to accelerate differentiation, whereas high levels abrogated differentiation and apoptosis of muscle cells.

PTPIP51 acts as a positive regulator of Raf-1 in transfected HeLa cells (Yu *et al.*, 2008) and alterations of PTPIP51 expression was triggered in immortalized human keratinocytes (HaCaT-cell line) by EGF application (Stenzinger *et al.*, 2006).

Barop and colleagues (2009), however, found a positive correlation between PTPIP51 expression and the degree of muscle cell differentiation, which seems to be in variance with the reported ability of PTPIP51 to activate the Raf–ERK cascade that is well known to negatively regulate muscle differentiation upon growth factor ligation. Nevertheless, the synopsis of these data suggests a regulatory function of PTPIP51 in myotube differentiation, either mediated by a negative influence on the MAPK pathway or by influencing other signaling cascades, which contribute to skeletal muscle cell differentiation. Moreover, the function of PTPIP51 and its role as signaling partner certainly depends on the ligand of the growth factor receptor, as well as on the extent and timing of PTPIP51 activation, possibly leading to the promotion of distinct cellular processes, such as proliferation and differentiation.

The regulatory role of Src-kinase in myogenic differentiation is well established and its promoting or repressing effects seem to be mediated by the spatiotemporal alteration of Src activity (Falcone *et al.*, 1991; Schneider and Olson, 1988; Yoon and Boettiger, 1994). PTPIP51 may influence the grade of activity by direct interaction with c-Src. As shown by Wang and Forsberg (2000) CNTF, which was found to govern PTPIP51 transcription in rat retina cells (Roger *et al.*, 2007), influences protein turnover in skeletal muscle cells. CNTF also has a myotrophic, protective effect on denervation-induced morphological changes (e.g., muscle size, fiber type) of skeletal muscle (Vergara and Ramirez, 2004). The role of PTPIP51 in these processes needs further investigation.

4.3. Nervous system

The Raf–ERK pathway, as well as 14-3-3-γ and 14-3-3-β proteins are essentially required for neuronal maintenance, neurite outgrowth and axonal transport (Dougherty and Morrison, 2004; Markus *et al.*, 2002a,b; Planchamp *et al.*, 2008). Alterations in 14-3-3 and Raf–ERK signaling lead to multiple neurological disorders, including Alzheimer's and Parkinson's disease, as well as malignant transformation (Seger and Krebs, 1995; McCubrey *et al.*, 2007; Lyustikman *et al.*, 2008). As shown by Roger and colleagues (2007) for rat retina, PTPIP51 expression is regulated by CNTF. CNTF promotes neuronal survival, proliferation, and differentiation and is, among other trophic factors, an essential cytokine for the development and maintenance of the nervous system, as well as for axonal growth (Fuhrmann *et al.*, 2003; Sleeman *et al.*, 2000; Weisenhorn *et al.*, 1999).

Immunostaining experiments revealed the expression of PTPIP51 in the cerebrum of adult mice (Koch *et al.*, 2009b). More specifically, PTPIP51 was detected in cells of the nucleus accumbens, that is, the most ventral portion of the striatum. A weaker signal was observed in the piriform cortex and its connections to the anterior commissure. A detailed morphological analysis of the diencephalon revealed expression of PTPIP51 mRNA and protein in the paraventricular (Fig. 6.8D) and supraoptical nuclei of the hypothalamus and the neurohypophysis. In the neuroendocrine nuclei and their connections to the neurohypophysis PTPIP51 was found to be co-localized with vasopressin and its transport protein neurophysin II. Vasopressin and oxytocin derived from magnocellular neurons (Russell and Leng, 2000) are transported together with their neurophysin carriers, neurophysin I and II (Fotheringham *et al.*, 1991; Trembleau *et al.*, 1994) in neurosecretory vesicles along the axon toward the neurohypophysis for secretion (Brownstein *et al.*, 1980; Dreifuss, 1975). As PTPIP51-positive neurons and fibers displayed an identical localization of neurophysin II, PTPIP51 may act as a signaling partner mediating processes required for the axonal transportation of neuropeptides.

Axonal transport requires the attachment of neurophysin–vasopressin complexes to the microtubular cytoskeleton (Gainer and Chin, 1998; Senda and Yu, 1999). Since PTPIP51 (FAM82C) harbors a TPR domain, which was shown to mediate microtubular association of RMD-1, another protein of the FAM82 family (FAM82B) (Oishi *et al.*, 2007), PTPIP51 may also directly associate to microtubule thereby anchoring various proteins (e.g., neurophysin) or mitochondria through the mitochondrial target sequence (Lv *et al.*, 2006) to the microtubular transport system. A detailed analysis of the biochemical structure of PTPIP51 is given in Section 2.1.

Perturbation and defects in the axonal transport, however, can lead to diverse diseases including Amyotrophic lateral sclerosis, Alzheimer's and

Huntington's disease (Gerdes and Katsanis, 2005; Goldstein, 2001). In the cerebellar cortex, all regions including the inferior cerebellar peduncle were found to express PTPIP51. The strongest immunosignal, however, was detected in the nodulus and uvula comprising the archicerebellum.

Positive immunoreactivity for PTPIP51 was found in the brain stem of mice in the superior colliculus, the nucleus and genu of facial nerve, as well as the spinal trigeminal tract (Koch *et al.*, 2009b). Histological analysis and double staining experiments with antibodies against the glial fibrillary acidic protein (GFAP) antigen (glia cells) and against PGP9.5 (neurons), respectively, revealed expression of PTPIP51 in the cell body of neurons and their extensions (i.e., neurites and axons). PTP1B, the *in vitro* signaling partner of PTPIP51 is known as a regulator of axonal growth (Pathre *et al.*, 2001). Since neurites display a strong PTPIP51-immunosignal, one may speculate that PTPIP51 plays a role in this process.

Lv and coworkers (2006) demonstrated PTPIP51 mRNA expression in fetal brain. Our own experiments confirmed this finding and, more specifically, PTPIP51 expression was observed in those regions of the developing mouse brain, which evolve into the PTPIP51-positive brain structures detected in adult animals. In the adult peripheral nervous system, PTPIP51 was observed in axons and cell bodies of neurons of the spinal cord (Stenzinger *et al.*, 2005). Ganglion cells of the sympathetic chain and their corresponding nerve fibers running to organs such as heart, spleen, kidney, and liver were also found to be PTPIP51-positive. This was also observed for branches of the vagal nerve. The enteric nervous system of the gut was also found to express PTPIP51.

4.4. Liver

The PTPIP51 transcript could be detected in rat and pig liver. In rat liver, the encoded protein is predominately expressed in non-parenchymal cells, that is, Kupffer cells, specialized macrophages of the liver, stellate cells (Ito cells), and resident large granular lymphocytes (Pit cells) but not in sinusoidal endothelial cells (Stenzinger *et al.*, 2007). Besides their well-known physiological functions in normal liver (Ramadori *et al.*, 2008), the complex crosstalk between all types of non-parenchymal cells greatly contributes to the inflammatory response following liver damage (Wallace *et al.*, 2008). Interestingly, the activity of all three PTPIP51-positive non-parenchymal cells relies on signaling molecules, which are known to interact with PTPIP51 (see Section 2.6.). For instance, Che and colleagues (2007) reported a PKA–Src–ERK1/2 cascade to promote collagen I expression in the hepatic stellate cells. Collagen expression is the key event for fibrotic transformation of liver tissue and subsequent liver cirrhosis. MEK1/2–ERK1/2 serve as signaling partners for the PDGF- and galectin-mediated induction of stellate cell proliferation (Britton and Bacon, 1999; Maeda *et al.*, 2003). Kupffer cells, cultured

under hypoxic conditions showed a significant increase in ERK1/2 and Src levels and concomitant activation of the Src kinase resulted in increased IL-6 production through increased p38 MAPK activation (Thobe *et al.,* 2006). The MEK1/2/ERK pathway is also involved in activation and proliferation as well as in the regulation of cytotoxicity of natural killer cells (Benson *et al.*, 2008; Chuang *et al.*, 2001; Yu *et al.*, 2000).

