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CHAPTER ONE

Origin and Differentiation of Ionocytes in Gill Epithelium of Teleost Fish

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Contents

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Abstract

This paper focuses on the environmental cues that transform the gills of euryhaline teleost fish from an oxygen exchange structure into a bifunctional organ that can control both gaseous movement and water/ion transport. The cellular development that allows this structure to accomplish these tasks begins shortly after fertilization of the egg. It involves alterations of structure and function of embryonic cells [ionoblasts (IB)] that are shed from the pharyngeal anlage area of the embryo. These IB contain unique protein-receptor domains in the plasma membrane. These receptors respond specifically to the environmental cues effecting a calcium-binding protein receptor [calcium-sensing receptor (CaSR)]. The CaSR containing IB act as stem cells and are acted upon by isotocin, a heteroprotein regulator which induces them to form progenitor ionocytes (pIC). The pIC form two types of cells. The first type becomes an aquaphilic ionocyte which regulates uptake of ions and through aquaporin molecules transports water out of the cell and controls body fluids of the fish. This mechanism is essential for freshwater living. The second type becomes a halophilic ionocyte and transports ions out of the cell and controls cell shrinkage by uptake of water via aquaporin molecules. This mechanism is essential for seawater living. These differentiating events in the pIC are controlled by the cross talking of genomic mechanisms found in the precursor IB. To unravel the cross talking events it is necessary to uncover how these genetic pathways are regulated by transcriptional and translational events coming from complementary DNA. Various gene families are involved such as those found in apoptosis mechanisms, regulatory volume regulators and ionic transport systems (cystic fibrosis transmembrane conductance regulator).

1. INTRODUCTION

The adult euryhaline teleost fish contains an epithelium in the gill arch that undergoes molecular rearrangements which provide the fish with physiological mechanisms to live in both freshwater (FW) and seawater environments (Evans et al., 1999, 2005; Marshall and Bellamy, 2010; Kaneko et al., 2008; Hwang et al., 2011). These investigations have not focused on how the genetic pathways underlying these physiological events occur during various stages of fish development. The purpose of this chapter is to review how the environmental cues that act upon the surface membrane osmoreceptor sites activate plasma membrane phosphorylation kinases to initiate differentiation in embryonic epidermal cells [ionoblasts (IB)]. These IB are stem cells which form mature ionocytes (IC). The kinases begin the intracellular events in IB via cross talking of genetic families, such as GCM2, FoxO, Notch, Grainyhead, etc. These gene products start restructure of the intracellular membrane network which is responsible for water and ion movement across or into the cell from the environment.

These intracellular events appear to involve in apoptosis mechanisms or its inhibition by autoregulatory mechanisms that occur between nuclear and mitochondrial genes.

2. BIOLOGY OF EPITHELIAL CELLS OF THE GILL

2.1. Morphology of Gill Epithelium

The filamental epithelial cells found in the region between respiratory leaflets are of three types of mature cells. They are the pavement cell (PVC), the mitochondria-rich cell (MRC), and the accessory cell (AC). The anatomical and ultrastructural features of these cells were described in detail by Wilson and Laurent (2002).

2.1.1. MRC in FW-Fish

The role of MRC in both FW and saltwater (SW) fish had been called earlier as being a "chloride cell" by Keys and Wilmer (1932) and involved in ion regulation. Lignot et al. (2002) and Watanabe et al. (2005) demonstrated the presence of large amounts of an isoform of aquaporin molecules (AQP3) in these cells. This molecule regulates water permeability. Subsequently, Perry (1997) and Hwang and Perry (2010) reported that in FW-fish, MRCs contain large numbers of proteins that transport ions. They have been described as being proteomic ion pumps (Wheatly and Gao, 2004). These proteomic ion pumps are proteins that are translated from messenger RNA (mRNA) which are subjected to a wide variety of chemical modifications in differentiating cells. In FW-fish, the MRCs facilitate sodium ion (Na^+) and calcium ion (Ca^{2+}) uptake while removing protons (H^+) and bicarbonate ions (HCO_3^-). Therefore, these MRCs are involved in several complex ion regulatory mechanisms. In addition, these cells regulate water movement via control of plasma volume (Hoffman et al., 2009). While it appears that MRCs also deal with changes of fluid volume and acid-base balance, they clearly are not just chloride cells or MRCs. They function as IC but due to the complex array of functions should more appropriately be called aquaphilic ionocytes (aqIC).

2.1.2. MRC in SW Fish

In SW fish, the role of the MRCs has been shown to be the cells which contain other types of proteomic ion pump (Wheatly and Gao, 2004). These protein pumps handle several other types of ions, such as sodium (Na^+),

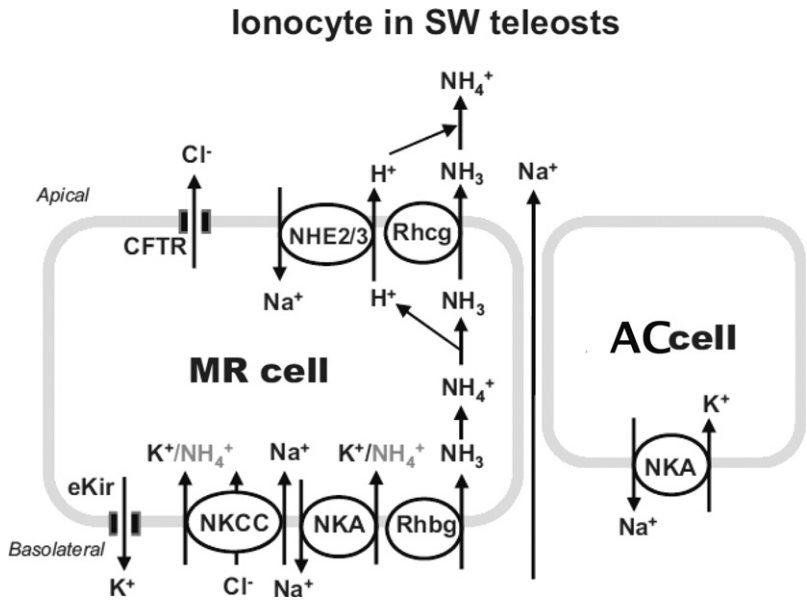

Figure 1.1 Model of IC in seawater. For details refer Section 1.2.2 in the text. *Ref. Hwang et al. (2011).*

calcium (Ca^{2+}), potassium (K^{+}) and chloride (Cl^{-}) ions. A currently accepted model (Fig. 1.1) for salt excretion by MRC cells consists of the cooperative action of three major enzymatic ion transporters: Na/K-ATPase, Na/K/2Cl co-transporter, and cystic fibrosis conductance regulator [cystic fibrosis transmembrane conductance regulator (CFTR)] at the apical pit which together with NHE2 and NHE3 apical membrane transporters maintain ion movement via the intracellular membrane network forming the chloride channel (Hwang et al., 2011). Therefore, it is more appropriate to refer to these epithelial cells as not being just large cells containing numerous mitochondria or MRCs. These are complex IC which have many intracellular mechanism(s) required for salt excretion and water retention and should be called halophilic ionocytes (haIC).

2.2. Origin of IC in Adult Gill Epithelium

Ionocytogenesis was a term first proposed by Conte (1980), in which any type of noninvasive physical or chemical force that can damage the gill arch initiates a replacement of the damaged cells. These replacement cells were derived from MRC found in the filamental region and not from the

respiratory leaflet area. Thus, the gill's ability to function as an ion-excretory organ depended upon this cellular renewal within the filamental region (Conte, 1965; Conte and Lin, 1967; Motais, 1970). These new MRCs required DNA replication to form postmitotic daughter cells. These daughter cells, containing newly formed DNA, could continue to follow genetic transcriptional and cellular translational pathways to conclude differentiating events that eventually produced mature IC. These pIC cells were located in the undifferentiated MRCs in contact with the basement membrane (Chretien and Pisam, 1986; Uchida and Kaneko, 1996). Thus, there was an urgency to find out what was the environmental cue that initiates cellular differentiation and makes them behave as if they were inducible adult stem cells. Unfortunately, this concept languished due to the inability of investigators at that time to carry out the necessary experiments which would prove the existence or nonexistence of adult stem cells. What turns out to be an important event was the finding that turnover time of the newly formed mature IC was about the same number of days (5–7) in order to complete the full formation of numerous mature IC needed in long-term SW residency.

2.3. Progenitor Ionocyte and Origin in Embryonic Tissues

Recent investigations of the various forms of MRCs in zebra fish embryos have led to the role of presumptive epidermal blastocysts (IB) being transformed into wandering progenitor cells [progenitor ionocyte (pIC)] for both aqIC and haIC. It was thought that the epidermal cell, being a blastocyst, could act as a pluripotential type of stem cell. This cell could then form all types of mature IC (Hwang et al., 2011; Jancike et al., 2010; Chou et al., 2011).

2.3.1. Yolk Membrane MRC Acting as pIC for Larval Skin

Prior to the work on the development of zebra fish embryos, the early chum salmon embryo had demonstrated that the embryo had the physiological ability to function hypoosmotically. In the late embryonic state (eyed stage) the embryo could regulate perivitelline fluid osmotically and ionically following transfer to full seawater (Kaneko et al., 1995, 2008). These results suggested that the eyed-stage embryo of chum salmon had already acquired some type of IC mechanism.

Since the experimental evidence for the substantiation of adult stem cell differentiating into pIC in these fish had not yet been found, it was believed

that the yolk sac membrane that contained MRC had furnished a mobile pIC. It ultimately would travel and differentiate into a mature IC and locate in the larval skin (skIC). These cells would be used for ion transport. Thus, it appeared that there was a need to find other species that would demonstrate identical cellular behavior. At the same time, it would be useful to provide a method for determining the identity of the stem cell and its pathway of differentiation into both pre-gill and post-gill epithelial cell populations.

2.3.2. IB in Pre-gill Epidermis and Formation of pIC

There was mounting morphological and physiological evidence that the pre-gill zebra fish larvae (<72 h postfertilization) while facing variable levels of oxygen and salinity were primarily able to breathe and osmoregulate through the skin of the operculum and abdomen. At this early stage, the pharyngeal gill buds 1 and 4 and not gill buds 2 and 3 have initiated development and appear to be the only source of blastocysts cells which could act as pIC and migrate into the epithelium of the skin and operculum (Kimmel et al., 1995). Subsequent to >72 h postfertilization and upon hatching, all the gill buds appear to be forming pIC cells and therefore could furnish all the necessary auxiliary ionoregulatory and respiratory structures for the larval fish. Hwang and his colleagues began to follow this lead and started investigating embryonic zebra fish at the stages where early differentiation of cells involved in pre- and post-gill development was taking place (Hwang et al., 2011). It was found that after post-gill development, extrabranchial epidermal cells (pIC) from any of the pharyngeal gill buds were present in epithelium of various organs. Therefore, these pIC have continued their migration into both skin, operculum and gill filaments of juvenile fish. These investigators found pIC had formed several subpopulations of IC. They were responsible for acid-secretion/Na^+ uptake, Ca^{2+} uptake and Cl^- uptake respectively. All the types of cells are very important in establishing ionic and osmotic regulation in FW-fishes (Hwang and Perry, 2010). They were especially devoted to H^+ ion regulation and acid-base balance but also involved in Na^+ ion uptake during early embryogenesis (Lin et al., 2006). These findings have been verified by Esaki et al. (2007) who found similar results in zebra fish larvae. These data have placed cellular and developmental physiologists at a crossroad in fish gill morphogenesis. For instance, could there be two different sources of stem cells for IC in the gill buds? Does one type come from the extrabranchial IB populations that are found in the early epidermal germ layers and later in the yolk sac membranes? These cells are known to furnish mature IC for skin, opercular

and gill tissues. Does a second source of IC stem cells reside in the region occupied by the later gill buds (2–3) of epidermal germ layers? If so, do they serve to generate dormant, nonfunctional pIC population in adult tissue serving as an inducible stem cell? This adult stem cell could in some temporal fashion respond to a stimulus to reproduce and synthesize appropriate enzymatic isoforms, such as those forming NKA, NKCC, NEH-2, and NEH-3, enzyme complexes needed in salt/proton-bicarbonate exchange and ammonia excretion. This hypothesis would provide an explanation as to the earlier findings that reconstruction of the gill from single-cell suspension obtained from gill fragments taken from adult eel, *Anguilla japonica*, could take place without the presence of IB cells (Naito, and Ishikawa, 1980).

2.4. Cellular Renewal in Gill Epithelium

The cellular turnover rate can be defined as the temporal interval occurring between the rapid versus slow appearance of mature and functional IC. The observed turnover rate in SW of labeled cells (IC) was found in filamental epithelium to be 4-5 days (Conte and Lin, 1967; Chretien and Pisam, 1986; Uchida and Kaneko, 1996). The time interval was reinvestigated by Wendelaar-Bonga and van der Meij (1989) through the analysis of ultra-structural features of the pleomorphic forms of MRC found in the adult gill epithelium of African cichlid teleosts during and after FW to SW adaptation. They found that the observed increase in branchial cellular turnover was due to the process of apoptosis rather than necrosis.

2.4.1. Apoptosis Mechanism

Intrinsic cell death or apoptosis has been shown to be caused by numerous pro-apoptotic signal transducing molecules acting upon mitochondria and provoking permeability changes in the outer mitochondrial membrane (Cande et al., 2002). The increases in pore size of the outer membrane allow for release of potentially toxic mitochondrial proteins which are classified as being associated with B cell CLL/Lymphoma 2 (Bcl-2) family of proteins. The Bcl-2 family are functionally classified as containing either anti-apoptotic or pro-apoptotic proteins. The regulation of their interactions will dictate whether cell survival or commitment to cell death takes place (Chipuk et al., 2010). In turn, once apoptosis is initiated then the pathway of apoptosis becomes the physiological mechanism that is part of the genetic program in controlling the balance between cell division and deletion in many types of differentiating epithelia (Tran et al., 2004).

2.4.2. Apoptosis in Adult Gill IC

Kammerer and Kultz (2009) continued the investigation of gill epithelial apoptosis by studying the time course program of branchial cell death in salinity-stressed tilapia using laser scanning cytometry of dissociated gill cells. Apoptosis in IC were distinguished from other cell types by utilizing antibody markers of the alpha subunit of Na,K-ATPase and for apoptotic enzymes caspase-3 and caspase-7 activity through a microplate luminesence assay. The results showed that salinity stress prolonged apoptosis in IC from 1 day to 5 days and plays a more active role in adaptive re-differentiation of gill epithelium than previously thought as only being a process for removal of nonspecifically damaged cells. Hsieh and Nguyen (2005) have summarized the variety of physical and chemical forces that appear to induce the molecular mechanisms that trigger apoptosis.

2.4.3. Non-apoptosis in Embryonic skIC

Hiroi et al. (1999), using tilapia embryos, followed the temporal fate of large numbers of MRCs (75%) in days after transfer from FW to SW. It was observed that many of these cells survived for at least 4 days. It was postulated that functional and morphological changes fostered by the osmotic and ionic environmental cues would have to act upon the preexisting IC (aqHRC). They did not demonstrate in FW-fish the need for recruitment of new progenitor cells (pIC) for salt-secreting IC. Hwang et al. (2011) postulate that changes in gill cell turnover rates appear to be species dependent and may depend upon environmental cues that stress the fish. It was cited that the tilapia skIC (pIC) took 3 days for acclimation of aqHRC to become haNaRC or acclimation from low-Cl^- to high-Cl^- FW environments it took 1-2 days. Additionally, in acidic waters, FW-fish after 4 days in pH 4 waters demonstrated a proliferation of epidermal cells with p63 marker to increase in number but did so without activation of apoptotic mechanisms (Horng et al., 2009). Therefore, if the presence of p63 mitogen-activated protein kinase (MAPK) being found in epidermal cells of the gill bud anlage and in some fashion can control cell proliferation, this would indicate that the various types of apoptotic receptors may play an important role in the formation of functional dualism seen in the IC aqHRC and haNaRC.

2.4.4. Apoptotic Receptor Molecules Located in the Apical Plasmalemma

It has been shown that apoptotic forces act through receptor-like molecules found in the cell surface membranes or within intracellular membranes of

the cytoskeleton. One of these apoptosis-inducing factors (AIF) is a protein molecule that activates the release of caspase enzymes from the mitochondria (Hong et al., 2004). Another activator is poly (ADP-ribose) polymerase or PARP family of nuclear proteins which are involved in repair of complementary DNA (cDNA) damage. It has found that one member of the family (PARP-1) involves the molecular mechanism that is essential for AIF activation of caspase being released from mitochondria. Both these factors are acting together to bring about the death of the cell by apoptosis. How the nuclear and mitochondrial interaction take place remains to be resolved. The question arises "Does increase or decrease in osmotic pressure due to various types of aquatic environments act as a physical cue for signaling adult stem cells to start IC replication and differentiation?"

2.5. Subcellular Differentiation in Gill IC

Kultz and Burg (1998) have shown that osmotic stress, such as that found in the environments occupied by euryhaline fishes, could impart a change in cell volume due to either cell shrinkage or cell swelling. There is evidence that osmoregulatory epithelia of euryhaline fishes respond to changes in osmolality due to variations of environmental salinities with phenotypic changes that alter the direction and/or the capacity of transepithelial ion transport. An example is the changes seen in mature IC of gill epithelium (Jurss and Bastrop, 1995). It has been suggested that MAPKs are the transducers of osmotic signals (Kultz, 1998). Surface receptors for destruction or stimulation of stem cell differentiation in gill epithelial cells could be brought about by one of the isoforms of p53 cascade family of tumor suppressors as suggested in a paper by De Nadal et al. (2002) wherein they describe a family of stressed-activated Phospho-kinase (SAPKs). Those cells when faced with increases in extracellular osmolarity lose water and shrink, and afterward they initiate the proper protein kinase responses that will eliminate these stresses. Recently, Kueltz (2011) has studied the basic principles of osmosensory signal transduction mechanisms and has shown that calcium ion plays a central role in these pathways. He describes how extracellular calcium is sensed by a large membrane-bound glycoprotein [calcium-sensing receptor (CaSR)]. This CaSR is a dimeric seven-protein membrane domain that is linked to many types of ion regulatory systems, such as phospholipase C pathways and MAPKs. Newly discovered p53 homologs, p63 and p73, which are proteins that recognize the same p53 DNA-binding sites, were found that if overproduced by surface receptors can activate p53 genes to induce apoptosis in

mammalian cells (Kaelin, 1999). Since some of these homologs (p63) are found in zebra fish (Kultz, personal communication), are they found in all species of teleost fish? Marshall et al. (2005) have found that hypotonic shock signals the involvement of the isoform p38 MAPK which is interacting with the protein kinases of the surface membrane. Is there an isoform of MAPK which will respond to hypertonic shock? It has been suggested that after acute swelling, cell volume is regulated by the process of regulatory volume decrease (RVD) which involves the activation of KCl cotransport and of channels mediating K^+, Cl^-, and taurine efflux. Thus, RVD could be interacting with an unknown isoform of p53 MAPKs (Hoffman et al., 2009). In addition to CaSR, several other membrane proteins, including Transient Receptor Potential (TRP) channel, adenylate cyclase and aquaporin have been implicated as osmosensors of fish (Fig. 1.2).

2.5.1. *Aquaporin Domain as Osmosensor Receptor Site in Apical Crypt*

The water channel molecule aquaporin has been investigated in euryhaline fishes, such as the European and Japanese eel, tilapia and the dace. The

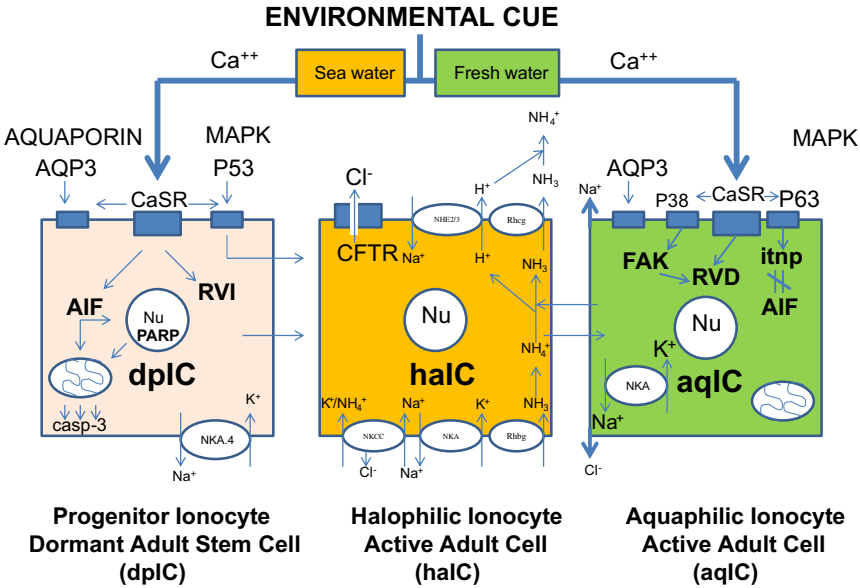

Figure 1.2 Model of environmental cues initiating different receptor sites. These protein domains release factors for either aqIC or halC development. In addition they assist in the formation of dormant progenitor stem cells (dpIC). For details see Section 2.5 in text. *Ref. Conte (present journal).* For color version of this figure, the reader is referred to the online version of this book.

structure of the aquaporin molecule shows it to be made of proteins that are in the form of six transmembrane alpha helices that span the width of the lipid bilayer of the plasma membrane. Aquaporins form tetramers in the cell membrane, with each monomer acting as a water channel (Gonen and Walz, 2006). In teleost fishes, the isoform of aquaporin (AQP3) is found in the epithelial tissues of the gill. Genetic sequencing of fish aquaporin suggests that there are many locations on the cell surface. Thus, the major differences between AQP3 mRNA may be more than one AQP3 homologues and these homologues are oriented in different locations on the cell surface. Thus, the major differences between AQP3 mRNA expression levels obtained from two different species of teleost gills could be explained by these differences. However, SW-adapted fish had a much lower level than FW-adapted fish which correlates with a decrease in water permeability of gill epithelial cells. Immunochemistry and electron microscopical studies by Lignot et al. (2002) reveal the thought to occur in the basolateral tubular network and the apical pole of the cell (Cutler et al., 2007).

2.5.2. Recycling of Apical Plasma Membrane in Adult Gill Epithelium

It has been found that CFTR chloride channel regulator and its activation by cyclic Adenosine Mono Phosphate cAMP-dependent protein kinase (PKC) undergo rapid and efficient recycling at the apical plasma membrane in polarized epithelial cells (Silvis et al., 2009). The cellular mechanisms that facilitate CFTR recycling are diverse among vertebrates and poorly understood. In euryhaline fish, the apical portion in polarized MRCs forms crypts or pits and the basal/lateral portion forms internal channels or infoldings into the cytoplasm. These areas of plasma membrane differentiation have also shown enzymatic differences as viewed by the immunochemical probes which have the apical membrane housing the CFTR chloride proteins (Marshall et al., 2002) and the basal/lateral portions housing NKA, NKCC, NHE2/NH3 apical transporting proteins (Ura et al., 1996; Witters et al., 1996). These findings have aided in the understanding of the functional differences in FW-IC and SW-IC (Hwang et al., 2011).

2.5.3. Recycling of Intracellular Membrane Network in Skin and Opercular Epithelium

The most understood mechanism involving apical crypt formation has been derived from the use of cells taken from opercular skin [opercular ionocyte (opIC)] obtained from the adult euryhaline killifish (*Fundulus heteroclitus*). Karnaky and Kinter (1977) have shown that on the inside of the opercular

skin lies a flat polarized epithelium composed of numerous chloride cells (kfMRC). Identification and quantification of kfMRC by fluorescent dyes specific for mitochondria permitted Karnaky et al. (1984) to establish the richness of kfMRC cells in this tissue. However, there has been little if any measure of cell replication in kfMRC as demonstrated with BrUridine/H3-thymidine labeling of kfMRC as being present in opercular epithelium. A vast number of investigations have used this preparation to establish the physiological and biochemical mechanisms underlying the role of how kfMRC cells provide euryhaline fish with osmotic and ionic balance (Evans, 2005). Marshall and his colleagues used this preparation to develop a theory as to how this epithelium undergoes cellular differentiation to provide killifish SW-FW transitions (Marshall, 2003). What is very important in their findings is that (1) the apical crypts contain not only the CFTR chloride channel regulator and its activation by cAMP-dependent protein kinase but also co-inhabits the crypt with the focal adhesion kinase (FAK) complex which is a tyrosine kinase. This kinase complex interacts at the phosphorylation site Y407 and provides for actin filament interactions that may regulate cell volume by increasing or decreasing size of the kfMRC cell. Thus, the osmotic shock of hypoosmotic media interacts with mechanical receptor sites and via inhibition of p38 MAPK allows expansion of the cell to occur (Marshall et al., 2005). This is our first understanding of osmotic stress sensing in fish tissue.

The next important finding was the presence of FAK complex being housed in intracellular vesicles that apparently arise from the basolateral tubular system (Marshall et al., 2002). These vesicles travel between basolateral poles to the apical poles of the cell. Therefore, these migration events could be part of the recycling of components used in differentiation of anchorage-dependent actin filaments occurring between kfMRC cells and PVC or other AC. This interpretation is supported by addition of the actin filament inhibitor (Cytochalasin D) which reduces the number of crypts being formed. Interestingly, Marshall (2003) illustrates in tabular form how all the effects of hormones acting upon chloride secretion in both killifish opercular (kfMRC) cells and marine teleost gill filamental MRC could occur. His conclusion is that the estuarine marine fish, which can temporarily reside in FW, can return to the stable marine environment and can call upon the aqIC, after sensing hyperosmotic stress cues, to resume salt secretion as haIC. They do not need to synthesize new proteins for the proteomic ion pumps but only need to recycle existing polypeptides into appropriate pump structures. In this manner, the estuarine marine fish need

not invoke permanent adaptation strategies, but use the short-lived devices to cope with entering and exiting hypoosmotic media (FW). However, what is still needed to be understood is why the process of long-term adaptation of seawater requires four to five days for the differentiation pathways to be completed in order for other species of euryhaline FW-fish to have enough haIC formed in the gill filamental population before these fish can have an extended marine life.

3. SALINITY ADAPTATION IN DEVELOPMENT OF OPERCULAR/SKIN EPITHELIUM VERSUS FILAMENTAL GILL EPITHELIUM

The dynamics of basolateral membranes involve many types of membrane kinases. Marshall et al. (2005) observed the disappearance of CFTR from the subapical locus of vesicles by immunocytochemistry and it apparently was part of the rapid turnover (<1 h) by apical crypt components used in the recycling mechanism. It was believed that de novo synthesis of protein components in these vesicles by transcriptional and translational processes could not have occurred due to time restraints. However, serum and glucocorticoid-inducible kinase-1 was shown to be in some manner involved in this mechanism but as yet not be resolved. Details of the mechanism of membrane kinases with Golgi have not been made in fish. However, recent studies on membrane kinases in Golgi apparatus taken from mammalian clones have shown that receptor tyrosine kinases function as key regulators in the trafficking of membrane components between Golgi and the basolateral tubular network (Charest et al., 2003). In conflict with the above interpretation, there are data obtained from other euryhaline species: coho salmon, tilapia, and guppy, that proteomic ion pumps needed for salt excretion are made by the mechanism of protein synthesis. This protein synthesis mechanism had been a focus for early investigations. It was shown that SW fish had increased levels of polyribosomes and tyrosinyl- and aspartyl-transfer RNA (Conte and Murray, 1973: Conte, 1976) and elevated levels of protein biosynthesis (Tondeur and Sargent, 1979) and enzymatic protein abundance (Tang and Lee, 2007). These results suggest that gill filamental IC were making new proteins and kinetic analysis indicated that it takes more than 1 week to be completed. The evidence that hypo- and hyperosmotic environments initiate cues for two different osmotic receptors supports the hypothesis that two developmental pathways may exist in gill

filamental epithelium. The most recent evidence for the existence of two pathways was obtained using new tools of molecular genetics, such as in translational gene knockout techniques and immunocytochemistry to unravel the role of protein pump domains necessary for maintaining ionic and osmotic balance in the embryonic fish. Hwang and Lee (2007) using antibodies to isoform subunits of Na,K-ATPase, coupled co-transporter NKCC and chloride channel transporter CFTR for seawater fish and in FW-fish, the isoform subunits of vacuolar H^+-ATPase, NHE2 and NHE3 sodium ion uptake, together with carbonic anhydrase complex. It was shown that extrabranchial stem cells (IB) were found to exist in various populations of differentiated pIC and in the completely functional (aqIC or haIC). Based upon these data, they interpreted that the extrabranchial stem cells (IB) are responsible for at least three subpopulations of mature IC: (1) Na pump type with apical calcium channel or NaRC IC, (2) proton pump type with acid secretion connected with carbonic anhydrase complex and sodium uptake or HRC IC, and (3) Co-transporter of sodium-potassium and chloride absorption or NCC IC (Fig. 1.3).

The skin/opercular IC contain all the molecular mechanisms which appear to be responsible for acid-base regulation in the early embryonic pre-gill larval stages while the embryo resides in FW. Later, Hiroi et al. (2005) using immunofluorescence staining for ion transport enzymes, such as Na/K-ATPase, NKCC and CFTR, found that intracellular localization of these three enzymes would yield four types of IC in tilapia embryos (Figs 1.4 and 1.5).

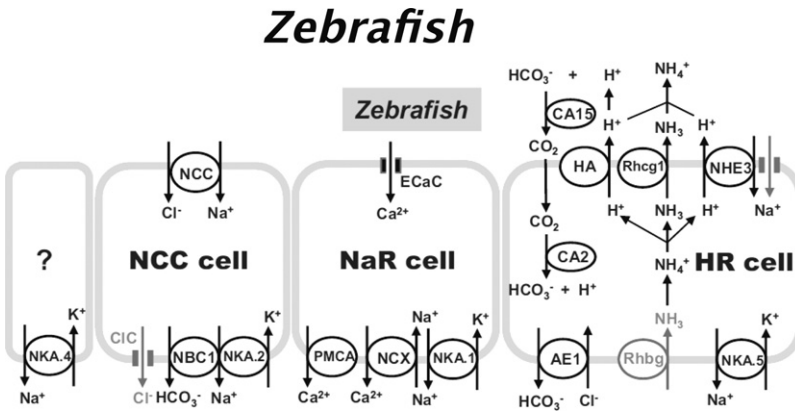

Figure 1.3 Model of IC differentiation in different FW species. In Zebrafish type, there are four cell types. For details see Section 7 in the text. *Ref. Hwang et al. (2011).*

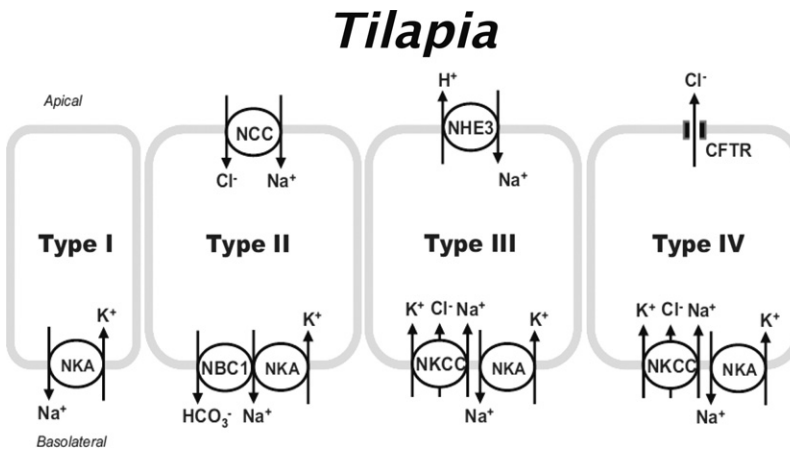

Figure 1.4 Model of IC differentiation in Tilapia. Schematic diagram of the four MRC types, showing intracellular localization of the ion transporters for control of ionic movements within the cell. For details see Section 7 in the text. *Ref. Hiroi et al. (2005).*

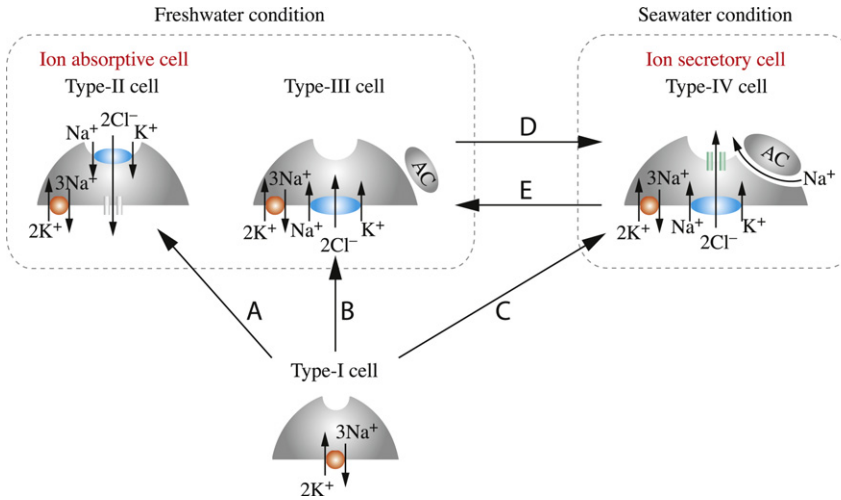

Figure 1.5 Model of the four types of MRCs as shown in Fig. 1.4 and their presumptive interrelationships. These pIC would then lead to mature IC which would be found in filamental epithelium of fish residing in either FW conditions or seawater conditions. For details refer to Section 7 in the text. *Ref. Hiroi et al. (2005).* For color version of this figure, the reader is referred to the online version of this book.

Type I cell was a nonfunctional immature pIC. Type II cell was a FW HRC using a proton pump in the formation of an ion absorption cell. Type III cell was a prototype and/or dormant type (pIC) of the salt secretory cell

(NaRC). Type IV was the active seawater-secreting NaRC containing the sodium pump and chloride channel which handle the movement of sodium and chloride ions. Since the classification of these four types of cells were obtained from embryonic tissue, the issue of whether the filamental IC only come from embryonic stem cells (IB) which are differentiated into migrating pIC that locate in the interlamellar region and become mature IC or does the Type I and Type III come from a dormant form of a progenitor (pIC) and can act as an adult stem cell. It is an important aspect in our understanding of how the integrative and regulatory physiology of transepithelial ion transport in teleost fishes takes place. Because, as was shown earlier, the time course data in seawater adaptation are under the influence of apoptosis factors that control destruction of differentiating pIC that permit the full induction of the adult stem cells to take place which is 5 days in length. This delay appears to provide the needed cell population of mature haNaRC for long-term adaptation. Can the initiation of apoptosis act upon surface receptors of non-NaRC or HR IC prototypes and cause their intrinsic death? Recently, Nguyen et al. (2006) studying the interaction between SPAK p63 and Notch-1 genes found that elevation of p63 counteracts the ability of Notch-1 genes to promote growth and differentiation of epithelial cells. Therefore, complex cross talk between these gene families could alter the selective inhibition of apoptotic factors. This genetic pathway becomes very important in the coordination of the various genes that provide regulatory processes for osmotic and ionic balance in fish body fluids.

4. GENOMIC PATHWAYS UNDERLYING FUNCTIONAL DUALISM IN FILAMENTAL GILL IC

A major breakthrough in elucidation of transcriptional and translational pathways involved in regulation of gill IC differentiation came from the work of several genomic developmental biologists interested in the differentiation of pharyngeal primordia in vertebrates. They found that neurons and glial cells have a common precursor, the neuroblast. If this blast cell is missing the glial cell factor, then it transforms into neurons. This factor is called the "glial cells missing" (GMC) gene. In land vertebrates, the transcriptional and translational pathways of the pharyngeal primordial blast cells give rise to the mature cells forming the parathyroid glands. In contrast, aquatic vertebrates, such as fish, lack parathyroid glands but contain pharyngeal primordial blast cells. Where do these blast cells differentiate and

finally locate? Hogan et al. (2004) found that the murine transcription factor (GCM2) was located in the pharyngeal blast cells that were destined to form the embryonic gill buds which ultimately develop into the gill filaments of the adult fish. Following this lead, Chang et al. (2009) investigated the role of GCM2 to see if it is involved in the regulation of the transcription and translational pathways that control the differentiation of the subsets of IC found in the fish gill filaments. The results showed that Type HRC skin/gill IC are regulated by the GCM2 gene, but that the NaRC cells were not. Thus, the mature HRC IC has messenger transcription and translation factors which can (1) increase or decrease the number of HRC cells and (2) can augment or inhibit acid secretion as a compensatory mechanism for maintaining acid-base balance within the body fluids of the fish. In addition to the murine transcription family of GCM2 genes, another breakthrough in gill IC regulation allowed by the use of transcription factors came about with the finding that FoxO transcription factors were also involved.

4.1. FoxO Genes and Initiation of Apoptosis

These FoxO transcription factors produce an important family of proteins that are used in the enzymes which initiate apoptosis, the cell cycle, oxidative stress, cell differentiation and other cellular functions (Huang and Tindall, 2007). FoxO factors were identified from genetic studies performed on fork head gene found in *Drosophila* (Weigel et al., 1989). Subsequently, Janicke et al. (2007) using the epidermal precursors of the zebra fish embryo found that the Foxi3b factor and Notch signaling factor controlled the formation of skIC Similarly, Hsiao et al. (2007) found that the epidermal IC destined for the gill filaments have a positive regulatory loop existing between transcription factors Foxi3a and Foxi3b and that this link is essential for the specification and differentiation in the formation of the functional and mature IC.

4.2. Grainyhead/CP2 Genes and Intercellular Junctional Complexes

Recently, Janicke et al. (2010) have found the Foxi3 transcription factor is effected by the murine transcription factors in the Grainyhead/CP2 family. The diversity of the developmental roles played by the Grainyhead genes is mediated through the formation of tissue-specific protein isoforms and also through the formation of target gene-specific heteromeric protein complexes, such as adherens junction, E-cadherin and the tight junction gene claudin 4 (Werth et al., 2010). The *grh/1* gene, which is expressed in

the epidermal stem cell (IB) and progenitor cells of IC (pIC), is switched off during IC differentiation. This downregulating of *grh/1* in pIC is performed by an autoregulatory mechanism during IC differentiation. However, this negative feedback mechanism is inhibited upon the knockdown of the gene. Cell numbers of both haIC and aqIC were analyzed for *grh/1* gene and ATPase enzyme. Double stainings showed large increase in cell numbers after knockdown of the *grh/1* gene. However, a knockdown of Foxi3 gene leads to a loss of a number of IC.

4.3. Isotocin (Isotocin-Neurophysin) or Osmopoietin as Heteroprotein Regulator

It has been proposed that the protein regulator of filamental IC differentiation is by isotocin heteroprotein regulation (Chou et al., 2011). They have demonstrated that isotocin-neurophysin (itnp) modulates the genetic operons that regulate the transcriptional and translation pathways. The experimental evidence presented shows that SPKA p63 markers of epidermal stem cell (IB) can be induced in early embryos (2hpf to 72hpf) to contain itnp and its receptors. These stem cells continue to differentiate and form progenitor cells (pIC) that contain various mRNAs which will form the isoform polypeptides such as NaK-ATPase (atp1b1b), the epithelial Ca^{2+} channel, Na^{+}-Cl^{-} co-transporter (slc12a10.2) and Na/H hydrogen exchanger (NHE3b). Additionally, using translational knockdown antisense morhpholinos and in situ hybridization techniques, they showed that IC densities via cell proliferation or via dedifferentiation of transitional IC could be modified. However, the transcription RVD mRNA was not investigated and, therefore, it may be involved in these pathways. It will require additional investigation.

5. CONCLUDING REMARKS

It has been demonstrated that at least two or more developmental pathways exist in the filamental gill epithelium of euryhaline fishes. In response to hyperosmotic cues, embryonic epidermal cells form IB. They are the stem cell origin of gill/skin/opercular IC. These IB, by replication and migration, move into various tissues and organs which then can maintain acid-base balance and osmotic and ionic balance in both larval and adult fish while residing in FW. These IB cells form progenitor cells (pIC). The pIC upon locating into specific tissues continue active differentiation and form

mature and functional IC or they might remain as dormant cells. These pIC cells are acting as stem cells and can respond through various types of genes to form Foxi3, p63, itnp and GRH-1 transcriptional and translational factors that create at least two types of functional IC, such as aqIC and haIC, dependent upon the need in the fish to changes of environmental conditions. In the case of seawater adaptation in some species of fish (salmonids) where parr-smolt modifications take place in older age juveniles these pIC must remain dormant. The finding of dormant IB cell in these older juveniles has not been established. The finding of a dpIC (acting as an adult stem cell) in the filamental epithelium has been suggested from earlier data but as yet have not been verified experimentally. If these dpIC were present, they would have to be organized in such a manner that the subcellular machinery needed for the secretion of sodium chloride and regulation of plasma fluidity for controlling cell shrinkage would be manufactured. This activity by the dpIC is derived from the synthesis of new proteins. These newly formed isoform polypeptides can structurally reassemble within the intracellular cytoplasm to become the active enzymes that form the sodium and chloride pump domains. There are three major enzymatic systems that form these domains: Na/K-ATPase, Na/K/2Cl co-transporter, and CFTR/cystic fibrosis conductance regulator of the chloride channel. These cells, although few in number and estimated at being between 10 and 25% of the total nonfunctional IB population, appear to be the functional epithelial cells used to maintain long-term homeostasis of internal body fluids of the SW-adult fish. Experimental evidence showing how circulating protein itnp being released from the neurohypophysis acts as an inducer for pIC differentiation either on preexisting IC and cause re-differentiation or to stimulate the proliferation of epidermal stem cell IB via MAPK (p63) to increase numbers of pIC to become new halophilic cells. Therefore, is the long-term adaptation of various fish species to seawater (days or weeks) dependent upon the ability of each developmental stage to cope with salinity through osmoregulation (Varsamos et al., 2005) as depicted in the theoretical model shown in Fig. 1.6 due to one of two strategies? (1) The first strategy could be for the development of functional IC, during acclimation to environmental changes, be initiated through the action of MAPK (p38, p53, p63) cues. These cue surface receptors would call for stem cells, either epidermal or adult, to form new cells via cDNA replication followed by new isoform polypeptide differentiating pathways. If there are dormant IB-type cells, then the initiation to continue differentiation at any time would be caused by an unknown osmopoietin factor (p53?). This action would be similar to

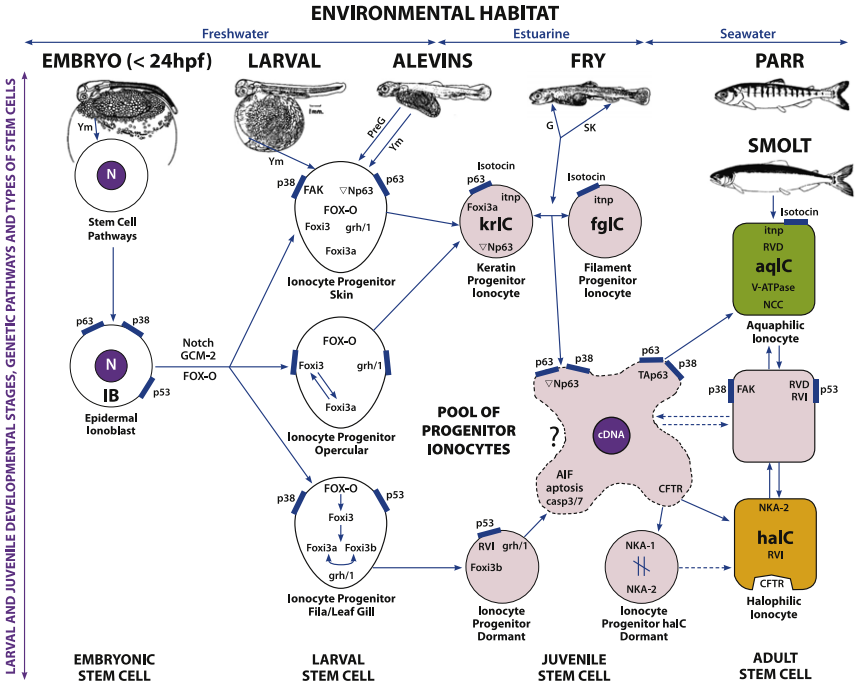

Figure 1.6 Theoretical model of anatomical stages of teleost fishes showing the modifications of progenitor stem cells and their molecular development into IC. The final location of the haIC is in the filamental epithelium of branchial arch of teleost fish. Whereas the aqIC can be found in various locations, such as in gill filamental epithelium, abdominal and opercular skin epithelia, note the focus on stem cell diversity and genetic pathways cross talking in pIC. This concept supports the idea of functional IC dualism that regulates ionic and osmotic levels of body fluids. *Ref. Conte (this journal)*. For color version of this figure, the reader is referred to the online version of this book.

the hypoxia cue that causes the release of erythropoietin to act upon an erythroblast (adult stem cell). The erythroblast begins the cytodifferentiation of the erythroid cell series via cell transcriptional and translational pathways. The end result is the formation and replacement of mature erythrocytes. (2) The second strategy could be for the development of functional IC, during acclimation to environmental changes, from the epidermal stem cells containing the itnp factor (IB-itnp) and can be acted upon by the GMC-2 genes. These modified epidermal IB-itnp cells become pIC that undergo the cytodifferentiation that follows the Fox1-3-mediated pathways. These cells would produce the molecular and physiological network of water permeability and ion-secreting channels needed in the formation of either aqIC or

haIC. These Foxi3-mediated pathways being controlled by the release of protein isotocin (itnp) from the anterior pituitary could be acting as the IC chalone postulated earlier by Conte (1980). This protein could increase or decrease the number functional cells with or without having to invoke the mechanism of apoptosis (Chou et al., 2011). The future research in this field will be of enormous interest to fish physiologists and to cellular and molecular biologist interested in the functional complexities performed by epithelial cells of vertebrates.

GLOSSARY OF TERMS

Ionocytogensis the morphological and biochemical pathways that form a mature and functional ionocyte.

Dormant Ionoblast (dpIB) an immature and dormant adult cell found in the epithelial populations of gill filaments. These inducible stem cells contain the genetic and biochemical pathways for the manufacture of ion pumps needed to regulate the ionic and osmotic stress created by seawater.

Ionocyte (IC) a mature and functional cell found in the gill filamental epithelium containing ion pumps that can regulate osmotic and ionic stresses created by either freshwater or seawater environments. (Replaces MRC or chloride cell nomenclature.)

Ionoblast (IB) embryonic stem cell (epidermal in origin) that can migrate to form ionocytes in any adult tissue/organ.

Progenitor Ionocyte (pIC) migratory embryonic cell that can differentiate or re-differentiate a mature type of IC into various types of ionocytes without undergoing cellular mitosis.

Aquaphilic Ionocyte (aqIC) the functional IC in the gill filament which contains ion pumps that regulate acid-base balance, regulate water permeability through aquaporin channels that regulate cell volume via apical and basal-lateral tubular network. These cells sustain fish in freshwater environments.

Halophilic Ionocyte (haNaRC) the functional IC of the gill filament which contains ion pumps that regulate salt excretion and ion uptake of calcium ion, and controls cell volume shrinkage and plasma fluid balance. These cells sustain marine fish in seawater environments.

Skin Ionocyte (skIC) the functional IC which are found in the skin tissue of larval and juvenile fish.

Opercular Ionocyte (opIC) the functional IC that is found in opercular skin of larval, juvenile or adult euryhaline fish.

CFTR cystic fibrosis transmembrane conductance regulator found in apical region of ionocyte and serves as the chloride excretory site.

RVI Regulatory Volume Increase factors for control of body fluids.

RVD Regulatory Volume Decrease factors for control of body fluids.

AIF Apoptosis Initiation Factors for proliferation or diminution of Ionocyte populations.

ACKNOWLEDGMENTS

The author wishes to apologize to those scientists who contributed to our understanding in comparative physiology of ionic and osmotic regulation by fish gills and who were not cited in this review. To Professor Tetsuya Hirano and Professor Toyoji Kaneko for inspiring the students at the University of Tokyo to continue the cellular investigations that began under Professor Seitro Utida in the early 1960s, my congratulations and thanks for a job well done.

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CHAPTER TWO

Cellular Mechanisms for the Biogenesis and Transport of Synaptic and Dense-Core Vesicles

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Abstract

The release of intercellular messengers from synaptic (SVs) and dense-core vesicles (DCVs) constitutes the primary mechanism for communication between neighboring or distant cells and organs in response to stimuli. Here we review the life span of SVs and DCVs found in the brain, neuroendocrine and exocrine tissues. These vesicles must be formed, trafficked, and their contents secreted; processes which require orchestrated actions of a great repertoire of lipids, proteins, and enzymes. For biogenesis and vesicular budding, lipids that influence curvature and aggregation of cargo are necessary for pinching off of vesicles. Vesicles travel on cytoskeletal filaments powered by motors that control the dynamics: location, speed, and directionality of movement. Regardless of mechanisms of traffic, vesicles arrive at sites of release and are docked for exocytosis, followed by membrane fusion, and release of vesicular content to exert physiological responses. Neurological disorders with pathology involving abnormal vesicular budding, trafficking, or secretion are discussed.

1. INTRODUCTION

All eukaryotic cells contain a constitutive secretory pathway (CSP), which is used to deliver soluble (e.g. albumin, growth factors) and membrane

proteins (e.g. receptors) to the plasma membrane (PM) to maintain growth, survival and differentiation of cells (Kelly, 1985). Proteins transported in vesicles of the CSP to the PM are secreted continuously and do not require an external stimulus (Gumbiner and Kelly, 1982; Dumermuth and Moore, 1998). Endocrine cells, neurons and exocrine cells, in addition to the CSP, have a regulated secretory pathway (RSP) which transports peptide hormones, neuropeptides and digestive enzymes, respectively, in large dense-core vesicles/granules (DCVs/DCGs) to the cell surface for secretion to mediate higher physiological function. Secretion of content from these DCVs is dependent upon external stimulation of the cell (Gumbiner and Kelly, 1982; Kelly, 1985; Dumermuth and Moore, 1998). Characteristic budding and trafficking behavior of the CSP and RSP are demonstrated in Fig. 2.1 and contrasting properties are listed in Table 2.1. For neurons, in addition to DCV function within the RSP, regulated release of neurotransmitters packaged in synaptic vesicles (SVs) is key for communication. Thus, neurons use both DCVs and SVs to mediate and modulate neurotransmission (De Camilli and Jahn, 1990).

This review is organized into four main segments. First, we introduce the types of vesicles found in professional secretory cells with an emphasis on neurons, where behavior and function of DCVs and SVs can be well contrasted. We examine non-neuronal professional secretory cells where regulated secretion is prominent, and discuss the components of the neuroendocrine system, with an emphasis on the stress response axis (Sections 2.2–2.4). Secondly, we present the current state of research for DCV biogenesis inclusive of sorting mechanisms at the trans-Golgi network (TGN) and genetic, posttranscriptional and posttranslational regulation of DCV biogenesis (Section 2.5). This is followed by an in-depth analysis of SV biogenesis at the synaptic terminal where protein and lipid constituents involved in the process of sorting and assembly are paramount (Section 2.6). In the third part, mechanisms of transport used by immature and mature DCVs, Piccolo–Bassoon transport vesicles (PTVs), and synaptic protein precursors/transport vesicles are discussed (Section 2.7). The involvement of DCV-specific and SV-specific mechanisms of exocytosis is briefly reviewed. Throughout this third segment (sections 2.5–2.8), several knockout (KO), mutant or dominant-negative cellular or animal models of members of the molecular machinery involved during vesicular biogenesis, trafficking and exocytosis illustrate the necessity of each component in these processes. Defective vesicle biogenesis and trafficking give rise to or is associated with various neuroendocrine disorders in humans. In the last segment

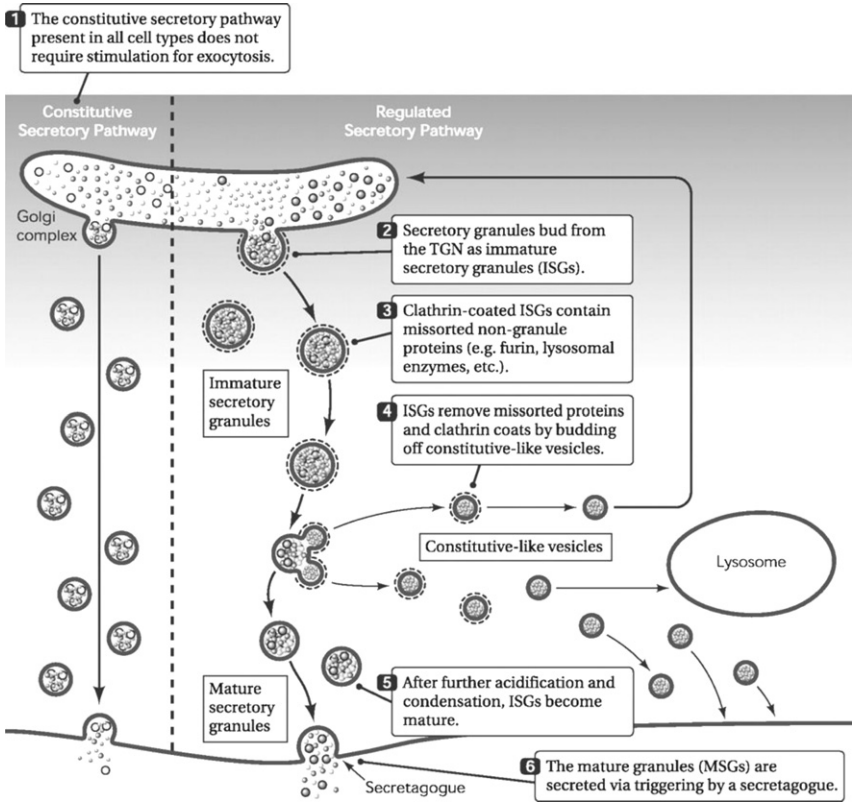

Figure 2.1 ***Formation and exocytosis of CVs and DCVs.*** Two distinctive secretory pathways are present in neurons, neuroendocrine and endocrine and exocrine cells, for constitutive and regulated secretion of proteins and peptides. Constitutive vesicles (CVs), release their content rapidly, while DCVs in the RSP undergo several maturation steps and storage prior to releasing their content.

(Section 2.9), although scientific information is sparse and slowly emerging, we discuss examples of neurodevelopmental, psychiatric and neurodegenerative disorders where defects in DCV and SV biology and function contribute to the pathogenesis of disease.

2. SVs AND DCVs IN NEUROTRANSMISSION

The most prevalent way that one neuron communicates with another is by releasing an excitatory, inhibitory or modulatory factor into the synapse, and eliciting a response from the second neuron. With the

Table 2.1 Comparison of constitutive versus RSP vesicles

	CVs	DCVs
Properties	Small, clear Diameter: 80−100 nm	Large, dense core Diameter: 100−300 nm
Assembly	No sorting signal required for cargo entry into CVs No aggregation of proteins before vesiculation Assembly not dependent on acidic pH at TGN	Sorting signal/motif generally required for entry into DCVs Aggregation is a prerequisite before vesiculation Clathrin coat and APs necessary for budding Assembly dependent on acidic pH at TGN and for DCV maturation
Secretion	Transported along microtubules from TGN to PM Independent of an external signal Non-calcium triggered Rapid transit and release from Golgi ~10 min; No storage pool Membrane components not recycled	Transported along microtubules from TGN to proximity of PM and actin-based transport to release site where they are docked Exocytosis requires external signal and intracellular calcium Exocytosis requires external signal and intracellular calcium Long residence time 7−10 h, stored in cytoplasm Membrane components recycled back to TGN after exocytosis

exception of constitutive inputs whereby neurons are constantly exposed to a particular environment, most neurotransmission occurs as a result of sufficient presynaptic stimulation to induce release of vesicular content into extracellular space—i.e. via a RSP. The vesicles that transport neurotransmitters or neuromodulators are classified as either SVs or DCVs, respectively. Whereas both SVs and DCVs are prominent in brain (Salio et al., 2007; Dieni et al., 2012), neuroendocrine tissues such as the pituitary and adrenal glands, endocrine pancreas, and gonads are primarily enriched with DCVs (Gorr et al., 2001). These neuroendocrine organs regulate communication, growth, food intake, mood, stress and cognition (Guan et al., 1997; Pacak and Palkovits, 2001; Stanley et al., 2005; Kenna et al., 2009). Because of these important functions, the abnormal biogenesis, transport and secretion of SVs and DCVs could lead to severe, debilitating disorders. Our focus is on the shared mechanisms used by secretory cells to generate and traffic DCVs and SVs to sites of regulated exocytosis.

2.1. SVs and Classical Neurotransmitters

SVs predominantly carry classical neurotransmitters classified as amino acids [glutamate, γ-aminobutyric acid (GABA), acetylcholine (ACh) or monoamines (dopamine, serotonin, epinephrine, norepinephrine, etc.)] and are specifically found in neurons. At the electron microscopic level, these vesicles have a translucent, circular appearance and are often found clustered and docked at the active zone of an axonal terminal, near a synapse demarcated by an electron dense region (Fig. 2.2E). Upon sufficient depolarization of the presynaptic neuron, the resultant increase in intracellular calcium causes SVs to fuse with the axon terminal membrane, assisted by anchoring soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins to exocytose their content into the synapse (Sollner, 2003; Malsam et al., 2008). SVs are recycled in the axon terminal, interact with synaptic protein transport vesicle (SPTVs), and are refilled locally with neurotransmitter in preparation for a subsequent depolarization (Parsons et al., 1999; Fei et al., 2008). Mechanisms of fusion and regulated exocytosis are only briefly discussed, and are more extensively reviewed elsewhere (Lin and Scheller, 2000; Sollner, 2003). The closely apposed dendritic spines of the postsynaptic cell are competent to respond to this signal if they express the appropriate neurotransmitter receptor to induce postsynaptic signaling. Activation of postsynaptic receptors can either result in the postsynaptic cell's depolarization or hyperpolarization depending on the receptors expressed at the dendritic membrane.

2.2. DCVs and Neuropeptides

In general, in secretory tissues, DCVs, also referred to as DCGs, have a characteristic opaque core, which appears as an electron-dense area encased within the vesicular lipid bilayer when observed with an electron microscope (Fig. 2.2A–D). The DCV sizes vary between 100 and 300 nm in diameter in different cells types. DCVs in exocrine cells (e.g. in the pancreas) are larger than those in endocrine cells, while the neurotrophin-containing vesicles in neurons, e.g. brain-derived neurotrophic factor (BDNF), are smaller and less electron dense (Fig. 2.2) (Sadakata et al., 2004; Gondre-Lewis et al., 2006; Kim et al., 2006). Contents of DCVs are highly condensed and concentrated, and DCVs abundantly express at least one member of the chromogranin or secretogranin class of acidic Ca^{2+}-binding proteins. At the light level, these DCVs have a heavily granulated appearance when stained for protein content, hence the reference as granules (Table 2.1).

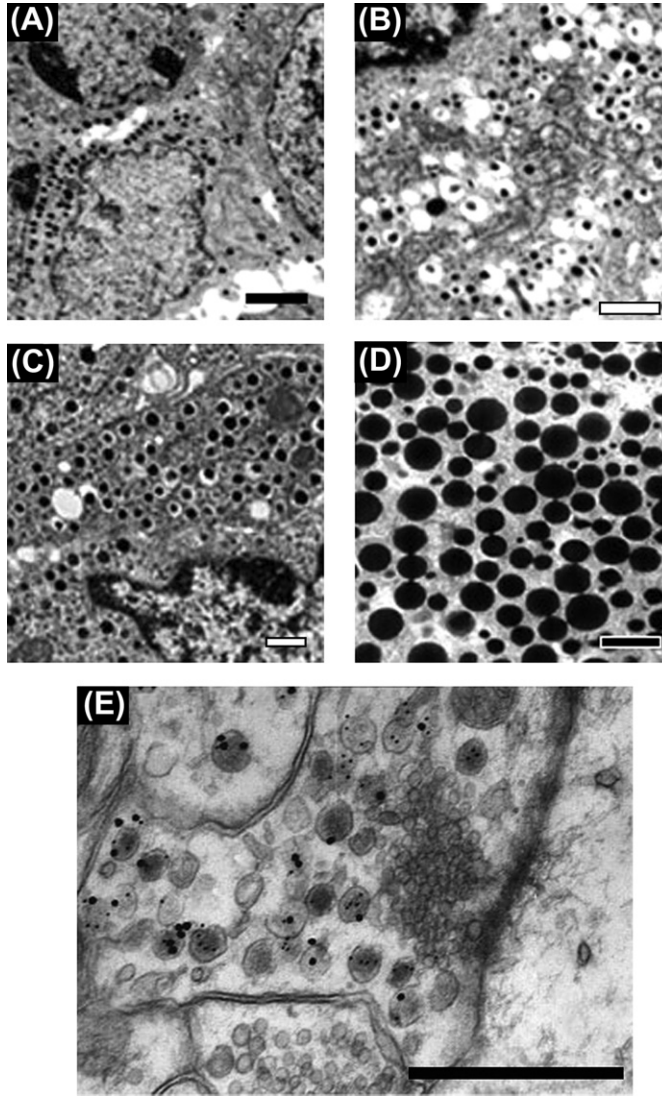

Figure 2.2 ***The many faces of DCVs in neurons, endocrine and exocrine tissues.*** Electron micrographs of DCVs in newborn mouse anterior pituitary (A), adrenal medulla (B), endocrine pancreatic islet cells (C), and exocrine pancreas packaging zymogen granules (D). (E) is a synaptic terminal with both SVs—small, clear, clustered—and DCVs, clearly larger than SVs with a dense-core and co-packaging BDNF and substance P (SP) as revealed with immuno-EM labels; BDNF 10 nm particles, SP, 20 nM. Note the size, morphology and distribution across tissues, with neurons and the pituitary having the smallest DCVs. *Panel E is from Salio et al., Devel Neurobiol., 67:3, 326–338, 2007, and is reproduced with the permission of John Wiley & Sons, Inc. Bars: A, 1 μm; B,D, 2 μm; C,E, 500 nm.*

DCVs in the nervous system package small neuropeptides and peptide hormones such as endorphins, neurotrophin family of growth factors, inclusive of BDNF, tachykinins, and somatostatin, to name a few. DCVs in the nervous system are said to be peptidergic vesicles. Unlike SVs, DCVs generally package their cargo in the soma and not at the synaptic terminal. Similarly, both SVs and DCVs bud from the TGN by specific molecular and physical mechanisms that will be discussed in the next sections. DCVs travel to the periphery using cellular motors, and once in the periphery, release their cargo into the extracellular space. Fusion with the PM and release of content represents their final action although some of the DCV membrane can be recycled and reutilized (Arnaoutova et al., 2003).

Neuropeptides are the most diverse, most numerous and most common signaling molecules in the brain with more than 100 neuropeptides having been identified thus far. Neuropeptides are synthesized in the nervous system and can function as neurotransmitters, neuromodulators and neurohormones (Hokfelt et al., 1980; Ogren et al., 2010). When released from neurons, those that function as neurotransmitters have receptors at the synapse, likely at extrasynaptic sites, whereas those that function as neuromodulators may have receptors distributed all along the target cell in addition to extrasynaptic sites. Neuropeptides packaged in DCVs respond to much stronger calcium signals than SVs, and thus for DCVs to release their content, they require robust, high frequency stimulation and not just a single action potential (Tallent, 2008).

Neuropeptides range in approximate size from 2 to 80 amino acids, and as mentioned above, are packaged and secreted via the RSP. Similar to peptides and hormones secreted by other cells in the body, they are synthesized as precursors (pre-pro-neuropeptide) and are cleaved by proteolytic enzymes to generate specific signal peptides. One precursor can produce different sets of mature peptides depending on tissue expression of proprotein convertases and other enzymes necessary for processing (Burbach, 2011). Neuropeptides constitute a very diverse group of neurotransmitters and constitute the majority of signaling in the brain (Ogren et al., 2010). In humans, there are approximately 90 different genes encoding 100s of peptides. Pro-opiomelanocortin (POMC) is the prototypical proneuropeptide, encoded by one gene, but depending on which cleavage site is targeted by processing enzymes and the neuron type in which it is expressed, it can give rise to several mature biologically active peptides.

2.3. DCVs in Astrocytes for Modulating Neuron Function

It is widely accepted that glia, especially astrocytes, secrete neurotransmitters such as glutamate in response to changes in calcium dynamics in order to modulate neuron function (Parpura and Haydon, 2000; Jourdain et al., 2007). Both the presence of DCVs and mechanisms of regulated secretion in astrocytes and other glial cell lines have recently been investigated. Secretory granules positive for the DCV markers, chromogranin B (CgB), secretogranin II (SgII), and Secretogranin III (SgIII), were detected in primary astrocytes (Calegari et al., 1999; Hur et al., 2010; Paco et al., 2010). In hippocampal astrocytes, SgII was demonstrated to undergo stimulated release in the presence of various secretagogues (Calegari et al., 1999), and in cortical astrocytes an unprocessed form of SgIII was found to be robustly expressed and continually secreted (Paco et al., 2010). These DCVs are proposed to function similarly to DCVs in other cells as internal inositol 1,4,5-trisphosphate (IP_3)-sensitive calcium stores. They respond to low levels of IP_3 via three different IP_3 receptors (IP_3Rs)expressed on their DCV membrane (Hur et al., 2010). Furthermore, the precursor of glia-derived neurotrophic factor (GDNF), (beta) pro-GDNF but not the constitutively secreted (alpha) pro-GDNF, was co-localized in secretory granules with the DCV marker, SgII, in these glial cell lines. The beta precursor protein was found to undergo enzymatic processing to produce mature GDNF and both precursor and mature forms were secreted in a regulated fashion in response to depolarization of the cell with KCl (Lonka-Nevalaita et al., 2010). It has been reported that astrocytes also synthesize and secrete neuropeptides and the processing enzyme carboxypeptidase E (CPE), but in a constitutive manner (Klein et al., 1992).

3. DCVs AND SVs IN TISSUES

Both neurotransmitters and small peptides act as signaling molecules that mediate synaptic transmission in the brain. These signaling molecules traffic to their site of release as cargo inside clear SV or large DCV. They are subsequently released into the synaptic cleft where they exert their function. Unlike SVs which are locally filled with neurotransmitters at the synapse, DCVs travel long distances from the cell body, along microtubule-based motors (Burbach, 1982; Yajima et al., 2008), to axon terminals and dendrites to release their cargo. Furthermore, at the Golgi, immature DCV cargos are packaged along with processing enzymes which cleave and generate the mature, active form of the protein inside the DCVs. Similar to SVs, DCVs

release their cargo upon stimulation. In the past decade, it has been shown that neurons and other professional secretory cells like the acinar cells use "porosomes"—previously termed depressions—as a cup-like lipoprotein portal to the extracellular space that facilitates emptying of DCV content without expanding the PM (Anderson, 2006; Jena, 2009a, 2009b; Trikha et al., 2010). This is especially important in polarized secretory cells like the acinar cells of the pancreas or in neurons. The limited fusion of the DCVs with porosomes is mediated by the same SNARE complex mechanism described for SVs (Sutton et al., 1998).

3.1. Brain

3.1.1. Hippocampus

This interesting brain region is intensely studied for its role in acquisition of new memories, and its function is influenced by exercise, emotion, hormones, and social interactions. In addition to its own highly organized synaptic connections, the hippocampus receives and integrates afferents from the basal forebrain, subcortical regions, the amygdaloid nuclei, and the thalamus. Thus, neurons in the hippocampus express receptors for a great many neuromodulators and neurotransmitters—not just glutamate and GABA. Perforant path projections from the entorhinal cortex and mossy fibers from the dentate gyrus co-express glutamate and the opioids enkephalin and dynorphin, and modulate function of neurons in the dentate gyrus and CA3 neurons, respectively, via their receptors (Schwarzer, 2009). Dynorphin is produced in the RSP by actions of endopeptidases PC1, PC2 and CPE (Dupuy et al., 1994; Ogren et al., 2010). Somatostatin and neuropeptide Y (NPY) expressing GABA-ergic neurons regulate hippocampal glutamate release via their pre- and postsynaptically localized receptors (Mancillas et al., 1986; Boehm and Betz, 1997); a mechanism that can aid in the control of epileptic seizures. In response to stress, the hippocampus abundantly expresses receptors for cortisol and its releasing hormone, and under healthy conditions, provides cortisol-induced inhibitory feedback to the hypothalamic–pituitary–adrenal axis (HPA) axis (Stokes, 1995; Meaney et al., 1996).

The interplay of SV- and DCV-mediated responses of the RSP in the hippocampus, which serves as a model system for studying enhancement or repression of synaptic inputs, is still unclear and is being intensively studied. Although small SVs have been localized and can be induced to exocytose at dendrites (Ovsepian and Dolly, 2011), traditionally, SV neurotransmitter release and vesicle recycling takes place in synaptic and extrasynaptic sites of

axonal terminals (Staras et al., 2010; Ratnayaka et al., 2011) whereas peptidergic or BDNF-containing DCVs are trafficked bidirectionally toward both axons and dendrites (Washburn et al., 2002; Lochner et al., 2008). This has even been found for DCV-localized vesicular monoamine transporter-2 (VMAT2), a monoamine transporter, which would implicate monoamines as being packaged and released to the somatodendritic domain (Li et al., 2005) as has been reported for NPY (Danger et al., 1990; Ramamoorthy et al., 2011). Later, we discuss the bidirectional dynein and kinesin motors responsible for DCV transport to both axons and dendrites (Kwinter et al., 2009; Lo et al., 2011) in hippocampal neurons.

3.1.2. Hypothalamus

The hypothalamus is in the brain but considered a neuroendocrine organ because like other endocrine organs, it secretes hormones into the bloodstream. Axons from the nuclei of the hypothalamus travel via the median eminence located ventral to the third ventricle, and when activated, secrete peptide hormones and dopamine into small capillaries that are connected to the pituitary via the infundibulum. Visceral organs in the periphery, circadian rhythm, sleep, temperature regulation, fluid homeostasis, stress, food intake, growth, spermatogenesis and ovulation are all regulated by the hypothalamus in collaboration with the autonomic nervous system. Thus, the hypothalamus integrates input from almost all brain regions as well as the circumventricular organs to produce a response in the form of secretion of neuropeptides.

3.2. The Neuroendocrine System

The neuroendocrine system is tripartite, consisting of the hypothalamus, which surrounds the third ventricle, the pituitary gland which sits just outside the brain, but protected in the sella turcica of the sphenoid bone in the interior of the cranium, and the target organ located within the abdominal cavity in the body. These target organs include the adrenal, mammary and thyroid glands, the gonads, and the liver. The specialized neurons clustered in nuclei within the hypothalamus control hormone release from the anterior pituitary by secreting hypothalamic-releasing or inhibiting hormones packaged in DCVs. The stimulated pituitary subsequently releases hormones from its DCVs into the bloodstream. These hormones travel long distances to reach their target organs and stimulate or repress their activity. In this manner, the hypothalamus works as the master regulator of appetite, growth, sex drive, reproduction, stressful situations, and other physiological behaviors.

To exert its function, the hypothalamus integrates afferent input from other parts of the central nervous system (CNS) that may also respond to circulating hormones released by target organs.

3.2.1. The Hypothalamic–Pituitary–Adrenal Axis

The manner in which an individual responds to stress can influence homeostatic regulation of a number of related physiological processes. The HPA axis is responsible for the neuroendocrine stress response by regulating gene expression and transcription in the hypothalamus, pituitary and adrenals to induce corticosteroid release from the adrenals (Elkabes and Loh, 1988; O'Connor et al., 2000, 2004). Upon stressful stimulation, corticotropin-releasing hormone from the paraventricular nucleus of the hypothalamus alone or in synergistic action with arginine vasopressin activates corticotrophs in the anterior pituitary to secrete adrenal corticotropic hormone (ACTH), a product of alternative cleavage of POMC precursor protein, into the peripheral circulation. ACTH binds to its receptor on the adrenals and induces glucocorticoid production and release. The effects of the HPA stress response is not limited to those three regions because for each of the hormones released, there are receptors on other brain areas or organs with the capacity to respond. For example, corticotropin releasing hormone (CRH) receptors are also localized throughout the CNS and abundantly expressed in the cerebral cortex, striatum, and cerebellum (Potter et al., 1994). Glucocorticoid receptors are widely distributed in conjunction with the high-affinity mineralocorticoid receptors (Reul and de Kloet, 1985; Han et al., 2005). Importantly, they are localized locally in the hypothalamus for self-regulation of CRH release. However, they are abundant in nearly all areas of the brain (Morimoto et al., 1996), especially in the hippocampus, amygdala and prefrontal cortex, regions intimately involved in mediating the stress response (Dedovic et al., 2009; Mora et al., 2012) as well as many organs in the periphery such as the heart, liver, etc. Therefore, in many ways the HPA axis regulates not only stress but also learning and memory and pleasure centers of the brain. It also regulates other H–P axes' functions such as growth and reproduction.

3.2.2. Insulin-Secreting Islet Cells

Islets are interspersed within a sea of exocrine acinar cells in the pancreas. They function as endocrine cells that secrete the hormone insulin from DCVs into the bloodstream in response to circulating blood glucose. Insulin secretion is also modulated by neuronal inputs or circulating hormones, and

the secretory response occurs in the same manner described for other neuroendocrine tissues and neurons (Wheeler et al., 1996; Anderson, 2006; MacDonald and Rorsman, 2007). Islet endocrine cells also contain synaptic-like microvesicles (SLMVs) similar to SVs in neurons, and these SLMVs have been shown to store and secrete the neurotransmitter GABA by regulated exocytosis in response to stimulation (Anhert-Hilger et al., 1996). SLMVs are similar to SVs in membrane composition, biogenesis and life cycle. The neuron-specific cytosolic adaptor protein (AP), AP-3B complex, associated with GABA-ergic vesicle biogenesis has recently been found to mediate SLMV biogenesis in pancreatic β-cells, in a mechanism similar to neurons (Suckow et al., 2010). The β-cells of the endocrine pancreas abundantly express glutamic acid decarboxylase and have a proton pump-dependent GABA transporter (GAT) supporting the idea that GABA can be synthesized, trafficked and released in these cells which use the SNARE proteins, SNAP25, synaptobrevin and syntaxin to aid in exocytosis (Thomas-Reetz et al., 1993; Anhert-Hilger et al., 1996). These SLMVs are present in anterior pituitary and adrenals, and have been well studied in the PC12 tumor cell line derived from adrenal chromaffin cells (Park et al., 2011). PC12 cells package and store ACh in addition to DCV cargo, and use synaptophysin, synaptotagmin and the same trafficking and exocytosis machinery used by neurons (Thomas-Reetz and De Camilli, 1994; Park et al., 2011).

3.3. Exocrine Tissues

Whereas endocrine tissues secrete hormones and peptides that act to regulate physiological homeostasis via the bloodstream, exocrine tissues secrete enzymes and fluid via ducts, and these are eventually excreted into the external environment. Zymogen granules from acinar cells of the exocrine pancreas secrete alpha amylase, trypsinogen, chymotrypsinogen, elastase, and lipase, and other digestive enzymes into the small intestine to aid in digestion (Bendayan and Ito, 1979). Likewise, in addition to saliva, the salivary glands produce alpha amylase which helps in the onset of digestion to breakdown complex carbohydrates to maltose and glucose (Jacobsen et al., 1972). Similar to the endocrine system, secretion of enzymes from pancreatic acinar and other exocrine cells occurs in a regulated manner in response to secretagogues (Jamieson and Palade, 1971). Contents of the DCGs of exocrine pancreas are processed and packaged at the Golgi (Fig. 2.2D) similarly to the endocrine pancreas, but the DCVs have a circular, black appearance at the electron microscopic level and are large

compared to endocrine granules, with areas that measure up to 2 μm^2 (Gondré-Lewis et al., 2006). Because of the ease of identification, these exocrine cells are often used to study stimulated secretion.

4. IMPORTANT CARGO IN DCVs ACTING ON BRAIN FUNCTION

4.1. Neurotrophins—BDNF

Neuronal birth, function and survival are dependent on the proper trafficking, processing, and release of neuropeptides packaged inside of DCVs. Neurotrophins constitute a class of growth factors necessary for neuronal survival, growth, and plasticity during development. They are modulators of synaptic transmission and influence synaptic efficacy during learning and in response to stressful stimuli in adults (Patterson et al., 1996; Poo, 2001). Deficiency in neurotrophin signaling due to either reduced growth factor or their tyrosine receptor kinases has been cited in the pathogenesis of neuropsychiatric, neurodegenerative and neurodevelopmental disorders. Neurotrophins are packaged in DCVs (Fig. 2.2E) and are trafficked to and secreted at release sites in response to calcium signaling, in a regulated manner, similar to other neuropeptides packaged within DCVs. We pay special attention to BDNF because it is critical for synaptic mechanisms related to learning and in fact is necessary for long-term synaptic plasticity in CA1 neurons in the hippocampus (Patterson et al., 1996; Matsumoto et al., 2008), cerebral cortex and throughout the brain. Similar to other DCVs that do not necessarily release contents at the synapse, BDNF has been shown to release its contents presynaptically from axons, postsynaptically from dendrites, or perisynaptically, thus endowing it with great versatility of action. Because this DCV growth factor is such a potent modulator of synaptic signaling, we will address its DCV biogenesis at the TGN, its retrograde and anterograde trafficking on microtubule motors (section 7.1.2) and its release at synaptic and non-synaptic sites.

Co-packaging and release of neuromodulators is an efficient means of regulating synaptic functions and plasticity in brain. For example, the growth factor BDNF is co-stored with calcitonin gene-related peptide (CGRP) and with substance P in the visual system and amygdala, as well as in the trigeminal ganglion and dorsal root ganglia of the peripheral nervous system (Berg et al., 2000; Buldyrev et al., 2006; Salio et al., 2007). Its co-release with CGRP has also been demonstrated. When co-packaged with

these neuropeptides, BDNF DCVs were more granular in appearance and BDNF was present at 36 times the rate of more agranular vesicles in the amygdala (Salio et al., 2007). Thus, the influential power of this growth factor at the synapse is amplified. In hippocampal neurons, plasminogen, tissue plasminogen activator which converts plasminogen to plasmin, and pro-BDNF for which the cleavage to BDNF is facilitated by plasmin, are co-packaged and co-released at dendritic spines to influence synaptic efficacy (Lochner et al., 2008). Although not necessarily co-packaged with neurotransmitters, BDNF has been shown to increase SV docking and enhance quantal neurotransmitter release (Tyler and Pozzo-Miller, 2001; Tyler et al., 2006).

It is now well established that neuropeptides including BDNF and NPY are often co-expressed and can be co-released from a given neuron along with catecholamines, GABA, other neurotransmitters, or other neuropeptides in order to facilitate neurotransmission (Hokfelt et al., 1980; Danger et al., 1990).

4.2. Granins in Brain

A recent review extensively characterizes the structure, processing, packaging, function and most other aspects of the granin family members (Bartolomucci et al., 2011), and therefore, our discussions here will be limited. Granins are traditionally considered markers for DCVs and the RSP, and although they are peptides that can be synthesized in the brain, they are not classified as neuropeptides primarily because their specific function in neuronal signaling is not clear. More recently, however, three hormones derived from proteolytic processing of chromogranin A (CgA), vasostatin (CgA 1–76), catestatin (CgA 344–364), and serpinin (bCgA 403–428) were shown to be regulators of not only cardiac function but were also important for cell survival and neuroprotection (Helle, 2010; Loh et al., 2012). Whether oxidative stress or low K^+ induced apoptosis in CNS neurons or in the AtT-20 pituitary cell line, exposure to serpinin effectively rescued neurons and neuroendocrine cells from the apoptotic cascade, possibly by regulating the transcription factors involved in its induction (Koshimizu et al., 2011a, 2011b).

SgII is a precursor for the neuropeptide manserin, expressed in the hypothalamus, cerebral cortex, pituitary, adrenal glands and pancreatic islets (Yajima et al., 2004; Tano et al., 2010). Because of Manserin's expression profile, it has been postulated that it may be involved in stress responses.

Manserin was recently identified in neuronal terminals of the organ of Corti and type II spiral ganglion cells in the auditory system, and in the synapses associated with the vestibular system (Yajima et al., 2004; Ida-Eto et al., 2012). That SgII itself is localized in secretory vesicles throughout the nervous system and endocrine tissues implies that its dysregulation can influence a number of functions. Nerve growth factor (NGF)-mediated differentiation of neuroblastoma cells requires SgII expression, and this expression is also protective against nitric oxide (NO)-induced apoptosis (Li et al., 2008). The functional role of granins in neurons and CNS function is a still evolving field.

5. OVERVIEW OF SECRETORY VESICLE BIOGENESIS

Secretory proteins upon synthesis at the rough endoplasmic reticulum (RER) are directed via the signal peptide into the RER cisternae and transported in COP II-coated vesicles to the Golgi apparatus. The proteins traverse from the cis through medial Golgi stacks to the TGN. Within the Golgi apparatus, secretory proteins are posttranslationally modified with carbohydrate modifications and proteolytic cleavage carried out by enzymes concentrated in distinct cisternae in the Golgi stack. The proteins are then sorted and packaged into constitutive or regulated secretory vesicles (Fig. 2.1). RSP vesicles are formed at the TGN while CSP vesicles can bud off from medial Golgi stacks as well as the TGN (Emr et al., 2009). Newly formed CSP and RSP vesicles are transported by a microtubule-based motor system to the proximity of the cell surface (Park and Loh, 2008). CSP vesicles are constantly transported and fuse with the PM without forming a storage pool. In contrast, RSP vesicles are stored in the actin-rich cortex just underneath the PM. Some of these vesicles are tethered or docked at the PM and poised for activity-dependent secretion.

5.1. Biogenesis and Properties of Constitutive Secretory Vesicles

Constitutively secreted proteins travel from the site of synthesis in the RER through the Golgi complex and is packaged into constitutive vesicles (CVs) formed by budding at medial Golgi stacks or the TGN. Additionally, AP-1/clathrin-coated constitutive-like vesicles are formed by budding from immature RSP vesicles to selectively remove cargo that does not belong to the RSP vesicles, but entered the RSP inadvertently during sorting

(Kuliawat et al., 1997; Klumperman et al., 1998). These missorted proteins such as lysosomal enzymes are delivered to endosomes. There are no specific sorting signals on constitutively secreted proteins that direct them into the CSP, rather they enter the CVs by "default". There is no concentration of constitutive vesicle (CV) proteins at the TGN prior to vesiculation, and moreover, the assembly of CVs is not associated with a clathrin coat or acidification (Orci et al., 1987). CVs transport both soluble secretory proteins, e.g. growth factors, as well as membrane proteins such as neurotransmitter transporters and receptors to the cell surface for secretion or incorporation into the PM, respectively. They are clear vesicles with a diameter of 80–100 nm (Walworth and Novick, 1987). CV membranes do not contain a proton pump and the internal pH of CVs is presumed to be neutral, since in yeast, pro-α-mating factor processing is carried out in CVs by *kex2*, an enzyme that functions at neutral pH (Julius et al., 1984). Secretion of CV content is independent of an external signal and is non-calcium triggered. The secretion rate of CV proteins depends on the rate at which they are synthesized. Since they do not have a storage pool, the transit time between the ER and the Golgi complex is about 20 min and from there to the cell surface is ~10 min except in cells that have long processes, such as neurons. The CV membrane contains all the exocytotic machinery necessary for fusion to the PM and for secretion of its contents. In a live-cell imaging study of PC12 cells, it was observed that membrane vesicular acetylcholine transporter (VAChT) moves from the Golgi compartment into a distinct Golgi subcompartment together with DCV proteins briefly, before being incorporated into constitutive-like vesicles, known as SPTVs that bud off this compartment (Park et al., 2011). It was proposed that in this sorting compartment, SPTVs acquire the exocytic machinery which is enriched in this subcompartment, for fusion and delivery of VAChT to the PM. The membrane containing VAChT is then recycled to form the SV.

5.2. Biogenesis and Characteristics of RSP Vesicles

Formation of DCVs requires a number of steps that are common to both endocrine and exocrine cells, and neurons (Kim et al., 2006). Proteins destined for the RSP are posttranslationally modified by glycosylation, sulfation and phosphorylation. They are sorted away from other proteins by aggregation in the TGN at an acidic pH in the presence of calcium, and are engulfed in budding vesicles to form immature DCVs (Orci et al., 1987). Maturation of DCVs involve further acidification to ~pH 5.5, and

processing of precursor hormones in endocrine cells and neurons, followed by storage of mature DCVs until release upon stimulation of the cell (Molinete et al., 2000; Kim et al., 2006). Storage time of DCVs within the cytoplasm is long, 7–10 h, compared to the short transient times of CVs, and depends on an external signal followed by entry of Ca^{2+} to trigger release (Orci et al., 1987). Differences in assembly and secretion of DCVs and CVs are illustrated in Table 2.1. The various steps and the lipids involved in DCV biogenesis is discussed below and summarized in Table 2.2.

5.2.1. Mechanisms of Sorting of DCV Proteins at the TGN

For some time, there has been a debate as to whether sorting of DCV proteins at the TGN to the RSP is a "passive" or an "active" process (Kelly, 1985; Chanat, 1993). The passive process involves aggregation of proteins to form a condensing aggregate, which is then trapped and packaged into the DCV. The active process involves a sorting signal on the protein binding to a sorting receptor located in the lumen of the TGN membrane, which then facilitates entry into the budding immature DCV (Dikeakos and Reudelhuber, 2007). Current evidence indicates that aggregation alone may not be sufficient to sort proteins into DCVs (Gorr et al., 2001; Garcia et al., 2005), but that both aggregation and sorting receptors/binding proteins are necessary for efficient sorting to the RSP.

5.2.1.1. Aggregation and Calcium-Dependent Sorting

Electron microscopic studies by Orci and his colleagues (Orci et al., 1987) have shown that DCV proteins aggregate at the TGN in endocrine cells (Dannies, 2012). They and others (Hutton, 1982; Orci, 1986; Demaurex et al., 1998; Rindler, 1998; Machen et al., 2003) have also demonstrated that the pH at the TGN is mildly acidic (~6.0–6.5). Numerous in vitro studies have shown that DCV proteins such as prohormones and granins aggregate at an acidic pH, with either itself or other DCV proteins (Rindler, 1998; Sobota et al., 2009). Acidification is achieved by membrane proton pumps (V/H-ATPase) present at the TGN and DCV membranes (Wu et al., 2001). Treatment of PC12 cells with bafilomycin A1, a specific inhibitor for H-ATPase, caused a decrease both in numbers of DCVs and secretion of CgA, supporting the importance of acidification at the TGN for granin aggregation and DCV biogenesis (Taupenot et al., 2005). In addition to an acidic environment, in general, calcium present in DCVs is required for DCV protein aggregation as has been demonstrated for prohormones, PCs, CPE and granins (Shennan et al., 1994; Rindler, 1998; Dikeakos et al., 2009).

Table 2.2 Protein and lipid components involved in different steps of DCV biogenesis

Step	Function	Proteins	Lipids	Discussed in section
Cargo sorting at TGN	Cargo aggregation★, sorting "receptors", acidification	Granins, CPE, SgIII, muclin, V-ATPase	Cholesterol, lipid rafts†	2.5.2.1
TGN budding	Membrane curvature	PLD, DAG kinase, protein kinase D, PI kinases, GTPases (Arf1, Rabs), FAPPs, clathrin, APs (AP-1, GGA)	PAs, phosphoinositides, DAG, cholesterol, lipid rafts	2.5.2.3
Vesicle fission at TGN	Vesicle release	Dynamin, actin, γ-adducin, myosin II, GTPases (Rabs)		2.5.2.4
Vesicle maturation	Removal of missorted cargo, constitutive-like vesicle budding, acidification, condensation Coat shedding	GTP-ase (Rab-3D), myosin Va, V-ATPase proton pump, AQPs 1 and 5		2.5.2.2, 2.5.2.4

†Lipid rafts consist of platforms of lipids rich in cholesterol and sphingolipids.
★Soluble cargo such as prohormones and proneuropeptides co-aggregate with chromogranins.

However, not all DCV proteins require calcium for self-aggregation (Cawley et al., 2000).

Ca^{2+} exists in the free and bound form in DCVs. The mechanism of uptake of Ca^{2+} into acidic Ca^{2+} stores which include DCVs and the Golgi complex is unclear but likely driven by mammalian homologues of Ca^{2+} ATPases and Ca^{2+}/H^{+} exchangers since such activities have been detected, but the molecules have yet to be identified (Patel and Muallem, 2011). By contrast, Ca^{2+} release from DCVs is mediated by members of the TRP (transient receptor potential) channel superfamily and the inositol trisphosphate receptors IP_3Rs/calcium channels (Patel and Muallem, 2011; Yoo, 2011; Yoo and Hur, 2012). CgA and CgB both bind the IP_3Rs and activate the IP_3Rs/calcium channels at the intragranular pH of 5.5. This coupling is proposed to play key roles in the IP_3-mediated Ca^{2+} signaling mechanisms in the cytoplasm. Thus, Ca^{2+} in the Golgi complex and DCVs are important sources of intracellular Ca^{2+} for DCV protein aggregation and perhaps also cell signaling.

5.2.1.2. Sorting Signal Motifs

Over the last 20 years, a large number of sorting signals have been proposed for sorting DCV proteins at the TGN into DCVs (Dikeakos and Reudelhuber, 2007). The signals are complex and vary with different proteins and with cell type. In our discussion here, we will divide DCV proteins into two classes: soluble proteins and membrane proteins that are either tethered to or traversing the membrane, respectively. The first group of proteins includes prohormones in endocrine cells, BDNF in neurons, granins in both cell types and in exocrine cells. The second group includes proprotein/hormone convertases (PCs), CPE, phogrin and muclin.

Sorting signals within prohormones, proneuropeptides and granins have been the most studied. Site-directed mutagenesis studies have identified a three-dimensional conformation-dependent consensus sorting motif consisting of two acidic residues, 12–15 Å apart from each other, exposed on the surface of the molecule, and two hydrophobic residues, 5–7 Å away from the acidic residues which is necessary for sorting of POMC, proinsulin and proenkephalin to the RSP (Cool et al., 1997; Dhanvantari et al., 2003). This sorting motif was found to interact with a sorting receptor, membrane CPE (see next section) to mediate sorting to the RSP. For POMC, this motif resides in the N-terminus disulfide loop (Cool et al., 1995). For proenkephalin, the motif lies within the N-terminal residues 1–32 (Loh et al., 2002). In the case of proinsulin sorting, while such a motif is present in

the monomeric form contributed by residues from the A and B chain, the motif was also found in the hexameric aggregated form of insulin in two adjacent A chains (Dhanvantari et al., 2003). In pancreatic cells, proinsulin appears not to be sorted at the TGN, but enters immature vesicles along with lysosomal enzymes and constitutive proteins and is processed. Insulin is retained in the immature vesicle while constitutive-like vesicles bud off from the vesicle to remove non-DCV proteins (Tooze and Tooze, 1986). In this case, the motif acts as a retention signal by binding to a sorting receptor, membrane CPE, in the vesicle (see next section, and Dhanvantari et al., 2003). Such a three-dimensional sorting motif has also been found to direct BDNF (Lou et al., 2005) to the RSP at the TGN. The conformation of BDNF and NGF are very similar, except that NGF is missing one amino acid residue to complete such a motif. Introduction of the missing residue by mutagenesis (Val20Glu) redirected NGF to the RSP, indicating the importance of the identified sorting motif in targeting BDNF to the RSP. This sorting motif can only be recognized in molecules that have a conformation model or a crystal structure available, and hence have been identified in only a limited number of prohormones and proneuropeptides.

Paired basic residues have also been suggested to act as sorting signals for targeting some neuropeptides to the RSP. These include pro-renin (Brechler et al., 1996), proneurotensin (Feliciangeli et al., 2001), progastrin (Bundgaard et al., 2004) and prothyrotropin-releasing hormone (Mulcahy et al., 2005). However, the mechanism is unclear. It is postulated that a certain pair of basic residues must be cleaved by one of the prohormone convertases (PCs), which usually acts in DCVs at an acidic pH, before it can be correctly sorted to/retained in the DCVs since mutation of the pair of basic residues to a furin (which cleaves proproteins early in the Golgi complex) cleavage site redirects them to the CSP (Brechler et al., 1996).

Various sorting domains have been identified for targeting granins, such as CgA, CgB, and secretogranins to the RSP. These domains have been extensively reviewed by Bartolomucci et al. (2011), and a few will be highlighted here. Taupenot et al. (2002) found that the N-terminal domain of CgA (bovine/human residues 40–115) is necessary for targeting CgA into the secretory granules of PC12 cells. This region concurred with the findings of Hosaka et al. (2002) who showed that the N-terminal domain (rat/bovine residues 48–111/48–95) of CgA was essential for binding to secretogranin III (SgIII), a membrane-associated sorting receptor for CgA (see next section). In contrast, studies on CgB identified a disulfide bonded loop structure located within the first 37 amino acids of the N-terminal of the

molecule that was essential for sorting to the RSP in PC12 cells (Kromer et al., 1998). Two putative α-helix-containing domains, hSgII 25–41 and hSgII 334–348, that can act independently, have been shown to target SgII to the RSP in PC12 cells (Courel et al., 2008). SgII has been shown to interact with SgIII in yeast two-hybrid studies, raising the possibility that SgII sorting may be through interaction with SgIII similar to CgA.