PTPIP51 could also be detected in the endothelium of blood vessels comprising the portal triad, as well as in cells comprising the bile ducts, portal ductules as well as interlobular bile ducts and terminal ductules (canals of Hering). Cholangiocyte proliferation is associated with increased ductal secretion (Alpini *et al.*, 1994; Lesage *et al.*, 1996) and seems to be mediated through changes in the PKA/Src/MEK/ERK1/2 pathway as shown by Francis and colleagues (2004). Proliferation of cultured cholangiocarcinoma cells is also controlled by the signaling molecules comprising the MAPK signaling cascade (Park *et al.*, 1999).

4.5. Placenta

Expression of PTPIP51 protein and mRNA was observed in the syncytiotrophoblast and cytotrophoblast layer of human placentae from the first, second, and third trimesters (Stenzinger *et al.*, 2008). The syncytiotrophoblast of third trimester placenta specimen, however, showed only an attenuated signal to the PTPIP51-antibody indicating a downregulation of PTPIP51 in mature chorionic villi (Fig. 6.8F). PTP1B expression was restricted to the syncytiotrophoblast during all gestational stages. The trophoblast epithelium surrounding the stroma of chorionic villi is the main structural and functional component of the placenta, which allows close contact of the fetal and maternal blood system. The inner layer consists of proliferating cytotrophoblasts, which differentiate and individually fuse into the syncytiotrophoblast (outer layer). Interestingly, Src family protein tyrosine kinases have been reported to be differentially expressed during trophoblast differentiation. With ongoing differentiation, an increasing mRNA expression was particularly observed for c-Src kinase (Daoud *et al.*, 2006). Moreover, experiments performed with cultured cells isolated from the trophoblast suggest that MAPK seem to serve as signaling partners in trophoblast differentiation (Daoud *et al.*, 2005).

Syncytial fusion also requires a finely regulated apoptotic cascade that is completed with the formation of syncytial sprouts ultimately being released into the maternal circulation (Huppertz *et al.*, 1998). We observed that cells of the syncytiotrophoblast show a strong signal to the PTPIP51 antibody and undergo apoptosis as determined by TUNEL assay, cytokeratin 18f, and caspase 3 expression.

PTPIP51 protein and mRNA were also found in the endothelium and smooth muscle cells of placental arterial and venous vessels comprising the

stroma of chorionic villi together with mesenchymal cells and macrophages (Hofbauer cells) (Fig. 6.8F). PTPIP51 was also detected in a small population of placental CD14-positive macrophages and mesenchymal cells within the villous stroma. Interestingly, Maruyama and colleagues (1999) reported endometrial stromal cells to express activated c-Src kinase during *in vitro* decidualization. This was accompanied by a subcellular translocation from the perinuclear to the cytoplasmic compartment.

Proliferative cells of the vascular smooth muscle layer in placenta displayed high PTPIP51 reactivity. This immunohistochemical finding may reflect the promotion of mitosis and cell cycle by PTPIP51 as suggested by Oishi *et al.* (2007) and Dephoure *et al.*, (2008). Although the functional correlate of this expression pattern has to be investigated in detail, PTPIP51 may be involved in signaling processes required for placental angiogenesis that is mandatory for the development of both the placenta and the fetal organism. As demonstrated by Reynolds and Redmer (2001), VEGF and FGF are the major growth factors governing placental angiogenesis and vascularization. Binding of the VEGF family members to the VEGFR-2 leads to proliferation mediated by the PKC–Raf–MEK–MAPK cascade (Kerbel, 2008). The Ras–MAPK pathway is also activated upon FGFR ligand binding and probably by PTPIP51 by a 14-3-3-dependent mechanism. Cell migration induced by VEGF-2 is executed by downstream activation of Src kinase which also phosphorylates PTPIP51.

4.6. Male reproductive epithelium

Human spermatogenesis is a complex, sequentially organized process, which encompasses (1) proliferation and differentiation of the spermatogonia, (2) meiosis of the spermatocytes, and (3) transformation of haploid round spermatids to the terminally differentiated spermatozoon and, finally, its release into the lumen of seminiferous tubules (Amann, 2008). The morphological correlate of spermatogenesis is the seminiferous epithelium, which also harbors Sertoli cells forming the blood–testis barrier. Immunohistochemical examination of PTPIP51 in human testis (Stenzinger *et al.*, 2005) revealed only differentiating germ cells (round and elongated spermatids, as well as spermatozoa) of the seminiferous epithelium to display a strong immune reaction, whereas stem cells (spermatogonia) remained negative (Fig. 6.8E). Human sperm cells showed a characteristic distribution of PTPIP51 with the protein present in the neck, mid, and principal piece. PTPIP51 mRNA was detected somewhat earlier in the differentiation process, namely, in spermatogonia type B, but not in type A pale and dark spermatogonia. Type B spermatogonia, which ultimately divide and differentiate to form primary spermatocytes, evolve by a complex process of differentiation from their type A precursors (Kostereva and Hofmann, 2008). The expression of the PTPIP51 gene could be traced up to round spermatids of stage III.

This PTPIP51 expression profile resembles that observed for epidermis with the protein being translated during cell cycle exit and in all stages of cellular differentiation. ERK1 and ERK2, signal transducers of the MAPK pathway are expressed in all types of germ cells and seem to influence germ cell survival (Bhattacharya *et al.*, 2005). The ERK proteins also serve a critical signaling partners in chromosome condensation (Di Agostino *et al.*, 2002) and meiotic progression (Sette *et al.*, 1999) of spermatocytes. Successful germ cell development, however, also relies on the careful regulation of cell adhesion and motility during the epithelial cycle.

In germ cells, the apical ectoplasmic specialization (ES), a testis-specific actin-based adherens junction type confers Sertoli-spermatid cell anchoring (Siu and Cheng, 2004; Vogl *et al.*, 2000). Assembly and disassembly of the apical ES have been shown to depend on the functional integrity of the ERK cascade (McLachlan *et al.*, 2002; Xia and Cheng, 2005). Another signaling molecule modulating junction dynamics is $P130^{Cas}$, which once phosphorylated by c-Src acts as a docking protein thereby recruiting other proteins to the ES site (Siu *et al.*, 2003a,b, 2005). One may speculate that PTPIP51 influences ES-dynamics through direct interaction with c-Src and/or indirectly by modulating the MAPK cascade on Raf level. Interestingly, Berruti (2000) reported a ternary complex of rap1, B-Raf, and 14-3-3 theta to influence germ cell differentiation and morphogenesis. Although physical interaction with 14-3-3 isoforms other than beta and gamma has to be experimentally confirmed, one could hypothesize a role for PTPIP51 in this complex formation.