For membrane-associated DCV proteins, sorting to the RSP occurs by interaction with TGN membranes. The TGN has cholesterol–sphingolipid-rich microdomains known as "lipid rafts" where budding is thought to occur to form immature DCVs (see Section 2.5.2.3) consistent with these organelles being highly enriched with cholesterol in their membranes (Dhanvantari and Loh, 2000). SgIII has a domain at the N-terminus (residues 23–186 in the rat sequence) of the molecule that binds cholesterol and this domain is essential for sorting of SgIII to the RSP (Hosaka et al., 2004). Membrane-binding domains containing short α-helical structures residing in the C-terminus of CPE (Zhang et al., 2003) and proprotein convertases PC1/3 (Jutras et al., 2000; Lou et al., 2007) and PC2 (Assadi et al., 2004) have been demonstrated to be essential for targeting these prohormone-processing enzymes to the RSP. The membrane-binding domains interact with lipid rafts of the TGN membrane to effect sorting to the RSP. In the case of membrane CPE, the membrane-binding domain appears to traverse the DCV membrane (Dhanvantari et al., 2002; Zhang et al., 2003) despite the lack of a typical transmembrane amino acid sequence. This has led to some debate about the transmembrane orientation of CPE, but the evidence supporting the existence of a cytoplasmic tail in CPE is strong (Cawley et al., 2012). Phogrin, an integral membrane DCV protein, also has a sorting domain located at the C-terminal cytoplasmic tail necessary for targeting to the RSP (Wasmeier et al., 2002; Torii et al., 2005). These membrane-associated DCV proteins are ideal candidate sorting receptors for soluble DCV proteins (see next section).

5.2.1.3. Membrane Sorting Receptors

It is clear that sorting of soluble aggregates of DCV proteins such as prohormones, proneuropeptides or granins to the RSP requires tethering/binding to a membrane receptor at the TGN. It has been shown that SgIII is a sorting receptor for targeting CgA to the RSP. The CgA sorting domain binds strongly to the SgIII domain comprising of residues 214–373 at pH 5.5 in the presence of 10 nM calcium (Hosaka et al., 2002).

An RSP sorting receptor that binds to the conformation-dependent sorting signal described above for POMC, proinsulin, proenkephalin and BDNF was identified as the transmembrane form of CPE. The two acidic residues in the prohormone/pro-BDNF sorting motif specifically interact with two basic residues, R255 and K260, of the sorting receptor, CPE, to effect sorting to the RSP (Cool et al., 1997). In vivo studies using a CPE KO mouse model showed that BDNF was not sorted to the RSP (Lou et al., 2005). Instead it was secreted constitutively in cortical and hippocampal neurons of the CPE KO mice. Constitutive secretion of proinsulin from isolated pancreatic islets was elevated and plasma levels of proinsulin were significantly higher in these mice, indicating a role of CPE in sorting of proinsulin to the RSP (Naggert et al., 1995; Irminger et al., 1997; Cawley et al., 2004). Recent studies of POMC sorting in primary anterior pituitary cultures from CPE KO mice showed defective regulated secretion of ACTH, consistent with CPE acting as a sorting receptor in vivo. However, some stimulated secretion of POMC was observed. Further investigation of pituitaries revealed elevated expression of SgIII (Cawley, N. X., and Loh, Y.P., unpublished data). an RSP sorting receptor, for CgA at the TGN. Previous work reported in the literature (Hosaka et al., 2005) has shown that SgIII binds to POMC, albeit with lower affinity than CPE, and can potentially act as a sorting receptor for POMC. Thus higher expression of SgIII in the pituitary of CPE KO mice may compensate as a sorting receptor for POMC in these mice.

Peptidyl-α-amidating monooxygenase (PAM), another type 1 membrane spanning DCV protein which function to amidate peptide hormones, has been suggested to play a role in sorting of atrial natriuretic peptide to the RSP since it binds membrane PAM very tightly in myocytes (O'Donnell et al., 2003). Muclin, a DCV membrane protein in exocrine pancreatic acinar cells, has been shown to mediate sorting of zymogens including amylase, prolipase, procarboxypeptidase A1, pro-elastase II, and chymotrypsinogen B to the RSP (Boulatnikov and De Lisle, 2004). It acts by binding to these zymogen aggregates in a mildly acidic pH present at the TGN through its sulfated, *O*-linked oligosaccharide groups.

5.2.1.4. Glycosylation, Sulfation and Phosphorylation in DCV Protein Sorting

Secretory glycoproteins undergo glycosylation during transit through the cis-medial-trans Golgi cisterni. At the TGN, some glycosylated proteins are further modified by sulfation. Protein glycosylation or sulfation could affect protein–protein interactions required for sorting some proteins to the RSP.

For example, in pancreatic acinar cells, sulfated, *O*-glycosylated Muclin, a type 1 membrane spanning protein, binds aggregated zymogens at the TGN to facilitate sorting to the RSP (Boulatnikov and De Lisle, 2004). Blockage of sulfation and glycosylation of Muclin inhibited sorting of the zymogens to the RSP. Another molecule, VMAT2, has an *N*-glycosylated luminal loop domain necessary for sorting to the RSP (Yao and Hersh, 2007). Inhibition of glycosylation of the luminal loop with 1-deoxymannojirimycin, a specific alpha-mannosidase 1 inhibitor, resulted in missorting of VMAT2 to CVs in PC12 cells.

However, protein glycosylation does not always affect protein sorting to the RSP. Sorting of cathepsin G to granules for its processing, activation, and secretion does not depend on its glycosylation (Garwicz et al., 1995). The mutant form of cathepsin G that lacks a functional glycosylation site can be processed to an enzymatically active form and released in a regulated manner in neutrophil granulocytes. Sorting of POMC/ACTH to the RSP was also not affected by inhibition of its glycosylation or sulfation (Moore, 1987). In addition, sorting of cerebral dopamine neurotrophic factor (Sun et al., 2011), pancreatic lysosomal enzymes (Chu et al., 1990), and renin (Kuliawat and Arvan, 1994) to the RSP were not inhibited by prevention of glycosylation. Thus, glycosylation-based sorting appears to be limited for some RSP proteins.

Evidence for phosphorylation of secretory proteins as a signal for guiding sorting to the RSP is sparse. In classical cholinergic neurons, chromaffin, and PC12 cells, VAChT is transported constitutively to the PM and recycled into SLMVs while VMATs are found mostly in DCVs (Fei et al., 2008). In protein kinase A (PKA)-deficient PC12 cells, VMATs are found in SLMVs (Yao et al., 2004). Treatment of wild-type (WT) PC12 cells with a PKA inhibitor, H89, also caused accumulation of VMATs in SLMVs. While VMATs undergo phosphorylation of the cytoplasmic tail (Krantz et al., 1997), it does not appear that it is carried out by PKA but rather by casein kinase II. It is not clear that this phosphorylation is important for sorting VMATs to the RSP. Instead, PKA facilitates sorting of VMAT to DCV indirectly. On the other hand, PKC-mediated phosphorylation of a serine residue in the cytoplasmic domain (R<u>S</u>ERDVLL) of VAChT prevents sorting of VAChT to SLMVs (Cho et al., 2000; Krantz et al., 2000). A phospho-mimicking mutation of serine to glutamate in the VAChT cytoplasmic tail caused sorting of VAChT to DCVs, maybe because the mutation renders the VAChT tail similar to the VMAT2 tail (KEEKMAIL). Despite these studies, there is no clear evidence indicating that phosphorylation coordinates sorting of VAChT and VMATs to SV/SLMVs and DCVs, respectively.

In another example, furin, a Golgi-resident protein that has inadvertently entered immature DCVs can be removed from these DCVs via clathrin-mediated constitutive-like secretion (Fig. 2.1). The cytoplasmic tail of furin interacts with AP-1 or Golgi-localized, gamma-ear-containing, ADP-ribosylation factor-binding proteins (GGA) that, in turn, recruits clathrin coat for removal of furin from immature granules (Park and Loh, 2008). Casein kinase II mediates phosphorylation of the cytoplasmic tail of furin, which is a prerequisite step for the interaction of AP-1 or GGA with furin (Dittie et al., 1997). Hence phosphorylation of the furin tail is important for its sorting and removal from immature granules.

In summary, all the findings reviewed above indicate that sorting of various proteins from the Golgi complex to DCVs in the RSP is mediated by a mechanism involving aggregation and a sorting signal/domain within the protein, interacting with a membrane-associated sorting receptor/binding protein, in endocrine, exocrine cells and neurons. Posttranslational modifications such as glycosylation, sulfation and phosphorylation do play a role in facilitating sorting of some DCV proteins.

5.2.2. Granins and Membrane Proteins in DCV Assembly

Granins have long been proposed to be important in DCV assembly since they are generally present in a high molar ratio relative to other proteins in the granule. Indeed, it has been demonstrated that overexpression of granins such as CgA, CgB and SgII induced DCG-like structures in fibroblast cells that have no RSP (Kim et al., 2001; Huh et al., 2003; Beuret et al., 2004). These structures are not likely to be "bona fide" DCVs with all the components necessary for regulated secretion, although addition of calcium ionophores has resulted in secretion of their contents in some studies (Beuret et al., 2004). Furthermore, antisense RNA downregulation of CgA expression inhibited granule structures in PC12 cells (Kim et al., 2001), and depletion of SgII by small interfering RNA (siRNA) in these cells resulted in a decrease in size and number of DCVs (Courel et al., 2010). In WT neuroendocrine PC12 cells, downregulation of CgA impaired both DCV formation and activity-dependent secretion of an exogenous cargo molecule, the prohormone POMC (Kim and Loh, 2006). Additionally, in 6T3 cells, a variant of the AtT-20 pituitary cell line, which lacks CgA and DCVs, upon exogenous expression of bovine CgA, DCV biogenesis was induced and regulated secretion was restored (Kim et al., 2001). Antisense RNA downregulation of CgA expression in transgenic mice and CgA KO mice showed significant decrease in the size and numbers of DCVs in adrenal

chromaffin cells (Mahapatra et al., 2005; Kim and Loh, 2006). However, a CgA KO mouse generated by Hendy et al. (2006) showed no difference in numbers of chromaffin granules compared to WT mice. Other granins such as CgB and SgII–VI were increased 2- to 3-fold in these mice, suggesting that they may compensate for the lack of CgA. All these studies taken together demonstrate that the granins are granulogenic proteins and play a very important role in providing the "driving force" for vesicle budding at the TGN to form immature DCVs.

DCVs also contain various membrane components necessary for their function and here we highlight the key ones. These include integral membrane proteins such as aquaporins (AQPs) and various ion-gated channels. AQPs are a family of water channels expressed in epithelial cells to transport water bidirectionally to mediate fluid absorption and secretion. AQP1 has been found in DCVs in pituitary, chromaffin granules from adrenal medulla and pancreatic zymogen granules, while AQP5 has been found in parotid gland DCVs (Cho et al., 2002; Arnaoutova et al., 2008; Francone et al., 2010). AQP1 has also been found in the TGN (Francone et al., 2010). Its function in TGN and granules is to remove water to facilitate condensation of aggregated DCV proteins at the TGN and during DCV maturation. Studies have shown that downregulation of AQP1 expression in AtT-20 cells, an anterior pituitary cell line, resulted in a significant decrease of ACTH-containing DCVs. Pulse-chase labeling studies showed that while POMC synthesis was unaffected, in these AQP1-deficient cells there was a major decrease in newly synthesized DCV proteins 1 h after synthesis, indicating that in the absence of DCV biogenesis these proteins were degraded. These findings corroborated with decreased POMC and its processing enzymes in anterior pituitary of AQP1 KO mice. Hence AQP1 appears to be a key component in condensation of DCV proteins and maintaining DCV biogenesis.

DCV membranes also contain a number of ion channels. These include calcium channels (see Section 2.5.2.1.1) for calcium uptake and sequestration, and a calcium-independent K+ selective channel found in chromaffin granules that may play a role in ion movement during granule assembly (Arispe et al., 1992). There are also proton pumps (H-ATPase) in the DCV membrane required for acidification during DCV maturation (Apps et al., 1989; Saroussi and Nelson, 2009). In addition there are components of the exocytic machinery such as vesicle associate membrane proteins (VAMPs), GGAs and SNAP25 present in immature DCVs (Eaton et al., 2000). These components have recently been found in a post-TGN-Golgi subcompartment from which DCVs and SPTVs bud (Park et al., 2011) in PC12 cells,

and are presumably assembled in microdomains where DCVs bud. However, the distribution of these components along the TGN membrane is unclear and it remains to be determined if they are randomly distributed or concentrated in lipid rafts (see Section 2.5.2.3).

5.2.3. Role of Membrane Lipids and Proteins in DCV Budding

Subsequent to assembly of DCV cargo and membrane components at the TGN, the next step is vesicular budding of the membrane to form the immature DCVs. Lipids play a significant role in this budding event, facilitated by proteins. Most studies on the role of lipids in vesicle budding at the TGN and membrane curvature pertain to CV budding in mammalian cells and yeast (Corda et al., 2002; Roth, 2004; van Meer and Sprong, 2004; Kim et al., 2006), and only a few relate specifically to DCV budding in endocrine/exocrine cells (Tooze and Tooze, 1986; Kim et al., 2006). Nevertheless, the lipid-mediated mechanisms for vesicle budding for both these two types of vesicles are likely to be similar.

For both constitutive and DCV vesicle budding at the TGN, two types of lipids are necessary, phosphatidic acid (PA) (Siddhanta and Shields, 1998; Siddhanta et al., 2000) and cholesterol (Wang et al., 2000). Inhibition of PA synthesis altered the structure of Golgi apparatus and quantitatively inhibited secretion of growth hormone (Siddhanta et al., 2000). In in vitro studies, when PA levels were increased by diacylglycerol (DAG) kinase or phospholipase D (PLD) 1 in GH3 cells, there was significant increase in secretory vesicle budding (Siddhanta and Shields, 1998).

PA can be derived from DAG or phosphatidylcholine by DAG kinase or phosphatidylcholine-specific PLD1, respectively. DAG is present in the TGN membranes and participates in CV budding (Yeaman et al., 2004), but its role in DCV budding is not known. PA has a cylindrical shape at neutral pH but assumes a cone structure under acidic pH and low calcium concentration, conditions present within the lumen of the TGN (Kooijman et al., 2003). This conical molecular structure apparently provides enough force to induce negative curvature in the Golgi membrane, leading to pearling of the bilayer and then fission (Corda et al., 2002; Shemesh et al., 2003).

Phosphatidylinositol (PI) is another lipid that promotes membrane curvature (Bryan and Hagen, 1991). In its phosphorylated form, phosphatidylinositol 4-phosphate (PI4P) has been shown to mediate budding of CVs at the TGN (Santiago-Tirado and Bretscher, 2011). PI4P is enriched in the TGN and recruits four-phosphate-adaptor protein (FAPP) 1 and FAPP2, which bind the small GTPase [ADP-ribosylation factor (Arf)] Arf1-

guanidine triphosphate (GTP). Through interaction of the pleckstrin homology (PH) domain of FAPPs with PI4P and Arf1-GTP, vesicle formation and transport of cargo to the PM is regulated (Godi et al., 2004; Santiago-Tirado and Bretscher, 2011). Phosphoinositol kinases are differentially localized in the Golgi complex to control local production of PI4P. This allows for additional control of cargo selection and vesicular subtype specification during vesicle transport (Weixel et al., 2005). While the role of PI4P in DCV budding has not been studied, it is likely also involved.

Cholesterol is a lipid that is of major importance in DCV budding. Pituitary DCV membranes are reportedly highly enriched in cholesterol (Dhanvantari and Loh, 2000). It has also been shown that the TGN is enriched with cholesterol, glycosphingolipids and sphingomyelin, and together they are packed in microdomains known as "lipid rafts" (Orci et al., 1981; van Meer, 1998). Because of the high cholesterol content of the DCV membranes, these microdomains are proposed to be the site of DCV budding in endocrine cells. Consistent with this is the finding that when cholesterol synthesis was blocked in AtT-20 cells, a pituitary endocrine cell line, DCV formation was halted, and condensed dense cores were found accumulated at the TGN. However, upon addition of cholesterol, DCV biogenesis resumed (Wang et al., 2000). Cholesterol also assumes a conical-shaped structure that promotes a negative curvature and therefore facilitates vesicle budding (Orci et al., 1980; Corda et al., 2002; Bacia et al., 2005). It would appear that cholesterol cannot be substituted by other sterols (e.g. precursors of cholesterol) in this function. In mouse models of human genetic diseases, Smith–Lemli–Opitz syndrome (SLOS) and lathosterolosis, where cholesterol synthesis is impaired due to defective synthesizing enzymes, DCV numbers were decreased in the pancreas, pituitary and adrenal gland. Moreover, the DCVs in the exocrine pancreas were malformed due to decreased membrane curvature as a result of increased rigidity of the cholesterol precursors in these mice (Gondré-Lewis et al., 2006). From the above studies reviewed, it is evident that lipids, especially cholesterol, are critical for secretory vesicle budding and biogenesis.

5.2.4. GTPases, Clathrin and Cytoskeletal Proteins in DCV Biogenesis

Small GTPases such as the Arf and Rab families play a role in vesicle budding while the large GTPase, dynamin, which assembles at the neck of budding vesicles, functions in membrane fission and vesicle release. These GTPases cycle between an inactive guanidine diphosphate (GDP)-bound form and the active GTP-bound form. As mentioned above, small GTPases such as Arf1

interact with FAPP-PI4P to mediate constitutive secretory vesicle budding at the Golgi complex (Santiago-Tirado and Bretscher, 2011). Arfs (Arf1 and Arf6) function to induce membrane curvature by insertion of an amphipathic helix into the cytosolic leaflet of the membrane (Lundmark et al., 2008). Moreover, GTP-induced dimerization of Arf1 appears to be an essential step for inducing Golgi membrane curvature during the formation of coated vesicles (Beck et al., 2008).In addition, two members of the Rab family, Rab2 and Rab6 are involved in vesicle biogenesis at the Golgi complex. In *Caenorhabditis elegans*, the unc-108 (equivalent of mammalian Rab2) is present in the soma of neurons, but not at the synapses, and it co-localizes with Golgi but not ER markers. *Unc108*/Rab2 mutants showed deficits in locomotion and abnormalities in the size and content of the DCVs in peptidergic neurons. Their DCVs were slightly larger and more variable in size, but the numbers, the dense-core diameter, and ability to secrete the contents were no different from the WT worms. While the DCVs in these mutants contained normal neuropeptide cargo, labeling studies indicate that two thirds of the cargo in immature DCVs, both soluble and membrane components, are moved into endosomes. It seems the defect in these *unc108* mutants is not in the initial generation of immature DCVs, but in the cooperative function of *unc108* with its effector, RIC-19, in retaining all the appropriate cargo during DCV maturation for normal physiological function (Edwards et al., 2009; Sumakovic et al., 2009). Rab6 has been shown to promote fission of Rab6-positive transport vesicles at the Golgi complex (Miserey-Lenkei et al., 2010). Myosin II and F-actin which are associated with Rab6-positive vesicles are essential for this process. Depletion of myosin II or depolymerization of F-actin led to inhibition of fission and appearance of Rab6-positive long tubules connected to the Golgi complex, concomitant with a decrease in Rab6-containing vesicles. Thus, myosin II and F-actin are effectors of Rab6-GTPase in vesicle fission at the Golgi. It has been proposed that contraction of short actin filaments by myosin II may increase membrane tension locally, which in turn could facilitate fission by the vesicle being pulled along microtubule tracks by the motor kinesin I. This study suggests an important role of Rab6 in cooperation with cytoskeletal proteins in vesicle fission. Rab3D working cooperatively with myosin Va has been implicated in DCV maturation by facilitating constitutive-like secretion and removal of, e.g. missorted furin, from the immature DCV (Kogel and Gerdes, 2010).

Dynamin was first discovered as a GTPase involved in clathrin-mediated endocytosis at the PM (Hinshaw, 2000), but more recently, evidence is emerging that it also plays a role in membrane fission and vesicle release at

the TGN. Dynamin has five functional domains: an N-terminal GTPase domain, a middle domain, a PH domain with variable hydrophobic loops, the GTPase effector domain (GED) and the C-terminal proline/arginine domain (PRD). The GTPase domain binds and hydrolyzes GTP (Chappie et al., 2010), while the middle domain and GED interact with each other to mediate self-assembly (Ramachandran et al., 2007) (http://onlinelibrary.wiley.com/doi/10.1111/j.1600-0854.2011.01250.x/full-b25). The PRD binds to SH3 domain-containing partners and is involved in targeting dynamin to clathrin-coated pits (CCPs) at the PM (Okamoto et al., 1997; Hinshaw, 2000). Subsequent to vesicle budding, in the presence of GTP, dynamin organizes into self-limited assemblies that continuously cycle at the membrane and drive vesicle release (Hinshaw, 2000; Pucadyil and Schmid, 2008). Current evidence suggests that shallow insertion of the hydrophobic loop in the PH domain of dynamin into the lipid bilayer leads to membrane deformation and fission (Ramachandran et al., 2009). There is also evidence suggesting that activity-dependent dephosphorylation of dynamin 1 is involved in fusion pore collapse during DCV exocytosis in chromaffin cells (Chan et al., 2010). What is known about the role of dynamin is mainly in endocytosis from the use of in vitro model membranes, liposomes or Hela cells. However, dynamin has been shown to be essential for transport of p75, a neurotrophin receptor, from the Golgi complex to the PM in neurons (Kreitzer et al., 2000), indicating that it is also involved in constitutive secretory vesicle formation at the TGN (Jones et al., 1998). While there have been no studies on the role of dynamin in DCV release at the TGN, it is likely that it also participates in membrane fission and release of DCVs subsequent to budding at the TGN, in (neuro)endocrine cells.

Clathrin coating is involved in CV and DCV budding at the TGN (Tooze and Tooze, 1986; Teuchert et al., 1999). Evidence that budding DCVs have a clathrin coat has been clearly demonstrated by electron microscopy of (neuro)endocrine cells (Orci et al., 1985; Tooze and Tooze, 1986). AP-1 and GGA are major APs that mediate clathrin coating on budding CVs, either independently or cooperatively (Bonifacino, 2004). Both AP-1 and GGA are found on immature DCVs, suggesting that they likely mediate clathrin coating of budding DCVs (Dittie et al., 1996; Kakhlon et al., 2006). An AP-1 adaptor complex bound to clathrin-coated immature vesicles in PC12 neuroendocrine cells has been reported and this binding is regulated by Arf1 (Dittie et al., 1996). Recently, it has been demonstrated in *Drosophila* epithelial cells of the larval salivary gland that AP-1 and clathrin co-localize at the TGN and in the mucin-containing immature secretory granules. Loss of either AP-1 or

clathrin inhibits formation of these secretory granules at the TGN, further indicating an essential role of AP-1 and clathrin in DCV biogenesis (Burgess et al., 2011). A number of other cytosolic proteins which have AP-1 or GGA-binding motifs may also assist in clathrin coating. These include phosphofurin acidic cluster sorting protein-1 (PACS-1) which facilitates binding of AP-1 to the acidic residues of the cytoplasmic tail of the integral membrane protein furin (Crump et al., 2001), and epsin-R that binds to PI4P, clathrin, and AP-1 (Mills et al., 2003). Subsequent to formation of the immature granule, the clathrin coat is removed by budding of constitutive-like clathrin-coated vesicles from the maturing DCVs (Tooze and Tooze, 1986).

SNARE proteins VAMP2, VAMP4, syntaxin 6, synaptotagmin I and IV are also recruited to immature DCV membranes and are subsequently removed, except for VAMP2 and synaptotagmin I, as the DCV matures to render them responsive to secretagogues (Eaton et al., 2000). The role of these SNARE proteins in DCV biogenesis is unclear. However, in some cells, such as PC12 cells, homotypic fusion of immature DCVs occurs and syntaxin 6 is required for the fusion (Tooze et al., 1991; Urbe et al., 1998).

Cytoskeletal proteins such as F-actin are found covering the cytoplasmic face of the Golgi complex and have been proposed to play a role in vesicular exit from the TGN (Godi et al., 1998; Fucini et al., 2000; Dubois et al., 2005; De Matteis and Luini, 2008; Miserey-Lenkei et al., 2010). Recently, we have found high levels of F-actin and the cytosolic actin-binding protein, γ-adducin, localized at the peri-Golgi area in AtT-20 endocrine cells. γ-Adducin has been shown to interact with the cytoplasmic tail of a transmembrane form of the prohormone-processing enzyme, CPE (Lou et al., 2010), and in POMC/ACTH-containing DCVs in AtT-20 pituitary cells. Transfection into AtT-20 cells of a dominant-negative construct containing a C-terminal domain of γ-adducin disrupted actin filaments and the interaction of γ-adducin with the CPE tail, resulting in inhibition of POMC/ACTH exit and accumulation of POMC in the Golgi complex. POMC/ACTH-containing DCVs were greatly diminished along and at the tips of the cell processes of these transfected cells, compared to control cells (Lou et al., 2012; Richter et al., 2008). We propose that γ-adducin may serve as an intermediary molecule to attach the budding immature DCV to actin filaments at the TGN to facilitate fission/release, analogous to caveolae being attached to actin filaments via actin-binding proteins during non-clathrin-dependent endocytosis at the PM (Richter et al., 2008). Furthermore, as described above, myosin II/F-actin has been shown to play a role in membrane fission to release Rab2-positive vesicles at the Golgi membrane.

Thus evidence is accumulating that cytoskeletal proteins are important in vesicle biogenesis at the Golgi complex.

5.3. Regulation of DCV Biogenesis

The biogenesis of DCVs can be regulated at the genetic, posttranscriptional and posttranslational levels (Fig. 2.3). Regulation of DCV biogenesis at the genetic level would be expected to be most important during development when progenitor cells differentiate to become specialized secretory cells that secrete hormones or neuropeptides, or non-DCV-containing cells. Another example where genes involved in regulating DCV biogenesis is of significance, is during endocrine, exocrine and neuronal cell regeneration from progenitors after injury in adults. In contrast, regulation at the posttranscriptional and posttranslational levels is likely used by differentiated secretory cells to replenish DCVs that have undergone exocytosis and released their cargo upon stimulation.

5.3.1. Genetic Regulation of DCV Biogenesis

The induction of DCV biogenesis at the transcriptome level is poorly understood. The receptor element 1-silencing transcription factor (REST)/the neuron-restrictive silencing factor (NRSF) has been proposed to be a negative regulator of DCV biogenesis. REST/NRSF binds a 23-bp DNA sequence element to cause suppression of the expression of various neuronal genes, including those necessary for secretion, as well as DCV proteins (Chong et al., 1995; Schoenherr and Anderson, 1995; Bruce et al., 2004). REST/NRSF expression is repressed in neuronal cells, but after epileptic and ischemic insults, REST/NRSF expression is derepressed, causing cell death (Palm et al., 1998; Calderone et al., 2003). WT PC12 cells do not express REST/NRSF, but in mutant cell lines that express REST/NRSF, such as PC12-REST and PC12-HZ4, expression of REST/NRSF is derepressed leading to inhibition of expression of various genes required for regulated secretion (Bruce et al., 2006). When a dominant-negative REST/NRSF construct was expressed in A35C cells, a REST/NRSF-expressing PC12 variant cell line without regulated secretory granules, messenger RNA (mRNA)-encoding proteins that are necessary for regulated secretion were elevated. However, DCV and SV proteins, such as CgA and synaptophysin, were not reexpressed at the protein level, and no DCV formation was observed (Pance et al., 2006), suggesting that REST/NRSF does not regulate expression of all genes necessary for DCV biogenesis in these cells.

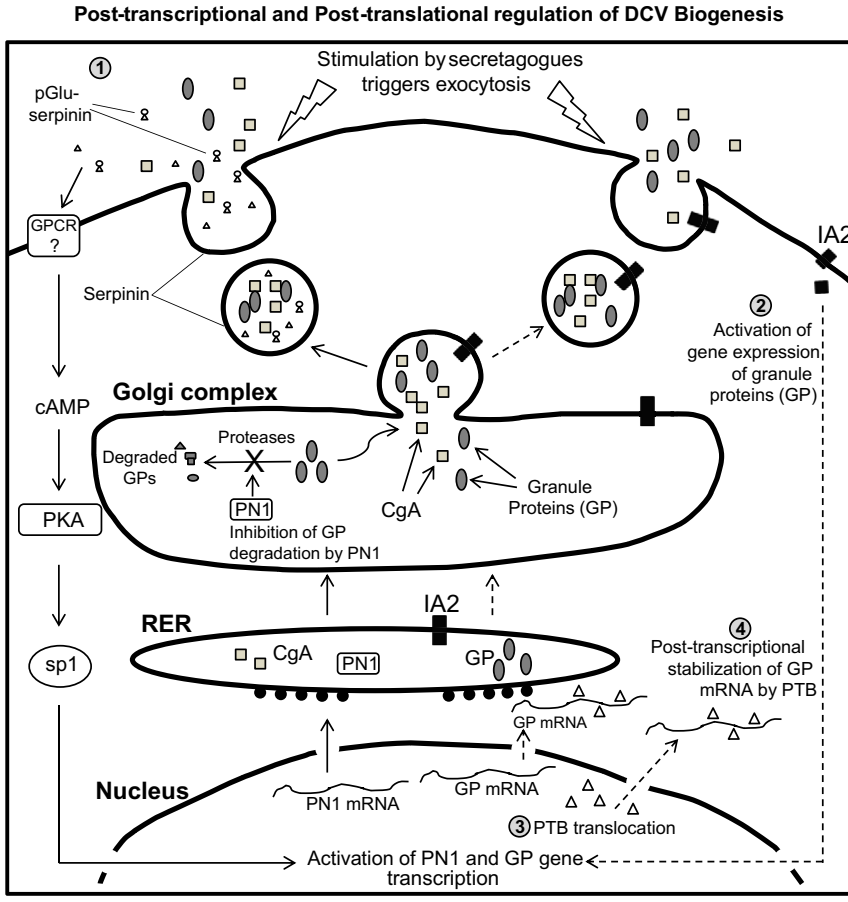

Figure 2.3 ***Multilevel mechanisms regulate DCV biogenesis.*** Formation of dense-core secretory granules in neuroendocrine and endocrine cells is regulated at multiple levels to maintain a steady-state pool of DCVs and to replenish stored pools after secretion. These mechanisms promote the level of granule proteins to enhance DCV biogenesis. Transcriptional (2) and posttranscriptional events (3, 4) regulating DCV biogenesis are illustrated by dotted arrows. The events involved in serpinin-induced posttranslational regulation of DCV biogenesis (1) are illustrated by solid arrows. In this posttranslational pathway, secreted serpinin and pGlu serpinin (CgA-derived peptides) bind to a putative G protein-coupled receptors (GPCR) which then leads to activation of a cAMP–PKA pathway, translocation of transcription factor sp1 into the nucleus and upregulation of PN-1 expression. This in turn inhibits granule protein (GP) degradation in the Golgi complex, increasing GP levels and enhancement of DCV biogenesis. For a color version of this figure, the reader is referred to the online version of this book.

Some astrocytes express high levels of REST/NRSF and do not contain DCV markers such as granins or neuropeptides (Prada et al., 2011). However, when the dominant-negative construct of REST/NRSF was expressed in these astrocytes, DCV formation was induced. Moreover, the DCVs were filled with NPY and SgII and released their contents in a Ca^{2+}-dependent manner. Astrocytes also form secretion-competent clear vesicles that contain glutamate (Haydon and Carmignoto, 2006; Parpura and Zorec, 2010) but formation of these vesicles seems to be independent of REST. This study also showed that not all proteins related to DCVs and secretion were increased after stable transfection of REST dominant-negative construct into astrocytes: SNAP25 was increased significantly, the other t-SNARE, Stx1a was only increased slightly in these astrocytes. SgII was elevated significantly, although all three proteins were well below that found in PC12 WT cells. In PC12–27 cells, a mutant cell line that is secretion incompetent, the SNAP25, Stx1a and SgII proteins were increased when stably transfected with the REST dominant-negative construct. In these cells, the rise of Stx1a was 12-fold, and that of SgII was only 2-fold (D'Alessandro et al., 2008). These differences indicate that control of gene expression by REST is cell specific.

Studies thus far would suggest that the negative regulation (suppression of expression) of genes involved in DCV biogenesis and secretion by the REST/NRSF transcription factor may be of significance during differentiation of cells to yield specific phenotypes. For example, for non-DCV-containing astrocytes during development, REST expression is high, and for astrocytes and neurons that contain DCVs, REST expression is low. REST may also play a role in maintaining the differentiated phenotype. However, in adults, after injury, differentiation of progenitors occurs to regenerate endocrine/exocrine and neuronal cells. Under these circumstances, another signal that downregulates REST expression in these progenitors may be present to facilitate differentiation into DCV-producing secretory cells. However, at present, not much is known about the level of REST in progenitors of endocrine/exocrine or neuronal cells, and more studies are necessary to investigate such a possibility.

5.3.2. Transcriptional Regulation of DCV Biogenesis

Another level of control of DCV biogenesis is at the transcriptional level. In insulin-secreting pancreatic β-cells, insulinoma-asssociated protein 2 (ICA512/IA-2), a transmembrane protein localized on DCV membranes, was shown to regulate DCV biogenesis (Harashima et al., 2005; Kim et al., 2006). When pancreatic β-cells are stimulated by glucose to release insulin,

the membrane of the exocytosed DCV is incorporated into the PM after the fusion process, thereby localizing ICA512/IA-2 to the cell membrane. The cytoplasmic tail of ICA512/IA-2 is then cleaved off and translocated into the nucleus where it induces expression of insulin (Fig. 2.3) (Trajkovski et al., 2004). Furthermore, when ICA512/IA-2 was overexpressed in MIN-6, an insulin-secreting β-cell line, insulin content and quantity of DCVs were increased (Harashima et al., 2005).

In another example, induction of exocytosis in sympathoadrenal chromaffin cells and PC12 pheochromocytoma cells resulted in upregulation of transcription of various DCV-related genes such as CgA, tyrosine hydroxylase, phenylethanolamine-*N*-methyltransferase, dopamine β-hydroxylase, and proenkephalin (Eiden et al., 1984; Rausch et al., 1988; Kilbourne et al., 1992; Hiremagalur et al., 1993; Tang et al., 1996; Mahapatra et al., 2003, 2005). Upregulation of expression of these genes and hence their proteins would be expected to drive DCV biogenesis. Indeed, it has been demonstrated that overexpression of granins and prohormones in fibroblasts can induce DCV biogenesis (Kim et al., 2001; Huh et al., 2003; Beuret et al., 2004; Stettler et al., 2009). However, the mechanism by which these genes are upregulated after an exocytosis event is unknown. It could be mediated by a peptide signal released from the vesicle, which then activates a signal transduction pathway leading to gene transcription (see Section 2.5.3.4 and Figure 2.3).

5.3.3. Posttranscriptional Regulation of DCV Biogenesis

Regulation of DCV biogenesis at the posttranscriptional level has been demonstrated in pancreatic insulin-producing cells (Knoch et al., 2004). In this mechanism, mRNA stability is increased leading to elevation of DCV protein biosynthesis and promotion of DCV formation (Fig. 2.3). Polypyrimidine tract-binding protein (PTB) is an RNA-binding molecule that regulates mRNA splicing, polyadenylation, 3′ end formation, internal ribosomal entry site-mediated translation, localization, and stability (Auweter and Allain, 2008). mRNAs of DCV proteins, such as insulin, insulinoma-associated protein 2 (ICA512/IA-2), CgA, and proprotein convertases, PC1/3 and PC2, all possess a PTB-binding site in their 3′-UTR (Knoch et al., 2004). Upon stimulation of secretion of insulin from pancreatic β-cells with glucose, PTBs were translocated from the nucleus to the cytoplasm where they bound the 3′-UTR of mRNAs encoding DCV proteins to stabilize them. As a consequence, translation and DCV protein levels were increased, leading to enhanced insulin secretory granule biogenesis (Knoch et al., 2004).

5.3.4. Posttranslational Regulation of DCV Biogenesis

Studies have indicated that CgA plays a very important role as a granulogenic protein in DCV biogenesis (see Section 2.3.2.2.). More recently, it was found that a C-terminal 26 amino acid fragment of CgA, named serpinin, which is secreted in an activity-dependent manner from AtT-20 cells, is an autocrine signal that regulates DCV biogenesis at the posttranslational level (Koshimizu et al., 2010). Investigations into the mechanism by which serpinin regulates DCV biogenesis revealed that it induced transcription of an mRNA encoding a protease inhibitor, protease nexin-1 (PN-1) that was found to be resident in the Golgi apparatus, although it was first identified as an extracellular protease inhibitor (Kim and Loh, 2006). DCV proteins appear to be constantly synthesized and degraded in the Golgi complex at steady state in PC12 and 6T3 cells. Enhanced degradation of DCV proteins occurred in CgA-deficient PC12 and 6T3 cells, and was prevented by reexpression of CgA. Furthermore, expression of PN-1 in 6T3 cells, in the absence of CgA, rescued DCV protein degradation and induced granule biogenesis. Serpinin and its pyroglutaminated form (pGlu-serpinin), which was more potent, enhanced PN-1 transcription through a receptor-mediated cyclic adenosine monophosphate (cAMP)–PKA signal transduction pathway (Koshimizu et al., 2011a, 2011b). Exogenous serpinin treatment of AtT-20 cells resulted in an increase in cAMP levels and PKA activity and caused translocation of the transcription factor, sp1, from the cytoplasm to the nucleus. Sp1 activated PN-1 transcription in the nucleus (Koshimizu et al., 2010, 2011b). This led to protection of DCV proteins from degradation, an increase in DCV proteins in the Golgi apparatus and promotion of secretory vesicle biogenesis. Treatment of PC12 cells with serpinin also increased PN-1 mRNA expression in PC12 cells, indicating that this mechanism is not unique to AtT-20 cells (Koshimizu et al., 2010). It was proposed that upon stimulation of DCV exocytosis and release of serpinins from (neuro)endocrine cells, expression of PN-1 would be increased, thereby stabilizing and increasing DCV protein levels in the Golgi complex and DCV biogenesis. This post-translational mechanism represents an efficient way to increase levels of all the proteins needed for DCV biogenesis after stimulated secretion (Fig. 2.3).

6. SV BIOGENESIS

SVs are formed from the PM by recycling of SV-specific proteins and lipids that are delivered to the PM via synaptic vesicle precursors (SVPs) or

SPTVs. The steps and protein and lipid components involved in SV biogenesis are reviewed below and summarized in Table 2.3. Three different modes of SV recycling from the PM have been proposed: kiss-and-run, complete fusion followed by clathrin-mediated endocytosis, or by bulk endocytosis (Ceccarelli et al., 1973; Heuser and Reese, 1973; Royle and Lagnado, 2003; Matthews, 2004). Kiss-and-run is thought to refill a readily releasable pool of SVs during mild stimulation while bulk endocytosis is for generation of SV reservoir after intense stimulation. In kiss-and-run mode, SV releases its content upon stimulation by forming a transient pore with the PM and then recycles by disconnecting itself from the PM (Ceccarelli et al., 1973; Fesce and Meldolesi, 1999; Harata et al., 2006). During kiss-and-run, SV membrane proteins and lipids are not intermixed with those in the PM. The kiss-and-run process has been observed during recycling of SVs in neurons and neuroendocrine cells as well as peptidergic vesicles in neuroendocrine cells (Klyachko and Jackson, 2002; Gandhi and Stevens, 2003; Zhang et al., 2007). In complete fusion followed by clathrin-mediated endocytosis, SVs collapse into the PM and release all their content. Subsequently, SV membrane proteins such as neurotransmitter transporters, are endocytosed via clathrin coating from the PM or early endosomes to form

Table 2.3 Protein and lipid components involved in SV/SLMV biogenesis

Step	Type	Proteins	Lipids	Discussed in section
Sorting at TGN	Sorting receptors, sorting adaptors	synaptophysin (VAMP2), ZnT3 (ClC-3), AP-2, AP-3, AP-4	Cholesterol, lipid rafts★	2.6.1, 2.6.2.2.2, 2.6.2.2.1
Vesicle endocytosis at PM	Clathrin based	Clathrin, AP-2, Stoned B, stonin 2, AP180, epsin, Dab2, PIPKIγ, PI4KIIα	PIP_2	2.6.2.2.1/ 2.6.2.1.2, 2.6.2.1.2
Vesicle sorting at EE	Non-clathrin based	AP-3, BLOC-1, Arf1, Casein kinase 1α-like kinase		2.6.2.2.1
Membrane curvature		Endophilin, amphiphysin	Cholesterol, lipid raft	2.6.2.2.1
Vesicle fission		Dynamin	PIP_2	2.6.2.2.1

EE= early endosome.
★Lipid rafts consist of platforms of lipids rich in cholesterol and sphingolipids.

empty SVs. In bulk (slow) endocytosis, endosomal intermediates are formed directly from the PM during intensive stimulation that causes excessive SV exocytosis (Richards et al., 2003; Royle and Lagnado, 2003; Evans and Cousin, 2007). Bulk endocytosis uptakes a relatively large PM area to balance sudden expansion of membrane during excessive exocytosis triggered by intense stimulation (Granseth et al., 2006; Wu and Wu, 2007). In the following sections, we will further discuss how proteins and lipids are sorted to SVs for biogenesis.

6.1. Sorting and Assembly of SV Proteins and Lipids

SVs contain a distinct set of proteins and lipids different from those in other membranous compartments, implying that there is selective sorting of SV-specific proteins and lipids away from non-SV components. The sorting appears to occur during formation of SVs from donor membranes such as the PM or early endosomes, or during formation of SVPs from the TGN. However, how SV-specific proteins and lipids are sorted to the SV is unclear. The selective sorting of SV proteins and lipids may start from specific protein–protein or protein–lipid interaction. SV-specific transmembrane proteins may recruit SV-specific proteins, interact with lipids across bilayer membranes, and bind to the cytoplasmic proteins involved in SV formation and localization. For example, synaptophysin mediates sorting of the SV-resident protein, synaptobrevin (VAMP2), but not syntaxin-1 and VAMP1, to synaptic vesicle precursors for transport to the presynaptic terminals in hippocampal neurons (Pennuto et al., 2003). The synaptophysin-based sorting of VAMP2 to the SVP is mediated by an interaction of synaptophysin with VAMP2 in the lumen of the TGN (Edelmann et al., 1995; Washbourne et al., 1995; Bonanomi et al., 2007). Then, the cytoplasmic domain of synaptophysin mediates targeting of synaptophysin/VAMP2-containing vesicles to the axonal terminal. Even in non-neuronal cells, synaptophysin reroutes VAMP2, but not synaptotagmin and syntaxin-1, from the PM to recycling endosomes (Bonanomi et al., 2007), suggesting that synaptophysin specifically mediates sorting of VAMP2 during its trafficking between membrane compartments. In addition, the interaction of synaptophysin with PM-localized cholesterol contributes to biogenesis of SLMVs, the SV counterpart in endocrine cells (Thiele et al., 2000), partly by mediating co-sorting of synaptophysin and VAMP2 (Galli et al., 1996; Mitter et al., 2003). Photoactivatable cholesterol specifically labels synaptophysin in the PM of MDCK (Madin Darby Canine Kidney) and PC12

cells upon UV irradiation while photoactivatable choline and inositol do not. Conversely, depletion of cholesterol reduces not only the levels of synaptophysin in SLMVs in PC12 cells but also the steady-state pool of SLMVs, suggesting that cholesterol is required for sorting of synaptophysin to SLMVs and for biogenesis of SLMVs. Hence, cholesterol-rich microdomains such as a lipid rafts appear to play a role as a platform for selective assembly of SV-specific membrane proteins and lipids.

The selective sorting or assembly of proteins and lipids during SV biogenesis may not necessarily lead to formation of SVs of uniform composition. Some microheterogeneity with respect to the types of proteins and lipids exists among SVs even within a single presynaptic bouton. The small differences in the composition of membrane proteins and lipids in donor membranes also contribute to generate the microheterogeneity in SVs. As a result, individual SVs with slightly different compositions are formed and have different functional characteristics, perhaps yielding different exocytosis rates (Valtorta et al., 2001; Newell-Litwa et al., 2007; Voglmaier and Edwards, 2007). However, even with different compositions, the majority of SVs in presynaptic boutons are usually of uniform size. Thus, one would expect that there should be some maturation process that would facilitate the generation of SVs of similar sizes. At present, little is known about the maturation process for SVs, however, high levels of continuous recycling of SVs during synapse maturation is proposed to contribute to vesicle size uniformity (Young and Poo, 1983; Matteoli et al., 1992; Antonov et al., 1999; Diefenbach et al., 1999). Another possibility is that SVs may be formed from uniformly sized microdomains on the donor membrane. In the following section, our discussion will focus on each protein and lipid sorted to SVs.

6.2. Molecules Involved in SV Biogenesis

6.2.1. Lipid Constituents

6.2.1.1. Bulky Lipids

Both SVs and synaptosomal PMs are rich in polyunsaturated fatty acids, such as docosahexanoic acid (Breckenridge et al., 1972). Gangliosides are usually found in synaptosomal membranes and bulky lipids such as cholesterol and sphingolipids are enriched in SVs. Depletion of cholesterol from the presynaptic membrane by treatment with methyl-β-cyclodextrin (MCD) reduces SV exocytosis at frog motor nerve endings, maybe by inhibiting the binding of proteins involved in docking and priming to the presynaptic

membrane (Petrov et al., 2010). Likewise, depletion of cholesterol from SVs by treatment of the nerve with MCD during prolonged stimulation decreases SV endocytosis, resulting in reduction in SV biogenesis at the motor nerve endings. Thus, cholesterol is required for both exocytosis and endocytosis of SVs. Additionally, cholesterol is involved in clustering of SNARE proteins (Chamberlain et al., 2001; Lang et al., 2001), SV proteins (Jia et al., 2006), and phosphatidylinositol 4,5-bisphosphate (PIP_2) (Pike and Miller, 1998) on the PM for facilitating SV biogenesis. Finally, the bulkiness of cholesterol appears to contribute to generation of higher curvature in the membrane (Gondre-Lewis et al., 2006; Kim et al. 2006), which facilitates formation of small sizes of SVs (Deutsch and Kelly, 1981; Martin, 2000; Thiele et al., 2000; Gondre-Lewis et al., 2006; Jia et al., 2006; Kim et al., 2006; Wasser et al., 2007). Since both cholesterol and sphingomyelin are major components of lipid rafts (Barenholz, 2004), lipid raft-like patches in the PM or in early endosomes are likely where most SV biogenesis occurs.

6.2.1.2. Phosphoinositides

Among several types of phosphoinositides, PIP_2 is the major lipid component of the vesicles and PM in SV exocytosis and endocytosis, i.e. SV biogenesis. PIP_2 in the PM provides a platform for assembly of clathrin coats during endocytosis (Cremona and De Camilli, 2001) and mediates actin nucleation via its interaction with actin-interacting proteins (Sechi and Wehland, 2000). AP-2 and the accessory clathrin adaptor protein, AP180, bind to the PM via their interaction with PIP_2 and initiate clathrin coating. Inhibition of the interaction of AP-2 with PIP_2 blocks recruitment of AP-2/clathrin to the PM, decreasing SV biogenesis (Jost et al., 1998). In the next steps, dynamin, a GTPase necessary for membrane fission and vesicular release, forms a polymeric stalk around the neck of endocytosed SVs and, upon GTP hydrolysis, mediates the pinching off of endocytosed SV from the PM (Cremona and De Camilli, 2001). The binding of dynamin to the neck of invaginated vesicle is also mediated by PIP_2 (Vallis et al., 1999).

The brain appears to have two major phosphatidylinositol 4-kinases (PI4K) that generate PIP_2: PI4K type II α (PI4KIIα) (Guo et al., 2003) and phosphatidylinositol phosphate kinase I-γ (PIPKIγ) (Wenk et al., 2001; Nakano-Kobayashi et al., 2007). PI4KIIα is associated with SVs at synapses and also found in other membranous compartments, such as the PM and Golgi complex in neurons (Guo et al., 2003). PIPKIγ, another PI4K concentrated in the brain, mediates formation of the clathrin coat and F-actin meshwork around SVs (Wenk et al., 2001). Synaptojanin-1 (synj-1),

a phosphatase of PIP_2, competes with PIPKIγ for binding to the PM and SVs to control the levels of PIP_2 during SV recycling (Wenk et al., 2001). Overexpression of the AP-2-interacting C-terminal domain of PIPKIγ inhibits SV endocytosis, suggesting that the interaction of PIPKIγ with AP-2 is important for clathrin coat formation and SV endocytosis. PIPKIγ KO (*PIPKIγ*$^{-/-}$) mice show various defects in neuronal activity and die shortly after birth (Di Paolo et al., 2004). *PIPKIγ*$^{-/-}$ mice have severe synaptic defects, such as decreased miniature current frequency and increased synaptic depression. Neurons from these knockout mice show defective SV endocytosis, fewer readily releasable SVs coated with clathrins and enlarged endosomes. It suggests that PIPKIγ is important for production of PIP_2 for clathrin-dependent biogenesis of SVs.

Stimulation of hippocampal neurons by membrane depolarization activates PIPKIγ that, in turn, increases the levels of PIP_2 at the presynaptic membrane, thus enhancing SV endocytosis (Nakano-Kobayashi et al., 2007). Similarly, NO generated by *N*-methyl-D-aspartate receptor activation increases SV endocytosis in a cyclic guanidine monophosphate (cGMP)-dependent manner, possibly by increasing the levels of PIP_2 in the presynaptic membrane of hippocampal neurons (Micheva et al., 2001, 2003) while decreasing PIP_2 in the cell body and dendrites (Micheva et al., 2001). The rate of SV endocytosis is increased in proportion to the levels of cGMP in stimulated neurons, while removing available PIP_2 suppresses the cGMP-induced SV endocytosis (Micheva et al., 2003). Thus, PIP_2 appears to be increased at the presynaptic terminals in an activity-dependent manner, thus enhancing SV endocytosis to prevent excessive expansion of the PM by SV exocytosis.

There are several phosphatases that decrease the levels of PIP_2 or other phosphoinositol phosphates in the vesicles and PM. Synj-1 is the major phosphatase important for SV recycling. Synj-1 is expressed predominantly in the nervous system and mediates conversion of PIP_2 and phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) to PI4P and $PI(3,4)P_2$ (McPherson et al., 1994; Haffner et al., 1997). The C-terminal SH3 domain of synj-1 interacts with various proteins involved in SV biogenesis and actin organization at the presynaptic terminals. Mice lacking synj-1 (*Synj1*$^{-/-}$ mice) die early after birth and show greater synaptic depression and accumulation of PIP_2 and clathrin-coated SVs at nerve terminals (Cremona et al., 1999; Luthi et al., 2001). In neurons, deletion of synj-1 causes slower SV endocytosis in response to both short and repetitive stimuli while having little effect on the exocytosis rate (Mani et al., 2007). Another study using *Synj1*$^{-/-}$ mice

shows that deletion of synj-1 decreases the pool of SVs available for the next round of exocytosis while increasing the pool of clathrin-coated SVs (Kim et al., 2002). It suggests that accumulated PIP_2 interferes with transition from clathrin-coated SVs to readily releasable SVs maybe by inhibiting removal of the clathrin coat. Furthermore, knockdown of synj-1 ($Synj1^{-/-}$) in zebra fish causes abnormal synaptic transmission at the cone photoreceptor synapses (Van Epps et al., 2004). The cone photoreceptor terminals of $Synj1^{-/-}$ zebra fish show accumulation of irregular actin polymers, uneven distribution of ~50% fewer readily releasable SVs, and enlarged endosomes. This finding suggests that abnormal accumulation of PIP_2 at the photoreceptor terminals increases random actin polymerization and inhibits SV biogenesis. Taken together, the function of synj-1 in lowering the levels of PIP_2 seems to be important not only for regulation of actin polymerization at presynaptic terminal but also for clathrin uncoating to generate the readily releasable pool of SVs.

6.2.2. Protein Constituents

6.2.2.1. Cytoplasmic Proteins in SV Biogenesis

Two AP complexes, AP-2 and AP-3, mediate biogenesis of SLMVs and SVs in endocrine cells and neurons; however, they use different mechanisms and donor membranes (Desnos et al., 1995; Faundez et al., 1998; Blumstein et al., 2001). AP-2 aids clathrin-mediated endocytosis of SVs from the PM while AP-3 mediates clathrin-independent SV biogenesis mostly from early endosomes. AP-3 mediates early recycling and maturation of SVs during synapse maturation in developing nerves (Zakharenko et al., 1999) whereas AP-2 facilitates SV recycling from the PM in mature presynaptic boutons.

AP-2 was well reviewed by Traub (2003). Briefly, the AP-2 heterotetramer consists of two large (α and β2), one medium (μ2), and one small (δ2) subunit. AP-2 binds to the PM via the interaction of its α subunit with PIP_2. Both α and β2 subunits interact with clathrin heavy chain, thus initiating formation of the clathrin lattice around the budding vesicle. The μ2 subunit of AP-2 functions as a cargo selector while the δ2 subunit is primarily structural, and recognizes YXXØ signals in the cytoplasmic domain of transmembrane protein cargo, thus facilitating endocytosis of the cargo. Various scaffolding and actin-interacting proteins such as AP180, epsin, and Dab2 collaborate with AP-2 for clathrin recruitment and assembly.

In *C. elegans*, unc-11, a homolog of mammalian AP180, is expressed specifically at nerve terminals (Nonet et al., 1999). Loss of unc-11 by nonsense mutations causes defective synaptic transmission at cholinergic,

glutamatergic, and GABA-ergic synapses. The unc-11 mutations specifically inhibit the targeting of synaptobrevin (VAMP2), but not other presynaptic proteins (e.g. synaptogyrin, synaptotagmin, Rab-3), to SVs. Moreover, the sizes of SVs at the presynaptic terminals in unc-11 mutants are larger than those in WT. These results suggest that AP180 may mediate the targeting of synaptobrevin to the presynaptic terminals and may control the size of SVs for proper synaptic transmission at cholinergic, glutamatergic, and GABA-ergic synapses in *C. elegans*.

In *Drosophila*, the loss-of-function mutation of Stoned B, an AP-2-interacting protein, causes formation of irregular shapes of vesicles and depletion of functional SVs at the larval motor nerve terminals (Stimson et al., 2001). The Stoned B mutation increases the retention of cysteine string protein and synaptotagmin at the presynaptic membrane and slows the rate of endocytosis of SV proteins from the membrane. Stonin 2, a human ortholog of *Drosophila* Stoned B, is enriched at the presynaptic terminal and is involved in clathrin-mediated endocytosis in rat cortical neurons (Walther et al., 2001). Stonin 2 directly interacts with and co-localizes with AP-2 and synaptotagmin 1 at the presynaptic terminal to facilitate AP-2-dependent internalization of synaptotagmin 1 to SLMVs and SVs in PC12 cells and cortical neurons, respectively (Diril et al., 2006).

AP-3 consists of δ, β3A/β3B, μ3A/μ3B, and σ3A/σ3B, mutations of which are linked to defects in biogenesis of endosomes, lysosomes, and lysosome-related organelles (Faundez et al., 1998; Dell'Angelica, 2009). AP-3 is expressed throughout the brain with especially high levels in the striatum and hippocampus, and is primarily localized to presynaptic axonal terminals (Newell-Litwa et al., 2010). AP-3 mediates SV biogenesis in an Arf1-dependent manner (Faundez et al., 1998). AP-3 was shown to mediate biogenesis of SLMV from early endosomes in a clathrin-independent manner in PC12 cells (Shi et al., 1998) while a later study showed that subunits β3A and β3B of AP-3 can interact with clathrin heavy chain (Peden et al., 2002). The recruitment of AP-3 to early endosomes for SLMV biogenesis is regulated by casein kinase 1α-like kinase that phosphorylates the β3A/β3B subunit of AP-3 (Faundez and Kelly, 2000). Inhibition of the kinase blocked recruitment of AP-3 to early endosomes and thus SLMV formation in PC12 cells. Mice deficient in AP-3 showed altered SV biogenesis in brain (Blumstein et al., 2001; Newell-Litwa et al., 2010). Mice lacking the AP-3 δ subunit showed enlarged SVs in the dentate gyrus but had minimal effects on presynaptic compartments in CA3 pyramidal neurons in the hippocampus and in the striatum (Newell-Litwa et al., 2010).

Thus, AP-3 seems to mediate SV or SLMV biogenesis from early endosomes in an Arf1-dependent and clathrin-independent manner, but its influence on these processes may be different depending on the brain region.

The protein complex, "biogenesis of lysosome-related organelles complex 1 (BLOC-1)", appears to collaborate with AP-3 to mediate SV biogenesis from early endosomes. BLOC-1 is an octamer made up of BLOS1–3, cappuccino, dysbindin, muted, pallidin, and snapin subunits (Di Pietro and Dell'Angelica, 2005). Both BLOC-1 and AP-3 are required for biogenesis of lysosomes and lysosome-related organelles. Mice with loss-of-function mutations of BLOC-1 or AP-3 develop Hermansky–Pudlak syndrome due to defective sorting of membrane proteins from endosomes to lysosomes and lysosome-related organelles (Li et al., 2004; Di Pietro and Dell'Angelica, 2005). AP-3 directly interacts with BLOC-1 via the interaction of its μ subunit with dysbindin, a subunit of BLOC-1 (Taneichi-Kuroda et al., 2009). Vesicular co-localization of AP-3 with dysbindin was observed in neurons of the dentate gyrus and throughout CA1 to CA3 subfields, and at presynaptic terminals and axonal growth cones of cultured hippocampal neurons. These findings suggest that AP-3 and BLOC-1 may collaborate to mediate SV biogenesis in the hippocampus. A recent study (Newell-Litwa et al., 2010) shows that mice lacking either pallidin (*Pldnpa/pa*) or muted (*Mutedmu/mu*) display similar neurological defects to those lacking AP-3, and BLOC-1 and AP-3 formed a complex in fractions enriched in brain nerve terminals (synaptosomes). However, BLOC-1 and AP-3 appeared to work sometimes in an incoherent manner (Newell-Litwa et al., 2010). Loss of function of the BLOC-1 alleles, Pldn(pa/pa) and Muted(mu/mu), had no effect while loss of AP-3 enhanced SV biogenesis in the dentate gyrus. Loss of AP-3 inhibited SV biogenesis in the striatum while loss of BLOC-1 had no effect. In addition, lack of dysbindin, the BLOC-1 subunit, in PC12 cells and hippocampal neurons showed various defects in neurosecretion (Chen et al., 2008). Overall, these mice showed depletion of readily releasable SVs, slower and fewer exocytic events, and larger sizes of SVs, while no change in the levels of proteins involved in the SV exocytic machinery was evident.

Some cytoplasmic proteins are involved in mechanical processes of SV biogenesis, e.g. invagination and fission of SVs. Endophilin-1, a protein enriched in brain presynaptic nerve terminals, participates in multiple stages in clathrin-coated endocytosis, from early membrane invagination to SV uncoating (Reutens and Begley, 2002). Using its C-terminal SH3 domain, endophilin binds to the proline-rich domains of synaptojanin and dynamin.

The N-terminus of endophilin binds directly to lipids on the membrane and mediates generation of membrane curvature by using its lysophosphatidic acid acyl transferase activity (Reutens and Begley, 2002). Synj-1, described in Section 2.6.2.1.2, regulates recruitment of dynamin, amphiphysin, and other proteins onto budding vesicles by changing PIP_2 levels in the PM (Mani et al., 2007). Amphiphysin is a major dynamin-interacting protein that is recruited to the neck of the endocytosing vesicles (Wu et al., 2009). Amphiphysin enhances membrane curvature as well as the GTPase activity of dynamin, thus facilitating invagination and fission of SVs from the PM. Dynamin is a GTPase required for biogenesis of SVs or SLMVs from the PM and early endosomes (Scaife and Margolis, 1997), and consists of four different domains—an N-terminal GTPase domain, a PH domain, a GED, and a C-terminal proline/arginine-rich domain. Dynamin is recruited to CCPs via the interaction of its proline/arginine-rich domain with the SH3 domain of amphiphysin (Hill et al., 2001). It mediates constriction of the neck between budding vesicles and the PM by forming spiral rings around the neck, resulting in vesicle fission.

In addition to the above mechanical proteins, the following proteins also affect SV biogenesis. α GDP dissociation inhibitor (αGDI) that regulates the GTPase activity and membrane targeting of Rabs is also important for SV biogenesis. Loss of function of αGDI impairs several steps in SV biogenesis and recycling in the hippocampus by causing abnormal accumulation of different Rabs throughout the intermediate compartments (Bianchi et al., 2009). Mice deleted of αGDI show defects in glutamate secretion from hippocampal synaptosomes, and short-term plasticity and memory. The small GTPase, Arf6, a mediator of endocytosis, affects SLMV biogenesis in PC12 cells (Powelka and Buckley, 2001). Expression of the constitutively active mutant of Arf6 increased the number of SLMVs while that of the dominant-negative Arf6 mutant decreased it.

6.2.2.2. Membrane Proteins in SV Biogenesis

The SV membrane contains several transmembrane proteins, such as synaptophysin, synaptobrevin, SV protein 2 (SV2), etc., and several types of neurotransmitter and ion transporters. There are four different classes of neurotransmitter transporters: VAChTs (ACh), vesicular glutamate transporter (VGLUT: glutamate), vesicular GAT (GABA/glycine), and VMATs (monoamines). The first three transporters are targeted to SVs while VMATs are found mostly in small peptidergic vesicles that are morphologically and dimensionally different from SVs. The specificity of targeting of SV

membrane proteins appears to be determined by specific motifs in the cytoplasmic domain of the proteins. For example, the di-leucine and Met-Leu (degenerate di-leucine) sequences are used for sorting of SV membrane proteins to SLMVs in an AP-3/Arf1-dependent manner (Blagoveshchenskaya et al., 1999). AP-3 is known to bind to the di-leucine motif directly (Odorizzi et al., 1998). In PC12 cells, point mutations of the di-leucine or Met-Leu motifs in the cytoplasmic domain of synaptotagmin-1 significantly reduce sorting of the proteins to SLMVs. Given that synaptotagmin-1 can interact with AP-2 via an interaction of its C2B domain with the μ2 subunit of AP-2 (Grass et al., 2004), sorting of synaptotagmin to SLMVs or SVs appears to be mediated by AP-2. We also cannot rule out the possibility that AP-3 (Odorizzi et al., 1998) may affect SV sorting of synaptotagmin-1 via its interaction with the di-leucine motif of synaptotagmin-1. VAMP2 appears to contain an SV-specific motif in its cytoplasmic domain (Hao et al., 1997). Deletion of VAMP2 amino acid residues 31–38 inhibited sorting of VAMP2 to SLMVs without affecting its endocytosis. By contrast, deletion of the VAMP2 amino acid residues 41–50 inhibited both SLMV targeting and endocytosis of VAMP2 whereas a point mutation (N49A) of VAMP2 increased the extent of targeting of VAMP2 to SLMVs by 200-fold. Thus, VAMP2 (synaptobrevin) appears to contain an SV-specific motif that is functionally involved in endocytosis.

The cytoplasmic domain of the zinc transporter, Zn transporter (ZnT) 3, has an AP-3-interacting domain that helps sorting of ZnT3 to SLMVs in PC12 cells (Salazar et al., 2004b). Inhibition of AP-3 blocked the sorting of only ZnT3 but not synaptophysin, but inhibition of AP-2 decreases the sorting of only synaptophysin but not ZnT3 to SLMVs. ZnT3 and synaptophysin are indeed found in different SLMV populations, accordingly, the SV sorting signals of synaptophysin and ZnT3 may be differentiated to bind specifically to AP-2 or AP-3, respectively, resulting in sorting to different SLMV pools. ZnT3 also helps sorting of a chloride channel, ClC-3, to SLMVs in an AP-3-dependent manner (Salazar et al., 2004a). The co-sorting of ZnT3 and ClC-3 to SLMVs and, likely, SVs is required to establish proper ionic composition inside of SLMVs/SVs. The nerve terminals of mossy fiber hippocampal neurons in mice deficient in AP-3 show significantly lower levels of ZnT3 and ClC-3. Thus, some neurological defects found in mouse models deficient in AP-3 may be caused by failure of co-sorting of ClC-3 and ZnT3 to SVs in the brain.

However, it appears that the cytoplasmic domains of SV membrane proteins do not share motifs specialized for sorting to SV (Prado and Prado,

2002; Voglmaier and Edwards, 2007). The cytoplasmic tail of synaptophysin has tyrosine-based repeats, which are similar to the internalization and lysosomal targeting sequences of lysosome-associated membrane proteins (Marks et al., 1996). VGLUT1 binds to endophilins via its proline-rich cytoplasmic domain and co-localizes with endophilins at the synaptic terminals of differentiated rat neocortical neurons in primary culture (De Gois et al., 2006; Vinatier et al., 2006). The interaction of VGLUT1 with endophilin is, however, not required for VGLUT1 endocytosis under moderate stimulation but is required under intensive stimulation. As such, deletion of the endophilin-binding domain of VGLUT1 inhibited endocytosis of VGLUT1 only during prolonged stimulation at high frequency.

Mutation or deletion of SV membrane proteins interferes with biogenesis and recycling of SVs in addition to loss of their innate functions. For example, mutations of VAMP2 or synaptotagmin-1 affected formation of SVs in glutamatergic nerve systems (Deak et al., 2004; Fremeau et al., 2004; Poskanzer et al., 2006; Wallen-Mackenzie et al., 2006). Loss of VAMP2 causes a delay in the refilling of readily releasable pools of SVs after stimulation without any drastic effect on the total number of presynaptic SVs (Deak et al., 2004). Synaptotagmin-1 was shown to regulate both the rate of formation and the size of SVs during endocytosis by using its calcium-sensing C2B domain (Poskanzer et al., 2006). The study showed that mutations of the polylysine motif in the synaptotagmin-1 C2B domain alter vesicle size while mutations in Ca^{2+}-binding aspartate residues affect only the rate of SV exocytosis. Moreover, loss of VGLUTs reduced the reserve SV pool and caused formation of larger, more elongated vesicles and tubulovesicular structures in neurons, which results in a decrease in fast excitatory synaptic activity (Fremeau et al., 2004; Wallen-Mackenzie et al., 2006). This decrease could not be reproduced by inhibition of vesicle filling or release (Augustin et al., 1999; Parsons et al., 1999; Verhage et al., 2000). Therefore, loss of VGLUTs appears to physically interfere with biogenesis of glutamatergic SVs.

7. GOLGI-TO-PM VESICLE TRAFFICKING

Post-Golgi transport of vesicles from the cell body where they are synthesized to the PM is microtubule dependent (Goldstein and Yang, 2000; Rudolf et al., 2001; Neco et al., 2003; Smith et al., 2003; Guzik and Goldstein, 2004). A recent review gives a good perspective of the functional significance of the orientation and posttranslational modification of

microtubules and actins, and microtubule motors in vesicular transport (van den Berg and Hoogenraad, 2012). Briefly, the plus ends of microtubules are oriented toward the axonal terminals and the minus ends toward the cell body. In distal dendrites, the plus ends of microtubules are oriented toward the dendritic terminals, but in proximal dendrites, the plus and minus ends of microtubules are intermingled. Conversely, F-actins are enriched in the axonal terminal boutons and dendritic spines and are not abundant along the axons and dendrites.

The microtubule motors include microtubule plus end-directed motors, the kinesins, which are the major conveyers for anterograde transport toward axonal and dendrite terminals, and cytoplasmic dynein, a minus end-directed motor, responsible for retrograde transport to the cell body or dendrite (Goldstein and Yang, 2000; Yano et al., 2001). Cytoplasmic dynein interacts with the multiprotein complex, dynactin, for proper function (Gill et al., 1991; Schroer and Sheetz, 1991). Dynactin is involved in both retrograde and anterograde transport on microtubules (Deacon et al., 2003; Dell, 2003). At the actin-rich matrix beneath the PM, myosin Va mediates transport of vesicles on actin filaments (Bridgman, 1999; Varadi et al., 2005). In the following section, and Fig. 2.4, we will provide an overview of post-Golgi transport of DCVs and SVs.

7.1. DCV Transport

7.1.1. Neuropeptide/Hormone-Containing Vesicles

The molecular mechanisms by which DCVs are transported along microtubules in peptidergic neurons and endocrine cells are poorly understood, but more insights are gradually emerging. In paraventricular nucleus neurons of the hypothalamus, DCVs containing thyroid-releasing hormone are transported by unidentified microtubule motor(s) (Alexander et al., 2005). In *C. elegans*, DCVs containing phogrin (IDA-1) and PC2 (Egl-3) are transported along both axons and dendrites by kinesin superfamily protein (KIF) 1A [uncoordinated (Unc)-104] (Zahn et al., 2004). Loss of Unc-104 prevents localization of phogrin and PC2 to nerve terminals. In two endocrine cell lines, AtT-20 and PC12, immature DCVs containing CgB are transported from the TGN to the proximity of the PM in a microtubule- and actin-dependent manner and mature at the actin-rich cortex beneath the PM (Rudolf et al., 2001). In anterior pituitary cells, both anterograde and retrograde transports of POMC/ACTH are mediated by a microtubule motor complex comprised of dynactin, cytoplasmic dynein, kinesin-2, and

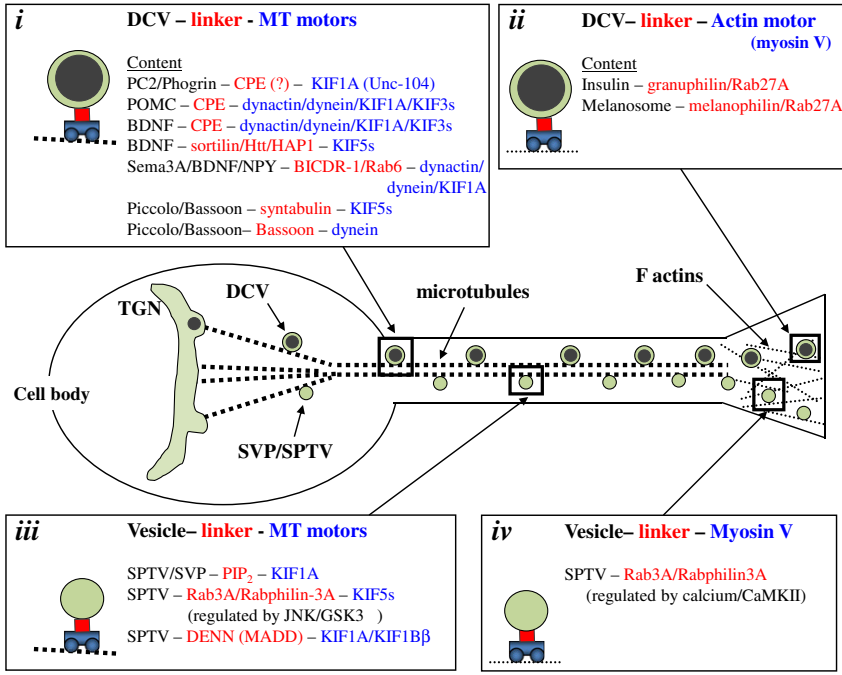

Figure 2.4 ***Proteins involved in post-Golgi transport of DCVs and SVs.*** Panel (i): Secretory and membrane proteins in DCVs are shown in black. CPE functions as a receptor (in red) for microtubule motors (in blue) on DCVs containing PC2/phogrin, POMC, and BDNF. Sortilin/Htt/HAP1 links BDNF vesicles to microtubule (MT) motors. BICDR-1 and Rab6 recruit motor proteins onto DCVs containing Sema3A/BDNF/NPY. PTV contains Piccolo, Bassoon, SNAP25, N-cadherin, Rim1, CgB, Rab3A, Munc18, syntaxin-1, and syntabulin (see Section 2.7.2.1). The interaction of syntabulin with kinesin-1 (KIF5s) mediates anterograde transport of PTV, and that of Bassoon with cytoplasmic dynein for retrograde transport. Panel (ii): Myosin Va mediates transport of DCVs along F-actins beneath the PM. Granuphilin and melanophilin connect myosin Va to DCVs via their interaction with Rab27A on DCV. Panel (iii): SVP and SPTV are transported by microtubule motors to maturing and matured synapses, respectively. SVPs and SPTVs contain SV proteins including synaptophysin, SV2, VAMP2, PIP_2 and/or DENN/MADD (see text). These proteins mediate recruitment of kinesin-3 (KIF1A) onto SVP and SPTV. Rab3A and rabphilin-3A recruit kinesin-1 onto SPTVs, which is under control of JNK and GSK3β. Panel (iv): Rab3A and rabphilin-3A connect myosin V to SPTV, which is regulated by calcium levels and CaMKII. For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

KIF1A (Park et al., 2008). The motor complex is linked to DCVs via their interaction with the cytoplasmic tail of CPE on DCVs. Competitive inhibition of overexpressed CPE cytoplasmic tail decreases both the velocity and real-time advancement of movements of POMC/ACTH vesicles.

Overexpression of CPE also decreases the localization of ACTH vesicles and regulated secretion of ACTH itself at terminals of anterior pituitary cells. In hippocampal neurons, the speed, but not the advancement of DCVs along neurites (both axons and dendrites), can be enhanced by PKA activation but not by depolarization with high K^+ (Washburn et al., 2002). A recent study reported an interesting observation: The polarity of DCV movement toward either axon or dendrite is determined not by the type of neuropeptide in DCVs but by the type of neuron (Ramamoorthy et al., 2011). In hippocampal GABA-ergic neurons of transgenic mice expressing NPY-green fluorescent protein (GFP), DCVs containing NPY-GFP were transported to both axons and dendrites whereas in hypothalamic GABA-ergic neurons, NPY-GFP-containing DCVs are found only in the axons. Hypothalamic primary neuron cultures transfected with NPY-Venus or immunostained for endogenous Agouti-related peptide showed specific targeting of these DCVs to axons only, unlike in hippocampal neurons.

In early neuronal differentiation, DCV transport required for neurite outgrowth in neurons is temporally regulated by Bicaudal-D-related protein 1 (BICDR-1) that interacts simultaneously with Rab6 on DCVs and with the microtubule-based motor complex that consists of dynactin, cytoplasmic dynein, and kinesin-3 (Schlager et al., 2010). In zebra fish, during early nerve development, the expression of high levels of BICDR-1 holds Rab6-containing DCVs at the pericentrosomal area causing inhibition of anterograde secretory transport and neuritogenesis. In fully developed nerve fibers, the expression of BICDR-1 is decreased, thus releasing Rab6-DCV for anterograde transport to future sites of neurite outgrowth.

In addition to microtubule motors, the F-actin motor, myosin Va, also affects movement of DCVs along axons and dendrites in cultured hippocampal neurons (Bittins et al., 2009). The dominant-negative form of myosin Va reduces retrograde, but not anterograde, transport of DCVs, suggesting that myosin Va facilitates only retrograde DCV transport. Rab27A connects myosin Va to DCVs for F-actin-based transport in pancreatic β-cells (Brozzi et al., 2011). Granuphilin-a, granuphilin-b, and rabphilin-3A are also a part of the Rab27A–myosin Va complex. Inhibition of binding of the granuphilins to Rab27A blocks myosin Va-mediated DCV transport. The role of rabphilin-3A in DCV transport remains unclear. In melanocytes, Rab27A connects myosin Va to melanophilin on melanosomes for transport of melanosomes to the PM (Izumi et al., 2003; Seabra and Coudrier, 2004). An effector protein, Rab3 GTP/GDP exchange protein, mediates correct targeting of Rab27A to melanosomes (Tarafder et al., 2011). Subsequent to

granuphilin/myosin Va-mediated DCV transport to the periphery, myosin Va and its interacting protein, exophilin8 (also known as myosin VIIA and Rab interacting protein/Slac2-c), mediate clustering and immobilization of insulin vesicles on F-actin in pancreatic β-cells (Mizuno et al., 2011).

7.1.2. Neurotrophin Vesicles

The dendritic secretion of neurotrophins [e.g. NGF, neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and BDNF] is important for neuritogenesis and synaptogenesis (Lessmann et al., 2003; Ernsberger, 2009). NGF, NT-3, and NT-4 are sorted to the CSP (Goldstein and Yang, 2000) while BDNF is transported through the RSP (Lou et al., 2005). Microtubule-based transport system is thought to mediate anterograde transport of neurotrophins to the dendritic terminals for secretion.

The microtubule-based transport of BDNF is better understood than other neurotrophins. The study of Huntingtin (Htt), a protein whose polyglutamine (polyQ) form is implicated in the pathogenesis of Huntington's disease, elucidates some aspects of BDNF transport. Htt forms a complex with Htt-associated protein-1 (HAP1) and dynactin (Gauthier et al., 2004; Kwinter et al., 2009). These studies showed that the Htt/HAP1/dynactin complex mediates anterograde transport of BDNF in hippocampal neurons. WT Htt enhanced and polyQ Htt inhibited BDNF vesicle movement along neurites. Deletion of HAP1 by siRNA eliminated Htt-mediated BDNF vesicle movement. Moreover, polyQ Htt sequestered HAP1–dynactin from binding to microtubules, effectively inhibiting BDNF vesicle movement, and this appears to contribute to the pathology observed in Huntington's disease. Indeed, in the brains of Huntington's disease patients, the interaction of polyQ Htt with HAP1 and dynactin is significantly increased, which likely results in reduction of BDNF transport and secretion. A recent study shows that sortilin stabilizes the htt–HAP1–dynactin complex on BDNF vesicles, which is proposed to prevent degradation of pro-BDNF and enhance processing of pro-BDNF (Yang et al., 2011). In addition, insulin growth factor-1 (IGF-1)/Akt-induced phosphorylation of Htt appears to determine the directionality of BDNF vesicle traffic (Colin et al., 2008; Zala et al., 2008). Phosphorylation of Htt at serine 421 facilitates recruitment of kinesin-1 to the Htt/HAP1/dynactin complex and enhances anterograde BDNF transport and secretion (Colin et al., 2008). By contrast, dephosphorylation of Htt causes detachment of kinesin-1 from Htt/HAP1/dynactin complex. This detachment makes cytoplasmic dynein-mediated retrograde transport toward the cell body more dominant over anterograde

transport of BDNF to the dendritic terminals (Colin et al., 2008). Interestingly, Ser421 phosphorylation also suppresses inhibitory effects of polyQ Htt and causes polyQ Htt to work as an enhancer of BDNF transport (Zala et al., 2008). Likewise, activation of IGF-1/Akt signaling pathway can restore BDNF vesicle transport and release in neurons containing only polyQ Htt.

The lumenal domain of CPE mediates sorting of the precursor of BDNF, pro-BDNF, to the RSP at the TGN (Lou et al., 2005) while the cytoplasmic tail of CPE on BDNF vesicles recruits a microtubule-based motor complex comprising of kinesin-2, kinesin-3, cytoplasmic dynein, and dynactin to transport the BDNF vesicle along dendrites of hippocampal neurons (Park et al., 2008). Unc-104 mediates anterograde transport of secretory vesicles containing CPE (egl-21) and ACh to the neuromuscular junction in *C. elegans* (Jacob and Kaplan, 2003). Loss of Unc-104 led to failure in localization of CPE/ACh-containing DCVs to the neuromuscular junction, resulting in reduced ACh secretion. KIF1A was also found to mediate BDNF transport in cultured hippocampal neurons (Lo et al., 2011) and in this study BDNF was sorted to CgA-containing DCVs. In addition, inhibition of histone deacetylase (HDAC) by trichostatin A is reported to indirectly enhance vesicular transport of BDNF (Dompierre et al., 2007). The inhibition of HDAC6 increases acetylation of α-tubulins of microtubules, which enhances binding of microtubule motors to microtubules and facilitates microtubule-based transport of BDNF. In the brains of Huntington's disease patients, the acetylation of α-tubulins is decreased, which would decrease binding of microtubule motors to microtubules, thus inhibiting BDNF transport. This study provides some premise for use of HDAC inhibitors as therapeutic agents for treatment of Huntington's disease.

7.2. Synaptic Vesicles

7.2.1. Piccolo–Bassoon Transport Vesicle

Structural proteins (e.g. Piccolo, Bassoon, Rim, and the liprins-α) that constitute the active zone (Schoch and Gundelfinger, 2006) at the presynaptic terminals appear to be carried to nascent presynaptic sites by Piccolo-Bassoon transport vesicles (PTVs) (Fenster et al., 2000; Zhai et al., 2001; Shapira et al., 2003; Fejtova and Gundelfinger, 2006; Regus-Leidig et al., 2009). PTVs purified from developing E18 rat brain have characteristics of DCVs with a diameter of ~80 nm and contain Bassoon, syntaxin-1, SNAP25, N-cadherin, Rim1, and CgB but not VAMP2, synaptophysin,

synaptotagmin, and GAT1 (Zhai et al., 2001). Another study shows that PTVs also contain Munc18 and Rab3A and that two or three PTVs are required for constitution of a new active zone (Shapira et al., 2003). PTVs aggregate with small clear vesicles during axonal transport to the active zone (Tao-Cheng, 2007). This aggregate has a diameter of 0.13–0.22 mm, and consists of one or two PTVs and five or six clear vesicles that express SV2, synaptotagmin, synapsin-1, and VAMP2 (synaptobrevin). A recent study showed that calneuron, a family protein of neuronal calcium sensor-1, regulates formation of PTVs from the TGN by sequestering PI4Kβ which mediates the synthesis of phospholipids required for formation of PTVs at the TGN (Mikhaylova et al., 2009). High calcium levels release PI4Kβ from the inhibitory binding of calneuron, thus facilitating PI4Kβ-mediated formation of PTVs at the TGN.

Bassoon, itself, plays a role in assembly of PTVs at the TGN. Both Bassoon and Piccolo are associated with the TGN in rat hippocampal neurons and PTVs cannot be formed if association of Bassoon with the TGN is blocked by low temperature, or if a Bassoon mutation inhibits binding of Bassoon to the TGN (Dresbach et al., 2006). In transgenic mice containing mutated Bassoon, fewer PTVs are formed and the protein levels of Piccolo and other active zone proteins are decreased in retinal photoreceptor ribbon synapses (Regus-Leidig et al., 2009). Despite the importance of Bassoon and Piccolo in the assembly of the active zone, mice deficient in both Bassoon and Piccolo do not show any detectable electrophysiological defects (Mukherjee et al., 2010). Loss of both Bassoon and Piccolo also does not affect glutamatergic and GABA-ergic synaptic transmission in hippocampal slices and neurons, but clustering of SVs at the active zone is decreased. These findings suggest that Bassoon and Piccolo play a redundant role and are not necessary for neurotransmission.

Kinesin-1 is a major microtubule motor that transports PTVs from the cell body to the distal axonal terminals. Transport of SVPs, however, is mostly mediated by kinesin-3 (KIF1A) (Goldstein et al., 2008). Syntabulin links kinesin-1 to syntaxin-1 on PTVs in axons of developing neurons (Cai et al., 2007). Knockout of syntabulin causes accumulation of PTVs in the cell body and reduction of SVs at the axonal terminals, resulting in a reduction in the amplitude of postsynaptic currents. Conversely, Bassoon of PTVs directly interacts with light chains (DLC1 and DLC2) of cytoplasmic dynein for bidirectional movement of PTVs along the axon (Fejtova et al., 2009). Inhibition of this Bassoon–dynein interaction by using a competitive dominant-negative peptide of Bassoon interferes with correct

targeting of PTVs to nascent presynapses and the neuron consequently forms immature presynapses. This study also showed that myosin Va associated with PTVs along with cytoplasmic dynein.

7.2.2. Synaptic Vesicle Precursor/Synaptic Protein Transport Vesicle

In developing axons, SVPs also called SPTVs (Park et al., 2011) deliver SV proteins, such as synaptophysin, to the PM constitutively (De Camilli and Jahn, 1990; Kelly, 1991). In adult axons, SPTVs are still the major vesicles that deliver SV proteins to the PM. Rab3A and its effector, rabphilin-3A, were shown to mediate axonal transport of SPTV (Li et al., 1995; Li, 1996). Rab3A appears to mediate transport of synaptophysin- and synapsin-1-containing SPTVs in both cell bodies and axonal terminals of motor neurons (Li et al., 1995). Rabphilin-3A appears to be involved in SPTV transport at the proximal segments of axons but not at the distal segments in spinal nerves (Li, 1996). DENN (MADD), an Rab3 GTP–GDP exchange factor, interacts directly with the stalk of KIF1A or KIF1Bβ and regulates the function of KIF1s in SVP transport in hippocampal neurons (Niwa et al., 2008). Overexpression of the dominant-negative domain or knockdown of DENN inhibits transport of Rab3-containing SPTVs to the terminals. KIF1-bound DENN (MADD) binds via its N-terminal domain to SPTV-associated Rab3-GTP more strongly than free Rab3-GDP, and forms a KIF1–DENN–Rab3–GTP complex on SPTVs which enhances axonal SVP transport. This suggests that GDP/GTP exchange on Rab3 may regulate KIF1A-mediated SVP transport. PIP_2 is also involved in SV transport as noted in the previous section. PIP_2 is required for binding of Unc-104 (KIF1A: kinesin-3) to SVs for axonal SV transport along microtubules of *C. elegans* (Klopfenstein et al., 2002; Klopfenstein and Vale, 2004; Kumar et al., 2010). The PH domain of Unc-104 directly binds to PIP_2 on the SV membrane. Loss of binding of Unc-104 to PIP_2 not only blocks axonal SV transport but also causes ubiquitin-mediated degradation of Unc-104 (Kumar et al., 2010).

Fast axonal transport of VAChT-containing SPTVs has been observed in motor, sensory, and autonomic axons of the peripheral nervous system (Li et al., 1998). Using the method "stop-flow/nerve crush," the authors demonstrated that small SPTVs containing VAChT, SV2, and synaptophysin are present in motor axons and in autonomic postganglionic neurons. In cholinergic SN56 cells, VAChT appears to first travel to the PM in the soma, recycled to early/recycling endosomes, and finally departs to the axon for function at the terminal (Santos et al., 2001). A VAChT mutant inept for

endocytosis is retained in the PM of the cell body and is never routed to early/recycling endosomes and SPTVs in the neurites. Synaptophysin is carried in SPTVs to neurite terminals of sympathetic neurons but was shown to use an alternative route to the terminals through small DCVs that contain VMATs and dopamine β-hydroxylase (Bauerfeind et al., 1995). However, small DCVs do not appear to be the major vehicle for delivery of synaptophysin to the nerve terminals of sympathetic neurons. During exit from the TGN for axonal transport, synaptophysin guides sorting of VAMP2, but not VAMP1 and syntaxin-1, to SPTVs in hippocampal neurons (Pennuto et al., 2003). The cytoplasmic domains of VAMP2 and synaptophysin form heterodimers, which promote the sorting of VAMP2 to SPTVs. Since VAChT exists with synaptophysin in the same vesicles in cholinergic neurons and chromaffin cells (Varoqui and Erickson, 1998; Park et al., 2011), it is likely that synaptophysin and VAChT travel together in the same SPTV during axonal transport.

Axonal transport of SPTVs is decreased during the pathogenic processes of neurodegenerative diseases, such as Alzheimer's disease (AD). c-Jun N-terminal kinase (JNK), a signaling protein activated by neuroinflammation, appears to play a major role in inhibition of axonal SPTV transport (Stagi et al., 2005, 2006). It is postulated that tumour necrosis factor (TNF) activates JNK and increases the levels of phospho-JNK along the neurites of hippocampal neurons (Stagi et al., 2006). Activated JNK, in turn, phosphorylates and dissociates KIF5B, a heavy chain of kinesin-1, from microtubules, resulting in inhibition of kinesin-1-mediated axonal transport of SPTV containing synaptophysin and mitochondria in cultured primary neurons. Pretreatment of hippocampal neurons with a JNK inhibitor, SP600125, restores binding of KIF5B to microtubules and consequently axonal transport of SPTVs. NO that is generated excessively by activated microglia also activates JNK and inhibits axonal transport of synaptophysin-containing vesicles in primary neurons (Stagi et al., 2005). In addition, loss of presenilin-1 (PS1), which is implicated in AD neuropathology, interferes with kinesin-1-mediated axonal transport of synaptophysin-containing SPTVs (Pigino et al., 2003). Loss of PS1 in mice ($PS1^{-/-}$) generates excessive amounts of active GSK3β that over-phosphorylates the kinesin light chain of kinesin-1, resulting in continuous dissociation of kinesin-1 from SPTVs and inhibition of axonal SPTV transport.

In addition to microtubule-based motors, myosin Va also plays a role as transporter of SPTVs and SVs through the actin cortex at the axonal terminal. At nerve endings, under resting conditions, myosin Va associates

with SVs via the direct interaction of its tail domain with SV-localized synaptobrevin-2 and synaptophysin, and this interaction immobilizes SVs (Prekeris and Terrian, 1997). However, upon Ca^{2+} influx due to membrane depolarization, myosin Va dissociates from SVs, allowing free movement of SVs toward the PM for tethering and docking. The GTP-bound form of Rab3A directly binds both the tail domain of myosin Va and rabphilin-3A. This was shown in extracts of giant squid axoplasm as well as mouse frontal cortex (Wollert et al., 2011). Rab-GDI inhibits myosin Va-mediated SV movement while calcium/calmodulin-dependent kinase II (CaMKII) enhances the binding of Rab3A to myosin Va, and myosin Va-mediated SV movement. Thus, Rab3A may regulate myosin Va-mediated transport of SV and likely SVP/SPTV as well through the actin cortex at the active zone in a GTP/CaMKII-dependent manner.

8. VESICLE TETHERING AND DOCKING FOR EXOCYTOSIS

Since priming and exocytosis of DCV and SV are well reviewed elsewhere (Sorensen, 2005; Stojilkovic, 2005; Rizo et al., 2006; Malsam et al., 2008; Parpura and Mohideen, 2008; Martin, 2012), we will not discuss SNARE-mediated priming and exocytosis. Instead, we will review recent findings of molecular mechanisms involved in docking and tethering of DCVs and SVs.

8.1. Exocytosis of DCVs

During DCV exocytosis, the optimum levels of PIP_2 should be maintained for proper exocytosis (Milosevic et al., 2005). Overexpression of the phosphatase synj-1 in chromaffin cells decreases the levels of PIP_2 in the PM, reduces the readily releasable pool of DCVs, and inhibits regulated secretion of catecholamines, whereas increased PIP_2 enhances the priming of DCVs and DCV exocytosis.

Many of the resident proteins of the actin matrix involved in vesicular trafficking and localized on membranes of vesicles are also important for the docking and exocytosis process. In DCVs, Rab3A and Rab27 mediate tethering and docking of DCVs at the PM of neurons and endocrine cells (Darchen et al., 1995; Fukuda, 2008; Tsuboi, 2009). Rab27A, however, plays a dominant role in DCV exocytosis and is required, for example for glucose-induced insulin secretion in pancreatic β-cells (Aizawa and Komatsu, 2005; Kasai et al., 2005). The decreased

docking of insulin DCVs in Rab27A KO mice was attributed to a reduction in the interaction between granuphilin on DCVs and syntaxin-1a on the PM.

Granuphilins are specifically expressed in pancreatic β-cells, chromaffin cells, and the pituitary gland, but not in pancreatic α cells and brain, and appears to negatively regulate exocytosis (Wang et al., 1999; Fukuda et al., 2002; Yi et al., 2002; Fukuda, 2003; Kasai et al., 2008). Granuphilin-a may be specific for DCV and not SLMV exocytosis because it was found only in DCVs in pancreatic β-cells (Wang et al., 1999). Knockout of granuphilin increases both spontaneous and stimulated fusion of DCVs to the PM, which decreases the number of DCVs proximal to the PM. The inhibitory effect of granuphilin is also lost when binding of granuphilin to Rab27A or syntaxin-1 is blocked. These data and others (Brzezinska et al., 2008; Kariya et al., 2010) strongly suggest that loss of Rab27s or their effectors decreases the docking/fusion of DCVs to the PM.

In PC12 cells, rabphilin and Noc2, like granuphilin, are also recruited to DCVs via their interactions with Rab27A (Fukuda et al., 2004) and play a role in DCV docking. In one study, overexpression of rabphilin inhibits high KCl-dependent NPY secretion, but another shows that overexpression of WT rabphilin significantly increases DCV docking without affecting the exocytosis kinetics (Tsuboi, 2009). Hence, it is unclear whether rabphilin is a negative or a positive regulator of DCV exocytosis. In PC12 cells, Noc2 negatively regulates Rab3A-mediated DCV exocytosis (Tsuboi, 2009). Moreover, overexpression of Noc2, but not Noc2 mutants unable to bind Rab3a, inhibited Ca^{2+}-triggered exocytosis (Haynes et al., 2001). In addition to Rab3A and Rab27, there are other Rabs, such as Rab11B and Rab18, that contribute to the mechanisms of regulated DCV exocytosis (for more on these Rabs, see Khvotchev et al., 2003; Vazquez-Martinez et al., 2007).

CAPS, the Ca^{2+}-dependent activator protein for secretion, was shown in *C. elegans* to specifically dock DCVs (Hammarlund et al., 2008). It contains a dynactin-1 binding domain and a DCV binding domain which promote formation of the open form of syntaxin, important for tethering, docking and priming of DCVs (Hammarlund et al., 2008; Lin et al., 2010). In granule neurons of developing cerebellum, CAPS2 was found to be associated with DCVs containing NT-3, BDNF, and CgB (Sadakata et al., 2004), and shown to play a role in increasing activity-dependent secretion of these neurotrophins in granule neurons and PC12 cells (Wassenberg and Martin, 2002). This suggests that CAPS mediates the activity-dependent

secretion of neurotrophins for survival and differentiation of cerebellar granule neurons.