During their transit in the female genital tract, spermatozoa undergo a process called capacitation, which encompasses hyperactivated motility and changes in the cellular membrane enabling the human sperm to fertilize the released oocyte by acrosomal reaction. This process is accompanied by protein tyrosine phosphorylation, particularly in the principal piece. Capacitation-associated tyrosine phosphorylation is partly mediated by Src kinase (Mitchell *et al.*, 2008), the latter being activated by the PKA (Lawson *et al.*, 2008) (for details of both pathways see Sections 2.7.1 and 2.7.3), both of which are known to interact with PTPIP51. Additionally, the MAPK kinase cascade is also well known to orchestrate the level of tyrosine phosphorylation during capacitation and changes in sperm motility (Sun *et al.*, 1999). PTPIP51 function may also contribute to the motility of the sperm flagellum by direct interaction with microtubules via its TPR motif (Oishi *et al.*, 2007).

4.7. Mammalian development

This section presents preliminary data on the tissue-specific PTPIP51 expression during mouse development from embryological (post coitum) day E12 to E18 and is currently being investigated in more detail. Until now

only a part of the organs has been analyzed for PTPIP51 expression (Maerker *et al.*, 2008), hence, the following depiction is incomplete.

As outlined in Section 3, all interacting partners of PTPIP51 are involved in mammalian development. While, for example, PTP1B and TCPTP are required for normal function of the embryonic immune system, the 14-3-3 protein family seems to be implicated in eye and brain development. As has been demonstrated by several knockout studies, almost all members of the MAP kinase cascade (MEK1, Giroux *et al.*, 1999; Erk2, Hatano *et al.*, 2003; Raf1, Mikula *et al.,* 2001) are essential in placental formation and organ vascularization. As reviewed by Meier *et al.* (2000) and Twomey and McCarthy (2005) programmed cell death is a fundamental event in development. PTPIP51 has been shown to enhance apoptosis *in vitro* (Lv *et al.*, 2006) and may therefore be also implicated in programmed cell death during embryogenesis. With respect to developmental processes, one should keep in mind that PTPIP51 is phosphorylated during cell cycle transition (Daub *et al.*, 2008; Dephoure *et al.*, 2008) and RMD-1, another member of the FAM82 family has been reported to mediate chromosome segregation (Oishi *et al.*, 2007).

On day E12 PTPIP51 mRNA and protein are expressed in ectodermal cells forming the outer lining of the mouse embryo. Further differentiation leads to a steady increase of cell layers, eventually forming the fully developed epidermis of the adult animal. While during early epidermal development all cell layers express PTPIP51, in the evolving epidermis of late developmental stages only the suprabasal cell layers reacted positive with the antibody against PTPIP51. This reaction pattern corresponds to the one observed for the adult animal.

PTPIP51 protein was traced in the epithelium of the developing floor of the mouth cavity, palate, and tongue. From day E16 onward these epithelial cells showed a steadily stronger reaction with the PTPIP51 antibody. The columnar epithelial cells of the developing gut express the PTPIP51 antigen as a broad band at the apical cell pole (Fig. 6.9A). As observed for developing bronchi, surrounding mesenchymal cells also reacted positive to the PTPIP51 antibody. By day E18, the future myenteric plexus can easily be detected by its high reactivity to the PTPIP51 antibody.

The developing bronchial epithelia also displayed a strong reactivity to the PTPIP51 antibody (Fig. 6.9B). PTPIP51 was found to be localized at the apical cell surface of all cells, whereas the basal cell pole showed a somewhat weaker reaction (E12). The cellular localization of the corresponding mRNA detected by *in situ* hybridization resembles the immunohistochemical staining pattern. On day E18, as well as in the adult mouse PTPIP51 expression is restricted to the ciliated cells. Notably, the developing bronchial epithelium is surrounded by PTPIP51-positive mesenchymal cells, which will ultimately develop into smooth muscle cells, fibroblasts, and hyaline cartilage.

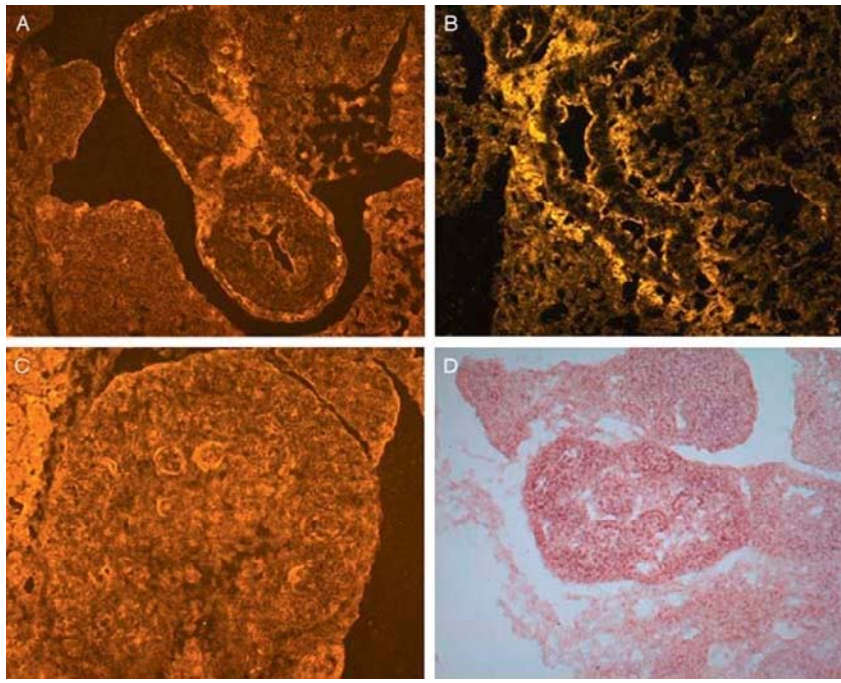

Figure 6.9 Localization of PTPIP51 in developing mouse tissues. (A) PTPIP51-immunostaining of gut day E15 (magnification: 10×). (B) PTPIP51-immunostaining of the lungs day E13 (magnification: 20×). (C) PTPIP51-immunostaining of the kidneys day E15 (magnification: 10×). (D) *In situ* hybridization with anti-sense probe to PTPIP51 of the kidneys day E15 (magnification: 10×).

From day E12 onward, the apical and basal part of cells comprising the choroid plexus were found to be PTPIP51 positive. In the adult mouse, however, only the apical cell pole showed a positive reaction to the PTPIP51 antibody. The developing ependymal lining of the brain surfaces reveals a steadily increasing expression of PTPIP51 in the apical cell pole oriented to the cerebrospinal fluid (D. Maerker, personal communication).

During eye development PTPIP51 protein was found in corneal epithelium and endothelium during all investigated stages. Moreover, from day E16 onward keratocytes also expressed PTPIP51 protein. From day E12 to the adult stage, PTPIP51 was found in the anterior lens epithelium. The evolving neuroretina displayed PTPIP51 protein in the inner neuroblastic as well as in the developing photoreceptor cell layer. Ongoing development led to an enhanced expression of PTPIP51 in both layers. In addition, PTPIP51 is upregulated in the formerly negative inner and outer plexiform layer (Maerker *et al.*, 2008). PTPIP51 expression persists also in the adult stage except for the outer neuroblastic layer. With respect to renal development PTPIP51 protein and mRNA can be observed in metanephric

derivatives giving rise to the Bowman's capsule (Fig. 6.9C and D). This reaction pattern corresponds to that in adult kidney, where podocytes of the visceral layer display a positive PTPIP51-reaction.