8.2. Exocytosis of SVs

Like DCVs, various Rab proteins are involved in tethering and docking of SVs to the presynaptic terminals. SV recycling and exocytosis is associated with 11 different Rab proteins, but Rab3a and Rab27b have emerged as the two major Rabs involved in Ca^{2+}-induced neurotransmitter release in the nervous system (Takai et al., 1996; Geppert et al., 1997; Mahoney et al., 2006; Pavlos and Jahn, 2011). Both Rab3A and Rab27B tightly associate with SVs under resting conditions (Yu et al., 2008; Pavlos and Jahn, 2011). This association is differentially regulated during Ca^{2+}-induced exocytosis: Rab3A is dissociated from SVs while Rab27B maintains its tight association (Pavlos et al., 2010). After Ca^{2+} influx, Rab-GDI easily retrieves dissociated Rab3A but not Rab27B from SVs, suggesting that only Rab3A is regulated by the GTP/GDP switch during Ca^{2+}-induced SV exocytosis.

In Rab3A KO mice, SV docking and exocytosis is impaired but not blocked. The readily releasable SV pool was intact but the rate of Ca^{2+}-induced fast phase SV fusion was enhanced (Geppert et al., 1997). After depolarization, however, Rab3A KOs had fewer docked SVs and delayed replenishment of these docked SVs compared to normal terminals. This is consistent with the phenotype of Rab3a KOs which display defective short- and long-term synaptic plasticity in the hippocampus, altered circadian motor activity with more active exploratory behavior (D'Adamo et al., 2004).

The major role of CAPS is in DCV docking/priming to the PM but they also function SV exocytosis. CAPS-1 and CAPS-2 are required for fast phasic transmitter release by enhancing vesicle priming (Jockusch et al., 2007). CAPS-deficient neurons have very few SVs competent for fusion, resulting in defects in fast phasic synaptic release, suggesting that CAPS is required for the makeup of readily releasable pools of SVs.

9. NEURONAL DISORDERS INVOLVING SV DEFECTS

9.1. Schizophrenia

Neuropeptide and neurotransmitter dysregulation is widely implicated in pathogenesis of schizophrenia (SCZ). Postmortem brains from schizophrenic patients had decreased levels of somatostatin, NPY, vasointestinal

peptide (VIP) and cholecystokinin (CCK) (Gabriel et al., 1996). These results were confirmed for somatostatin, NPY and CCK by microarray analysis studies, which also showed a significant reduction in transcripts of neuropeptides associated with GABA-ergic neurons and GABA metabolism (Hashimoto et al., 2008). These data indicate a role for impaired GABA synthesis and signaling, as well as neuropeptides, in patients with SCZ.

When the SV marker synaptophysin is studied, results are in conflict with the idea of a global presynaptic reduction of proteins, although VAMP was proposed to be downregulated in SCZ brains (Halim et al., 2003). DCV markers CgA, CgB and SgII, however, were shown to be altered in regionally specific ways in SCZ brain (Willis et al., 2011). Deficiency of these proteins known to be involved in DCV biogenesis (see Section 2.5.2) might cause impairment of packaging of neuropeptides and BDNF, resulting in feedback inhibition and downregulation of synthesis of these molecules. As previously discussed, BDNF is a key regulator of synaptic plasticity and therefore, cognitive function, and thus is proposed in the pathogenesis of SCZ. BDNF mRNA and protein are downregulated in the dorsolateral prefrontal cortex of schizophrenic patients, indicating that intrinsic and target neurons of the cortex may receive dampened trophic support provided by BDNF (Weickert et al., 2003; Wong et al., 2010). It remains to be investigated whether transcriptional control of BDNF genes and stabilization of its mRNA and protein at the TGN can influence packaging and availability at the synapse.

9.2. Alzheimer's Disease

From a histopathological point of view, vesicular deficiencies have been a hallmark in AD and it is precisely the lack of synaptic function that manifests in affected individuals (Shankar and Walsh, 2009; Waites and Garner, 2011; Willis et al., 2011). Cellular deficiencies include defective sorting and processing of amyloid precursor protein (APP) at the TGN and in endosomes, as well as defects in formation of vesicles (Small and Gandy, 2006). Lipid rafts are involved in aggregation of proteins at the TGN, in vesicles and at the PM. Via their aggregative function, these lipid rafts mediate the segration of γ-secretase and its substrate, the C-terminal fragment (CTF) of APP, for cleavage in the TGN. Depletion of cholesterol, a major constituent of lipid rafts, disrupts the proximity of association of γ-secretase and CTF, and induces non-plaque-forming alpha-secretase activity (Vetrivel and Thinakaran, 2010). Granins such as CgA, CgB, and SgII are extensively co-localized with amyloid-β plaques in the hippocampus

of mouse models of Alzheimer's and in human postmortem AD brain, suggesting a potential role of these granins in AD pathology. (Willis et al., 2011). CgA activates microglial stress cascades of neurotoxicity, an effect amplified when combined with Aβ (Twig et al., 2005; Hooper and Pocock, 2007; Hooper et al., 2009), and may be an avenue of intervention in AD patients to reduce neurotoxicity. A precise role for the granins in vesicular defects of Alzheimer's is yet to be revealed, however, recent analysis of granin peptides (CgA, CgB, SgII), compared to APP and Aβ in the cerebrospinal fluid supports the proposition that APP and Aβ may be primarily secreted through a RSP, with enzymes separate from BACE1 within the constitutive pathway (Mattsson et al., 2010). If validated, this idea will be consistent with the findings that APP was found at neuronal terminals in vesicular organelles not associated with SV markers (Ikin et al., 1996), and soluble APP undergoes stimulated release in neuroendocrine bovine chromaffin cells in response to cholinergic stimulation (Efthimiopoulos et al., 1996).

APP is processed by several proteases: α-secretase to generate non-amyloidogenic peptides, β-secretase to generate amyloidogenic competent peptides, followed by γ-secretase to cleave α-CTF or βCTF, respectively (Vetrivel and Thinakaran, 2006; Marks and Berg, 2010). It is the production and secretion of Aβ within the amyloidogenic pathway to form amyloid plaques that is detrimental to neuronal function in disease states. The next paragraphs will primarily address recent work addressing molecules functioning at the TGN.

In terms of vesicular budding and sorting at the Golgi, there are many Alzheimer protein-specific defects reported. A marked reduction in PLD activity resulting in dysregulated membrane phospholipid metabolism is a key feature in AD (Kanfer et al., 1986), and downregulation of PLD1 is associated with increased beta-amyloid plaque formation. Presenelin 1 (PS1), mutations in which causes inherited familial AD, is one of four members of the γ-secretase complex that regulates processing of β-amyloid precursor protein (βAPP) to generate Aβ42, the plaque-forming protein in AD (Citron et al., 1997; Chau et al., 2012). By binding to PLD1, PS1 recruits it to the Golgi/TGN as a negative regulator of Aβ formation. Overexpression of PLD1 decreased the association of γ-secretase components whereas down-regulation of PLD1 by short hairpin RNA (shRNA) increased the association of γ-secretase components as well as its proteolytic activity (Cai et al., 2006). Furthermore, PLD1 itself can increase vesicular budding and trafficking of βAPP, even while it reduced Aβ generation by inhibiting cleavage of βAPP at the TGN (Cai et al., 2006).

AP-4 is the non-clathrin-associated AP that mediates sorting of transmembrane cargo in post-Golgi compartments. Bonifacino's group recently showed that the μ4 subunit of AP-4 interacts with APP, and mediates trafficking of APP to endosomes (Burgos et al., 2010). Disruption of this interaction reduces localization to the endosome and increases γ-secretase activity to produce the pathogenic Aβ peptide (Burgos et al., 2010). Therefore, AP-4 can be said to be protective against Aβ production by targeting APP away from γ-secretase activity.

Estrogen's neuroprotective effects and effectiveness as reducing β-amyloid plaques in AD are attributed to its stimulation of vesicular budding of APP-containing vesicles from the TGN and into post-Golgi compartments. 17β-estradiol recruits the small GTPase, Rab11, to the TGN from the cytosol and can induce the budding of APP-containing vesicles from the TGN by influencing the redistribution of PI composition at the TGN membrane, and into post-Golgi vesicles. As discussed previously, changes to the lipid concentration in the TGN can induce increased membrane curvature so as to promote budding from the TGN (Huttner, 2000; Gondre-Lewis, 2006). Thus, PI was detected in post-Golgi compartments after treatment with estrogen.

The mechanisms discussed above provide a foundation to develop therapeutic strategies that manipulate lipid raft components, budding and processing at the Golgi and endosomes to ameliorate the severity of the disorder by decreasing Aβ formation. One strategy is to increase APP-containing vesicles. If less APP is available in the TGN to be later converted to Aβ, then the secretion of Aβ and plaque formation will be reduced, which could be neuroprotective.

9.3. Fragile X Syndrome

Fragile X is an x-linked inherited intellectual disorder caused by the silencing of the *fmr1* gene due to the hypermethylation of its promoter (Oberle et al., 1991). The resulting absence of fmr1 protein reduces synaptic plasticity by reducing long term potentiation of synaptic inputs, a condition that could be restored with exogenous BDNF although basal BDNF and pro-BDNF levels are comparable to control (Lauterborn et al., 2007). Based on later studies addressing regulated secretory release of neuropeptide and neuromodulators, it seems that the cargo packaged in DCVs, possibly including BDNF, does not undergo effective regulated secretion. The GTPase Rab3A involved in vesicle docking and fusion is significantly

downregulated in cerebral cortex of *fmr1*$^{-/-}$ mice, which exhibited severely impaired—no peaks by MALDI-MS—neuropeptide release even in the absence of significant differences in DCV density (Annangudi et al., 2010). GABA-ergic receptor- and somatostatin-expressing neurons and the size of pancreatic islets were significantly decreased in the *fmr1* KO mice which had higher plasma glucose levels, indicative of a more widespread effect on neuroendocrine function (El Idrissi et al., 2012). These effects on endocrine function, especially the stress response, are exacerbated in patients with both autism spectrum disorder and Fragile X (Roberts et al., 2009). Although a mental retardation disorder, Fragile X patients experience neuroendocrine symptoms such as abnormal stress responses, sleep abnormalities, and growth patterns, but the number of studies addressing the biology of these aberrations are few (Hessl et al., 2004; El Idrissi et al., 2012). Further studies in the biogenesis, morphology and secretory mechanisms in *fmr1* KO may reveal a significant role for the gene product in SV and DCV biology, although currently there is no evidence for such a role for fmr1.

9.4. Smith–Lemli–Opitz Syndrome

Smith–Lemli–Opitz syndrome is an autosomal recessive genetic disorder caused by a mutation in the gene encoding 7-dehydrocholesterol reductase (7-DHC), an enzyme necessary for the conversion of 7-dehydrocholesterol to cholesterol during cholesterol biosynthesis (Porter, 2008). SLOS is one of many congenital malformations, inclusive of lathosterolosis (Brunetti-Pierri et al., 2002), where patients make insufficient enzymes to catalyze the synthesis of the final product, cholesterol. Cholesterol plays an important role as precursor to steroids and secreted hormones in the adrenal medulla and gonads. When the proper synthesis of these hormones is impaired, packaging of cargo into secretory vesicles is reduced, and this in turn attenuatess the formation of DCVs. In addition, cholesterol is an important and integral component of membranes. In SLOS mice, there is a defect in the ability of DCVs to form properly (Gondré-Lewis et al., 2006). We have shown using artificial membranes that cholesterol is required for the rigidity of the membrane, a feature that cannot be duplicated by 7-DHC or lathosterol substitution. Consistently, a prominent feature of cholesterol-deficient mice was the decreased number or total absence of secretory vesicles in the anterior pituitary, adrenal gland, and endocrine and exocrine pancreas. DCVs in mice lacking cholesterol had abnormally large, immature, unpinched vesicles with no dense core. Cells from these mice were

functionally impaired in their ability to respond to stimulation via the RSP; however, this function could be restored when cholesterol was supplied to the cells (Gondre-Lewis et al., 2006).

10. CONCLUSIONS AND FUTURE DIRECTIONS

The works reviewed here highlight the dynamics of SV and DCV biology which allow the brain and professional secretory cells of the endocrine and exocrine systems to respond to physiological stimuli. Regardless of the tissue type, there is a shared mechanism for SV or DCV targeting to the PM. The content of DCVs are sorted to and concentrated in specific regions of the TGN by the actions of granins, CPE, and V-ATPases. DCV budding from the TGN is dependent on cholesterol, lipid rafts, PAs and/or DAG, as well as a number of kinases, GTPases and APs. Maturation of DCVs is facilitated by acidification by V-ATPase and condensation of contents by actions of water channels, the AQPs. DCVs are trafficked along microtubule motors, dynactin/dynein and the KIF proteins, and on actin motor, myosin V. When at the periphery, they release their hormone, neuropeptide, or enzyme cargo and the DCV membrane components are recycled.

Recently, we have identified a post-Golgi sorting compartment which carries membrane and soluble proteins associated with both DCVs and SPTVs, implying a shared machinery to deliver synaptic and peripheral proteins (Park et al., 2011). Studies in the future will provide more clarity on the sorting mechanisms of SPTV components at the TGN as compared to DCV components, and the extent of convergence and divergence of the pathways.

For the multitude of neuropeptides and hormones with biological action packaged within DCVs, understanding of the mechanisms of genetic, transcriptional, posttranscriptional, translational, and posttranslational regulation of DCV biogenesis is steadily emerging. Our group and others have begun to elucidate some steps where potential key regulators such as REST/NRSF at the genetic level in neurons and astrocytes and IA-2 at the transcriptional level in insulin-secreting β-cells are involved. Many DCV proteins have been identified as possessing a PTB-binding site, and thus PTB may function as a master posttranscriptional stabilizer of DCV-associated mRNA to increase translation. The CgA-derived peptide, serpinin, through PN-1 gene regulation, can increase availability of DCV proteins at the TGN

by controlling protein degradation and hence promote DCV biogenesis. Although these findings are important in understanding some mechanism of DCV biogenesis, there is great paucity of information regarding genetic control of this process.

SVs follow a somewhat similar path as DCVs in that a mature, filled SV has components from SVPs or SPTVs which were formed at the TGN and trafficked to the periphery with the aid of KIF1A and other KIF family of motors. The lipid PIP_2, along with cholesterol and lipid rafts, is critical for SVs to bud and pinch from the PM. These recycled SVs then interact with SPTVs and acquire components important for responding to subsequent stimuli. Unlike for DCVs, an abundance of research regarding the mechanisms involved in SV biogenesis has been conducted. Still, issues involving *how* SV-specific proteins and lipids are sorted to SVs need further research. There is also very little known about SVPs and transport vesicles, and the nature of their interaction with SVs.

We have discussed a few neurological disorders with impairment of vesicular budding and/or trafficking. In some cases, the defective, mutated proteins and lipids responsible for the various pathologies give insight into the complexity of the interplay required for vesicular biogenesis, trafficking, docking, and release. These disorders may expand the repertoire of molecules believed to be involved in vesicular function and, together with this expanding field in the future, may provide avenues of intervention for amelioration of disease.

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CHAPTER THREE

Effect of Viral Infection on the Nuclear Envelope and Nuclear Pore Complex

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Contents

Abstract

The nuclear envelope (NE) is a vital structure that separates the nucleus from the cytoplasm. Because the NE is such a critical cellular barrier, many viral pathogens have evolved to modulate its permeability. They do this either by breaching the NE or by disrupting the integrity and functionality of the nuclear pore complex (NPC). Viruses modulate NE permeability for different reasons. Some viruses disrupt NE to deliver the viral genome

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into the nucleus for replication, while others cause NE disruption during nuclear egress of newly assembled capsids. Yet, other viruses modulate NE permeability and affect the compartmentalization of host proteins or block the nuclear transport of host proteins involved in the host antiviral response. Recent scientific advances demonstrated that other viruses use proteins of the NPC for viral assembly or disassembly. Here we review the ways in which various viruses affect NE and NPC during infection.

1. INTRODUCTION

The nuclear envelope (NE) is a vital cellular structure that separates the cytoplasm from the nucleus. In the nucleus, DNA is protected from harmful reactions taking place in other parts of the cell. In addition, DNA is separated from proteins such as transcription factors, which may then be transported into the nucleus in a regulated manner. The NE consists of an inner nuclear membrane (INM) and an outer nuclear membrane (ONM) separated by the perinuclear space (Fig. 3.1). The ONM is continuous with the endoplasmic reticulum (ER), while INM contains a unique complement of proteins. Embedded in these membranes, where ONM and INM are fused forming a highly curved pore membrane (POM), are the nuclear pore complexes (NPCs). Underlying the NE is the nuclear lamina, a thin protein layer that is closely associated with both INM and chromatin (Fig. 3.1).

Because the NE is such a critical cellular barrier, many viral pathogens have evolved to modulate its permeability. They do this in one of two ways: by disrupting the NE or by altering the composition and function of the NPC. Viruses modulate NE permeability for different reasons. During their life

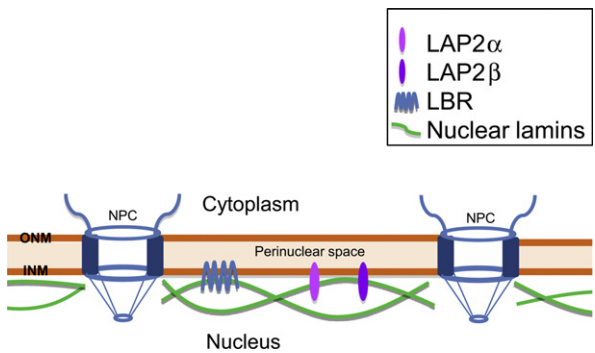

Figure 3.1 ***Components of the NE.*** Illustration of the NE showing the localization of its major components. Only INM proteins discussed in the text are included. For color version of this figure, the reader is referred to the online version of this book.

cycles, most DNA viruses must deliver their genomes into the host nucleus and utilize the cellular machinery for DNA replication and RNA processing. After DNA replication, the assembly of new viral particles often occurs in the nucleus, which requires the nuclear import of newly synthesized viral proteins. Thus, the genomes of nuclear-replicating viruses must enter the nucleus, while newly assembled viral particles must exit the nucleus. In order for these events to occur, it may be necessary for the virus to modulate the permeability of the NE. In addition, viruses that do not replicate in the nucleus may also modulate NE permeability (Gustin, 2003; Younessi et al., 2012). While the reasons for this are not entirely clear, it is thought that inhibition of nuclear transport is advantageous for these viruses because it prevents the host cell from mounting an antiviral response. Here we briefly review cellular mechanisms for nuclear transport and NE breakdown, and discuss the ways in which various viruses affect the NE and NPCs during infection.

2. NPC AND NUCLEAR TRANSPORT

NPCs are massive multiprotein complexes that act as passageways for the transport of molecules into and out of the nucleus. With a molecular mass of 66 MDa in yeast (Rout and Blobel, 1993) and 125 MDa in vertebrates (Reichelt et al., 1990), the NPC is one of the largest and most complex protein structures of eukaryotic cells. The NPC structure as revealed by electron microscopy (Fig. 3.2A) has been described as eight spokes symmetrically encircling a central channel. This assembly of spokes has a diameter of 120 nm and a height of 70 nm, and constitutes the central NPC framework or scaffold. The spoke substructure is sandwiched between a cytoplasmic and a nuclear ring. Attached to these peripheral rings are eight cytoplasmic filaments and a basket-like structure on the nuclear ring (Fig. 3.2B–D). The NPC structure has been refined over the years using state-of-the-art electron microscopy techniques and three-dimensional averaging software; the latest studies have resulted in three-dimensional models of the NPC with a resolution of 6 nm (Beck et al., 2007; Frenkiel-Krispin et al., 2010).

Two proteomic analyses using NPC from yeast (Rout et al., 2000) and mammals (Cronshaw et al., 2002) have revealed that the NPC is composed of 30 different proteins, termed nucleoporins, each one presented in multiple copies. It has been estimated that about 500–1000 nucleoporins make up the NPC (Hoelz et al., 2011). Most nucleoporins are denoted Nup followed by their molecular mass in kilodaltons (e.g. Nup62). Some

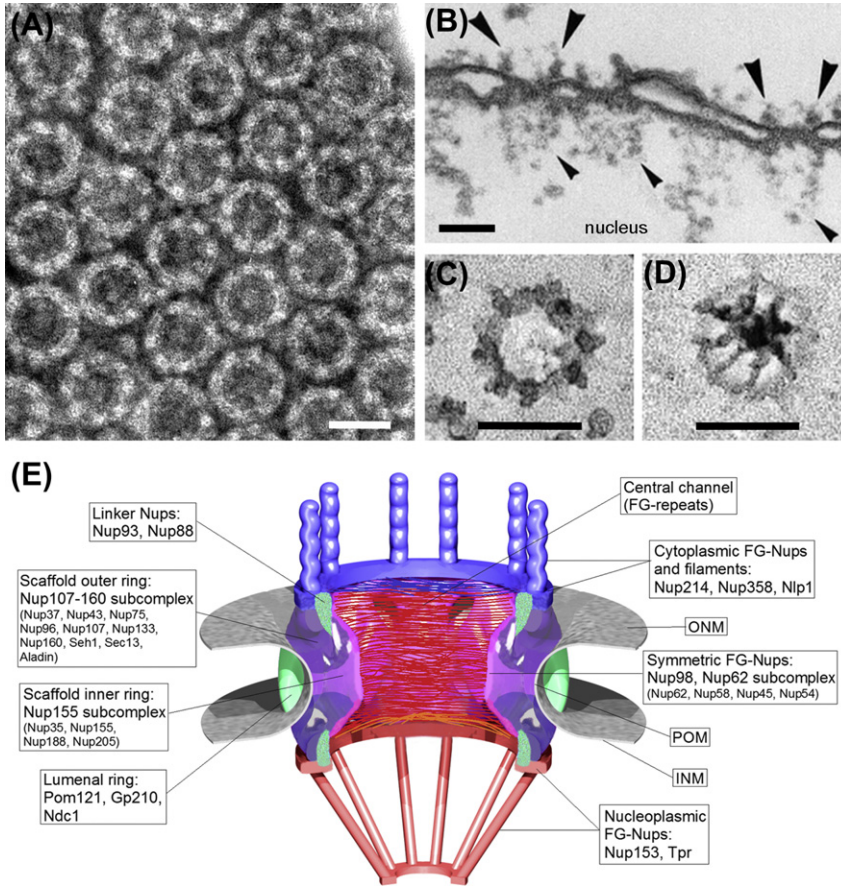

Figure 3.2 ***NPC structure and composition.*** (A–D) Electron micrographs from *Xenopus* oocytes NE that have been prepared for electron microscopy using different protocols. While preparation of isolated NE by negative staining (A) reveals the eight-fold symmetry of the NPC and the characteristic spoke complex structure, embedding–thin sectioning (B) reveals transverse sections of the NPC, which depict filamentous structures attached to both the cytoplasmic (large arrowheads) and nuclear (small arrowheads) side of the NPC. Visualization of the cytoplasmic and nuclear surface of the NPC after quick freezing, freeze-drying and metal shadowing reveals eight filaments attached to the cytoplasmic ring (C) and the nuclear basket attached to the nuclear ring (D). Scale bars, 100 nm. (E) Schematic diagram of the structure of the NPC indicating the location of nucleoporins, based on the model by Wente and Rout (2010). For color version of this figure, the reader is referred to the online version of this book.

nucleoporins are found in defined subcomplexes that remain intact during mitosis and serve as NPC building blocks. These subcomplexes have been very well characterized, even crystallographically [e.g. the Nup107-160 subcomplex (Whittle and Schwartz, 2009) and the Nup62 subcomplex (Solmaz et al., 2011)]. About one third of all nucleoporins contain numerous phenylalanine–glycine (FG)-repeat motifs. With 5–50 FG repeats per nucleoporin, it has been estimated that there are approximately 10,000 FG motifs per NPC (Peters, 2007). FG-nucleoporins are subdivided into symmetric, cytoplasmic and nucleoplasmic subtypes facing the NPC central channel, cytoplasm and nucleus respectively. The FG-repeat regions of the symmetric nucleoporins are thought to form a network of FG-repeat filaments that presumably populate the central channel of the NPC and form the permeability barrier of the NPC (Wente and Rout, 2010).

As illustrated in Fig. 3.2E, a detailed model for the position of each nucleoporin within the NPC structure has been proposed based on experimental data obtained from molecular, biochemical and structural analysis of the NPC and nucleoporins (Alber et al., 2007; Wente and Rout, 2010). In this model the scaffold of the vertebrate NPC is formed by two protein subcomplexes: the Nup107-160 and the Nup155 subcomplex. While several copies of the Nup155 subcomplex form the scaffold inner ring, several Nup107-160 subcomplexes form two scaffold outer rings facing the cytoplasm and the nucleus respectively (Fig. 3.2E). The scaffold is anchored in the POM by three integral membrane proteins that form a lumenal ring. FG-nucleoporins are attached to the scaffold through linker nucleoporins and scaffold inner ring nucleoporins. It is worth emphasizing that the position of some nucleoporins within the NPC is not stationary; while some proteins that form the NPC scaffold, such as members of the Nup107-160 complex, are stably embedded in the NPC structure, others such as Nup153 and Nup98 move on and off the NPC (Rabut et al., 2004) and can even be found in the nucleus (Griffis et al., 2002).

Transport of molecules into and out of the nucleus occurs through the NPC central channel, which allows passive diffusion of molecules smaller than 9 nm in diameter (Paine et al., 1975), and selective facilitated transport of larger cargos up to 39 nm in diameter (Pante and Kann, 2002). The selectivity for facilitated transport is dictated by short stretches of amino acids called nuclear localization signals (NLSs) or nuclear export signals (NES) residing on the transported molecule. These signals are recognized by soluble transport receptors (importins, transportins or exportins) derived from a family of proteins known as karyopherins (Chook and Suel, 2011;

Strambio-De-Castillia et al., 2010; Wente and Rout, 2010). Typically, a transport receptor binds to an NLS- or an NES-containing cargo in one compartment, targets it to and accompanies it through an NPC, releases it in the opposite compartment, and then shuttles back to the first compartment to repeat the cycle. The small GTPase Ran and its regulatory factors control the binding and release of the importins and exportins to their signals (Fried and Kutay, 2003). The detailed molecular mechanism of the translocation across the NPC central channel remains unsolved, but presumably involves transient interactions between nuclear transport receptors and the FG-repeat regions of nucleoporins (Wente and Rout, 2010).

3. NE BREAKDOWN

There are two physiological situations during which the NE breaks down: cell division and programmed cell death (Fig. 3.3). Because some of the viruses discussed here hijack these mechanisms, we will briefly review what is known about how NE breakdown occurs during mitosis and apoptosis.

3.1. NE Breakdown During Mitosis

During cell division, chromosomes must be partitioned between daughter cells. In some higher eukaryotes this is achieved through "open mitosis." In

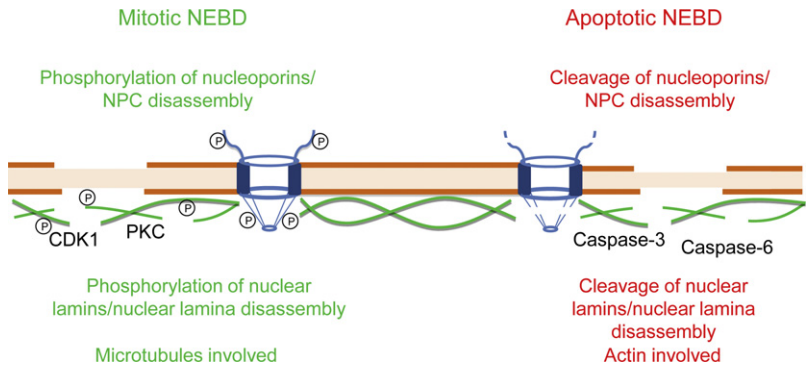

Figure 3.3 ***Mitotic and apoptotic NE breakdown (NEBD).*** During mitotic NEBD, phosphorylation of nucleoporins and nuclear lamins by CDK1 and PKC leads to NPC and nuclear lamina disassembly. Microtubules are involved in clearing the nuclear membranes from chromatin. In contrast, during apoptotic NEBD cleavage of nucleoporins and nuclear lamins by caspase-3 and caspase-6 leads to NPC and nuclear lamina disassembly. Actin is involved in removing nuclear membranes. For color version of this figure, the reader is referred to the online version of this book.

open mitosis, the NE is completely disassembled and removed from chromatin; this allows the cytoplasmic mitotic spindle to access chromosomes (Hetzer, 2010; Laurell and Kutay, 2011). In contrast, during the "closed mitosis" of yeast the NE remains intact and the mitotic spindle forms inside the nucleus. Intermediate forms of mitosis where the NE partially opens up near to centrosomes also exist, for example in *Caenorhabditis elegans* early embryos (De Souza and Osmani, 2009; Guttinger et al., 2009).

The NE breakdown of open mitosis proceeds via several steps: first NPCs disassemble into subcomplexes, then the nuclear lamina depolymerizes, INM proteins detach from chromatin, and ONM proteins retract into the ER (Guttinger et al., 2009). In addition, dynein and microtubules are involved in providing the force necessary to clear the nuclear membranes from chromatin (Beaudouin et al., 2002; Salina et al., 2002). Vesiculation of the nuclear membranes mediated by the COPI coatomer complex may also play a role in removing the nuclear membranes (Liu et al., 2003).

Many of these events are controlled by the activation of mitotic kinases. Of the kinases involved, the role of cyclin-dependent kinase 1 (CDK1) is currently the best understood. CDK1 is required for NPC disassembly (Muhlhausser and Kutay, 2007; Onischenko et al., 2005), and many nucleoporins are phosphorylated at CDK1 sites during mitosis (Fig. 3.3; Blethrow et al., 2008; Favreau et al., 1996; Glavy et al., 2007; Macaulay et al., 1995; Mansfeld et al., 2006). A recent study found that phosphorylation of Nup98 by CDK1 and Nek family kinases acts as a "molecular switch" to trigger NPC disassembly during mitosis (Laurell et al., 2011). In addition, phosphorylation of nuclear lamins by CDK1 leads to nuclear lamina depolymerization (Fig. 3.3; Heald and McKeon, 1990; Peter et al., 1990). Lastly, the INM proteins lamina-associated protein 2α (LAP2α; Dechat et al., 1998), lamina-associated protein 2β (LAP2β; Dreger et al., 1999) and lamin B receptor (Courvalin et al., 1992) are all targets of CDK1, suggesting that this kinase may also play a role in the release of INM proteins from chromatin and nuclear lamins. Protein kinase C (PKC) also plays a role in mitotic NE breakdown; lamin B is a target of PKCβ2, and inhibition of PKC results in G2 arrest (Thompson and Fields, 1996). In addition, aurora A (Hachet et al., 2007; Portier et al., 2007) and polo-like kinase 1 (Chase et al., 2000; Lenart et al., 2007) have also been implicated in NE breakdown.

3.2. NE Breakdown During Apoptosis

Apoptosis, or programmed cell death, occurs either developmentally or in response to stress such as DNA damage (He et al., 2009). One of the

hallmarks of apoptosis is NE breakdown. Like mitotic NE breakdown, the dismantling of the NE during apoptosis involves kinases. For example, lamin B is phosphorylated by PKCδ, resulting in nuclear lamina depolymerization (Cross et al., 2000). However, unlike mitotic NE breakdown, during apoptosis nucleoporins, nuclear lamins, and INM proteins are all cleaved by apoptotic proteases (Fig. 3.3; Fahrenkrog, 2006; Ferrando-May, 2005). Lastly, analogous to the requirement of mitotic NE breakdown for dynein and microtubules, apoptotic NE breakdown requires myosin and actin to generate the force required for nuclear disintegration (Croft et al., 2005).

Apoptosis is carried out by a family of cysteine proteases called caspases. Caspases are divided into two categories, initiators and executioners. While both types are constitutively present in the cytoplasm as latent precursors called zymogens or procaspases, they are activated by different mechanisms (Pop and Salvesen, 2009). Initiator caspases exist as monomeric zymogens, which require dimerization to become active; cleavage of initiator caspases is thought to stabilize the dimer without being necessary for formation of an active site (Pop and Salvesen, 2009). In contrast, executioner caspase zymogens are already dimers, and cleavage is thought to be the activating event (Pop and Salvesen, 2009). There are two main apoptotic pathways: the extrinsic pathway, initiated during development or by immune cells, activates initiator caspase-8 or -10, while the intrinsic pathway, initiated in response to cell stress such as DNA or mitochondrial damage, activates initiator caspase-9 (Pop and Salvesen, 2009). Both these pathways lead to activation of executioner caspases, which include caspase-3, -6, and -7; these executioner caspases then go on to cleave a wide variety of targets (Pop and Salvesen, 2009).

The executioner caspases implicated in the cleavage of nucleoporins, nuclear lamins, and INM proteins are caspase-3 and -6. The NPC proteins Nup93, Nup96, Nup153, Nup358, Nup214, and POM121 are all cleaved during apoptosis (Buendia et al., 1999; Ferrando-May et al., 2001; Kihlmark et al., 2001, 2004; Patre et al., 2006). Cleavage of nucleoporins takes place in sequential order, with the NPC linker nucleoporin Nup93 and the scaffold nucleoporin Nup96 cleaved first (Patre et al., 2006). This is followed by cleavage of the integral membrane nucleoporin POM121 and the cytoplasmic filament nucleoporin Nup358 prior to nucleosomal DNA degradation, and cleavage of the nuclear basket nucleoporin Nup153 slightly later (Kihlmark et al., 2001, 2004). Nup153 connects the NPC to the nuclear lamina, and it is thought that cleavage of Nup153 causes the clustering of NPCs typically observed during apoptosis (Fahrenkrog, 2006). Meanwhile, during apoptosis lamin A is cleaved by caspase-6, while lamin B is cleaved by

caspase-3 (Slee et al., 2001). Lastly, the INM proteins LAP2α and LAP2β are both substrates for caspases (Buendia et al., 2001; Gotzmann et al., 2000). The cleavage of all these proteins results in the clustering of NPCs, and the blebbing and detachment of the NE from chromatin.

4. VIRUSES THAT CAUSE NE DISRUPTION

Several virus families have been shown to induce NE disruption. The non-enveloped parvoviruses and the polyomavirus simian virus 40 (SV40) both induce disruption of the nuclear lamina in a caspase-dependent manner early during infection. In both cases, this disruption seems to facilitate nuclear entry of the viral genome and subsequent replication. In contrast, the enveloped herpes family viruses disrupt the nuclear lamina during nuclear egress of newly assembled virions, budding into the perinuclear space and then fusing with the ONM to release de-enveloped capsids into the cytoplasm (Johnson and Baines, 2011). These examples illustrate the importance of the NE as a barrier at different stages of the viral replication cycle. In this section we review our current understanding of how these three viruses breach the NE. The key features of these viruses are summarized in Table 3.1, and Fig. 3.4 summarizes their pathway through the NE.

4.1. NE Disruption Induced by Parvoviruses

Parvoviruses are non-enveloped, icosahedral, single-stranded DNA viruses (Berns and Parrish, 2007). At 18–26 nm in diameter, they are among the smallest DNA animal viruses; the ~5 kb parvovirus genome contains only two open reading frames, coding for two nonstructural proteins and two capsid proteins (Cotmore and Tattersall, 2006). After binding to cellular receptors, parvoviruses are taken up via clathrin-mediated endocytosis (Parker and Parrish, 2000). Following endocytosis, virions slowly escape from endocytic compartments to the cytosol (Vihinen-Ranta et al., 2000, 2002). The mechanism of parvoviral escape from endosomes likely involves a viral phospholipase. Parvoviruses contain a conserved phospholipase A2 (PLA2) motif in the viral protein 1 (VP1) (Zadori et al., 2001). Minute virus of mice (MVM) PLA2 mutant virions are not viable, but can be rescued by three different treatments that disrupt endosomes, suggesting that the parvoviral PLA2 functions in endosomal escape (Farr et al., 2005). Following release into the cytoplasm, parvoviruses are transported to the perinuclear region in a microtubule-dependent fashion (Seisenberger et al., 2001). Several lines of

Table 3.1 Summary of the major characteristics of the viruses discussed in this review

Virus Family	Virus Structure and Size (nm)	Capsid Structure and Size (nm)	Genome Architecture and Size (kb)	References
Viruses that cause nuclear envelope disruption				
Parvovirus-adeno-associated virus 2 (AAV2)-minute virus of mice (MVM)	Non-enveloped18-26	Icosahedral capsid18-26	DNA, single-stranded linear 5	Cotmore and Tattersall, 2006; Berns and Parrish, 2007
Polyomavirus-simian virus 40 (SV40)	Non-enveloped 40-45	Icosahedral capsid 40-45	DNA, double-stranded circular 5	Imperiale and Major, 2007
Herpesvirus-herpes simplex virus 1 (HSV-1)	Enveloped 180-225	Icosahedral capsid 120	DNA, double-stranded linear 152	Roizman et al., 2007
Viruses that alter the NPC				
Picornavirus-enterovirus/ cardiovirus	Non-enveloped 27-30	Icosahedral capsid 27-30	RNA, positive sense 7.2-8.5	Racaniello, 2007; Castello et al., 2011
Adenovirus-adenovirus 2	Non-enveloped 105	Icosahedral capsid 90	DNA, double-stranded linear 36	Berk, A., 2007
Retrovirus-human immunodeficiency virus 1 (HIV-1)	Enveloped120	Conical capsid Length: 100 - 120 Diameter of wide end: 50-60 nm	RNA, positive sense 7-13	Freed and Martin, 2007

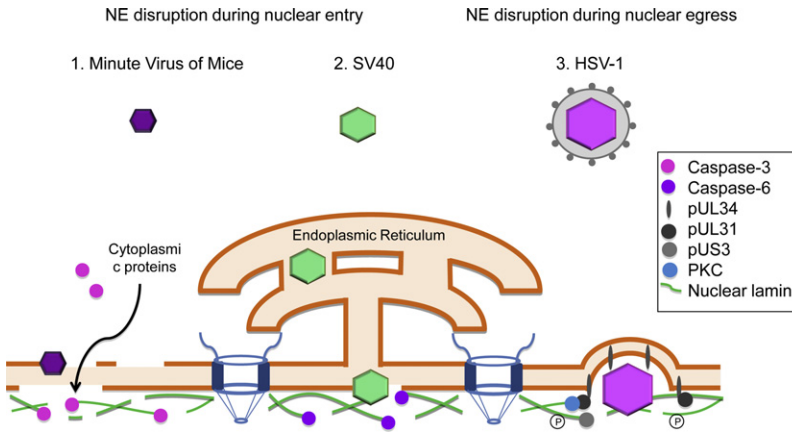

Figure 3.4 ***Viruses that disrupt the NE.*** Schematic representation of the NE indicating its disruption induced by infection with three different viruses. (1) MVM infection causes disruption of the nuclear membranes and nuclear lamina in a caspase-3-dependent manner. This allows MVM capsids to access the nucleus from the cytoplasm. (2) SV40 infection results in caspase-6-dependent disruption of the nuclear lamina, potentially allowing capsids to pass directly from the ER to the nucleus via the INM. (3) HSV-1 infection causes phosphorylation of the nuclear lamins by pUS3 and PKC, resulting in nuclear lamina disassembly during nuclear egress. For color version of this figure, the reader is referred to the online version of this book.

evidence indicate that the parvoviral genome enters the nucleus in association with an intact capsid after escape from endosomes. Multiple studies using immunofluorescence, or green fluorescent protein (GFP)- or fluorophore-conjugated virions have detected parvoviral capsid proteins in the nucleus of infected cells (Bartlett et al., 2000; Lux et al., 2005; Mani et al., 2006; Seisenberger et al., 2001; Vihinen-Ranta et al., 1998). In addition, microinjection of antibodies against the capsid of the human parvovirus adeno-associated virus 2 (AAV2) into the nucleus can inhibit productive infection of tissue culture cells (Sonntag et al., 2006). Lastly, immunogold electron microscopy has revealed apparently intact capsids of canine parvovirus in the nucleus of cells infected with this virus in the presence of cyclohexamide, which prevents the synthesis of new capsid proteins (Suikkanen et al., 2003).

At less than 26 nm in diameter, parvovirus capsids are small enough to enter the nucleus intact through the NPC, and it has been assumed that this is how the parvovirus genome accesses the nucleus. However, recent evidence supports a model in which parvoviruses enter the nucleus via an NPC-independent mechanism that involves disruption of the NE (Fig. 3.4). The first indication that parvoviruses can enter the nucleus independently of

the NPC came from studies with AAV2. It was found that AAV2 could enter purified nuclei in the absence of nuclear transport receptors and factors (Hansen et al., 2001). In addition, blocking the NPCs with the lectin wheat germ agglutinin or with antibodies against the NPC protein Nup62 did not prevent uptake of AAV2 capsids into purified nuclei, indicating an alternate nuclear entry route (Hansen et al., 2001).

Subsequent studies with the parvovirus MVM have also supported an NPC-independent nuclear entry pathway. Microinjection of *Xenopus laevis* oocytes is a popular system for visualizing cargo, including virus capsids, crossing the NPC (Au and Pante, 2012; Rabe et al., 2003). Microinjection of MVM into *Xenopus* oocytes visualized by electron microscopy revealed that MVM induces small (100–300 nm) disruptions in the ONMs and INMs (Fig. 3.5; Cohen and Pante, 2005). Microinjected virions were observed in close proximity to disruptions of the ONM, and in the perinuclear space (Fig. 3.5; Cohen and Pante, 2005). Similar results were obtained by microinjecting *Xenopus* oocytes with canine parvovirus (Cohen et al., 2011a) and rat parvovirus (Cohen, S., Pante, N. and Kann, M., unpublished results). In addition, in *Xenopus* oocytes pre-injected with wheat germ agglutinin to block transport through the NPC, microinjection of MVM caused NE disruptions that could support nuclear entry of proteins in an NPC-independent manner (Cohen and Pante, 2005).

Follow-up electron microscopy studies revealed that MVM causes small NE breaks in infected fibroblast cells as well, as early as 30 min after infection (Cohen et al., 2006). MVM infection was also associated with alterations in nuclear morphology, characterized by multiple amorphous invaginations of the NE, and with nuclear lamina disruption, visualized as distinct gaps in lamin A/C immunostaining (Cohen et al., 2006). The typical nuclear rim staining of the lamin A/C, however, reappeared at later times during infection (Cohen et al., 2011b), indicating that parvoviruses transiently disrupt the NE. The process of MVM-induced NE disruption was also reconstituted using digitonin-treated cells (Cohen et al., 2011b), whose plasma membrane was permeabilized but whose NE remained intact, and the permeability of the NE was visualized as a nuclear influx of a fluorescently labeled 155-kDa dextran that cannot diffuse through the NPC.

Recently it has been shown that MVM-induced NE disruption involves the hijacking of host enzymes. Microinjection of PLA2 mutant virions into *Xenopus* oocytes visualized by electron microscopy revealed that viral PLA2 activity is not required for this phenomenon (Cohen et al., 2011b). In contrast, screening of several kinase and protease inhibitors in the

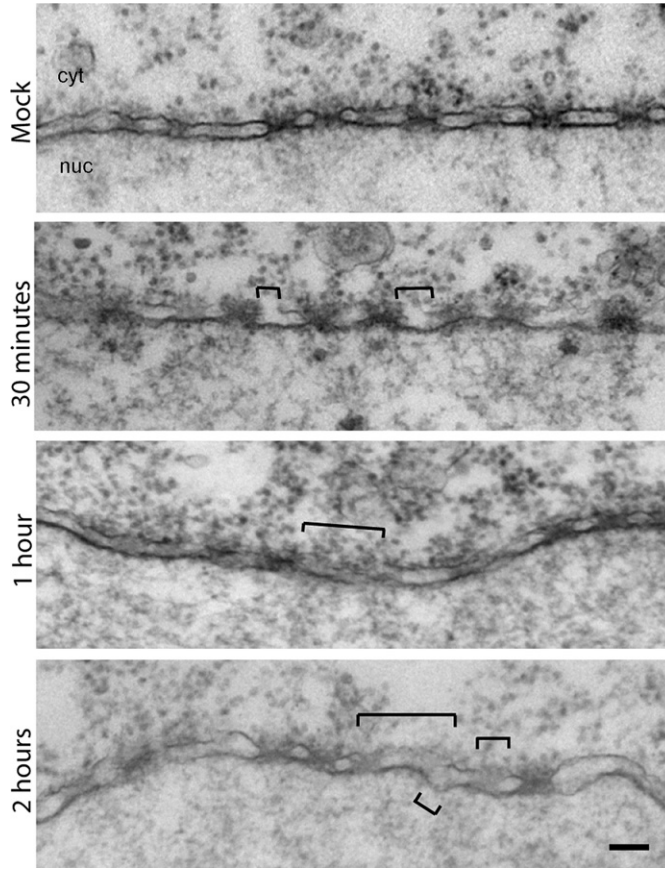

Figure 3.5 ***MVM induces disruption of the NE in microinjected*** **Xenopus** ***oocytes.*** Views of NE cross sections with adjacent cytoplasm (cyt) and nucleus (nuc) from *Xenopus* oocytes that have been mock injected or injected with MVM. After injection, oocytes were incubated for 30 min, 1 or 2 h at room temperature and processed for embedding and thin-section electron microcopy. A representative NE view is shown for each condition. Brackets indicate disruptions in the NE caused by MVM. Scale bars, 100 nm.

digitonin-permeabilized cell assay described above revealed that caspase-3 is necessary to facilitate MVM-induced NE disruption (Cohen et al., 2011b). *Xenopus* oocyte microinjection experiments as well as immunofluorescence of the nuclear lamina in cells infected in the presence of caspase inhibitors corroborated these results, and Western blot showed that MVM infection induces cleavage of lamin B in a caspase-3-dependent manner (Cohen et al., 2011b). Interestingly, caspase-3 was not activated above basal levels in MVM-infected cells, but was relocalized to the nucleus (Cohen et al., 2011b). Lastly, both immunofluorescence and immunogold electron

microscopy showed that MVM capsids accumulate at the cytoplasmic side of the NE in infected cells when NE disruption is inhibited with a caspase-3 inhibitor, and viral gene expression was also significantly reduced under these conditions (Cohen et al., 2011b). This is significant because it suggests that NE disruption is the primary nuclear entry route for MVM, and not just an alternative to NPC-mediated nuclear import.

In summary, several lines of evidence indicate that parvoviruses use a novel nuclear entry pathway that does not rely upon the host nuclear transport machinery, but rather upon apoptotic components. The detailed molecular mechanism of the parvovirus-induced NE disruption remains to be investigated.

The advantage to using a nuclear entry strategy that involves disruption of the NE is unclear. This is especially puzzling given that parvoviruses are small enough to enter the nucleus intact through the NPC using NLSs and nuclear import receptors. Interestingly, MVM-induced NE disruption results in localized changes in the compartmentalization of cellular proteins (Cohen et al., 2011b). It is possible that such changes in compartmentalization are beneficial for MVM, e.g. cytoplasmic proteins used by the virus for a replication step are able to leak into the nucleus. It is also possible that disruption of the ONM, which is continuous with the ER, results in release of calcium and that subsequent signaling plays a role in infection. These possibilities remain to be investigated.