4.8. Cancer

Expression studies revealed the presence of PTPIP51 mRNA and protein in human keratinocyte carcinomas (Koch *et al.*, 2008), prostate cancer (Koch *et al.*, 2009a), invasive breast cancer and glioblastoma (M, Petri, personal communication). In all these cases, PTPIP51 was predominantly found in the cells comprising peritumoral tissue and to a lower extent in clustered malignant cells invading the stroma. Particularly the endothelium and in some cases also the smooth muscle of the vasculature showed a strong immunosignal for PTPIP51. Most strikingly, cells of the myeloid and lymphoid lineage invading the peritumoral stroma also expressed high amounts of PTPIP51 (Fig. 6.10D). In healthy human bone marrow and peripheral blood, PTPIP51 is present in mature neutrophils (A, Brobeil, personal communication), but not in lymphocytes or other cells of the myeloid lineage. These findings suggest that PTPIP51 is upregulated in immune cells invading tumor microenvironment and requires further investigation since a chronic inflammatory state induced by cells of the innate and adaptive immune system is able to potentiate or even promote cancer development (de Visser *et al.*, 2006).

In some bone marrow specimen of patients suffering from acute myeloid leukaemia (AML), myeloblasts were found to express a detectable amount of PTPIP51 (A, Brobeil, personal communication). The underlying cause for this remains to be investigated. PTPIP51 is expressed in many human carcinoma cell lines (Lv *et al.*, 2006; Stenzinger *et al.*, 2005) isolated from colon cancer (HT29), hepatocellular carcinoma (HepG2), breast adenocarcinoma (MCF7), estrogen receptor negative breast cancer (modulator of drug activity, MDA), choriocarcinoma (BeWo), and cervical carcinoma (HeLa). Human glioblastoma cells (U87) were also found to express PTPIP51 (Fig. 6.10C) (M, Petri, and U, Nestler, personal communication). With respect to hematological malignancies, PTPIP51 expression was detected in human cell lines derived from Burkitt's lymphoma (Raji), promyelocytic leukemia (HL60), and acute T-cell leukemia (Jurkat).

These cell lines certainly provide a powerful tool to investigate the role of PTPIP51 in signaling cascades of malignant cells. Besides its functional role in cellular motility and apoptosis, we assume that PTPIP51 also serves as a signaling partner during mitosis. This hypothesis is based on the fact that (i) all cell cultures investigated so far express PTPIP51 and (ii) in HaCaT cells the amount of endogenous PTPIP51 mRNA alters during the cell cycle (unpublished results). Recent findings by Dephoure and colleagues (2008) support this hypothesis by showing that PTPIP51 is phosphorylated

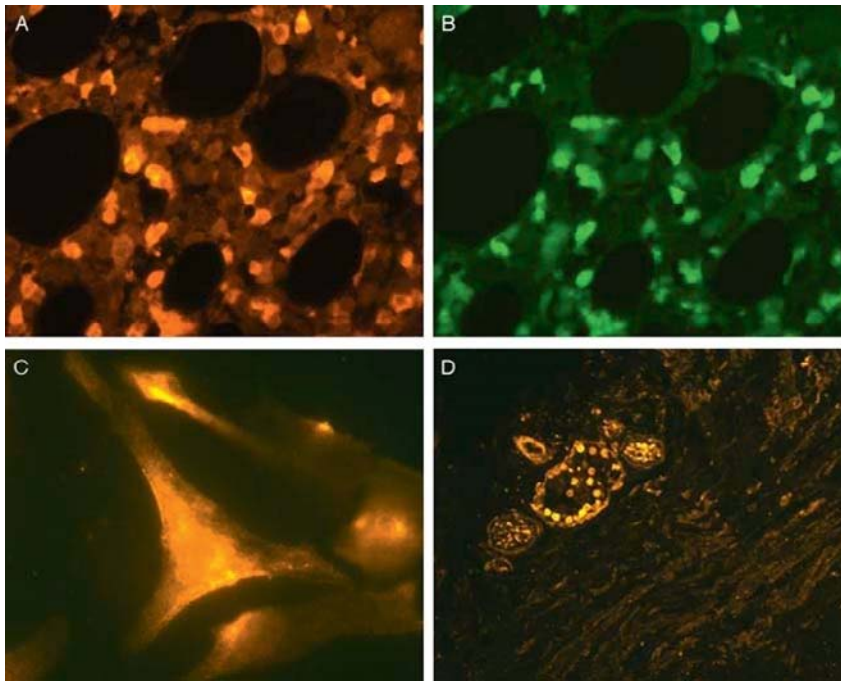

Figure 6.10 Localization of PTPIP51 in malignant tissue and cells of human origin. Double-immunostaining of PTPIP51 (A) and granulocyte marker (SPM250) (B) of human bone marrow from a patient with acute myeloid leukemia (magnification: 40×). (C) PTPIP51-immunostaining of human glioblastoma cells (magnification: 100×). (D) PTPIP51-immunostaining of human prostate cancer (magnification: 20×).

on distinct serine residues during the cell cycle (reviewed above). Since the structurally related TPR domain of RMD-1 (FAM82B) associates with microtubules and acts in chromosome segregation in *C. elegans* (Oishi *et al.*, 2007), it is tempting to speculate that PTPIP51 (FAM82C) may play a role in microtubular spindle formation during mitosis of higher vertebrates. Nevertheless, it is important to note that endogenous PTPIP51 is also and in some cases predominantly expressed in the peritumoral microenvironment rather than in the cells comprising the malignant tissue. Comparison of cell cultures models with *in vivo* conditions, therefore, should be done carefully.

Although we observed PTPIP51 expression in apoptotic and proliferative cells of normal and malignant tissue, one should qualify the statement by saying that PTPIP51 is not exclusively restricted to these processes (Koch *et al.*, 2008; Stenzinger *et al.*, 2008). The PTPIP51 effects on motility and migration of cells (Yu *et al.*, 2008) may match with the observation that PTPIP51 was detected in immune cells (Koch *et al.*, 2008) and clustered

prostate cancer cells invading the surrounding stroma (Koch *et al.*, 2009a). The data available so far suggest that PTPIP51 is possibly integrated in more than one signaling pathway and/or promotes crosstalk by acting at the interface of distinct signaling cascades (for details of the pathways see Section 3). Things get even more complicated when one takes into account splice variants of PTPIP51, which have been observed for healthy tissue (Stenzinger *et al.*, 2005) and HaCaT cells resembling keratinocytes in their early stages of squamous cell carcinogenesis (Ashton *et al.*, 2005; Stenzinger *et al.*, 2006). Further research is imperative to fit the observations into a bigger picture.

5. Conclusions and Further Perspective

In this chapter, the recent findings regarding the cell- and molecular biology of PTPIP51 are discussed. Although the data available are too preliminary to ascertain the precise role of PTPIP51 in cell biology, the biochemical properties and the expression profile, as well as a synopsis of both implicate PTPIP51 to operate in a cell- and tissue-specific manner in different cellular process, such as proliferation, differentiation, apoptosis, and motility. Besides its integration in distinct signaling pathways, splice variants may account for the diverse functions of PTPIP51. Subcellular localization of PTPIP51 possibly depends on the variants expressed in a given cell type and the phases of the cell cycle and may also be influenced by its interacting partners. PTPIP51 is phosphorylated on Tyr176 *in vitro* and *in situ* and is an *in vitro* substrate of Src and the PTP1B/TCPTP PTPs. It is phosphorylated by cAMP-dependent protein kinase on Ser212 and probably also by other protein serine/threonine kinases in cell cycle—dependent manner. In addition to PTPs, PTPIP51 interacts with 14-3-3 proteins, with the Nuf2 kinetochore protein, with the ninein-interacting CGI-99 protein, with diacylglycerol kinase alpha, and also with itself forming dimers and trimers.

Thorough further investigation is strongly needed to obtain a better picture on the functional properties of PTPIP51 *in vivo*. In this context, knockout models might be a helpful line of research to shed light on PTPIP51 biology, particularly in epithelial cell turnover, germ cell development, mammalian embryogenesis, and cancer formation.