4.2. NE Disruption Induced by SV40

SV40 is a small (40 nm) non-enveloped DNA virus in the polyomavirus family (Imperiale and Major, 2007). SV40 enters the cell by an unusual mechanism: the virus is taken up by caveolar endocytosis, and then traffics to the ER (Pelkmans et al., 2001). In the ER, the host chaperone protein ERp29 triggers a conformational change in the capsid (Magnuson et al., 2005). This results in the exposure of the capsid protein VP2, which then integrates into and perforates the ER membrane, releasing the capsid or subviral particle (Rainey-Barger et al., 2007).

It is unclear whether SV40 escapes from the ER to the cytoplasm and then enters the nucleus through the NPC or whether it enters the nucleus directly from the ER by penetrating the INM. The latter possibility would presumably require disruption of the nuclear lamina. Indeed, immunofluorescence microscopy has revealed that when nondividing cells are infected with SV40, deformation of the NE and gaps in lamin A/C immunostaining

are apparent starting at 2 h postinfection (Butin-Israeli et al., 2011). Western blot showed fluctuations in the levels of lamin A/C, but not lamin B, within the first 10 h of infection with SV40; dephosphorylation of an unknown epitope on lamin A/C and accumulation of lamin A in the cytoplasm were also observed (Butin-Israeli et al., 2011). These changes in lamin A/C levels were prevented by a caspase-6 inhibitor (Butin-Israeli et al., 2011). Interestingly, previous work had shown that caspase-6 is activated very early on during infection of cells with SV40, starting at 1 h postinfection and that inhibition of this caspase prevents expression of viral proteins (Butin-Israeli et al., 2010). Lastly, real-time polymerase chain reaction experiments determined that the SV40 genome enters the nucleus of quiescent cells at 6–8 h postinfection (Butin-Israeli et al., 2011). Taken together, these data are consistent with a model in which SV40-induced signaling leads to destabilization of the nuclear lamina, allowing virions delivered to the ER to perforate the INM in a VP2-dependent manner and enter the nucleus directly without passing through the cytoplasm in quiescent cells (Fig. 3.4). Notably, expression of the mouse polyomavirus proteins VP2 and VP3 has been shown to result in activation of caspase-3 (Huerfano et al., 2010). Immunogold electron microscopy also localized these proteins to the perinuclear space (Huerfano et al., 2010). Therefore, disruption of the NE may be a common feature of polyomaviruses.

Thus, at least two families of non-enveloped viruses—the parvoviruses and polyomaviruses—disrupt the NE in order to deliver their genomes into the host nucleus. In both cases nuclear lamins are cleaved in a caspase-dependent manner. However, MVM infection results in lamin B cleavage by relocalized caspase-3, while SV40 infection causes lamin A/C cleavage by activated caspase-6. The advantages to using a nuclear entry mechanism involving lamin disruption also differ between the two virus families. While infection with MVM alters the permeability of the NE to cytoplasmic proteins, infection with SV40 does not (Butin-Israeli et al., 2011). Thus, a main advantage of lamina disruption for the polyomaviruses may be the ability to enter the nucleus directly from the ER, avoiding detection and degradation in the cytoplasm.

4.3. NE Disruption During Exit of Herpesviruses from the Nucleus

Herpesviruses are enveloped viruses with an icosahedral capsid containing the viral DNA, and a proteinaceous layer consisting of tegument proteins between the capsid and the envelope (Roizman et al., 2007). The family of

herpesviruses is very large and includes alphaherpesviruses such as herpes simplex virus 1 (HSV-1), betaherpesviruses such as human cytomegalovirus, and gammaherpesviruses such as Epstein–Barr virus. Assembly and nuclear egress are best understood for the alphaherpesviruses, although the betaherpesviruses and gammaherpesviruses seem to use similar mechanisms (Lee and Chen, 2010; Morrison and Delassus, 2011).

The alphaherpesvirus HSV-1 is a large virus that codes for over 80 viral proteins by its UL1-UL56, US1-US12, RL1-2 and RS1 genes (Roizman et al., 2007). HSV-1 enters host cells by fusing its envelope with cellular membranes, either with the plasma membrane (which is thought to be the primary entry pathway) or endosomal membranes after internalization by endocytosis (Nicola et al., 2005). The capsid, with some tegument proteins still associated, is then transported along microtubules to the NPC (Dohner et al., 2002; Sodeik et al., 1997). After binding to the NPC, the HSV-1 capsid releases its DNA through the NPC into the cell nucleus, where viral DNA replication and assembly of progeny virions occur. Newly formed capsids, which at ~125 nm are too large to pass through the NPC, undergo an envelopment–de-envelopment process by budding through the INM and then fusing with the ONM (Johnson and Baines, 2011; Mettenleiter, 2002; Morrison and Delassus, 2011). The released capsids acquire tegument proteins in the cytoplasm, and are re-enveloped by budding into the trans-Golgi network, followed by fusion of exocytic vesicles to release mature virions from the cell (Johnson and Baines, 2011).

The envelopment–de-envelopment model of HSV-1 nuclear egress is supported by electron microscopy studies that have revealed newly assembled capsids in the nucleus (Fig. 3.6A), naked HSV-1 capsids associated with the nuclear side of the INM (Fig. 3.6B), enveloped capsids in the perinuclear space (Fig. 3.6C), and naked capsids associated with the cytoplasmic side of the ONM (Fig. 3.6D; Granzow et al., 2001; Mettenleiter, 2002). In addition, the tegument and envelope of perinuclear virions differ in ultrastructure from the tegument and envelope of extracellular virions, suggesting that re-envelopment is required (Granzow et al., 2001). Genetic and biochemical analyses have also shown differences in the protein and lipid composition of perinuclear versus extracellular virions (Mettenleiter, 2002; Skepper et al., 2001). Lastly, an in vitro nuclear egress assay performed in isolated nuclei resulted in the accumulation of enveloped capsids between the nuclear membranes and release of exclusively naked capsid (Remillard-Labrosse et al., 2006).

In order to bud through the INM during primary envelopment, herpesvirus capsids must bypass the nuclear lamina (Fig. 3.4). HSV-1 makes

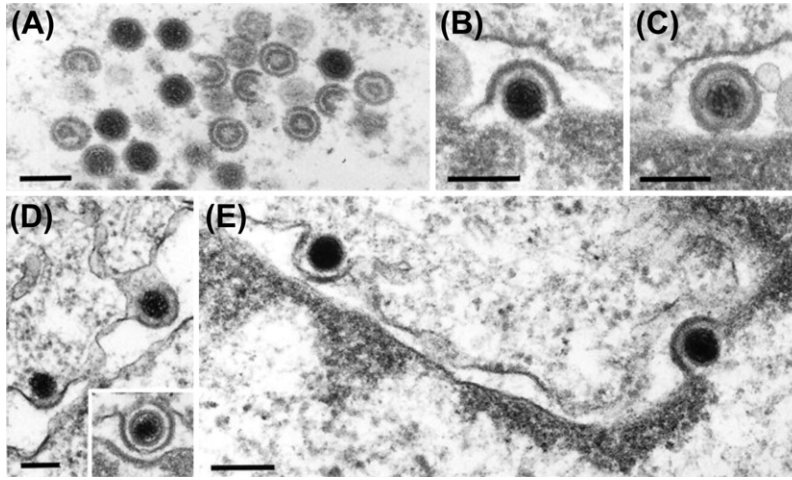

Figure 3.6 ***Assembly and egress of HSV-1.*** Electron micrographs showing newly assembled HSV-1 capsids in the nucleus (A), a capsid undergoing primary envelopment at the INM (B), a primary enveloped virion traversing the perinuclear space (C), and finally, fusion of the primary envelope with the ONM (D). Rarely, primary envelopment of HSV-1 at the INM and de-envelopment at the ONM have been captured in the same micrograph (E). Scale bars, 150 nm. *Reproduced with permission from Mettenleiter (2002); panel E is originally from Granzow et al. (2001).*

use of multiple viral and cellular proteins in order to overcome this barrier. Two key viral proteins involved in primary envelopment are pUL31 and pUL34 (Chang et al., 1997; Reynolds et al., 2001; Roller et al., 2000), which together are referred to as the nuclear envelopment complex (NEC). pUL34 is an integral membrane protein targeted to the INM (Shiba et al., 2000), which interacts with and recruits the tegument phosphoprotein pUL31 to the nuclear periphery (Chang and Roizman, 1993; Reynolds et al., 2002). Deletion of the pUL31 gene prevents primary envelopment and results in the accumulation of naked capsids in the nucleus (Chang et al., 1997). Both pUL31 and pUL34 are associated with virions visualized in the perinuclear space by electron microscopy, suggesting that these proteins form a bridge between the capsid and the primary envelope (Reynolds et al., 2002). The NEC is necessary but not sufficient to cause the disruption of the nuclear lamina that occurs during egress of HSV-1 (Reynolds et al., 2004). pUL31 and pUL34 have been proposed to bind to lamin A/C directly, which could disrupt lamin–lamin interactions through competition (Reynolds et al., 2004; Simpson-Holley et al., 2004). In addition, the NEC is responsible for recruitment of cellular kinases. Both the novel PKCδ and the

conventional PKCα are recruited to the nuclear periphery in an NEC-dependent manner (Park and Baines, 2006), where one or both these kinases phosphorylate lamin B (Leach and Roller, 2010). The HSV-1 kinase pUS3 can also phosphorylate lamin A/C at multiple sites (Mou et al., 2007), as well as phosphorylating the INM protein emerin (Morris et al., 2007). Deletion of the pUS3 gene results in the accumulation of virions associated with invaginations of the nuclear side of the NE (Reynolds et al., 2002), emphasizing the importance of pUS3 for nuclear egress. Thus, HSV-1 makes use of both viral and cellular kinases to phosphorylate both the A/C- and the B-lamins. This results in localized disruption of the nuclear lamina at sites where the concentration of pUL31 and pUL34 is high (Bjerke and Roller, 2006; Mou et al., 2007), in order to facilitate budding of progeny capsids through the INM.

As illustrated in Fig. 3.4, like the non-enveloped parvoviruses and polyomaviruses, herpesviruses also disrupt the nuclear lamina during infection. However, rather than disrupting the lamina to facilitate nuclear entry of the viral genome, herpesviruses must overcome this barrier during egress of progeny capsids from the nucleus. Recent evidence suggests that SV40 may also disrupt the NE to facilitate nuclear egress: VP4, which is expressed late during SV40 infection, has been shown to localize to the nuclear periphery and to permeabilize the NE (Giorda et al., 2012), likely by forming membrane pores (Raghava et al., 2011). Thus, NE permeabilization during nuclear egress of viruses may also be a common theme.

Unlike parvoviruses and polyomaviruses, the herpesviruses use phosphorylation but not cleavage of lamin proteins to disrupt the lamina. Interestingly, similar to early SV40 infection but unlike parvovirus infection, the nuclear permeability barrier is maintained during infection with HSV-1 (Hofemeister and O'Hare, 2008). This suggests that different viruses have different requirements when it comes to compartmentalization of host cell proteins.

5. VIRUSES THAT CAUSE STRUCTURAL AND FUNCTIONAL DISRUPTIONS OF THE NPC

Activation of the cellular innate immunity pathways and the establishment of a cellular antiviral response highly depend on transport through the NPC. Thus, several viruses have developed different strategies to shut

down this important cellular pathway as a way of inhibiting the nuclear transport of host proteins responsible for inducing the innate immune response, and thereby ensure the progression of the viral infection. As the nuclear transport machinery have different components and can be regulated at different stages, disruption of nuclear transport could arise as a consequence of the virus or viral proteins interacting with or altering different cellular components of this machinery. For example, the matrix protein of the vesicular stomatitis virus inhibits nuclear export of host cell messenger RNAs (mRNAs) by interacting with the mRNA export factor Rae1 (Faria et al., 2005), and the VP24 protein of Ebola virus inhibits nuclear import by binding to importin α1 (Reid et al., 2006). For a review on viral proteins that interfere with other nuclear import receptors, see Fulcher and Jans (2011) and references therein. In this section we review the mechanisms used by several viruses that target the NPC and alter its composition, structure and function (Fig. 3.7, and Table 3.2). The key features of these viruses are summarized in Table 3.1.

The phenomenon of virus-caused disruption of the NPC has been well documented for two genera of the *picornaviridae* family: enterovirus and cardiovirus (Castello et al., 2011; Gustin, 2003; Younessi et al., 2012). Research in the past few years has uncovered two other viruses that disrupt the NPC by displacement of nucleoporins from the NPC: adenovirus (Strunze et al., 2011) and human immunodeficiency virus 1 (HIV-1) (Monette et al., 2009, 2011). Future studies should also uncover more examples of viruses that cause structural and functional disruptions of the NPC.

5.1. Alteration of the NPC Composition and Traffic Capacity by Some Members of the *Picornaviradae* Family

Picornaviruses are non-enveloped viruses composed of a single-stranded, positive-sense RNA molecule (7.2–8.5 kb) enclosed within an icosahedral capsid of 27–30 mm in diameter. The genomic RNA has a single open reading frame, encoding a single polyprotein that is cleaved during translation by virus-encoded proteases. These proteases also target several cellular proteins, leading to the inhibition of host gene expression in infected cells (Castello et al., 2011). The overall genomic structure of all picornaviruses is evolutionarily conserved, with all picornaviruses encoding the protease $3C^{pro}$ and its precursor $3CD^{pro}$. However, members of the enterovirus genus encode the additional protease $2A^{pro}$, and members of the cardiovirus genus encode a leader (L) protein, which is unique for this group of viruses.

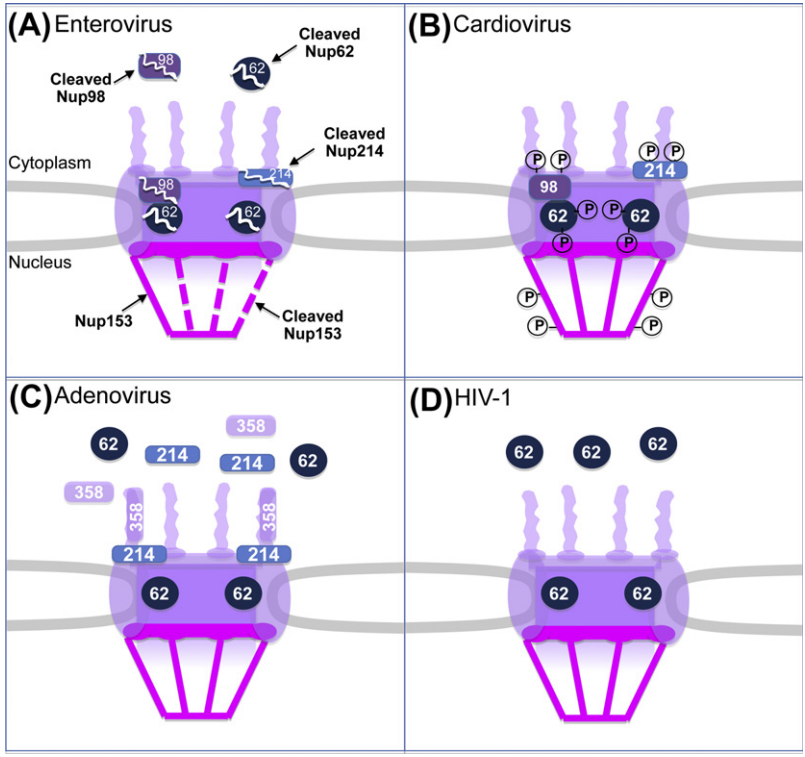

Figure 3.7 ***Viruses that alter the NPC.*** Schematic representation of the NPC and its alteration upon infection with four different viruses. (A) In enterovirus infection the FG-nucleoporins Nup62, Nup98, Nup153 and Nup214 are proteolytically cleaved by the unique enteroviral protease $2A^{pro}$. (B) During cardiovirus infection Nup62, Nup98, and Nup153 are hyperphosphorylated, a process that depends on the cardiovirus L protein. (C) Adenovirus infection induces displacement of the cytoplasmic filament nucleoporins Nup214 and Nup358, and the central channel nucleoporin Nup62 from the NPC. (D) HIV-1 induces displacement of Nup62 from the NPC, as well as alteration of the NPC composition (Table 3.2; Monette et al., 2011). For color version of this figure, the reader is referred to the online version of this book.

The picornavirus life cycle is entirely cytoplasmic, and the virus is able to replicate in enucleated cells or in a test tube in the absence of nuclei and in the presence of an in vitro translation extract (Follett et al., 1975; Molla et al., 1991; Svitkin and Sonenberg, 2003). Nevertheless, several host nuclear factors, such as polypyrimidine tract binding protein, La autoantigen and Sam68, interact with picornaviral RNA and proteins, apparently promoting the viral replication (Hellen et al., 1993; Meerovitch et al., 1993; Waggoner and Sarnow, 1998). Notably, these host nuclear proteins relocate

Table 3.2 Alteration of nucleoporins by viruses discussed in this review

Nucleoporin	Enterovirus	Cardiovirus	Adenovirus	HIV-1
Nup62	Proteolysis (Gustin and Sarnow, 2001; 2002)	Hyperphosphorylation (Porter and Palmenberg, 2009)	Displacement from NPC (Strunze et al., 2011)	Displacement from NPC Abundance decreased[1] (Monette et al., 2009; 2011)
Nup98	Proteolysis (Park et al., 2008)	Hyperphosphorylation (Ricour et al., 2009)	Unknown	Abundance increased[2](Monette et al., 2011)
Nup153	Proteolysis (Gustin and Sarnow, 2001; 2002)	Hyperphosphorylation (Porter and Palmenberg, 2009)	No effect (Strunze et al., 2011)	Abundance decreased (Monette et al., 2011)
Nup214	Proteolysis(Ghildyal et al., 2009)	Hyperphosphorylation (Porter and Palmenberg, 2009)	Displacement from NPC (Strunze et al., 2011)	Abundance unchanged (Monette et al., 2011)
Nup358	Proteolysis (Ghildyal et al., 2009)	No effect (Porter and Palmenberg, 2009)	Displacement from NPC (Strunze et al., 2011)	Abundance unchanged (Monette et al., 2011)

[1]Other nucleoporins whose abundance decreased during HIV-1 infection are: the FG nucleoporins Nup153, Nup58, Nup54 and Nup45; the luminal ring nucleoporins gp210 and POM121; the Nup107-160 subcomplex nucleoporins Nup160, Nup133, Nup107, Nup85, Nup43, Nup37; the Nup155 subcomplex nucleoporin Nup155; and the linker nucleoporin Nup93 (Monette et al., 2011).
[2]Other nucleoporins whose abundance increased during HIV-1 infection are: the Nup155 subcomplex nucleoporin Nup35 and the nuclear filament nucleoporin Tpr (Monette et al., 2011).

to the cytoplasm during picornavirus infection (Back et al., 2002; Gustin and Sarnow, 2002; McBride et al., 1996; Meerovitch et al., 1993; Waggoner and Sarnow, 1998). This abnormal relocation of nuclear proteins in cells infected with picornaviruses was first demonstrated in experiments using HeLa cells expressing GFP fused to a classical NLS (Belov et al., 2000; Gustin and Sarnow, 2001). This chimera protein localizes to the nucleus of uninfected cells, but is found in the cytoplasm of cells infected with several picornaviruses, including poliovirus (PV) and human rhinovirus (HRV), belonging to the enterovirus genus, as well as encephalomyocarditis virus (EMCV), mengovirus and Theiler's murine encephalomyelitis virus (TMEV) from the cardiovirus genus (Bardina et al., 2009; Belov et al., 2000; Delhaye et al., 2004; Gustin and Sarnow, 2001, 2002; Lidsky et al., 2006).

Although for the La autoantigen, it has been demonstrated that its cytoplasmic relocation is due to the cleavage of its NLS by the PV protease $3C^{pro}$ (Shiroki et al., 1999), the abnormal relocalization of nuclear proteins during infection with enteroviruses or cardioviruses has been explained by both inhibition of receptor-mediated transport through the NPC and increased permeability of the NPC. Experiments using the in vitro nuclear import assay consisting of cells semipermeabilized with digitonin (Adam et al., 1990), which permeabilizes the plasma membrane but not the NE, showed that GFP fused to a classical NLS was not able to enter the nucleus of semipermeabilized cells derived from PV- or HRV-infected cells (Gustin and Sarnow, 2001, 2002). On the other hand, experiments using cells expressing an NLS-fused fluorescent Timer protein, which distinguish between newly synthesized proteins and "old" proteins that are located in the nucleus, demonstrated that upon infection with the enterovirus PV or the cardiovirus EMCV the reporter protein that was in the nucleus before infection relocated to the cytoplasm presumably by diffusing through the NPC (Belov et al., 2004; Lidsky et al., 2006). Thus, there are several explanations for the abnormal cytoplasmic relocation of nuclear proteins induced by infection with several members of the *picornaviradea* family.

It is important to note that not all nuclear proteins are relocated to the cytoplasm after infection with enteroviruses or cardioviruses. Thus, there seems to be a specific mechanism of how these viruses induce cytoplasmic localization of nuclear proteins. In search for this mechanism it has been demonstrated that infection with enteroviruses or cardioviruses alters the NPC in different ways. While enteroviruses proteolytically cleave a subset of FG-nucleoporins, cardioviruses hyperphosphorylate a similar set of nucleoporins (Table 3.2, Fig. 3.7). Immunoblot analysis of cell lysates

prepared at various times after infection with the enteroviruses PV or HRV revealed that Nup62, Nup98 and Nup153 were degraded during the course of infection (Gustin and Sarnow, 2001, 2002; Park et al., 2008). The kinetics of proteolysis was, however, different for each nucleoporin: Nup98 was cleaved first (at 2 h postinfection), followed by Nup62 and Nup153 at 4.5 and 6 h postinfection respectively (Park et al., 2008). However, inhibition of nuclear import and cytoplasmic relocation of nuclear proteins occurred only after these three nucleoporins were cleaved (Park et al., 2008). Importantly, similar immunoblot analysis of cells infected with several cardioviruses revealed that these nucleoporins remained intact during infection (Lidsky et al., 2006; Porter and Palmenberg, 2009). However, Nup62, Nup153 and Nup214 were hyperphosphorylated in cells infected with the cardioviruses mengovirus or EMCV (Bardina et al., 2009; Porter and Palmenberg, 2009), and Nup98 was hyperphosphorylated during infection with the cardiovirus TMEV (Ricour et al., 2009).

Not surprisingly, such alterations of the NPC composition were also detected at the level of both fluorescence and electron microscopy. When compared with uninfected cells, cells infected with PV or HRV showed a decreased fluorescence staining of their NE after immunofluorescence labeling with antibodies against Nup62 or Nup153 (Gustin and Sarnow, 2001, 2002). When examined by electron microscopy after embedding and thin sectioning, cross sections of the NPCs of uninfected HeLa cells showed their typical morphology (Fig. 3.8A), with an electron-dense central channel that is supposed to be filled with natively unfolded FG repeats of a number of nucleoporins, including Nup62 and its interacting proteins Nup58, Nup54 and Nup45, making up the barrier of permeability within the NPC (Wente and Rout, 2010). In contrast, the central channel of NPCs from mengovirus or PV-infected cells appeared less electron dense (Fig. 3.8A; Bardina et al., 2009; Belov et al., 2004; Lidsky et al., 2006), most probably reflecting the proteolysis, in the case of PV infection, or the phosphorylation, in the case of mengovirus infection, of Nup62. This structural change of the NPC is better illustrated in electron micrographs of tangential sections of nuclei from cells infected with these viruses (Fig. 3.8B), which also document that PV and mengovirus induce different alterations of the NPC. While the NPC central channel appeared to be empty in mengovirus-infected cells, asymmetrical granules were identified in the middle of the NPC of PV-infected cells (Fig. 3.8B; Bardina et al., 2009). These granules have been proposed to be products of Nup62 proteolytic digestion that occur during PV infection (Bardina et al., 2009). Since it has been recently

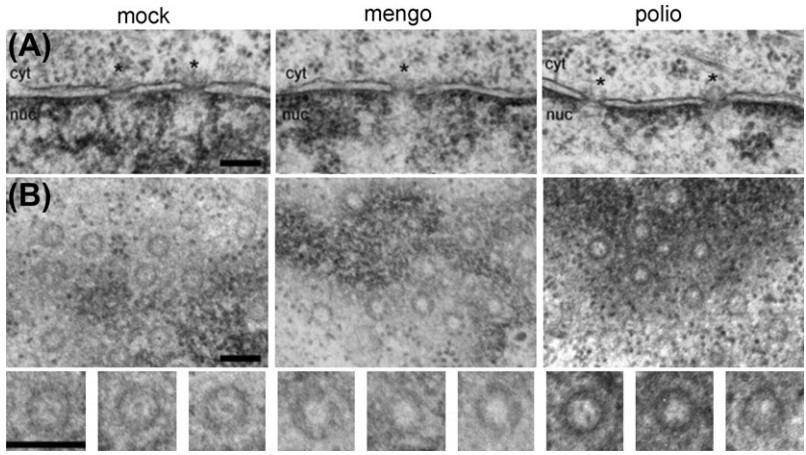

Figure 3.8 ***Structural alterations of the NPC caused by picornavirus.*** (A) Electron micrographs of NE cross sections with adjacent cytoplasm (cyt) and nucleus (nuc) from HeLa cells that have been mock infected or infected with mengovirus or PV for 4 h. NPCs are marked with asterisks. (B) Electron micrographs of in-plane tangential sections of the NE from the same experimental conditions as indicated in panel A. The lower rows are enlarged examples of NPC tangential sections documenting that the electron-dense granule in the central channel of control NPC is totally (mengovirus) or partially (PV) lost in infected cells. Scale bars, 200 nm. *Reproduced with permission from Bardina et al. (2009).*

reported that the Nup62 subcomplex located in the NPC central channel is composed of two independently interacting modules: Nup54•Nup58 and Nup62•Nup54 (Solmaz et al., 2011), these granules may represent the Nup54•Nup58 subcomplex. On the other hand, we speculate that the negative charges added upon phosphorylation of Nup62 might affect the interactions between FG repeats in the central channel, resulting in a retraction of these domains to the scaffold of the NPC leaving the NPC central channel empty in the mengovirus-infected cells. In any case, the electron microscopy data support both the hypothesis of enhanced NPC permeability induced by picornavirus infection and the hypothesis of inhibition of the NPC active transport by preventing the interaction of nuclear transport receptors with the FG-nucleoporins in the NPC central channel.

The next question addressed in this field has been how these picornaviruses achieve the chemical alterations of the NPC. Several studies have now established that the unique protease $2A^{pro}$ of the enteroviruses PV and HRV plays a direct role in the cleavage of nucleoporins and the unique L protein of cardioviruses is involved in the hyperphosphorylation of

nucleoporins. The involvement of the enterovirus $2A^{pro}$ was first inferred from experiments with inhibitors of this protease, which prevented the cytoplasmic relocation of nuclear proteins, as well as experiments with cells transfected with PV $2A^{pro}$ plasmids, which yielded similar NPC transport defects than cells infected with PV (Belov et al., 2004). More recently, it has been directly demonstrated that both expression of $2A^{pro}$ and addition of this protease to whole-cell lysates prepared from uninfected cells resulted in the cleavage of Nup62, Nup98 and Nup153 (Castello et al., 2009; Park et al., 2008). Moreover, purified $2A^{pro}$ was able to cleave purified Nup62 in vitro, and analysis of the cleavage sites indicated that proteolysis releases the Nup62's FG-repeat region (Park et al., 2010). However, experiments with $2A^{pro}$ proteases from different HRV clades indicated that each protease reacted at different sites within Nup62, Nup98, and Nup153 (Watters and Palmenberg, 2011). Interestingly, partial in vitro cleavage of Nup62 was also achieved by its incubation with high concentrations of purified HRV $3C^{pro}$(Park et al., 2010). Furthermore, expression of vector-delivered HRV $3C^{pro}$ and 3CD led to proteolysis of Nup153, Nup214 and Nup358, suggesting that this protease may as well participate in the NPC alterations induced by enterovirus (Ghildyal et al., 2009).

Several lines of experimental evidence point to the unique L protein of cardiovirus as the factor responsible for inducing NPC alterations. A mutant cardiovirus lacking this protein failed to trigger nuclear egress of GFP-NLS, while cells transiently expressing L protein triggered GFP-NLS nuclear egress (Lidsky et al., 2006). Moreover, disruption of the zinc finger domain of this protein abolished Nup62 and Nup98 hyperphosphorylation by mengovirus and TMEV, respectively (Bardina et al., 2009; Ricour et al., 2009). Finally expression of EMCV L protein led to hyperphosphorylation of Nup62, Nup153 and Nup214 (Porter et al., 2010), providing additional evidence that this protein is sufficient and crucial for cardioviral NPC alteration. Since L protein does not possess kinase activity it was suggested to hyperphosphorylate its target nucleoporins by hijacking cellular kinases that lead to NPC disassembly during mitosis (Bardina et al., 2009; Lidsky et al., 2006). Indeed, inhibitors of mitotic kinases were able to inhibit the effect of cardiovirus infection on the permeabilization of the NPC (Bardina et al., 2009). More recently, inhibitors of two mitogen-activated protein kinases (MAPKs), the extracellular signal-regulated receptor kinase and p38 MAPK were reported to block nucleoporin phosphorylation in both EMCV-infected cells and cells expressing L protein alone (Porter et al., 2010). However, whether nucleoporins are directly phosphorylated by any of these

kinases remains to be elucidated. It is also worth mentioning that the L protein of EMCV binds directly to the Ran-GTPase (Porter et al., 2006). Thus, cardiovirus may use different strategies to disrupt nuclear transport.

In summary, multiple experiments demonstrated that picornaviruses from enterovirus and cardiovirus genera alter the composition, structure and function of the NPC. As summarized in Fig. 3.7, this is achieved by different mechanisms leading to dissimilar types of NPC disruption: while enteroviral proteases cleave a set of FG-nucleoporins, the L protein of cardiovirus triggers hyperphosphorylation of the same set of nucleoporins (Table 3.2). Further characterization of these mechanisms at the molecular level will help us understand how picornaviruses and other viruses interact with the NPC.

5.2. Displacement of Nucleoporins from the NPC Induced by Adenovirus

Adenoviruses comprise a family of non-enveloped double-stranded DNA viruses containing a genome of 34–48 kb and icosahedral capsids of 90 nm in diameter. The capsid consists of 20 facets and 12 vertices, and is composed of three major proteins: the hexon protein (also called protein II), the penton base protein (also called protein III), and fiber protein (also called protein IV). Trimers of the hexon protein form the facets of the capsid, and a noncovalent complex of penton base and trimeric fiber proteins forms the vertices with fiber proteins making up the distinct fibers projecting from the vertices of the capsid (Berk, 2007). Four minor proteins (pIIIa, pIV, pVIII and pIX) are also located in the adenovirus capsid and play a role in stabilizing the interactions between hexons (Berk, 2007). Inside the capsid, the double-stranded DNA genome is associated with several copies of four viral proteins forming an inner nucleoprotein core.

The infection pathway of human adenoviruses (Ad2 and Ad5 serotypes) includes binding to primary and secondary plasma membrane receptors, internalization by receptor-mediated endocytosis in clathrin-coated vesicles and escape to the cytosol (Leopold and Crystal, 2007). During these events the virus is partially disassembled releasing its fibers and several capsid-stabilizing proteins, and some of the remaining viral proteins are proteolytically cleaved (Greber et al., 1993, 1996; Nakano et al., 2000; Wiethoff et al., 2005). The partially uncoated virus is transported through the cytoplasm to the nucleus along microtubules (Leopold and Crystal, 2007), and docks to the NPC through binding to Nup214 at the NPC cytoplasmic filaments (Trotman et al., 2001). NPC binding induces further capsid

disassembling, resulting in the subsequent nuclear import of the viral genome through the NPC (Greber et al., 1997; Trotman et al., 2001).

A recent study reported that infection of tissue culture cells with Ad2 induces dissociation of a set of nucleoporins from the NPC (Strunze et al., 2011). Indirect immunofluorescence microscopy using antibodies against Nup358, Nup214, and Nup62 showed displacement of these nucleoporins to the cytoplasm of Ad2-infected cells 3 h postinfection. Ad2 infection, however, did not induce proteolytic processing of these nucleoporins. Intriguingly, these nucleoporins were found colocalized with disassembled virus particles (detected with an antibody that recognizes disrupted but not intact Ad2 capsids) at the cell periphery (Strunze et al., 2011). Binding partners of these nucleoporins were also found in the cytoplasm of the infected cells: RanGAP1, which binds to Nup358, and the nuclear export receptor Crm1, which binds to Nup214 and Nup358, colocalized with Ad2 particles at the cell periphery. However, Nup153 located at the nuclear side of the NPC was not mislocalized in Ad2-infected cells.

Although the effect of the Ad2-induced displacement of nucleoporins on the NPC structure remains to be determined, Strunze et al. (2011) found that the NPC permeability increased in the Ad2-infected cells. HeLa cells microinjected with fluorescent dextrans of different sizes and then infected with Ad2 for 3 h yielded nuclear localization of a 500-kDa dextran, which is normally excluded from the nucleus of noninfected cells. The increase of NPC permeability was, however, transient: the fluorescent dextran was excluded from the nucleus when cells were first infected for 4 h and then microinjected with the dextran. The authors proposed that the increased permeability of the NPC allows nuclear entry of the viral genome.

To answer the question of how the disassembled capsids (which are produced at the NPC, and are still too large to diffuse through the cytosol) and nucleoporins reach the cell periphery, Strunze et al. (2011) examined whether disassembled capsids colocalized with the anterograde microtubule motor kinesin-1. They not only found colocalization of this motor protein with the Ad2 disassembled capsids but also demonstrated that the virus particles recruited kinesin-1 via its light chain and the binding occurs through the capsid protein pIX. Based on these results and the fact that the kinesin-1 heavy chain has been reported to interact with the cytoplasmic filament nucleoporin Nup358 (Cai et al., 2001; Cho et al., 2009), Strunze et al. (2011) proposed a model in which the Ad capsid disassembles at the NPC by the action of kinesin-1, which is activated by the binding of its

heavy chain to Nup358. They propose that the action of the Nup358-bound kinesin-1 on the Nup214-docked capsid results in a force that pulls the virion apart, facilitating disassembly of the capsid and release of virus genome, and leads to dissociation of Nup358, Nup214 and Nup62 (which occurs in a subcomplex with Nup214 and Nup88; Macaulay et al., 1995) from the NPC. In support of this model the authors show that kinesin-1 colocalized with Ad2 capsids at the NPC, knockdown of either Nup358 or Nup214 prevented adenovirus uncoating, and expression of the kinesin-1 heavy-chain binding domain of Nup358 or a dominant-negative construct of the kinesin-1 heavy chain significantly reduced Ad2 expression.

Thus, displacement of nucleoporins from the NPC during Ad2 infection appears to be a consequence of Ad2 capsid disassembly by the action of kinesisn-1. This alteration of the NPC also results in transient disruption of the permeability barrier at the NPC. It remains to be determined whether other nucleoporins (for example Nup88 which is a component of the Nup214–Nup88–Nup62 subcomplex, or other members of the Nup62–Nup58–Nup54–Nup45 subcomplex) are also dissociated from the NPC during Ad2 infection, whether these nucleoporins are reincorporated into the NPC later during infection to restore the permeability of the NPC, and whether the resulted disruption of the NPC opens access for the viral genome to enter the nucleus.

5.3. Displacement of Nup62 from the NPC and Alteration of NPC Composition Induced by HIV-1

The HIV-1 belongs to the genus lentivirus of the *Retroviridae* family. It is a spherical (120 nm in diameter) enveloped virus containing a linear, dimeric single-stranded RNA genome of about 9.8 kb with three major genes (*gag*, *pol*, and *env* encoding structural, enzyme and envelope proteins respectively), as well as several additional regulatory and accessory genes (Freed and Martin, 2007). The viral genome is packed with viral and host proteins into a conical capsid of 100–120 nm in length (Benjamin et al., 2005). The virus life cycle begins with virion binding to specific cellular receptors and fusion of the viral envelope with the plasma membrane of T cells and macrophages, resulting in the release of the capsid into the host cell cytoplasm. This is followed by a poorly understood uncoating step (Arhel, 2010) and the reverse transcription of the viral RNA (vRNA) by the viral reverse transcriptase into a double-stranded viral DNA molecule. Subsequently, the resulted DNA, associated with the viral integrase and additional viral and

host proteins (comprising the pre-integration complex), is imported into the nucleus using the nuclear transport machinery. This step is assisted by several viral proteins (Cohen et al., 2011a; Suzuki and Craigie, 2007). In the nucleus the viral DNA is inserted into the host genome by the viral integrase, and the integrated viral genome is transcribed to produce a number of spliced fragments, encoding regulatory/accessory proteins, and the full-length vRNA. This vRNA functions as the mRNA for structural proteins and enzymes (the *gag* and *pol* genes) and is also packaged into progeny virion. The vRNA is exported from the nucleus through the NPC in conjunction with the viral Rev protein as a vRNA–Rev complex. At the final phase of infection, after the synthesis of viral proteins, the vRNA is packaged into viral particles at the plasma membrane of the infected cell, and the virions are eventually released by budding from the plasma membrane (Jouvenet et al., 2006).

At every step of its viral life cycle, HIV-1 extensively utilizes several host cellular machineries and interacts with multiple host proteins, which leads to alterations in host cell morphology and cellular processes. Recently, several genome-wide small interfering RNA screens have identified over 1000 candidate host proteins that assist HIV-1 replication (Friedrich et al., 2011; Lever and Jeang, 2011). Not surprisingly, some of these cellular proteins are nucleoporins, nuclear transport receptors and cellular factors that are involved in transporting molecules through the NPC. The general understanding is that these host proteins are involved in the process of nuclear import of the pre-integration complex and several viral proteins, and/or the nuclear export of the vRNA. Thus, because several steps of the HIV-1 life cycle highly depend on nuclear transport it has largely been assumed that the virus does not block, alter or interfere with the nuclear transport machinery. However, recent studies by Mouland and colleagues indicate that this is not the case, and in fact HIV-1 alters both NPC function and composition (Monette et al., 2009, 2011). Examination of the cellular distribution of the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 protein and its nuclear import receptor transportin-1 revealed that HIV-1 causes cytoplasmic retention of both proteins (Monette et al., 2009). The hnRNP A1 protein, which is one of the major pre-mRNA/mRNA binding proteins with a wide range of roles in diverse cellular events including RNA processing and trafficking, localized to the nucleus but also shuttles continuously between the nucleus and the cytoplasm (Pinol-Roma and Dreyfuss, 1992; Pollard et al., 1996). Surprisingly, immunofluorescence staining as well as Western blots of nuclear and cytoplasmic fractions from cells transfected

with the proviral HIV-1 DNA showed that hnRNP A1 accumulated in the cytoplasm of HIV-1-infected cells (Monette et al., 2009). Similar immunostaining experiments showed cytoplasmic retention of transportin-1 in the HIV-1-infected cells (Monette et al., 2009).

Searching for the mechanism by which HIV-1 induces cytoplasmic retention of these proteins, Monette et al. (2009) demonstrated that their nuclear import was blocked due to alterations in the localization and abundance of Nup62. Using heterokaryon assays, they found that expression of the virus genome prevented import of hnRNP A1 into the nucleus, indicating that HIV-1 impaired the capacity of the nuclear transport machinery to translocate cargo from the nucleus to the cytoplasm. Importantly, examination of lamin A/C by immunofluorescence microscopy (Monette et al., 2009) as well as thin-section electron microscopy analysis of NE and NPC of infected cells (Monette et al., 2011) did not detect HIV-1-caused disruption of the NE or the NPC, pointing to the possibility that slight alternations in the NPC composition and/or structure could be the cause of the nuclear import block. Because transportin-1 was reported to interact with Nup62 in vitro (Bonifaci et al., 1997; Percipalle et al., 1997), Monette et al. (2009) then analyzed the expression level of this nucleoporin and found that it was significantly reduced in HIV-1-infected cells. They also examined the localization of this nucleoporin by immunofluorescence microscopy, and surprisingly found that instead of the typical NE rim staining, the Nup62 immunostaining was dispersed across the NE and extended markedly into both the cytoplasm and the nucleus of cells transfected with the proviral HIV-1 DNA. These results suggest that HIV-1 infection displaces Nup62 from the NPC. In agreement with this, immunogold electron microscopy studies using antibodies that predominantly recognized Nup62 yielded immunogold staining at the NPC but also throughout the cytoplasm of cells transfected with the proviral HIV-1 DNA (Fig. 3.9; Monette et al., 2011). Intriguingly, Nup62 immunogold staining was also found in budding HIV-1 virions (Fig. 3.9; Monette et al., 2011). Nup62 incorporation into the mature viral particles was corroborated using Western blot analysis of purified virion, pointing to the possibility that this nucleoporin may play an important role in the assembly and/or infectivity of HIV-1 (Monette et al., 2011). Indeed, small interfering RNA-imposed knockdown of Nup62 resulted in nuclear retention of vRNA, as well as reduction in Gag synthesis and progeny virus production. These results indicate that Nup62 is essential not only for vRNA nuclear export but also for HIV-1 replication and infectivity.

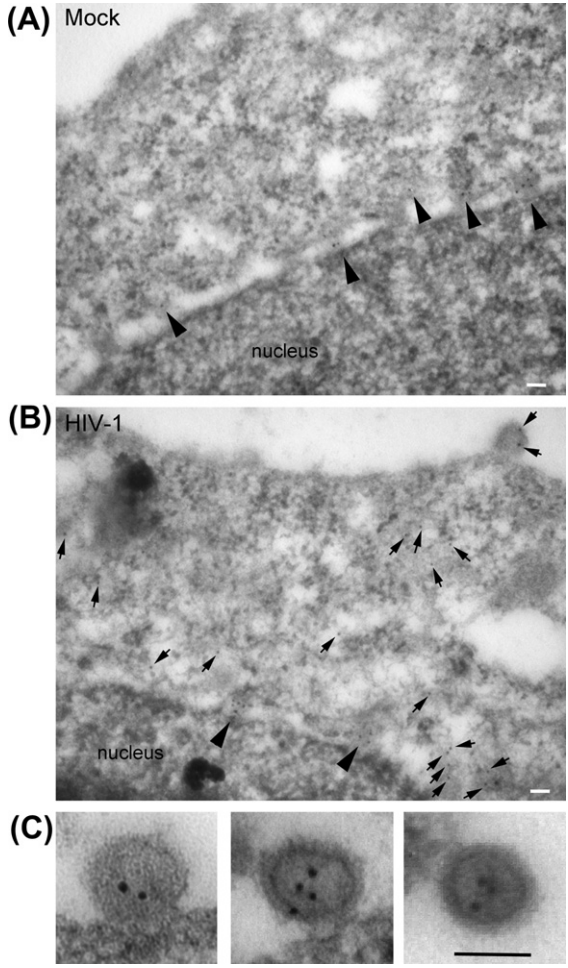

Figure 3.9 ***HIV-1 displaces Nup62 from the NPC.*** Electron micrographs of HeLa cells that have been mock transfected or transfected with proviral HIV-1 DNA, prepared for thin-section electron microscopy and immunolabeled with the anti-nucleoporin antibody mAb414, which recognizes primary Nup62, and 10-nm gold-conjugated secondary antibody (Monette et al., 2011). In mock-transfected cells, gold particles were exclusively observed at the NE associated with NPCs (arrowheads). In contrast, in cells transfected with the proviral HIV-1 DNA, gold particles were found at the NPCs (arrowheads), throughout the cytoplasm (arrows) and in budding virions (arrows). *Experiments were performed by Drs Anne Monette and Andrew Mouland (Monette et al., 2011). Scale bars, 100 nm.*

Another question addressed by Monette et al. (2011) was which viral gene was responsible for the altered Nup62 distribution. To answer this question, they tested several proviral DNA mutants, and found that only Rev-deficient mutants did not cause the dissociation of Nup62 from the NPC. Next, the authors performed experiments overexpressing Rev alone or together with a Rev(−) proviral DNA, and strikingly found that Nup62 was displaced from the NPC only when Rev was overexpressed in the presence of the mutant vRNA. Interestingly, they also found colocalization of Nup62 with Rev, the viral ribonucleoprotein (vRNP), and Gag. Based on this colocalization, Monette et al. (2011) suggested that Nup62 may be part of the vRNP complex, and proposed a model in which Nup62 associates with the vRNA–Rev complex as it is exported from the nucleus through NPC. In this model, the nuclear export of the vRNP results in dissociation of Nup62, and possible other nucleoporins from the NPC. This displacement of nucleoporins from the NPC alters the nuclear import capacity of the NPC, and explains the HIV-1-induced inhibition of the nuclear import of hnRNP A1. In agreement with this model, hnRNP A1 did not accumulate in the cytoplasm of cells transfected with a Rev(−) proviral DNA mutant (Monette et al., 2009). After being dissociated from the NPC by the vRNA–Rev complex, Nup62 then remains linked to the vRNP in the cytoplasm and is encapsidated in progeny virus.

To determine whether other nucleoporins may be depleted from the NPC by HIV-1, Monette et al. (2011) performed a proteomic analysis of purified NEs from mock and HIV-1-infected cells. Strikingly, they found that not only the concentration of Nup62 but also that of 14 other nucleoporins was decreased in the NE of cells transfected with the proviral DNA. It is very surprising that such an effect on the NPC composition does not seem to affect the NPC morphology, as detected by embedding–thin-section electron microscopy (Monette et al., 2011). This could be explained by the high abundance of some nucleoporins [e.g. it has been estimated that each NPC has 128 molecules of Nup62 in the Nup62–Nup58–Nup54–Nup45 subcomplex (Hoelz et al., 2011), and several other copies of Nup62 are in the Nup214–Nup88–Nup62 subcomplex], so that the amount of nucleoporins displaced from the NPC by the Rev–vRNA complex is not enough to disrupt the NPC structure and completely impair its transport function. Another possibility could be the apparently functional redundancy of nucleoporins, as has been concluded from experiments demonstrating that functional NPCs can be assembled in the absence of some nucleoporins (Powers et al., 1995; Stavru et al., 2006; Walther et al., 2002; Zhou and

Pante, 2010), and the existence of NPCs of different composition among different type of cells and different tissues (D'Angelo et al., 2012; Lupu et al., 2008; Olsson et al., 2004).

In summary, HIV-1 was found to displace Nup62 from the NPC (Fig. 3.8) to inflict significant alterations of the NPC composition and to cause defects in the nuclear import of the hnRNP A1 protein. The demonstrated association of Nup62 with cytoplasmic vRNA complex and progeny virions reveals new aspects of nucleoporin function, which may play an important role in HIV-1 replication/structure. It remains to be investigated whether the other nucleoporins whose abundance is modified during HIV-1 infection assist HIV-1 replication. Such insights could be exploited to targeting specific nucleoporins as a means to inhibit HIV-1 replication. Learning more about how HIV-1 and other viruses interact with the NPC can also help elucidate important aspects of NPC composition, assembly and function.