With respect to the latter, it is certainly of interest to carefully investigate the contribution of PTPIP51 to cancer biology, since disturbances and alterations in the MAPK pathway, as well as in Src kinase and PTP1B signaling have been extensively shown to promote and maintain human tumor development.

ACKNOWLEDGMENTS

The authors are indebted to their collaborators A. El-Zaher Mostafa, A. Porsche, T. Bürklen-Schneeweis, R. Büttel, S. Becker-Weimann. A. Brobeil, and M. Petri for the contribution of unpublished information.

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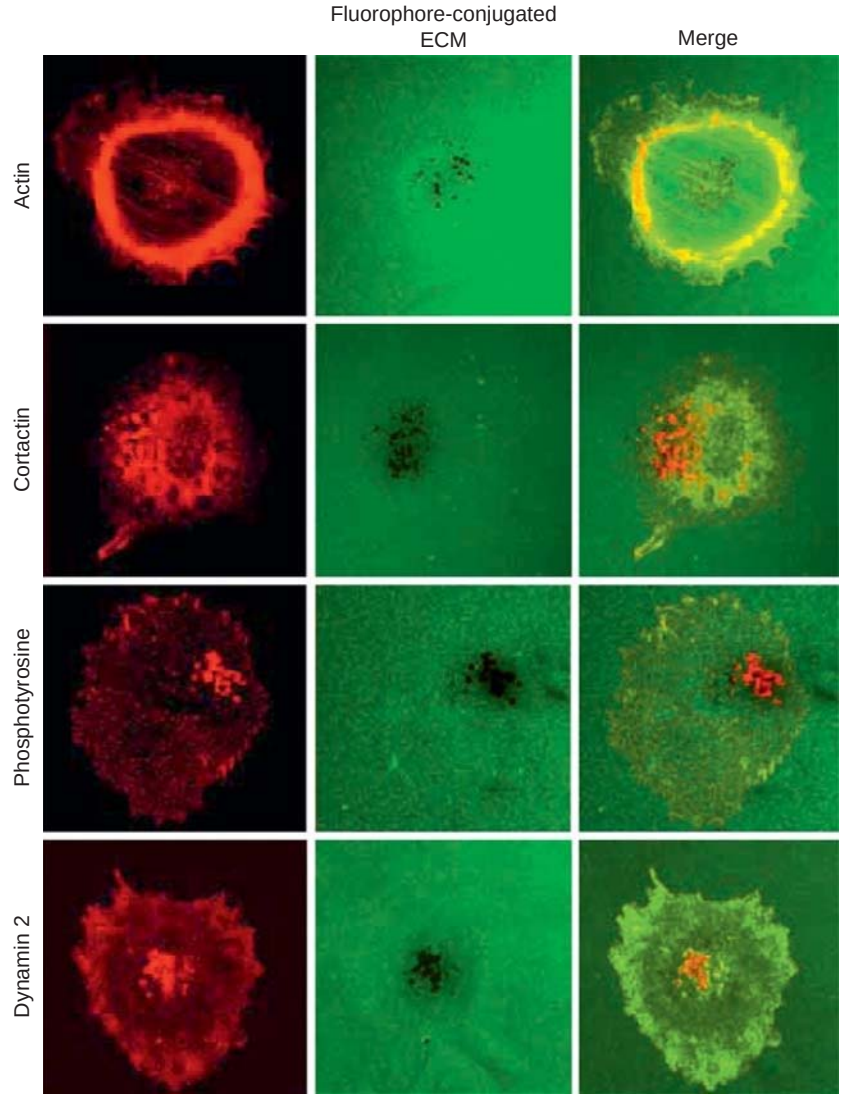
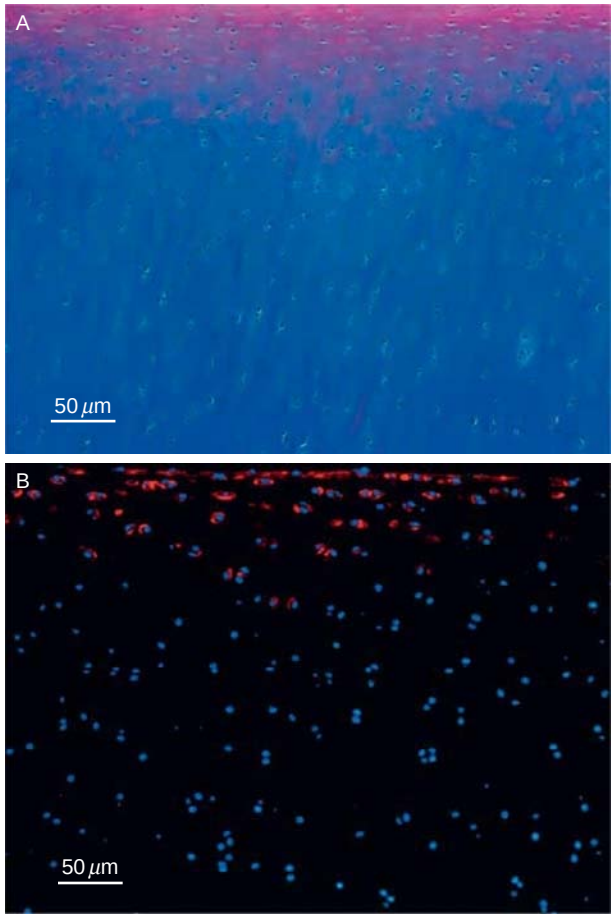
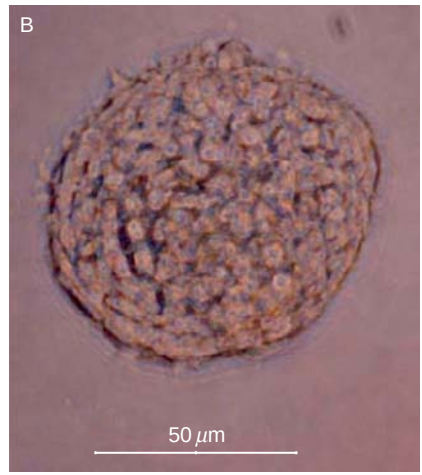


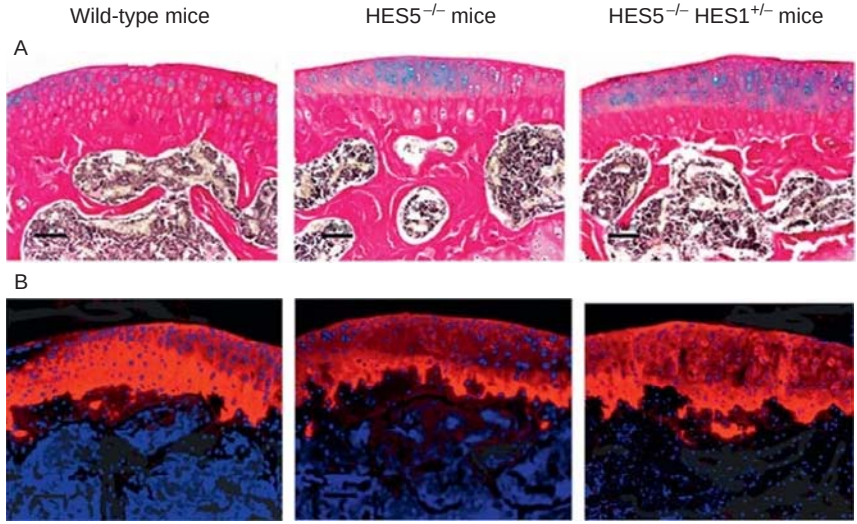
Giusi Caldieri *et al.*, Figure 1.1 Identification of invadopodia. A375MM melanoma cells grown on FITC-conjugated gelatin (green) and then fixed and stained with Alexa 546-phalloidin and anti-cortactin, anti-phospho-tyrosine and anti-dynamin 2 antibodies (red). Invadopodia match with underlying areas of degradation. Merged images are also shown.



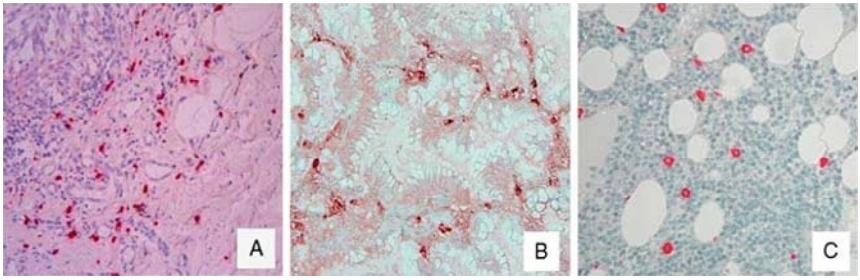
Camilla Karlsson and Anders Lindahl, Figure 3.3 Alcian Blue van Gieson staining of the articular cartilage (A) and immunohistochemical localization of Notch1 (B).



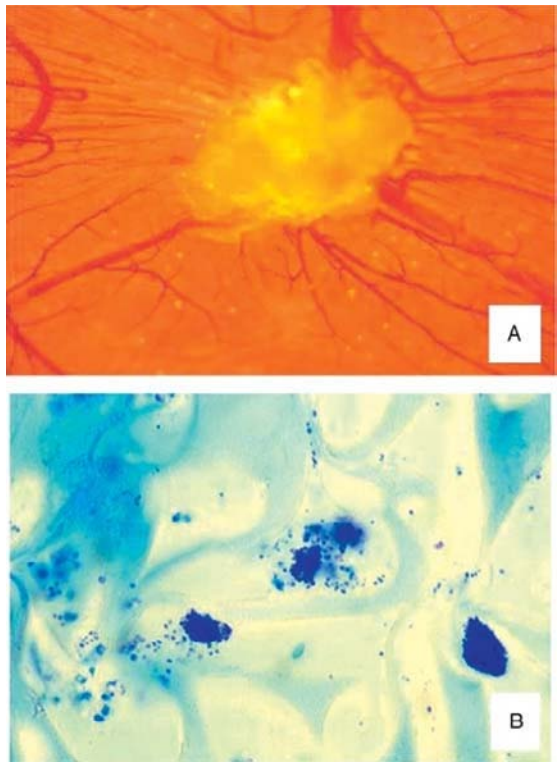
Camilla Karlsson and Anders Lindahl, Figure 3.4 Colony-forming efficiency of different sizes of chondrocytes as well as Notch1 positive and negative cells both directly after isolation and after expansion.



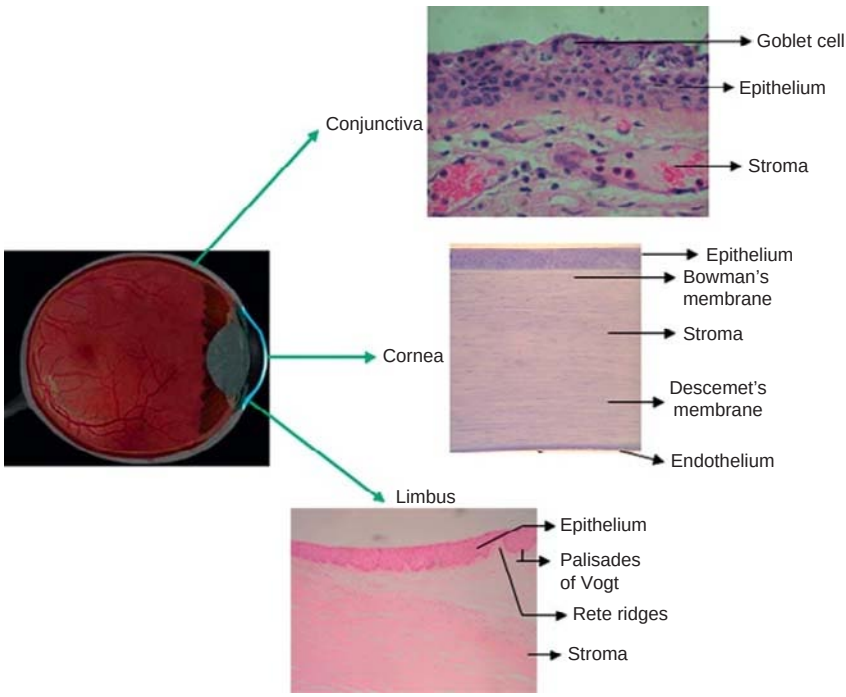
Camilla Karlsson and Anders Lindahl, Figure 3.6 Alcian Blue van Gieson staining (A) and detection of collagen type II (B) in wild-type and transgenic mice lacking the HES5 genes and one HES1 allele.



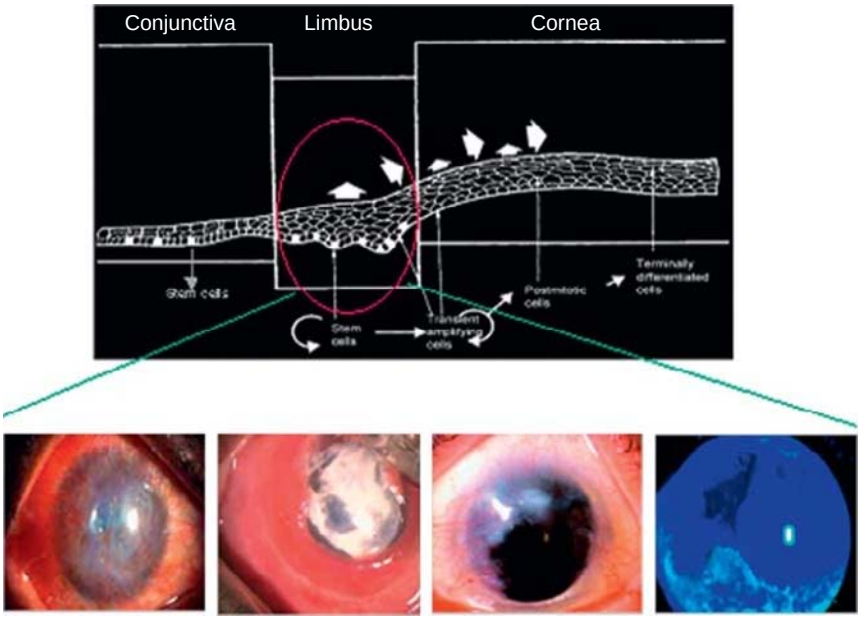
Domenico Ribatti and Enrico Crivellato, Figure 4.4 Tryptase-positive mast cells in bioptic specimens of human melanoma (A), nonsmall-cell lung cancer (B) and B cell chronic lymphocytic leukemia (C). Original magnification: (A)–(C), ×250.