6. CONCLUDING REMARKS

Clearly, the NE is a critical cellular barrier that poses challenges to invading viruses at multiple stages of the viral life cycle. Many viral pathogens have evolved ways to modulate its permeability. Parvoviruses and the polyomavirus SV40 disrupt the NE during delivery of the viral genome into the nucleus for replication, while HSV-1 disrupts the nuclear lamina during nuclear egress of newly assembled capsids. Other viruses affect NE permeability and NPC structure in order to alter the compartmentalization of host proteins. Picornaviruses cause alterations of the NPC resulting in both an increase in the passive diffusion through NPC, which contributes to relocalization of nuclear proteins that facilitate viral replication, and inhibition of certain nuclear transport pathways, which may prevent the host cell from mounting an antiviral response. HIV-1 may alter NPC composition and modulate transport through the NPC for the same reason: to facilitate viral replication and to inhibit host antiviral response. Another potential reason for HIV-1-mediated NPC remodeling is suggested by the observation that Nup62 is actually encapsidated in HIV-1 virions (Monette et al., 2011). Thus, encapsidated Nup62 may play a role in delivery of the pre-integration complex into the nucleus of newly infected cells. Lastly, the effect of adenovirus on the NPC seems to play dual roles in capsid disassembly and delivery of the viral genome into the nucleus.

In addition to enriching our understanding of viral pathogenesis, investigation of the interaction of viruses with their host cells can elucidate previously unknown cellular processes. Based on studies of herpes virus nuclear egress and on the observation that an *Saccharomyces cerevisiae* acetyl coenzyme A carboxylase mutant accumulates NE luminal vesicles (Schneiter et al., 1996), it has recently been proposed that cells may have an endogenous vesicular trafficking pathway between the INM and ONM (Burns and Wente, 2012). Similarly, studies of parvovirus and SV40 have revealed non-apoptotic roles of caspase-mediated nuclear lamin cleavage, which may be more widely applicable under physiological conditions. Indeed, caspase-3 is upregulated during mitosis (Hsu et al., 2006), indicating that this enzyme likely plays a role in cellular processes other than apoptosis. Finally, study of the effect of viruses on the NPC is likely to provide insight about NPC assembly, disassembly and function. We anticipate that further research in this area will lead to the development of novel antiviral therapeutics, as well as a better understanding of the biology of both the pathogens and their hosts.

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CHAPTER FOUR

New Insights into Cell Cycle Regulation and DNA Damage Response in Embryonic Stem Cells

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Contents

Abstract

Embryonic stem cells (ESCs) have unlimited proliferative potential, whilce retaining the ability to differentiate into descendants of all three embryonic layers. High proliferation rate of ESCs is accompanied by a shortening of the G_1 phase and the lack of G_1 checkpoint following DNA damage. The absence of G_1 arrest in ESCs after DNA damage is likely caused by a dysfunction of the p53-dependent *p21Waf1* pathway that is a key event for the maintenance of pluripotency. There are controversial data on the functional status of p53, but it is well established that one of the key p53 target—*p21Waf1*—is expressed in ESCs at a very low level. Despite the lack of G_1 checkpoint, ESCs are capable to repair DNA defects; moreover the DNA damage response (DDR) signaling operates very effectively throughout the cell cycle. This

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review covers also the results obtained with the reprogramming of somatic cells into the induced pluripotent stem cells, for which have been shown that a partial dysfunction of the p53Waf1 pathway increases the frequency of generation of pluripotent cells. In summary, these results indicate that the G_1 checkpoint control and DDR are distinct from somatic cells and their status is tightly connected with maintaining of pluripotency and self-renewal.

1. INTRODUCTION

Embryonic stem cells (ESCs) are currently attracting much attention because they can be used for detailed study of mechanisms of differentiation into descendants of all three embryonic layers that is necessary for the purposes of regenerative medicine and replacement cell therapy. A new impact for research of ESCs has been received through the development of innovative methods aimed at obtaining the induced pluripotent stem cells (iPSCs) from the normal somatic cells, which would be equivalent to the ESCs. This could eliminate many of the ethical issues associated with the use of the human embryo material in the regenerative medicine. For molecular and cell biologists, ESCs are of a great interest, because they are immortal cells and can divide indefinitely in culture at a high speed, while retaining the ability to differentiate into all cell types of the adult organism. Briefly saying, the unique properties of ESCs can be described in such fundamental characteristics as self-renewal and pluripotency. Since mutations, which could occur, are a threat to many developing tissues of the growing organism, it is conceivable that in ESCs some mechanisms should operate that would minimize the consequences of DNA damage. Available data suggest that the solution to these challenges is provided through a number of cellular mechanisms. First, the ESCs lack the cell cycle arrest in G_1 phase, so they do not stop in the cell cycle, otherwise they will be triggered to differentiate. Second, despite the absence of G_1 cell cycle arrest, the level of reparative processes in ESCs is even higher than in somatic cells, and the repair is taking place at all phases of the cell cycle. Third, despite the widely shared view that «p53: guardian of the genome and policeman of the oncogenes» (see Efeyan and Serrano, 2007), p53 functions in ESCs are not realized in full, as it would inevitably conflict with the necessity to maintain the most important property—pluripotency. Respectively, the main function of p53 in checkpoint control is compromised in ESCs. The study of mechanisms of molecular cross talk between cell cycle regulation, energy metabolism, functioning of the tumor-suppressor p53/pRb pathways and

DNA damage repair shows that in ESCs all these processes are closely coordinated and aimed at maintaining self-renewal and pluripotency.

2. CELL CYCLE AND ITS REGULATION IN ESCs

2.1. Unique Structure of Cell Cycle in ESCs

ESCs derived from the inner cell mass of blastocysts have two important features—the pluripotency, that is the ability to differentiate into all cell types of the adult organism and the self-renewal. Despite the unique ability to differentiate, ESCs have a high rate of proliferation with the cell cycle of approximately 10–12 h for the mouse and monkey ESCs and 15–16 h for human ESCs compared to somatic cells where the cell cycle varies from 24 to 32 h (Stead et al., 2002; Becker et al., 2006; Fluckiger et al., 2006). Such a high proliferation rate of ESCs is due to unusual structure of their cell cycle—a truncated G_1 phase with lack of the early part of G_1 prior to the so-called restriction point (R) (Jones and Kazlauskas, 2001). The proportion of cells in G_1 and S phases of the cell cycle in undifferentiated ESCs is respectively 15–20% and 60–70%, but this ratio changes during ESCs differentiation (Fig. 4.1). In the early G_1 phase of somatic cells the external growth factors activate the mitogen-activated protein kinase-extracellular signal-regulated kinase (MAPK-ERK) signaling pathway involved in phosphorylation of transcription factors (c-Fos, Elk, SRF, and many others) that regulate the transcription of early-growth responsive genes for the $G_1 > S$ transition. Correspondingly, MEK–Erk inhibitors effectively suppress proliferation of somatic cells, but not ESCs indicating the lack of the Erk-dependent part of G_1 phase in ESCs (Ying et al., 2008). Moreover, MEK–Erk inhibitors, when added to the ESCs, contribute to the maintenance of undifferentiated state and remove differentiated cells from the population (Burdon et al., 2002; Ying et al., 2008; Silva et al., 2008).

In case of somatic cells removal of mitogenic growth factors before the restriction point leads to the exit of the cell cycle and the transition to the quiescence state (G_0). However, if a cell has passed the restriction point, it is no longer sensitive to the absence of growth factors and transits to the S phase. It is believed that the restriction point coincides with the phosphorylation of pRb, and is absent in many human tumors. ESCs neither stop dividing nor go into the quiescence state G_0 after the removal of serum growth factors that is consistent with permanent pRb phosphorylation. ESCs are very similar to tumor cells, which are frequently defective in cell cycle regulation pathways

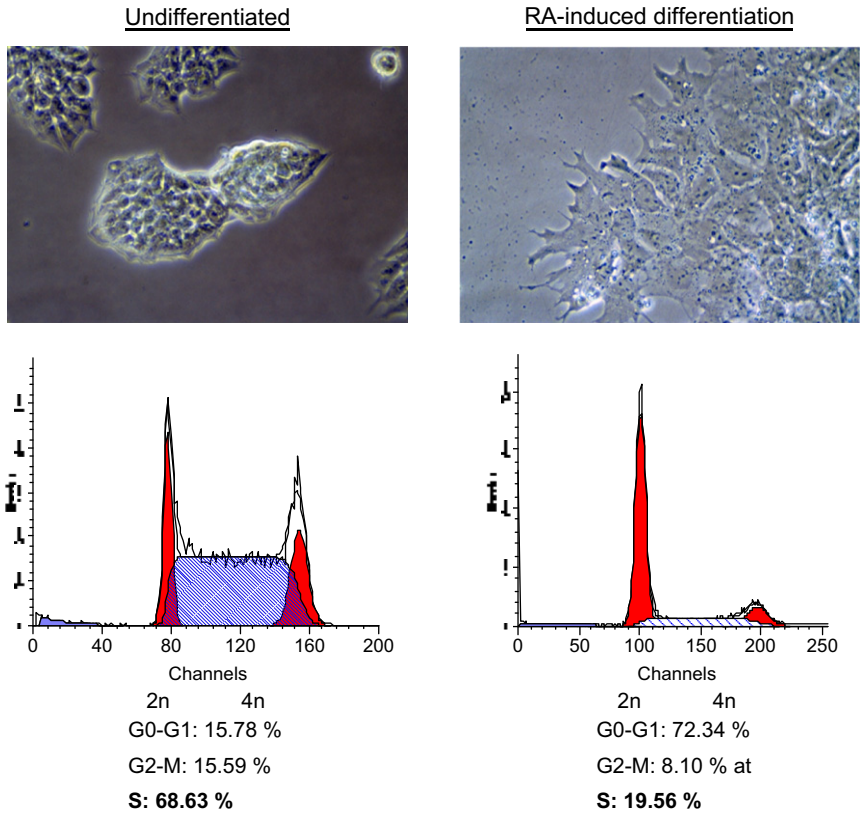

Figure 4.1 Flow cytometry analysis of cell cycle phase distribution of undifferentiated and differentiated mouse ESCs (bottom panel). Phase contrast microscopy of undifferentiated (left) and differentiated (right) mouse ESCs (upper panel). Differentiation was induced by 1 μM of all-*trans* retinoic acid for 4 days (magnification 40×). *Figure courtesy of Maria Lyanguzova.* For color version of this figure, the reader is referred to the online version of this book.

(p53/Arf and pRb/Ink4). Similarly, ESCs and embryonal carcinoma cells (ECCs) treated with DNA-damaging agents do not undergo G_1/S cell cycle arrest (Malashicheva et al., 2000, 2002; Schratt et al., 2001; Chuykin et al., 2008; Momcilovic et al., 2011). All these data may indicate that a reduction of G_1 phase and the absence of the restriction point as well as of the G_1/S arrest provide the unique organization of the cell cycle of ESCs and is directly related to their self-renewal and maintenance of the pluripotent state (Becker et al., 2006; Momcilovic et al., 2011). As in the early G_1 phase the cell is committed to respond to mitogenic growth factors by the progression of the cell cycle or differentiation, the functional significance of the absence of

mitogen-sensitive period in the G_1 phase of the ESCs is likely to avoid the stimulating action of the signals that trigger differentiation. Thus, the molecular mechanisms that regulate the cell cycle of ESCs differ significantly from those in somatic cells and relate primarily to the G_1 to S phase transition.

2.2. Regulation of Cyclin-Kinase Complexes in Mouse and Human ESCs

Cell cycle progression depends on the activity of complexes consisting of two subunits: a catalytic subunit represented by cyclin-dependent kinases (Cdks) and a regulatory subunit, which includes a variety of cyclins. In somatic cells Cdks are expressed throughout the cell cycle, whereas expression of cyclins is carried out in well-defined phases (cycling). The transition from one phase to another is regulated by the composition of the cyclin-kinase complexes. Thus, the passage of the G_1 phase and transition into S phase is dependent on the presence of active cyclin D/Cdk4, 6 and cyclin E/Cdk2 complexes, whereas the S and G_2 phase events are regulated by a cyclin A/Cdk2. Complexes of cyclin A and cyclin B with kinase Cdc2 provide entry into mitosis and control of early mitotic events. Regulation of the cyclin-kinase complexes is controlled by inhibitors (CKI), represented by two protein families: Cip/Kip1 and Ink4. The first family, Cip/Kip1, consists of *p21Waf1*, p27 and p57 proteins, which inhibit both Cdks and the cyclin–Cdk complexes. The second family, Ink4, consists of p16Ink4a, p15Ink4b, p18Inc4c, and p19Ink4d, which specifically inhibit only Cdk4 and Cdk6 kinase activity by preventing their association with cyclin D. It was shown that all the CKI inhibitors are expressed at very low levels in ESCs (Stead et al., 2002; Faast et al., 2004; White et al., 2005; Becker et al., 2006; Neganova et al., 2009). Due to low expression of Cdk4 kinase and therefore the low content of the complexes cyclin D1/Cdk4 (Stead et al., 2002), mouse ESCs are not sensitive to the mitogenic signals that operate in G_1 phase and are able to trigger differentiation. In contrast to somatic cells, in mouse ESCs the cyclins are synthesized throughout the cell cycle, except for cyclin B, the maximum expression of which takes place in mitosis (Stead et al., 2002; White et al., 2005). Mouse ESCs exhibit an extremely high activity of Cdk2 kinase, which is constitutive throughout the cell cycle and is the main driving force for rapid proliferation of these cells (Stead et al., 2002; Koledova et al., 2010a). Activity of Cdk2 is regulated, at least, by two oppositely directed phosphorylations: activating phosphorylation at Thr-160 by CAK Cdc7 kinase, while inhibitory phosphorylation occurs on Thr14/Tyr15 and is conducted by Wee1 kinase

(Sørensen and Syljuasen, 2012). Kinase Cdc7 is essential for the viability of eukaryotic cells and its inhibition or depletion leads to cell death specifically in cancer cells (Sawa and Masai, 2008). Knockout of Cdc7 alleles in mice leads to early embryo death; the mutant embryos die between E3.5 and 6.5 (Kim et al., 2002). The authors generated conditional Cdc7-defcient mouse ESC lines and showed that Cdc7 is essential for DNA replication of ESCs as the Cdc7 knockout ESCs demonstrate S-phase arrest and p53-dependent cell death. Inhibitory phosphorylation of Cdks 1 and 2 by Wee1 at Thr14/Tyr15 inhibits their activity. Knockout of *Wee1* is a lethal and the embryos die before embryonic (E) day 3.5. Cells from the 3.5 embryos are defective in the G_2/M cell cycle checkpoint induced by γ-irradiation and died of apoptosis (Tominaga et al., 2006). In mouse ESCs the Wee1 protein is not present or expresses on a low level, thus making the Cdk2 unphosphorylated at Thr14/Tyr15 and therefore active. But the Wee1 protein appears during differentiation (Lichner et al., 2011). Analysis of micro-RNAs, which participate in G_1/S regulation and highly transcribed in mouse ESCs, showed that transcripts of *Wee1* as well as *p21Waf1* are the targets for micro RNAs miR-290–295 cluster (Lichner et al., 2011) that results in reducing the level of negative Cdk2 regulators. In summary, these results shed light on the reasons for the high constitutive Cdk2 activity in mouse ESCs. Given the important role of Cdk2 in the proliferation, downregulation of Cdk2 in ESCs prolongs G_1 phase, leads to restoration of phase-dependent activation of cyclin/Cdk complexes and induces the expression of differentiation markers (White et al., 2005; Koledova et al., 2010a). Since Cdk2 and Cdk1 are functionally redundant in supporting DNA replication therefore both the G_1/S and G_2/M checkpoints appear to remain fully functional in Cdk2−/− mouse embryonic fibroblasts (Chung and Bunz, 2010). In addition to constitutive Cdk2 activity, mouse ESCs are characterized by relatively high activity of the cyclin D3/Cdk6 complex. Despite the low expression of cyclin D3, the cyclin D3/Cdk6 complexes formed have high catalytic activity and are not sensitive to p16 inhibitor (Faast et al., 2004). Mouse ESCs lack active Cdk4 kinase and therefore the complexes cyclin D1/Cdk4 are not detectable (Stead et al., 2002). The complexes formed by cyclin D2 are also absent due to the lack of cyclin D2 expression (Faast et al., 2004). Thus, cell cycle in mESCs is characterized by a lack of regulated expression of the key components—cyclins and Cdks. In contrast to use mouse ESCs and human ESCs, human ESCs exhibit phase-dependent expression of cell cycle cyclins and thus restrict the activity of Cdks. It was shown that the level of cyclin E increases during the transition from G_1 to S phase and the expression of cyclin A is maximal in S

and G_2 phases. Synthesis of cyclin B, like in mouse ESCs, reaches its maximum at the border from G_2 to mitosis (Neganova et al., 2009). In addition, the activity of Cdk2, Cdk4 and Cdk6 varies depending on the phase of the cell cycle (Neganova et al., 2009). As in mESC, Cdk2 kinase is most active and plays a significant role in the proliferation and maintenance of the pluripotent state of human ESCs (Becker et al., 2006; Neganova et al., 2009). Knockdown of Cdk2 causes changes in the morphology of human ESCs and according to the profile of gene expression they become similar to somatic cells (Neganova et al., 2009). Inhibition of Cdk4 activity did not result in any visible changes in morphology or expression pattern of genes specific for undifferentiated cells (Filipczyk et al., 2007). Thus, human ESCs exhibit cycling pattern of some cell cycle regulators (cyclins A and B), which is in a clear contrast to mESC. This probably is due to a longer cell cycle of human ESCs as compared with mouse ESCs. Despite that use mouse ESCs and human ESCs have common properties—pluripotency and self-renewal—human ESCs differ in the structure of the cell cycle. It is believed that the distinction is due to mESCs and hESCs originated from embryo cells at different stages of embryonic development. In fact, human ESCs are more similar to mouse ESCs derived from postimplantation epiblast than from the inner cell mass of blastocysts (Tesar et al., 2007; Hanna et al., 2010). Apparently, the conditions for cultivation of human ESCs might be not optimal and the emerging stress may thus influence the cell cycle in human ESC.

2.3. Status of pRb/E2F Pathway

The pRb protein plays a key role in regulating the transition from G_1 to S phase of cell cycle, so the loss of its function in most cases leads to uncontrolled cell proliferation (Conklin and Sage, 2009). The mechanism of cell cycle regulation is based on the ability of unphosphorylated pRb to form complexes with proteins of E2F transcription factor family, which are responsible for the transactivation of genes encoding the key components of the G_1/S transition and DNA replication. E2F transcription factor, being bound with pRb, is not able to activate transcription of target genes. In somatic cells, pRb is mainly hypophosphorylated and is found in complexes with E2F, blocking the passage of cells into S phase. In response to various mitogenic stimuli and growth factors operating in the early G_1 phase the active cyclin D/Cdk4, 6 complexes are formed and then phosphorylate pRb that leads to a partial release of E2F. This event triggers the expression of many target genes such as *cyclin E*, *pol* α, and *Cdc25A*. As a result active cyclin

E/Cdk2 complexes are formed, which further phosphorylate pRb with subsequently releasing the remaining E2F. These events mark the transition into S phase. Analysis of the synchronized mouse ESCs showed that pRb protein is constitutively hyperphosphorylated throughout the cell cycle and therefore is not able to bind to E2F (White et al., 2005). Accordingly, transcription of E2F-dependent genes proceeds independently on the phase of the cell cycle (Stead et al., 2002). Perhaps, the constitutive pRb hyperphosphorylation is due to a permanent activity of cyclin E/Cdk2 and cyclin A/Cdk2 complexes, as well as the high activity of the complex cyclin D3/Cdk6 (Faast et al., 2004). The mitogen-dependent cyclin D/Cdk4/pRb signaling pathway in mESCs is not regulated, since after serum removal they continue to divide and do not move into quiescent state G_0 (Schratt et al., 2001; Malashicheva et al., 2002). The presence of only hyperphosphorylated pRb form leads to a permanent expression of genes regulated by E2F, in particular, *cyclin E*. Correspondingly, constitutive expression of cyclin E supports the activity of the cyclin E/Cdk2 complexes and consequently phosphorylation of pRb protein throughout the cell cycle. Genes related to ESCs pluripotency may be also involved in providing unregulated activity of cyclin E/Cdk2 complexes (Boyer et al., 2005). Thus, it has been shown that Nanog binds to the promoters of *cdk6* and *cdc25A* genes; therefore, a decreased Cdk6 and Cdc25A expression facilitates S-phase entry in hESCs (Zhang et al., 2009). In addition, it should be noted that differentiation of mouse ESCs restores the active (unphosphorylated) pRb status, whereas the inactive (phosphorylated) pRb blocks differentiation of mESCs (Dannenberg et al., 2000; White et al., 2005). In human ESCs, the percentage of cells in G_1 phase exceeds that in mESCs, and, respectively, a larger part of pRb is represented in the hypophosphorylated form (Filipczyk et al., 2007). Like mESCs, human ESCs constitutively express cyclin E, as well as negative for the expression of cyclin D and do not respond to Cdk4 inhibitor p16. In a recent publication, however, certain human ESC lines still express D-cyclins (Neganova et al., 2009). This specifies a difference between mouse and human ESCs, primarily based on the fact that human ESCs originate from cells which are on the later stages of embryonic development.

2.4. Status of p53/*p21Waf1* Pathway

Transcription factor p53 plays an important role in maintaining genome stability of somatic cells, so the mutations of this gene are found in almost half of all human cancers. The functions of p53 are diverse: it is involved in

arrest of cell division (checkpoint control) and induction of cellular senescence, as well as in regulation of a number of genes controlling apoptosis. In response to genotoxic stress p53 is stabilized and activated that in turn triggers the transcription of several target genes, including *p21Waf1* gene, expression of which is required for the cell cycle arrest. The p21Waf protein, a key regulator of G_1/S checkpoint, directly inhibits the activity of cyclin-kinase complexes. Thus, the signaling pathway mediated through p53/*p21Waf1* executes the G_1/S checkpoint in somatic cells, during which the cells either repair DNA damages or die due to apoptosis, or undergo irreversible cell cycle arrest and senescence. ESCs do not demonstrate G_1/S checkpoint, there appears only a temporary block of G_2/M (Aladjem et al., 1998; Chuykin et al., 2008; Momcilovic et al., 2009) (Fig. 4.2). However, data of Barta's group argue that hESCs short wave ultraviolet C (UVC) irradiated in G_1 phase are capable of undergoing G_1/S cell cycle arrest and exhibit decreased Cdk2 kinase activity (Barta et al., 2010). In this paper, hESCs were synchronized by nocodazole at G_2/M phase, then washed and irradiated with ultraviolet light. However, there are convincing data that nocodazole-induced synchronization upregulates DDR signaling pathway and affects the expression of pluripotent markers thereby changing the status of undifferentiated cells. In particular, Kallas et al. reported the changes in the expression of pluripotent markers Nanog and Oct4 as well as ES-specific surface markers SSEA-3 and SSEA-4 in human embryonic cells after their treatment with nocodazole (Kallas et al., 2011). The nocodazole treatment activated p53, which triggered induction of a reversible cell cycle arrest accompanied by irreversible loss of expression of pluripotent markers Nanog and Oct4. Hence, the data cast doubt on the conclusions drawn from the

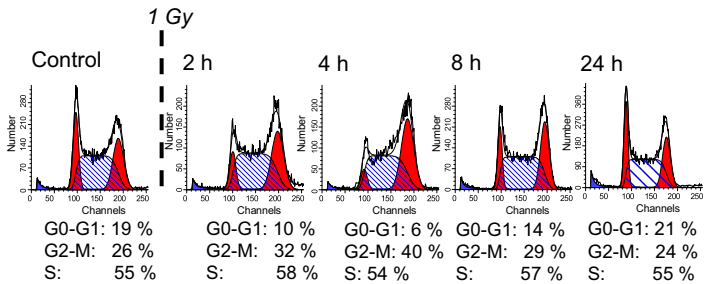

Figure 4.2 Flow cytometry analysis of cell cycle phase distribution of mouse ESCs at different time points after 1 Gy irradiation (2, 4, 8, and 24 h). The percentage of cells in G_0/G_1, S and G_2/M phases of the cell cycle is indicated below. For color version of this figure, the reader is referred to the online version of this book.

nocodazole-synchronized cells used to prove the existence of a functional G_1 checkpoint in human ESC (Barta et al., 2010).

As ESCs have very short duration of G_1 phase, it has been suggested that lengthening of G_1 phase of the ESC cycle can cause the loss of pluripotent state and the onset of differentiation. One may conclude that the lack of a functional G_1/S checkpoint is a mechanism that maintains the exclusive properties of ESCs—pluripotency and high proliferative potential (Becker et al., 2006). In order for this mechanism to be effective, the signaling pathways responsible for maintaining the genome stability of ESCs must operate differently than their counterparts in somatic cells. The most noticeable and significant difference is a decreased function of p53/*p21Waf1* pathway in ESCs (Stead et al., 2002; White et al., 2005). It has been shown earlier that although DNA damage activates *p21waf1* gene transcription in ECC line F9, *p21Waf1* protein is subjected to proteasomal degradation (Malashicheva et al., 2000). The same results were later obtained for mouse ESCs (Malashicheva et al., 2002) (Fig. 4.3). Thus, a very low expression level of *p21Waf1* protein has been demonstrated in mouse and human ESCs (Chuykin et al., 2008; Barta et al., 2010). It was recently suggested that the modulation of the *p21Waf1* expression may also occur on a post-transcriptional level regulated by a group of specific micro-RNA 290–295 (Wang et al., 2008; Lichner et al., 2011). Expression of the miR-290 cluster is downregulated as ESCs differentiate and consequently the G_1 phase becomes longer and the G_1/S checkpoint appears in the differentiated somatic cells (Wang et al., 2008). The issue of how other micro-RNAs, which are specific for ATM (miR-421), p53 (miR-125b/miR-504) and

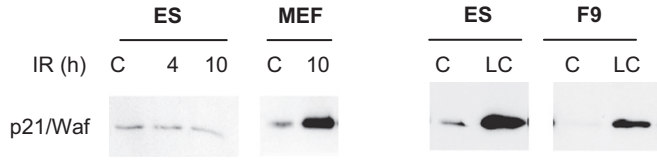

Figure 4.3 Proteasome inhibitor lactacystin causes accumulation of p21Waf1 in mouse ESCs. The p21Waf1 content was determined by Western blotting of protein extracts obtained from 6 Gy-irradiated cells or cells treated with lactacystin. Cells were γ-irradiated and after 4 or 10 h postirradiation were lysed to harvest protein extracts. Cells were grown in the presence of 1 μM lactacystin for 3 h, and then proteins were extracted and analyzed by Western blotting with antibody to p21Waf1 protein. C, control; LC, lactacystin; F9, embryonic carcinoma cell line F9. *This image (Malashicheva et al., 2002) is reproduced with permission from Tsitologiia.* For color version of this figure, the reader is referred to the online version of this book.

p21Waf1 (miR-106b), contribute to the G_1/S checkpoint of ESCs is an interesting open question and worthy of further investigation (Hu and Gatti, 2011).

Not only ESCs but also some types of adult tissue stem cells (ASC) fail to undergo cell cycle arrest due to a deficiency of *p21Waf1* pathway. It was noted, for example that neural stem and progenitor cells (NSPCs) do not initiate a detectable G_1/S block after DNA damage and continued to enter S phase at a similar rate to the nonirradiated control due to low expression of *p21Waf1* protein (Roque et al., 2012). A number of transcription factors such as FoxG1, Bmi1, Olig2, and RunX can be involved in *p21* gene repression to promote the amplification of NSPCs (Fasano et al., 2007; Ligon et al., 2007). Despite the absence of G_1 arrest, NSCs are capable of responding to X-ray irradiation by the activation of a robust canonical DDR. Interestingly, the activation of ATM and its downstream DDR factors were strongly inhibited on a transcriptional level in NSC descendants, astrocytes, as evidenced by a reduced phospho-ATM and the lack of detectable 53BP1 nuclear foci (Schneider et al., 2012). Unlike ESCs, which proliferate continuously, most adult tissue stem cells (ASC) maintain "stemness" being largely quiescent and reside primarily in G_0 phase of the cell cycle (Mandal et al., 2011). Since most of the tissue ASCs do not proliferate, for example as in case of quiescent hematopoietic stem cell, they attenuate DNA damage response (DDR) and permit DNA damage accumulation in response to genotoxic stress and during aging (Rossi et al., 2007). Only after the signal to proliferation of these cells the accumulated defects can be realized.

Surprisingly, a decreased *p21Waf1* expression is detected in the course of the tissue regeneration when the cells undergo dedifferentiation and acquire a stem-like phenotype. In their remarkable work, Bedelbaeva et al. compared the level of *p21Waf1* expression and ability to regeneration of unique MRL mice having high regeneration potential (Murphy Roths Large strain) versus wild-type counterparts. They found a direct relationship between the lack of *p21Waf1* expression in MRL mice and their capability to tissue regeneration (Bedelbaeva et al., 2010). In addition to the unusual property of MRL mice, in normal mice after 70% partial hepatectomy in vivo, the reprogramming factors Oct4 and Nanog were found to be upregulated and therefore may play a role in liver regeneration (Bhave et al., 2011). This confirms an issue that tissue regeneration in mice is mediated via formation of a stem cell-like phenotype (Bedelbaeva et al., 2010). Downregulation of *p21Waf1* expression seems necessary both for regenerating and for reprogramming cells; the necessity is based on the fact that the elevated

expression of *p21Waf1* promotes cellular senescence, which is inhibitory to both the above processes.

As for the status of the p53 in ESCs, the results are still controversial. In earlier studies it was shown that p53 is localized in the cytoplasm of mouse ESCs and not efficiently transported into the nucleus after DNA damage (Aladjem et al., 1998). The results obtained for some other ESC lines somewhat were different showing that p53 is still able to accumulate in the nucleus of mouse ESCs in response to DNA damage and thus activate target genes (Solozobova et al., 2009). Actually, a basal level of p53 in mouse EC and ESCs can be even higher than in somatic cells because of the elevated stability of its messenger (mRNA) and low expression of negative regulator micro-RNA 125a and 125b (Solozobova and Blattner, 2010). In summary, the p53 activation, subsequent nuclear accumulation of p53 protein and transactivation of specific target genes appear to be less efficient in mESCs as compared to somatic cells (Aladjem et al., 1998; Hong and Stambrook, 2004; Chuykin et al., 2008; Solozobova et al., 2009; Solozobova and Blattner, 2011) (Fig. 4.4).

While the above functional and mechanistic studies provide evidence of p53 attenuation in mouse ESCs, the studies on hESCs indicate that p53 is more active. Respectively, p53 activates and accumulates in the nuclei in response to genotoxic stress (Grandela et al., 2007; Momcilovic et al., 2009; Barta et al., 2010). Moreover, the p53 accumulation induced by nutlin, an

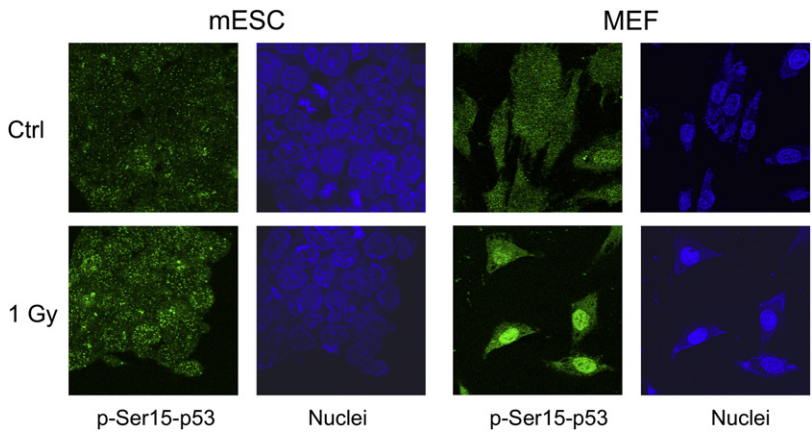

Figure 4.4 Gamma-irradiation (1 Gy) slightly induces phosphorylation of p53 in mESCs. Immunofluorescence of mouse ESC and MEF cells: nonirradiated (Ctrl) and irradiated (1 Gy). The cells were immunostained with antibody to phospho-p53 (green). Nuclei were stained with To-Pro3 (blue).

inhibitor of p53/MDM2 binding, causes differentiation toward primitive endoderm (Maimets et al., 2008). Nevertheless, the question of whether p53 is able to fully activate its target genes remains controversial (Qin et al., 2007). It is unclear what explains the conflicting data on the expression and intracellular localization of p53 in mESCs and hESCs as well as between different mESC lines. Without any doubt, p53 retains important functions, as deletion of both p53 alleles may lead to the genome instability (Song et al., 2010). The ability of p53 to function effectively as a transcription factor, at least in human ESCs, has been convincingly demonstrated by the fact that p53 can directly suppress the transcription of such pluripotent genes as *Oct3/4* and *Nanog* in response to DNA damage, thus initiating the differentiation program (Lin et al., 2005; Qin et al., 2007). In addition, p53 activates expression of small noncoding RNAs miR-34a and miR-145, which also repress expression of pluripotent factors Oct3, Klf4, Lin28A, and Sox2 (Jain et al., 2012). As it is now well known that Oct3/4 and Nanog play a key role in maintaining pluripotency of ESCs, their downregulation leads to the establishment of a gene expression profile characteristic of differentiated cells. Thus, in addition to the immediate induction of apoptotic death as a means to eliminate defective cells, an alternative mechanism for maintaining the genome integrity in ESCs can be realized through triggering the p53-dependent differentiation of DNA-damaged cells with subsequent death of the defected cells (Sherman et al., 2011). Consistently, accumulation of p53 by nutlin, which blocks the interaction of MDM2 protein with p53 thereby reducing its proteasomal degradation, causes accumulation of *p21Waf1*, restores the G_1/S checkpoint, slows down proliferation and initiates differentiation (Maimets et al., 2008).

There is a more complex feedback between p53/*p21Waf1* pathway and the expression of pluripotent factors, according to which the factor Oct3/4 was shown to directly repress promoter activity of the *p21Waf1* gene. Respectively, downregulation of the Oct3/4 expression increases the gene *p21Waf1* transcription and accumulation of *p21Waf1* protein without changing the p53 content (Lee et al., 2010a). However, although the level of *p21waf1* transcription increases in DNA-damaged mESC cells, but this is not followed by an increase of *p21Waf1* protein content.

Therefore, it is likely that the Oct3/4-dependent modulation of *p21Waf1* transcription is an additional mechanism for regulation of *p21Waf1* expression. The main conclusion from these results is that dysfunction of the p53/*p21Waf1* signaling pathway is closely related to the maintenance of the essential properties of ESCs—self-renewal and pluripotency. Mechanisms of

attenuation of the p53/*p21Waf1* pathway function may be different in different lines of mouse and human ESCs.

A novel function of p53 in maintaining the genome integrity of ESC population after DNA damage has been recently shown by an integrated genome-wide approach (Lee et al., 2010b). The authors used chromatin-immunoprecipitation-based microarray (ChIP-chip) and gene expression microarray assays to identify p53 target genes in UV-irradiated mESCs. Surprisingly, the Wnt signaling pathway was identified as one of the major downstream pathways of p53 in mESCs upon DNA damage. They hypothesized that the damaged cells, in addition to p53-dependent down-regulation of *Oct4* and *Nanog* genes, secreted the Wnt ligands that act on neighboring cells to prevent their differentiation. As we know now, active Wnt/β-catenin signaling pathway contributes to the maintenance of pluripotency (Sato et al., 2004; Sineva and Pospelov, 2010). The described above compensatory "altruistic" mechanism is intended to provide stabilization of the population cell number (Li and Huang, 2010).

2.5. Cdc25A and its Role in Acute but Temporary Cell Cycle Delay in ESC

Somatic cells respond to DNA damage by executing both a rapid Cdc25A-dependent but p53-independent cell cycle delay and a slower-operating p53 checkpoint that leads to a sustained cell cycle arrest (Lukas et al., 2004). The rapid but temporary response is mediated by Chk2-dependent phosphorylation and subsequent degradation of Cdc25A phosphatase. Cdc25A normally dephosphorylates Thr-14/Tyr-15 residues of Cdk2 thereby causing its activation. DNA damage induces Chk2-dependent phosphorylation of Cdc25A followed by its enhanced ubiquitination and proteasome-mediated degradation that makes the Cdk2 inactive. As a result, the acute and temporary cell cycle delay operating in the late G_1, S and G_2/M phases does occur. The slower-developing response to DNA damage involves a p53-dependent expression of the *p21Waf1* that suppresses cyclin–Cdk2 activity and provides a sustained cell cycle arrest, which operates in the early and late G_1 as well as in G_2/M. Both slow and rapid responses to DNA damage proceed through the activation of sensor kinases ATM/ATR that recognize the DNA damage and effector kinases Chk1/Chk2 that participate in activation of signaling intermediates. Data presented in the previous section show that ESCs do not stop in G_1 phase of the cell cycle after DNA damage or withdrawal of serum growth factors, i.e. they lack G_1/S arrest, which is

a convenient stop in the cycle for repair of defects without amplification of DNA defects in the case of ongoing DNA replication. A mechanism for the lack of G_1/S checkpoint seems to be connected with a dysfunction of p53/*p21Waf1* pathway as well as with attenuation of Chk2/Cdc25A pathway.

There is sufficient body of information suggesting that in mouse and human ESCs the ATM/Chk2/Cdc25A pathway may operate defectively, although the human ESC cells in this respect are closer to the somatic cells than mESCs. While in human ESCs the Chk2-dependent degradation of Cdc25A leads to inhibition of Cdk2 activity, as in case in somatic cells, the mouse ESCs do not demonstrate inhibition of Cdk2 activity after DNA damage (Barta et al., 2010; Koledova et al., 2010b). It has been shown that in mouse ESCs Cdc25A and Cdk2 proteins are spatially separated, the Chk2 kinase being localized at centrosomes and thus unable to phosphorylate the Cdc25A for subsequent proteasomal degradation (Hong et al., 2007). Hence, in mouse ESCs Cdk2 kinase does not lose activity under DNA damage and G_1 block does not take place (Koledova et al., 2010b). Interestingly, the ectopic expression of Chk2 kinase in mESCs restores the rapid Cdc25A-dependent response to DNA damage but does not lead to restoration of the slower-operating p53/*p21Waf1*-dependent signaling pathway (Hong and Stambrook, 2004). It has been shown that under certain conditions the GSK3β kinase may replace Chk2 for Cdc25A phosphorylation and its subsequent degradation in mouse ESC (provided that the principal effector kinases Chk1 and Chk2 are localized at the centrosomes). Nevertheless, this does not lead to the suppression of Cdk2 activity and cell cycle arrest (Koledova et al., 2010b). In addition to Chk2/Cdc25A-dependent pathway, there can be several alternative mechanisms for maintaining constitutive activity of Cdk2 in ESCs. It may be noted that both the G_1/S and G_2/M checkpoints appear to remain fully functional in Cdk2−/− mouse embryonic fibroblasts implying that Cdk1 can substitute Cdk2 in cyclin–Cdk2 complexes (Chung and Bunz, 2010). Insensitivity Cdk2 to genotoxic factors plays an important role in maintaining the pluripotent state and high proliferative potential of mouse ESCs. This opinion is supported by the data that the Cdk2 downregulation caused by pharmacological inhibitors or a specific small interfering RNA (siRNA) induces differentiation (Koledova et al., 2010a).

As for the human ESCs, these cells do not demonstrate constitutive active Cdk2; instead, it is effectively regulated by the Cdc25A phosphatase as in somatic cells (Neganova et al., 2009; Barta et al., 2010). According to available data, Cdc25A phosphatase is quickly degraded in irradiated human ESCs in a Chk1/2-dependent manner (Barta et al., 2010). In contrast to

mouse ESCs, Chk1 and Chk2 kinases are not localized at the centrosomes of human ESCs and are involved in phosphorylation and subsequent degradation of Cdc25A that leads to a transient cell cycle delay (Momcilovic et al., 2009; Barta et al., 2010). Cdk2 knockdown in human ESCs using siRNA-cdk2 decreases the Cdk2 activity that leads to the G_1 block, the activation ATM/Chk2 signaling pathway, the accumulation of CDK inhibitors p27 and *p21Waf1* and loss of pluripotency (Neganova et al., 2011).

2.6. P53-Dependent and p53-Independent Apoptosis

Human and mouse ESCs are very sensitive to DNA damaging and stress factors and undergo both p53-dependent and p53-independent apoptosis to eliminate potentially dangerous defect cells from population. Despite intensive research it is still unclear what the mechanisms of induction of apoptosis in stem cells. Earlier work of Aladjem et al. showed that p53 does not participate in the induction in mESC, in part because it does ineffectively move into the nucleus after DNA damage (Aladjem et al., 1998). However, targeting into the genome of mESCs the p53-tetramerization mutants resulted in delayed transcriptional activation of several p53 target genes in response of irradiation. Also, doxorubicin-induced apoptosis was severely affected in the mutant ESCs compared with wild-type cells (de Vries et al., 2002). There are other convincing data that the mESCs undergo a p53-dependent apoptosis. Thus, in mESCs DNA damage induced both p53-dependent apoptosis and differentiation, so that the cells started to differentiate may subsequently die (Sabapathy et al., 1997; Corbet et al., 1999).

In contrast to mouse ESCs, hESCs normally undergo high rates of spontaneous apoptosis and differentiation, making them difficult to maintain in culture. Emerging data suggest that the level of apoptosis is determined by the balance of Bcl2 family proteins: pro-apoptotic as Bax and Bak, and anti-apoptotic as Bcl-2 and Bcl-xL. Commitment of hESCs to apoptosis is provided due to the high level of expression of pro-apoptotic Bcl-2 family members, including NOXA, BIK, BIM, BMF, and PUMA, which regulate mitochondrial-dependent apoptosis (Madden et al., 2011). In addition, low level of pro-survival of Bcl-2 protein is expressed in hESCs; therefore, the overexpression of anti-apoptotic Bcl-2 or Bcl-xL proteins greatly promotes cell survival and increases cloning efficiency of hESCs (Ardehali et al., 2011; Bai et al., 2012). Also, hESCs undergo dissociation-dependent apoptosis (anoikis) to a greater extent than mESCs that poses one of the greatest obstacles to effective human stem cell cultivation (Ohgushi et al., 2010).

Given that the expression of *p21Waf1* in ESCs is at a low level, the function of DDR-activated p53 is not directed at the induction of cell cycle arrest, but rather on the induction of p53-dependent apoptosis, which can result either from transcriptional activation of pro-apoptotic genes or from p53-mitochondrial pathway. It is known that p53 is subjected to various posttranslational modifications, some of which define cell cycle arrest (Ser-15 and Ser-20 phosphorylation), whereas phosphorylation of p53 at Ser-46 regulates the ability to induce apoptosis (Oda et al., 2000). Particularly, DNA damage-induced and glucose deprivation-activated phosphorylation of p53 at Ser-46 might trigger the p53-dependent apoptotic program mediated by pro-apoptotic p53AIP1. Of note, phosphorylation of p53 at Ser-46 is AMP-activated protein kinase (AMPK) dependent and hence this kinase can play a crucial role in the induction of apoptosis (Okoshi et al., 2008). p53AIP1, which is localized within mitochondria, is capable of downregulating mitochondrial membrane potential thereby releasing cytochrome *c* from the mitochondria to the cytoplasm. Interestingly, although human ESC lines H1 and H9 demonstrate accumulation of p53 after DNA damage, the activated p53 is unable to transactivate a number of its target genes but still induces apoptosis through the mitochondrial pathway. In this way, p53 binds to the outer mitochondrial membrane that allows pro-apoptotic Bcl-2 proteins to induce membrane permeabilization and form complexes with the survival of BclxL and Bcl-2 proteins (Erster and Moll, 2004). Correspondingly, pifithrin-μ, an inhibitor that blocks the p53 binding to the mitochondria, significantly increased the survival rate of H1 cells after UV irradiation (Qin et al., 2007). Screening of small chemicals to identify compounds that induce mitochondria permeabilization-dependent apoptosis in mouse and human ESCs allowed finding those, which are very selective for undifferentiated cells as compared with somatic fibroblast cells (Conesa et al., 2012). This approach might have important applications by eliminating undifferentiated pluripotent stem cells from the differentiated derivatives used in the stem cell-based therapy.

3. DDR SIGNALING IN ESCs

3.1. Activation of ATM/ATR Pathway in ESCs

Activation of signaling pathways that regulate both checkpoints and DNA repair is triggered by phosphorylation of common sensor kinases ATM/ATR as a primary response to DNA damage. Since ESCs are characterized by the absence of a G_1/S cell arrest, it is essential that the DDR signaling

pathway would be very effective for DNA repair taking into account a high proliferation rate of these cells. The key DNA damage-activated kinases ATM and ATR phosphorylate the effector targets—Chk1/2, p53, 53BP1, and H2AX. All of them are involved in the formation of DNA repair foci marked with antibody to phosphorylated histone H2AX (γH2AX), one of the markers of double-stranded DNA breaks (DSBs). It is assumed that the ATM is mainly involved in signal transduction triggered by DSBs, while the ATR is activated by replication stress (replication stalling) or in the case of UV-induced lesions. Recent data, however, provided evidence that the ATR can be activated by DNA double-strand breaks and this activation is ATM dependent (Myers and Cortez., 2006). Data obtained on different mouse and human ESC lines unambiguously show that genotoxic stresses cause rapid ATM phosphorylation at Ser-1981 and its accumulation in the nucleus that is clear indication of its activation. The activated ATM kinase is capable of phosphorylating their targets—histone H2AX, a protein Nbs1, which is part of the complex MRE11, Chk2 kinase, and p53. In the absence of G_1 checkpoint, it is the activity of ATM which is necessary for the initiation of the G_2/M checkpoint in ESCs and DNA repair (Momcilovic et al., 2009). Although ATM is involved in phosphorylation of histone H2AX in the vicinity of DSB sites and formation of the irradiation-induced DNA repair foci (Valerie and Povirk, 2003), however, inhibition of ATM activity in human ESCs does not lead to a noticeable change in the number of repair foci. In contrast, in astrocytes inhibition of ATM blocks the formation of new foci and disassembly of the existing radiation-induced foci (Adams et al., 2010a). Hence, the role of ATM in the formation of repair foci in human ESCs is not critical, whereas knockdown of ATR leads to a significant reduction in the number of repair foci and suppression of DNA repair. Thus, DNA repair in human ESCs can be ATM independent and requires ATR for its effective execution (Adams et al., 2010b). This is consistent with the data showing that the ATM knockout in human ESCs though reduces the activity of ATM signaling pathway (as tested by phosphorylation of H2AX at Ser-139 and Chk2 at Thr-68), it does not lead to appreciable genetic instability. Taken together, it is suggested that the ATM has a more supporting role and, in the absence of ATR, can substitute for it (Song et al., 2010). Knockout of the *atm* gene is not lethal in mice, although the mice with the ATM knockout exhibit growth retardation and impaired fertility (Elson et al., 1996). Knockout of *atr* leads to embryonal death, moreover atr−/− blastocysts die in culture by a mechanism of mitotic catastrophe (de Klein et al., 2000). Nevertheless, ESCs with ATM knockout

efficiently repair DSBs but with an increased number of errors compared with the normal ATM-expressing cells (Banuelos et al., 2008). Less important role of ATM in the ESCs may be due to deregulation of ATM-dependent functions—Chk2 and G_1 checkpoint (Hong et al., 2007). To summarize, we can conclude that in mouse and human ESCs both ATM and ATR signaling pathways are functional albeit at different extent. However, despite the important role of ATM kinase in activation of p53 and Chk2 and in maintaining genome stability of ESCs, available data indicate the important role of ATR in DNA repair especially in mouse ESCs. It becomes especially clear given that ESCs have unique high proliferation and are therefore under a greater pressure of the replicative stress as an endogenous source of DNA damage (Murga et al., 2009).