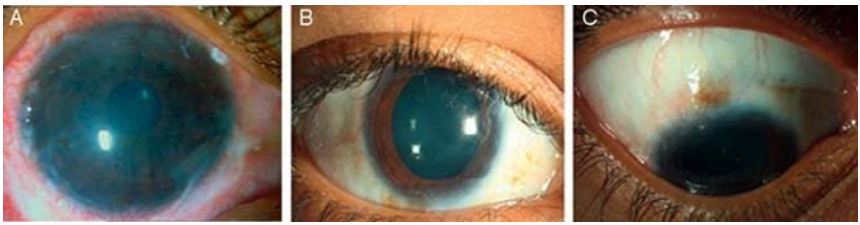
Domenico Ribatti and Enrico Crivellato, Figure 4.5 A mast cell suspension has been delivered on the top of the chick embryo chorioallantoic membrane on Day 8 of incubation using a gelatin sponge implant. Macroscopic observation on Day 12 shows the gelatin sponge surrounded by numerous allantoic vessels that develop radially towards the implant in a ''spoked-wheel'' pattern (A). The histological analysis shows among the sponge trabeculae metachromatic mast cells and their secretory granules (B). Original magnification: (A) ×50, (B) ×250 (adapted with modifications from Ribatti *et al.*, 2001).



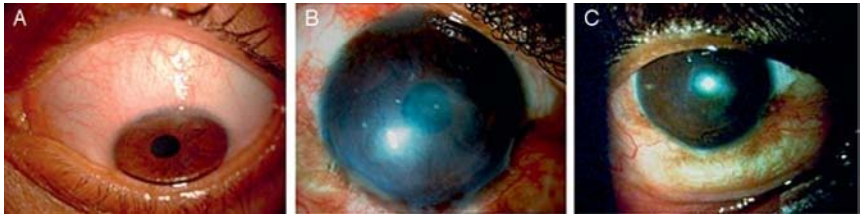
Geeta K. Vemuganti *et al.*, Figure 5.1 Anatomy of ocular epithelia. Location and histology of ocular surface epithelium conjunctival, corneal, and limbal epithelia. Note the nonkeratinized, stratified epithelium with underlying connective tissue stroma in all the three epithelia. Also note the presence of goblet cells with a vascular stroma in conjunctiva, and palisades of Vogt, which are absent toward the corneal epithelium, an avascular stromal bed with distinct basement membranes and a single layer of endothelium of the cornea.



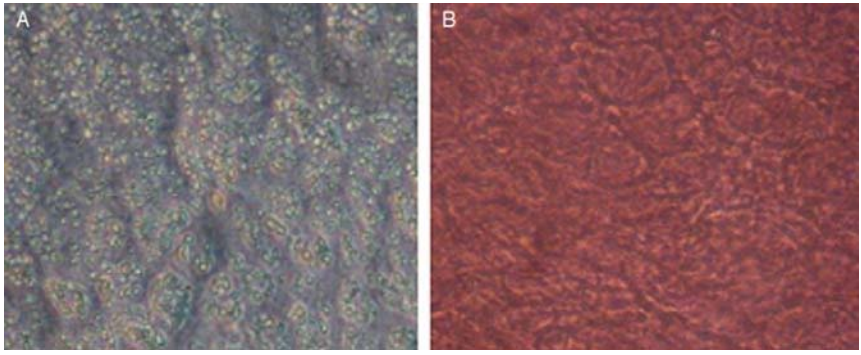
Geeta K. Vemuganti *et al.*, Figure 5.2 Regeneration of corneal epithelium and clinical signs of LSCD. Regeneration of corneal epithelium is by migration of stem cells (differentiation) from the basal layer of limbal epithelium toward the cornea. An insult to this process (marked in red) leads to limbal stem cell deficiency presented clinically with vascularized cornea, persistent epithelial defect, conjunctivalization, and stippled flourescein staining of the conjunctivalized cornea (from let to right).



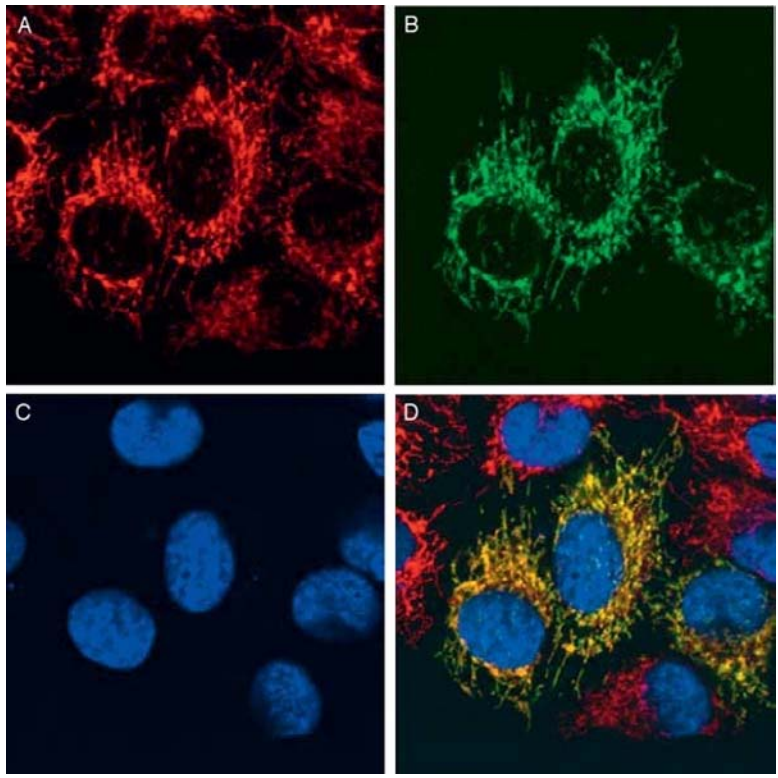
Geeta K. Vemuganti *et al.*, Figure 5.4 Amniotic membrane transplantation in LSCD. The clinical follow-up of a patient who underwent an amniotic membrane transplantation in the acute phase of chemical injury showing eye with AMT (A); 4 years posttransplant showing a clear cornea (B), and limbal pigmentation (C).



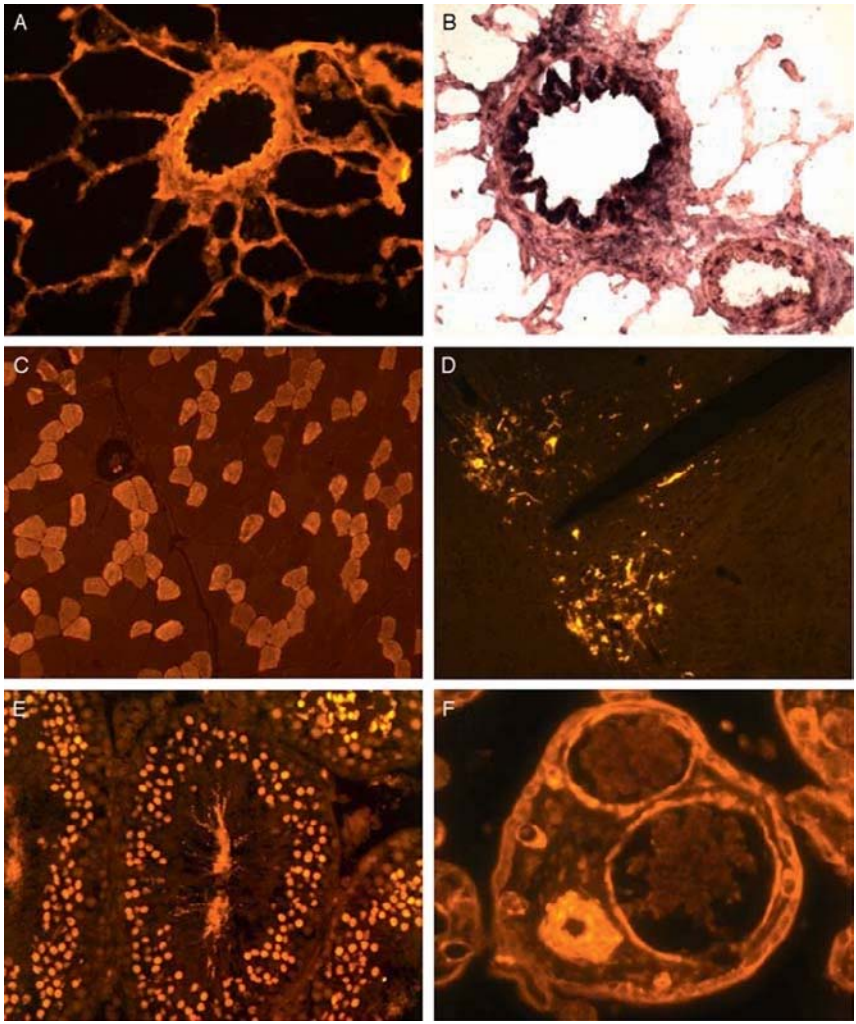
Geeta K. Vemuganti *et al.*, Figure 5.5 Cultivated limbal epithelial transplantation in LSCD. The clinical outcome in a patient with LSCD, who has undergone a CLET for visual rehabilitation showing the site of biopsy (arrow) (A); stable ocular surface with residual scarring 7 weeks postcultivated limbal autograft transplantation (B), reduction in density of scar and a stable ocular surface 10 months postoperatively (C).



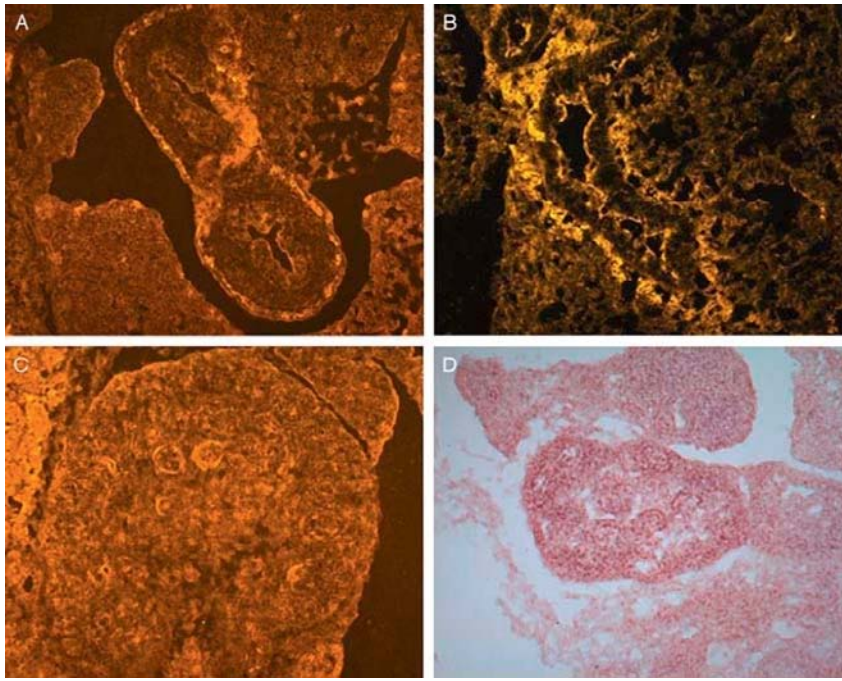
Geeta K. Vemuganti *et al.*, Figure 5.6 HAM with intact and devitalized epithelium. The morphology of HAM with the intact epithelium (A) and a devitalized epithelium (B) (devitalization by trypsin digestion and mechanical scrapping of the epithelium) as seen under a phase contrast microscope at a magnification of 100×.



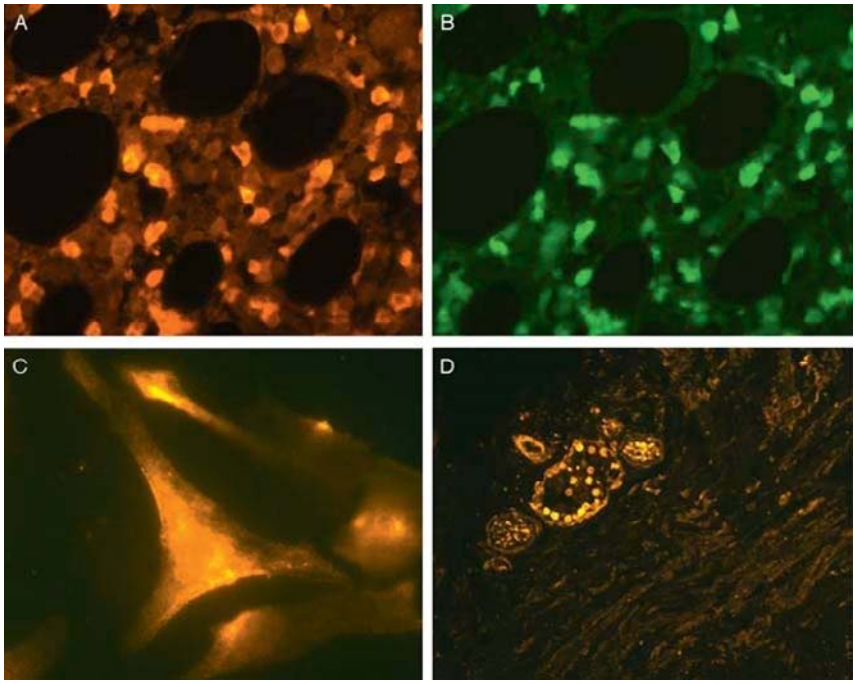
Albrecht Stenzinger *et al.*, Figure 6.4 Localization of a PTPIP51–EGFP fusion protein expressed in HEK293 cells. HEK293 cells expressing full-length PTPIP51 fused to EGFP (B) were stained with MitoTracker (A) and Hoechst 33342 (C). The merged images show co-localization of mitochondria and PTPIP51–EGFP in the transfected cells (D).



Albrecht Stenzinger *et al.*, Figure 6.8 Localization of PTPIP51 in tissues from adult human, rat, and murine origin. (A) PTPIP51-immunostaining of rat bronchus (magnification: 20×). (B) *In situ* hybridization with anti-sense probe to PTPIP51 of rat bronchus (magnification: 40×). (C) PTPIP51-immunostaining of rat skeletal muscle (magnification: 20×). (D) PTPIP51-immunostaining of mouse paraventricular nucleus (magnification: 10×). (E) PTPIP51-immunostaining of rat seminiferous epithelium (magnification: 20×). (F) PTPIP51-immunostaining of human placental villi from term placenta (magnification: 40×).



Albrecht Stenzinger *et al.*, Figure 6.9 Localization of PTPIP51 in developing mouse tissues. (A) PTPIP51-immunostaining of gut day E15 (magnification: 10×). (B) PTPIP51-immunostaining of the lungs day E13 (magnification: 20×). (C) PTPIP51-immunostaining of the kidneys day E15 (magnification: 10×). (D) *In situ* hybridization with anti-sense probe to PTPIP51 of the kidneys day E15 (magnification: 10×).



Albrecht Stenzinger *et al*., Figure 6.10 Localization of PTPIP51 in malignant tissue and cells of human origin. Double-immunostaining of PTPIP51 (A) and granulocyte marker (SPM250) (B) of human bone marrow from a patient with acute myeloid leukemia (magnification: 40×). (C) PTPIP51-immunostaining of human glioblastoma cells (magnification: 100×). (D) PTPIP51-immunostaining of human prostate cancer (magnification: 20×).