3.2. Homologous Recombination Repair Versus Nonhomologous End Joining

Potentially the most dangerous DNA lesions are DSBs because they can cause a variety of chromosomal rearrangements. Such damage can occur in response to genotoxic factors (such as γ-irradiation) or the collapse of the replication fork. At that, DNA repair process involves both nonhomologous end joining (NHEJ) and homologous recombination repair (HRR). In somatic cells, the choice of DNA repair mechanisms depends on the type of DNA damage and cell cycle phase. NHEJ repair occurs predominantly in early G_1 and S phases of the cell cycle and HRR pathway is more effective in S and G_2 phases when sister chromatids are available as the template. DNA repair mediated through HRR pathway is a more accurate mechanism, as it utilizes a DNA template of a sister chromatid or homologous chromosome. Mice with a knockout of some HRR genes (e.g. *rad51*) are not viable and cells derived from the *rad51*−/− blastocysts did not proliferate implying an important function of this gene in early development (Tsuzuki et al., 1996). In comparison with the effective HRR, DNA repair through NHEJ pathway requires the smaller of homologous sequences (sometimes they are not required), and depending on the type of DSB ends, the process can proceed with no errors or mistakes. Direct ligation of compatible DNA ends usually proceeds without errors, but if the ligation has to be preceded by the processing of DNA ends, the individual nucleotides will be added or removed from the ends, thereby creating mutations (Tichy and Stambrook, 2008). Nevertheless, some authors consider that NHEJ and HRR pathways are not competing, NHEJ being an immediate early repair pathway that

precedes a more prolonged attempt to repair persistent DNA lesions by HRR (Serrano et al., 2011). In general, the available data indicate that ESCs have a more effective system for repair of DNA damages caused by H_2O_2, UVC, ionizing radiation, psoralen and other agents. Accordingly, the level of expression of a number of repair genes in ESCs is higher than in somatic cells (Maynard et al., 2008). There are differences between somatic and ESCs in relation to the contribution of NHEJ and HRR pathways in repair of DNA damages. Emerging evidences suggest that ESCs have an increased activity of HRR that operates throughout the cell cycle (Adams et al., 2010a; Serrano et al., 2011). This correlates with a higher expression of the Rad51 protein in ESCs (Serrano et al., 2011). So, Rad51 protein levels are about 20-fold higher in ESCs than in mouse embryonic fibroblasts (MEFs) where NHEJ predominates. Co-localization of Rad51 foci with γH2AX is a direct evidence of involvement of HRR system in the repair of DSBs (Serrano et al., 2011). Quantitative analysis using two mutants of the neomycin resistance gene demonstrated that mouse ESCs repair about 80% of DSBs with HRR, whereas only 20% with help of NHEJ. This relationship is partly explained by predominance of S-phase cells and this value changes toward NHEJ during differentiation (Tichy et al., 2010). Thus, the HRR pathway is more active in ESCs and it is functional throughout the cell cycle independently on the source of damage (Serrano et al., 2011). However, ESCs can use the NHEJ repair pathway as well, but according to literature data the contribution of both pathways to DNA repair is different in mouse and human ESCs (Banuelos et al., 2008). In particular, human ESCs are able to utilize NHEJ pathway by a mechanism that may have differences from that in somatic cells (Bogomazova et al., 2011). The low level of NHEJ repair in mouse ESCs can be associated with a lack of expression of the DNA-PK protein Ku70 (Banuelos et al., 2008). Correspondingly, human ESCs have higher levels of the DNA-PK Ku70 protein expression that is consistent with their ability to repair the damaged DNA ends after irradiation at high dose (Banuelos et al., 2008; Bogomazova et al., 2011). However, an inhibition of DNA-PK activity did not always affect the level of repair via the NHEJ pathway, implying that there can be a DNA-PK-independent NHEJ repair in human ESCs, where the DNA-PK may play not a decisive role (Adams et al., 2010a). In summary, human ESCs utilize both the HRR and the NHEJ pathways, the latter being somewhat different from that in somatic cells. The NHEJ pathway of human ESCs is characterized by accurate repair of DNA damage albeit with a slower kinetics (Adams et al., 2010b). In addition, human ESCs demonstrate the high expression levels of genes that

belong to such repair systems as the base excision repair (BER), nucleotide excision repair (NER) and interstrand crosslink (ICL) repair, thereby indicating for elevated activity of the most repair pathways (Maynard et al., 2008). Nevertheless, not always hESCs demonstrate substantial changes in expression of DNA repair genes after DNA damage; in case of hESC line H9 cells there was no significant activation of DNA repair genes following 1 Gy of gamma ray exposures (Sokolov et al., 2011).

Thus, despite the lack of p53-mediated *p21Waf1* G_1 checkpoint, the ESCs use various DNA repair mechanisms operating throughout the cell cycle. While ESCs predominantly utilize the HRR pathway, the NHEJ may be also used as an additional mechanism in case of the absence of a template for HRR (Fan et al., 2011; Bogomazova et al., 2011).

3.3. Tolerance of ESCs to Endogenous γH2AX Foci

Recently, it has been shown that mouse ESCs have visible γH2AX foci which arise probably due to the preexisting uninduced by external factors DNA single-strand breaks (SSBs) identified by a single-cell DNA comet-assay (Chuykin et al., 2008) (Fig. 4.5). Interestingly, the γH2AX foci were previously considered as markers of DSBs produced in a cell by DNA-damaging agents. Emerging data indicate that the phosphorylation of histone H2AX may occur in the absence of DNA damage, in particular, in the cells treated with histone deacetylase inhibitors (HDACi) (Pospelova et al., 2009; De Micco et al., 2011) that leads to acetylation of nucleosomal histones, relaxation of chromatin structure and facilitates the phosphorylation of H2AX. In addition, γH2AX foci occur in heat shock-treated cells or during mitosis without the concomitant DNA breaks (McManus and Hendzel, 2005; Hunt et al., 2007; Ikura et al., 2007). Moreover, the overexpression of

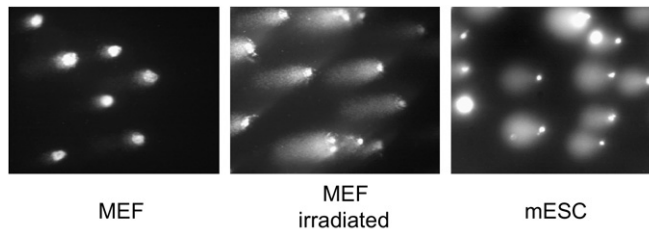

Figure 4.5 SSBs in mESCs. Mouse ESCs were subjected to single-cell gel DNA electrophoresis under denaturing conditions (comet assay). Control MEF cells were γ irradiated at dosage 1 Gy. Images of ethidium bromide-stained DNA comets were taken at magnification 200×. *This image (Chuykin et al., 2008) is reproduced with permission from Cell Cycle.*

DNA repair proteins can induce ATM- and DNA-PK-independent activation of the DDR signaling without visible DNA breaks (Soutoglou and Misteli, 2008). Also, infection of U2OS cells with an inactivated adeno-associated virus induces a stalled replication fork signal and causes pannuclear ATR-dependent H2AX phosphorylation without any signs of damage to the host genome (Fragkos et al., 2009).

In mouse ESCs H2AX foci are predominantly distributed in the S-phase cells thereby implying a correlation with an incomplete maturation of replication forks (Chuykin et al., 2008). However, many factors can affect the formation of H2AX foci and activation of DDR signaling. It is worth noting here that human fibroblasts activate a DNA damage-like signaling pathway in the absence of *p21Waf1*, as shown by phosphorylation of histone H2AX and Chk1 proteins (Perucca et al., 2009). The authors argue that in cells lacking *p21Waf1* the cell cycle checkpoint is induced by an unscheduled entry into S phase, followed by a reduced efficiency in DNA maturation that eventually activates DDR signaling pathway. As mESCs have very low *p21Waf1* expression and unverified by G_1 checkpoint entry into S phase, it is likely that these circumstances may provide a driving force of the H2AX foci formation. Thus, it appears that γH2AX foci are not only DSB markers but also occur in case of local changes of chromatin structure caused by the histone acetylation and DNA single-strand gaps. The SSBs are believed to occur as a consequence of incomplete maturation of replication forks provided by the high rate of proliferation (Chuykin et al., 2008). What is most surprising that the ESCs are tolerant to these endogenous γH2AX foci and single-stranded DNA breaks, as there is no activation of the sensor kinase ATM (Fig. 4.6). According to Banath's opinion (Banath et al., 2009), the presence of γH2AX foci in mESCs is not a result of DNA SSBs, but likely reflects the unusual chromatin structure in ESCs. In particular, mouse ESCs have higher overall levels of histone acetylation and, consequently, a more decondensed chromatin structure. The authors believe that the relaxed chromatin structure potentiates the formation of DNA SSBs during the alkaline single-cell DNA electrophoresis (Banath et al., 2009). No matter what the actual cause of the γH2AX foci formation, these data suggest the liability of chromatin may influence the DDR signaling pathway. Because the observed SSBs are not considered by the ESCs as the defects to be immediately repaired and their existence does not lead to chromosomal aberrations, it appears that the nature of the SSBs is associated with an increased exchange between sister chromatids. This, in turn, may increase the likelihood of HRR pathway which is active throughout the cell cycle (Serrano et al., 2011).

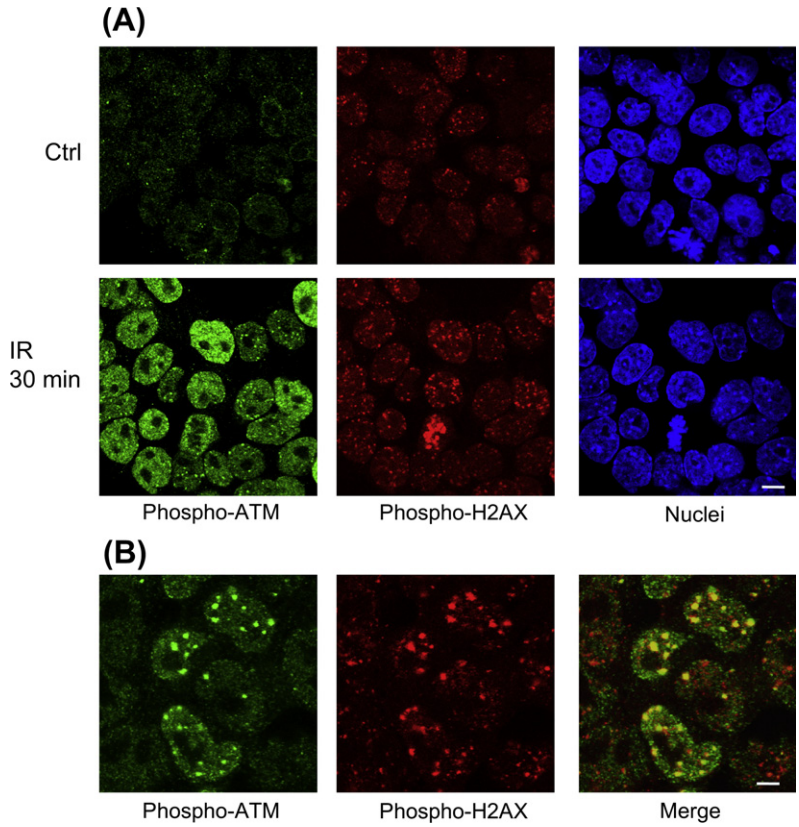

Figure 4.6 Undifferentiated mouse ESCs have noticeable γH2AX foci but no phospho-ATM staining. (A) immunofluorescent staining of γH2AX and phospho-ATM in mouse ESCs in control and 30 min after 1 Gy. Scale 8 μM. (B) γH2AX and phospho-ATM are mainly co-localized in γ-irradiated cells, scale 4 μM. *This image (Chuykin et al., 2008) is reproduced with permission from Cell Cycle.* For color version of this figure, the reader is referred to the online version of this book.

4. REPROGRAMING SOMATIC CELLS TO IPSCS AND ATTENUATION OF P53/WAF1/INK4 PATHWAYS

For the first time, the induced pluripotent cells (iPSCs) were obtained in 2006 from somatic cells by introducing the genes encoding so-called pluripotent factors Oct3/4, Sox2, Klf4, and c-Myc. There may be variations in the composition and number of the genes depending on the method of gene introduction and subsequent selection procedures to obtain the desired clones of iPS cells (Takahashi and Yamanaka, 2006; Okita et al., 2007; Park et al., 2008). The iPSCs share many common properties with routinely

cultivated ESCs. In particular, they express markers of pluripotency (Nanog, endogenous Oct4 and Sox2, ESC-specific surface antigen SSEA-4), form embryoid bodies, can differentiate toward cells of all germ layers, they incorporate in a 4-cell blastocyst and give rise to chimeric animals, form teratomas when injected subcutaneously, may give rise to germ cells and also participate in the viable offspring F2 (Betts and Kalionis, 2010). Currently, attempts are made to obtain mouse iPSCs from mouse fibroblasts by introducing a single gene *Oct4* with addition of small molecules. These small molecules include HDACi valproic acid and H3K4 demethylation inhibitor tranylcypromine that indicates for the role of epigenetic modulators in reprogramming (Li et al., 2011). The observed contribution of the epigenetic modulators suggests that H3K4 demethylation and histone deacetylation could be two critical epigenetic barriers to reprogramming, which may repress the establishment of a pluripotent state. Of note, a combination of small molecules as CHIR99021, a GSK3beta inhibitor, and 616452, a TGF-β inhibitor, efficiently replaces *Sox2* for reprogramming (Li et al., 2011).

Many of the obtained so far human iPSCs had somatic mutations that put doubts for their use in regenerative cell therapy and requires further researches to find out the nature of emerging defects (Stadtfeld and Hochedlinger, 2010; Gore et al., 2011). New approaches are currently developing to limit iPSC tumorigenicity and increase their safety through increased copy number of tumor suppressors in the established iPSCs. So, in some cases the iPS cells even containing an extra copy of the p53 or Ink4a/ARF locus show normal state of pluripotency, as determined by in vitro and in vivo differentiation assays (Menendez et al., 2012). The stochastic mechanisms involved in reprogramming somatic cells by the so-called "Yamanaka factors" OCT4, SOX2, KLF4 and c-MYC to a pluripotent-like state remain largely unknown (Hanna et al., 2009). To achieve a pluripotent-like state, there should be a blockage of natural barriers, which can be induced upon the expression of the pluripotent or oncogenic factors. The most powerful barriers include a p53-mediated cell cycle arrest and cellular senescence (Zhao and Xu, 2010). There are confirmed data that the unscheduled expression of oncogenes causes in normal somatic cells activation of G_1 checkpoint followed by senescence (Di Micco et al., 2006; Bartkova et al., 2006). By this reason, senescence is also a barrier to the derivation of iPSCs but this obstacle is successfully overcome. First, Lapasset's group showed that iPSCs generated from the senescent and centenarian cells have reset telomere size and gene expression profiles similar to that in hESCs implying that senescent program is reversible (Lapasset et al., 2011).

Four iPSC lines were generated from dermal fibroblasts derived from an 84-year-old woman, representing the oldest human donor so far reprogrammed to pluripotency. Despite the presence of karyotype aberrations, all aged iPSCs were able to differentiate into neurons, reestablish telomerase activity, and reconfigure mitochondrial ultrastructure and functionality to a hESC-like state (Prigione et al., 2011). Second, recent findings demonstrated that well-characterized mTORC1 inhibitor rapamycin, which effectively suppresses cellular senescence (Demidenko et al., 2009), notably improves the speed and efficiency of iPSC generation (Menendez et al., 2011; Chen et al., 2011). Third, data of Adjaye's group indicate that iPSCs in addition to the modulation of p53 signaling exhibit alterations of the senescence-related mitochondrial/oxidative stress and telomerase pathways. In particular, they showed that somatic mitochondria during formation of human iPSCs revert to an immature ESC-like state with respect to organelle morphology and distribution, expression of nuclear factors involved in mitochondrial biogenesis, content of mitochondrial DNA, intracellular ATP level, oxidative damage, and lactate generation (Prigione et al., 2010). Thus, iPSCs and ESCs share similar mitochondrial properties and bioenergetic metabolism, which are distinct from those of fibroblasts, and are capable of escaping cellular senescence (Prigione and Adjaye, 2010). In line with this, human and mouse ESCs rely mostly on glycolysis to meet their energy demands and seem to have lower overall OXPHOS mitochondrial activity. This places ESCs in line with a number of tumor cells, which often use glycolysis as a predominant source of energy (Varum et al., 2011; Folmes et al., 2011).

It should be noted here that the reprogramming is becoming more successful when there is a suppression of the expression of genes that negatively control the cell cycle: *p53/p21Waf1* and *Ink4/Arf* (Li et al., 2009; Kawamura et al., 2009; Utika et al., 2009; Marion et al., 2009). In fact, expression of the tumor-suppressor genes is a natural barrier for transformation of somatic cells into the self-renewing and pluripotent cells. Available data show convincingly that the p53/*p21Waf1* pathway operates in iPS cells in the same format and with the same decreased efficiency as in ESCs. This is especially noteworthy because iPSCs are produced by reprogramming somatic cells, which have powerful p53/*p21Waf1*-dependent checkpoints. From this it follows that in the process of reprogramming the p53/*p21Waf1* pathway must undergo a significant modulation in order to facilitate the dedifferentiation of somatic cells toward embryonic pluripotent cells.

Much attention has been drawn to the effect of modulation of p53/*p21Waf1* and Ink4/Arf pathways on the efficiency of iPSC generation. The

results show that a reduced expression of p53 facilitates somatic cell reprogramming to the iPSC as evidenced by using p53-null cells (Hong et al., 2009), p53-specific small hairpin RNA (shRNA) (Hong et al., 2009; Kawamura et al., 2009), and cells with introduced dominant-negative p53. In any case, 3- to 10-fold reduction of the p53 expression (Marion et al., 2009; Kawamura et al., 2009) increases the efficiency of iPSC generation. However, the iPSCs obtained from p53 knockout cells are genetically unstable and have elevated malignant tumor-forming potential (Sarig et al., 2010). Inhibition of the Ink4a/Arf locus also greatly increases the efficiency of reprogramming (Kawamura et al., 2009; Banito et al., 2009). The Ink4/Arf locus includes genes *Cdkn2a* and *Cdkn2b*, which encode three tumor-suppressor proteins p16Ink4a, p19Arf and p15Ink4b. This locus controls p53 and pRb signaling pathways. The p15 and p16 proteins associate with and negatively regulate the activity of the cyclin D-Cdk4/Cdk6 complexes thereby inhibiting the pRb that causes cell cycle arrest. The p19 protein inhibits Mdm2 function and by this manner indirectly activates p53. Dysfunction of p19 increases the efficiency of reprogramming by factors 2–4 (Kawamura et al. 2009), whereas inhibition of locus Ink4/Arf increases by factors 4–7 that is comparable with the efficiency of p53-null cells. Down-regulation of *p21Waf1*, which is an inhibitor of the cyclin-Cdk activity, also increases the efficiency of reprogramming albeit to a lesser extent than the downregulation of Arf/Ink4a. As for the effect of pRb inactivation, there are few conflicting data. Some evidence suggests that the inactivation of pRb does not increase the likelihood of the iPSC generation (Hong et al., 2009), but there are also contradicting data (Kawamura et al., 2009).

It should be noted that both ESCs and iPSCs respond to DNA damage very similarly. On one hand, iPSCs and ESCs to a greater extent than differentiated cells are sensitive to DNA damage and prone to activate apoptosis (Momcilovic et al., 2010; Fan et al., 2011). On the other hand, ESCs and iPSCs demonstrate the increased activity of various DNA repair systems—homologous (HRR) and nonhomologous (NHEJ) repair as compared with somatic cells. A detailed analysis was done to prove this point of view. Authors studied the efficiency of repair of DNA breaks as tested by a comet assay and the mRNA levels of repair proteins, as well as immunofluorescent detection of foci marked the repair proteins (Ku70 for NHEJ and Rad51 for HRR) (Fan et al., 2011). A conclusion is that both the repair mechanisms are functional, the HRR being slightly more preferential probably due to a longer S phase. The DNA-damaged iPS cells execute only a temporary G_2/M block, but not a long-term G_1/S arrest. Like in somatic parent cells, activation of DDR signaling involves

the phosphorylation of ATM at Ser-1981, Chk2 at Thr-68, NBS1 at Ser-343 and p53 in ATM-dependent site Ser-15 (Momcilovic et al., 2010). ATM kinase is becoming co-localized with DSB repair foci visualized by antibody to phosphorylated H2AX histone. Now it is well known that activation of oncogenes or overexpression of certain endogenous genes in somatic cells induce DDR signaling pathway that leads to cell cycle arrest and senescence (Di Micco et al., 2006; Bartkova et al., 2006). Not surprisingly that expression of the pluripotent factors used for cell reprogramming activates the DDR signaling pathway: p53 activation, the elevated expression *p21Waf1* and Arf/Ink4a, and phosphorylation of ATM and its effectors. As a result of the DDR activation, there can be either cell cycle arrest and senescence or p53-dependent apoptosis (Marion et al., 2009). From these data it follows that the activation of DDR signaling is a barrier not only for tumor formation but also for somatic cell reprogramming to the iPSC. To overcome the barrier of the cell reprogramming, there should be a suppression of DDR signaling by yet poorly understood mechanisms: compromised function of p53 and/or *p21Waf1*, and methylation and silencing of Arf/Ink4a locus. One mechanism that can be relevant is that the elevated expression of pluripotent gene *Oct4* attenuates transcription of *p21Waf1* (Lee et al., 2010), so it is likely that the exogenous Oct4 firstly downregulates *p21Waf1* transcription and then participates in establishing the stem-like phenotype and pluripotency. Another putative mechanism is based on the ability of miR-290–295 cluster (Lichner et al., 2011) to reduce the content of transcripts of *Wee1* and *p21Waf1* thereby making an impact to the proliferation of ESCs. Available data show that miRNAs are capable of abolishing the p53-mediated barrier to reprogramming, as DDR-activated p53 may enhance the processing and maturation of several miRNAs in human fibroblasts (Mallannaa and Rizzino, 2010). By combining effects of immortalizing (c-Myc, Klf4) and pluripotent factors (Oct4, Sox2), somatic cells get an impact to acquire the main properties of ESCs—self-renewal (immortality) and pluripotency. To maintain so vital properties as self-renewal and pluripotency, the ESCs are forced to lose such important functions as p53/*p21Waf1* status and G_1 cell cycle checkpoint.

5. CONCLUSION

ESCs have unique properties such as self-renewal and pluripotency, which must be maintained under the conditions of rapid proliferation, when a high probability of replication errors may occur as well as problems with

the DNA repair. The repair process is complicated by a dysfunction of the p53/*p21Waf1* pathway resulting in failure of G_1 arrest of the cell cycle needed for careful check of DNA damages followed by their repair. Nevertheless, the ESCs have developed several mechanisms that help to withstand the mutational pressure and thereby to provide self-renewal and pluripotency.

What have ESCs developed to better resist the mutations for maintaining pluripotency? Some of them are the following (see also Fig. 4.7): (a) ESCs have effective antioxidant mechanisms for removing alien compounds thereby protecting ESCs of potential agents that can generate radical oxygen species (ROS). For instance, mESCs have developed well working verapamil-sensitive mechanism for DCF-DA withdrawal, which is a substrate for the P-glycoprotein pump channel and is a widely used fluorogen for ROS detection (Saretzki et al., 2004). In addition, the level of ROS in ESCs is significantly lower than in differentiated derivative cells at least in part due to the fewer number of mitochondria and also because ESCs generate ATP by glycolysis rather than by mitochondrial oxidative phosphorylation (Saretzki et al., 2008). (b) Because ESCs generate less ROS, the level of spontaneous mutations is approximately 100 times lower than in differentiated cells. In that case, if the cell is not able to repair DNA defects, it moves to S phase unrepaired, so the defects are amplified thereby slowing down the cell cycle and stimulating processes of either differentiation or apoptosis (Hong et al., 2007; Serrano et al., 2011). (c) ESCs express a decreased level of p53 but in response to DNA damage p53 still can be activated at least in human ESCs and suppress transcription of *Nanog*—a gene which is responsible for pluripotency (Lin et al., 2005). Even a slight suppression of the *Nanog* expression is sufficient to start differentiation. (d) What is the functional role of the shortening of G_1 phase in ESCs? Functionally, the mechanisms of maintaining pluripotency and shortening the cell cycle (or more precisely G_1 phase) are tightly associated. Prolongation of G_1 (by inactivation of Cdk2) leads to a slowing down of proliferation, reduced expression levels of pluripotent genes and initiation of differentiation process (Becker et al., 2010; Neganova et al., 2011; Spike and Wahl, 2011). Therefore, such factors as p53 which influence the duration and progression of G_1 phase are under the pressure of negative selection that would ensure the pluripotency and self-renewal. (e) Maintenance of ESC pluripotency and the reprogramming of somatic cells to pluripotent iPSCs require attenuation of p53-dependent G_1 checkpoint. Partly contradictory information regarding the dysfunction of p53-dependent pathway is explained by the fact that the many

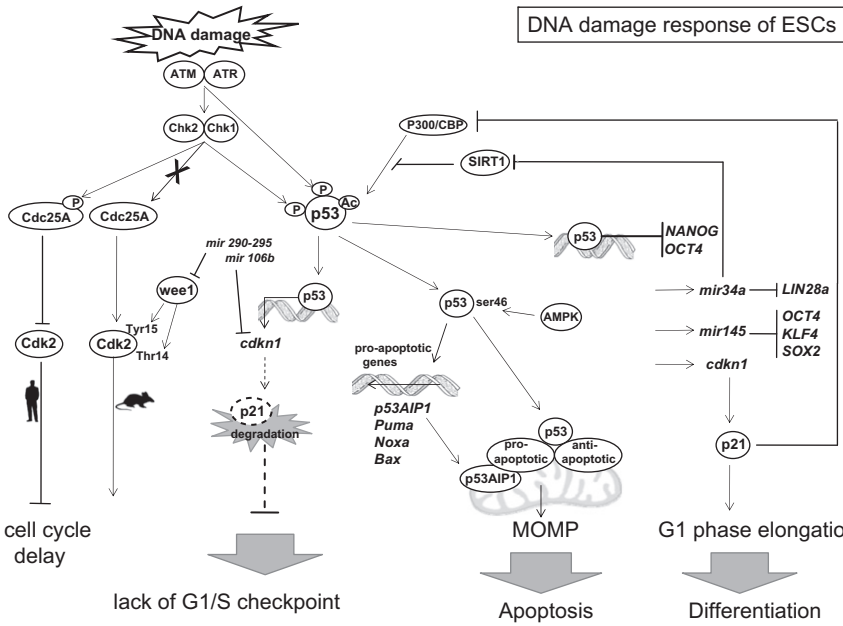

Figure 4.7 DNA damage activates the sensor ATM/ATR kinases, which in turn phosphorylate the downstream kinases Chk1/Chk2 and the main checkpoint protein p53 thereby triggering the DDR signaling. There are a rapid Cdc25A-dependent but p53-independent cell cycle delay and a slower-operating p53 checkpoint that leads to a sustained cell cycle arrest. For human ESCs, in case of the rapid response Chk2-phosphorylated Cdc25A phosphatase degrades and incapable to dephosphorylate Thr14/Tyr15 residues of Cdk2. As a result, Cdk2 remains inactive that leads to a cell cycle delay. In mouse ESCs, Chk2 being bound to centrosomes does not phosphorylate Cdc25A and therefore Cdk2 is permanently active (see text Section 4.2.5). The slow but more effective p53/p21Waf1-dependent checkpoint is triggered by ATM/ATR-mediated phosphorylation of p53 at Ser-15 and Chk1/Chk2-dependent phosphorylation at Ser-20. Acetylation of p53 at Lys-382 (Lys-379 in mice) by HATs p300/CBP provides the p53-regulated transcription of *cdkn1(p21*waf1). In ESCs p21Waf1 protein is detected at very low levels due to either posttranscriptional downregulation of its RNA by miR-106b and miR-290–295 cluster and/or through proteasomal degradation of p21Waf1 protein. Thus, the p53/p21Waf1 pathway is defective in mouse ESCs. DNA damage-activated p53 is involved in the induction of both apoptosis and differentiation. Phosphorylation of p53 at Ser-46 by AMPK determines the expression of pro-apoptotic p53AIP1 that regulates the membrane potential of mitochondria. Also, p53 activates the transcription of pro-apoptotic genes *bax*, *puma*, and *noxa* and migrates into the mitochondria where interacts with pro-apoptotic and anti-apoptotic proteins of Bcl2 family thereby inducing MOMP (mitochondrial outer membrane permeabilization). Active p53 induces differentiation through a repression of pluripotent genes *Nanog*, *oct4* as well as *Lin28*, *Klf4*, and *Sox2*. In turn, p53-dependent transcription of micro-RNAs miR-34a and miR-145 negatively affects the expression of pluripotent genes *klf4* and *sox2*. Besides, the miR-34a regulates the p53 acetylation by inhibiting the deacetylase SIRT1 activity that increases the p53 acetylation at Lys-382 (Lys-379 in mice).

components of this pathway can be compromised in various ESCs to provide the lack of G_1 checkpoint. The main reason for dysfunction of p53 pathway is that the ESCs are committed to rapid proliferation, while p53-dependent cell cycle arrest induces differentiation.

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CHAPTER FIVE

Angiogenic Effects of Erythropoietin

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Contents

Abstract

Erythropoietin (Epo) is a low-molecular weight glycoprotein hormone stimulator of erythropoiesis synthesized in the fetal liver and in the adult kidney. Moreover, Epo is a pleiotropic cytokine that exerts diverse biological effects in non-hematopoietic tissues, and angiogenesis is indicated as one of its extra-hematopoietic functions. The involvement of Epo in angiogenesis may be considered as subset of its role in improving overall tissue oxygenation and of its anti-apoptotic role. In this context, Epo may be considered as an endogenous stimulator of vessel growth during tumor progression, and inhibition of Epo signaling might be suggested as a new anti-angiogenic therapeutic approach. It is conceivable suppose that the effect of Epo is multifactorial, depending on the type of tumor and level of functionality of Epo

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receptor expression in tumor cells, as well as on other variables, such as hypoxic stress and degree of anemia.

1. HISTORICAL BACKGROUND

The idea of hormonal regulation of erythropoiesis was first formulated in 1906 by Carnot and Deflandre. They postulated that a humoral factor, which they called hemopoietin, regulated red blood cell production, and showed an increase in reticulocytes in normal recipient rabbits following the injection of serum from donor rabbits who have a bleeding stimulus. The concentration of red blood cells in the recipients increased by 20–40% within 1–2 days after a single injection of few milliliters of serum. Moreover, Carnot and Deflandre (1906) reported that the hemopoietin-containing serum was inactivated by heating to 56°C, whereas erythropoietin (Epo) is a heat-stable glycoprotein that loses little activity on short-term boiling. The suggestion of Carnot and Deflandre (1906) was supported by irreproducible and doubtful data and several years were required to give a definitive prove for their prediction.

In 1932, Sander (1932) demonstrated an increased red cell count and increased number of red cell precursors in the marrow of rabbits previously injected with serum from hypoxic donors. In 1936, Hjorte (1936) published confirmation of work by Carnot and Deflandre (1906). He removed plasma from rabbits following bleeding, injected this plasma into recipient rabbits, and produced a marked reticulocytosis. In 1943, Krumdieck (1943) demonstrated that erythropoietically active plasma from bled rabbits produced a reticulocytosis in recipient rabbits. Formerly in 1948, Bonsdorff and Jalavisto (1948) introduced the name "erythropoietin", implying restricted action of that humoral factor and red cell production.

In 1950, Reissmann (1950) showed that erythroid hyperplasia in bone marrow and reticulocytosis were induced in both parabiotic animals when only one partner was exposed to hypoxemia and the other partner breathed an atmosphere at normal oxygen tension. These data indicated that the hypoxic partner produced an erythropoietic substance that passed into the circulation of the partner breathing air at a normal atmosphere pressure and stimulate erythroid proliferation in the bone marrow. These data were confirmed by Ruhenstroth-Bauer (1950).

In 1949, the erythropoietic activity of exogenous Epo in humans was first demonstrated by Oliva et al. (1949), who transfused plasma from

patients with pernicious anemia into normal subjects. In 1953, Erslev (1953) induced reticulocytosis, and later increased hematocrit, in rabbits repeatedly injected with large volumes of plasma from anemic donor animals, and predicted the potential therapeutic role of Epo in treating anemia. In 1954, Stohlman et al. (1954) and in 1955 Schmid and Gilbertsen (1955) found that patients suffering from patent *ductus arteriosus* showed generalized erythroid hyperplasia, suggesting a link between the lower part of the body which is hypoxemic in this condition and the stimulation of erythropoiesis.

In 1957, Jacobson et al. (1957) demonstrated that bilaterally nephrectomy in rats almost completely abolished the effects of cobalt in increasing Epo titers. Jacobson had developed an assay for Epo that involved radioactive iron incorporation in red cells of starved rats and found that cobalt's erythropoietic effect was largely due to the effect of cobalt in increasing Epo titers in plasma. Gurney et al. (1957) reported the same observations in anemic patients affected by chronic renal failure. The explanation for these findings was that Epo is produced in the kidney in response to hypoxia or anemia and stimulates the production of erythroid cells in the bone marrow.

In 1961, Kuratowska et al. (1961) demonstrated an increased Epo production in isolated kidney perfused with hypoxemic blood and Fisher and Birdwell (1961) detected Epo in the blood perfusate of in situ perfused dog kidneys, respectively. In 1981, Jelkman and Bauer (1981) quantified Epo activity in renal extracts of rat exposed to hypoxia and showed that the hormone was mainly present in the renal cortex (at the level of proximal tubular cells, glomerular cells, mesangial cells, interstitial cells, and peritubular interstitial cells) and not in the medulla. These data were confirmed by Fried et al. (1981). In 1986, Beru and co-workers and in 1987 Schuster et al. (1987) demonstrated Epo messenger RNA (mRNA) in renal extracts. The first attempts to identify cells synthesizing Epo within the kidney were made with in situ hybridization of Epo mRNA expression in hypoxic animals (Koury et al., 1991). By this approach, Epo-producing cells were localized to the peritubular space of the cortex. Maxwell et al. (1993) showed that these cells were located between adjacent tubules or between tubules and vessels.

In 1968, based on their experience with the extraction of Epo from sheep plasma, Goldwasser and Kung (1968) calculated that a volume of 3250 l of urine from anemic patients was required to purify 10 mg pure human Epo. These authors purified after 9 years a few milligrams of Epo from over 2500 l of urine from patients suffering from aplastic anemia (Miyake et al., 1977). The pure human urinary Epo enabled the partial

identification of its amino acid sequence, which, in turn, allowed the deduction of the nucleotide sequences required for probes used in the attempts to clone the Epo gene. In 1985, Lin et al. (1985) and Jacobs et al. (1985) cloned and transfected in mammalian cells the Epo gene in Chinese hamster ovary cells and, respectively, in African green monkey kidney cells.

In 1986, Winearls et al. (1986) and in 1987 Eschbach et al. (1987) used successfully the DNA-derived recombinant human Epo (rHuEpo) to treat the anemia of chronic renal disease in clinical trials. In patients with end-stage renal disease, rHuEpo restores the hematocrit and eliminates the need for blood transfusion. Since the initial reports demonstrating cure of the anemia of chronic renal failure, well over a million of patients with uremia have been effectively treated with rHuEpo with remarkably few adverse side effects or complications. Currently, the main indication for rHuEpo is treatment of anemia due to Epo deficiency in patients with preterminal and terminal renal failure (Jacobs et al., 2000). In addition, rHuEpo is used in anemic patients with nonmyeloid malignancies treated with chemotherapeutic agents (Ferrario et al., 2004), in AIDS patients with anemia due to zidovuline therapy, and for autologous blood donation in perioperative surgical patients.

2. BIOLOGICAL PROPERTIES AND PRODUCTION OF EPO

2.1. Biological Properties

Epo, belonging to the family of class I cytokines, is a glycoprotein of molecular mass 30.400 Da (Lacobe and Mateux, 1998). Carbohydrate accounts for a high proportion (39%) and is required for activity in vivo, but not in vitro. The remainder is a 165 amino acid polypeptide. The glycosylated chains are necessary for the biological activity of Epo and can protect the Epo protein from damage by oxygen radicals (Uchida et al., 1997). The human Epo gene is located on chromosome 7 and occupies a 5.4-kb region of genomic DNA, composed of five exons and four introns. It encodes a 193 amino acid protein, and cleavage of the 27 amino acid leader sequence gives a mature protein which undergoes posttranslational processing.

In 1974, Chang and co-workers and in 1984 Krantz and Goldwasser first provided evidence that Epo binds to a transmembrane receptor, a member of the cytokine receptor superfamily, which is mainly expressed on erythroid colony-forming units (CFU-E) and consists of an extracellular domain,

a transmembrane domain and an intracellular domain (Moritz et al., 1997; Lacombe and Mateux, 1998).

In 1989, D'Andrea and co-workers cloned and characterized the murine Epo receptor (EpoR). EpoR is a 484 amino acid glycoprotein and a member of the type I superfamily of single transmembrane cytokine receptor. EpoR contains two polypeptide chains on its extracellular component, which becomes homodimerized after interaction with their ligand (McCaffrey et al., 1989). A single Epo molecule binds to two adjacent EpoR on the target cell membrane and begins an intracellular signaling cascade. Unlike many other receptors, EpoR has no intrinsic tyrosine kinase activity to activate receptor signaling. Major signal transduction pathways activated by Epo include the Janus kinase 2 (JAK2)/signal transducer and activator of transduction (STAT5) and the Ras/mitogen-activated protein kinase pathways involved in the inhibition of apoptosis and the stimulation of cell proliferation in response to Epo (Moura et al., 1994; Wojchowski et al., 1999). In 1999, Wu et al. (1999) demonstrated that the complete knockout of the EpoR gene results in a phenotype of severe cardiac malformations and fetal death at day 13.5 in mice probably due to anemia and tissue hypoxia.

Epo is essential for the survival, proliferation and differentiation of erythroid precursors in the bone marrow and leads to an increased expression of the anti-apoptotic proteins (Silva et al., 1996) and inhibition of apoptosis (Tilbrook and Klinken, 1999), by controlling the dynamic balance between erythropoiesis and erythrocyte loss in order to maintain red cell volume (Moritz et al., 1997; Lacombe and Mateux, 1998). When the concentration of Epo rises in the blood, many more burst-forming unit erythroid and CFU-E escape from apoptosis and proliferate favoring the maturation of proerythroblasts and normoblasts (Wu et al., 1995). The importance of the Epo–EpoR system in primary and definitive erythropoiesis has been determined by generating lines of mutant mice lacking either the Epo or the EpoR gene (Wu et al., 1995; Lin et al., 1996). Both lines died of severe anemia between embryonic days 13 and 15.

Epo also prevents apoptosis through the PI3-K/Akt pathway, which maintains the mitochondrial membrane potential, prevents the cellular release of cytochrome C, and modulates caspase activity (Chong et al., 2002, 2003).

2.2. Extrarenal Sites of Production

In 1990, D'Andrea and Zon (1990) demonstrated that EpoR mRNA is expressed in various non-hematopoietic tissues, such as endothelium,

neuronal cells and placenta. Although the kidney has been viewed as the major site of Epo production under normal conditions, extrarenal sites of Epo production are present, notably the liver (Koury et al., 1991; Maxwell et al., 1994) and the tissue-resident bone marrow macrophages (Vogt et al., 1989). The macrophage is the sole source of Epo in the fetal liver, where it forms the control of the erythropoietic blood islands.

Epo and EpoR were found to be expressed in other sites besides liver and kidney: neurons, astrocytes, brain endothelial cells, microglia and oligodendrocytes (Buemi et al., 2003), trophoblast cells of the human placenta (Conrad et al., 1996). Considerable amounts of Epo are also present in human milk (Kling et al., 1998). EpoR is expressed by a variety of non-hematopoietic cell types, including neurons (Juul et al., 1998) and trophoblast cells (Farichil et al., 1999). In the endocrine system, EpoR expression has been demonstrated in insulin-producing cells of the pancreatic islets (Fenjves et al., 2003), as in parathyroid cells (Oztürk et al., 2007) and in pituitary gland (Jelkmann, 2005). In the digestive tract, gastric mucosa (Sereno et al., 2006) and enterocytes (Juul et al., 2001) express EpoR. In the female reproductive tract, Epo is expressed in the cervix, endometrium, ovary and oviduct (Yasuda et al., 2001; Masuda et al., 2000). EpoR is expressed in vascular endothelial and smooth muscle cells, endometrial decidual cells, glandular epithelial cells, and ovarian follicles at various stages of development (Yasuda et al., 2001a). In the male reproductive system, Epo and EpoR are expressed in the testis, Sertoli and peritubular myoid cells, Leydig cells, and epididymis (Magnanti et al., 2008; Yamazaki et al., 2004; Kobayashi et al., 2002).

In 2010, Sinclair et al. (2010) reported that EpoR is barely detectable in non-hematopoietic tissues, casting doubt on the role of Epo as a pleiotropic hormone. They reported that EpoR mRNA was consistently expressed in human neuronal, cardiac, endothelial and renal cell lines, albeit at low levels compared with cells in the erythroid lineage.

3. EPO AND ANGIOGENESIS

3.1. Cross Talk between Hematopoiesis and Angiogenesis

The relationship between endothelial and hematopoietic cells has been seen as an indication that a common progenitor, the hemangioblast, gives rise to both cell types in the yolk sac, the initial site of hematopoiesis and blood

vessel formation during mammalian development (Ribatti, 2008 Bikfalvi and Han, 1984).

Hematopoiesis is regulated by several cytokines and interleukins (IL) with pleiotropic activity, including granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-3, IL-4, IL-6 and IL-8 (Pellettier et al., 2000).

Several evidences accumulated in the last 20 years have clearly demonstrated that these molecules, formerly regarded as specific for the hematopoietic system, also affect certain endothelial functions and that hematopoietic factors clearly influence angiogenesis (Ribatti, 2012). G-CSF and GM-CSF receptors have been detected on the surface of endothelial cells (Bussolino et al., 1989). These cytokines induce endothelial cells to migrate and proliferate and are angiogenic in the rabbit cornea (Bussolino et al., 1989) and in the chick chorioallantoic membrane (CAM) (Valdembri et al., 2002).

Accordingly, endothelial cells exposed to recombinant human vascular endothelial growth factor (VEGF) manifest increased mRNA for several hematopoietic growth factors, including G-CSF, GM-CSF, stem cell factor and IL-6, which may act as growth factors for myeloid and lymphoid cells (Chen et al., 2000). VEGF may thus play an important role in the growth of hematological neoplasms via a paracrine mechanism. Fibroblast growth factors (FGFs) positively regulate hematopoiesis by acting on stromal cells, early and committed hematopoietic precursor cells and some mature blood cells, exerting both autocrine and paracrine functions in these biological processes (Moroni et al., 2002).

The ability of various hematopoietic cytokines to affect endothelial cell and angiogenesis (Table 5.1) may be a reflection of a common ontogenesis of the vascular and hematopoietic system. In fact, the presence of progenitors that give rise to both hematopoietic cells and endothelial cells in the early mesoderm population in the blood islands of the yolk sac and endothelial cells lining the vascular lumen is very likely. Hemangioblasts derived from the extraembryonic mesoderm are at the origin of vasculogenesis and primitive blood formation in the yolk sac, whereas those derived from the intra-embryonic splanchnopleural mesoderm generate definitive hematopoietic cells in the dorsal aorta through a hemogenic endothelium intermediate.

The relationship between the two systems is reasonable, because the two systems must develop together in order to establish a functional oxygen-deliver system during organogenesis. Moreover, the generation of this link between vascular and hematopoietic systems may be related to the evolution

Table 5.1 Pro- and antiangiogenic effects of hematopoietic cytokines

Pro-angiogenic
Epo
G-CSF
GM-CSF
ILs -1, -3, -4, -6, -8, -10, -15, -17
Antiangiogenic
ILs -2, -4, -12, -13

of the closed circulatory system of vertebrates. The problem of how hematopoietic precursor cells could be incorporated in the closed vascular system appears to be solved by generating endothelial cells and hematopoietic precursor cells from a common progenitor.

3.2. Effects of Epo on the Cardiovascular System

The role of Epo has been demonstrated also in the cardiovascular system, where Epo promotes various effects in endothelial cells. Epo stimulates both proliferation and migration of human and bovine endothelial cells in vitro and of endothelial cells isolated from rat mesentery (Ribatti et al., 1999a; Anagnostou et al., 1990; Yamaji et al., 1996; Ashley et al., 2002), as well as in the rat aortic ring model (Carlini et al., 1995). Moreover, Epo induces endothelin-1 expression in endothelial cell cultures (Carlini et al., 1993; Vogel et al., 1997). rHuEpo induces a increased cell proliferation, matrix metalloproteinase-2 expression and differentiation into vascular tubes of human endothelial cells in vitro (Fig. 5.1) (Ribatti et al., 1999a). In vivo, in the CAM assay, rHuEpo exerted an angiogenic activity comparable to that of FGF-2, and CAM's endothelial cells expressed both EpoR and factor VIII-related antigen (Figs 5.2 and 5.3) (Ribatti et al., 1999a). EpoR mRNA is expressed in human umbilical vein endothelial cells (HUVEC) (Anagnostou et al., 1994), bovine adrenal capillaries (Anagnostou et al., 1990) and rat brain capillaries (Yamaji et al., 1996). A differential display analysis of HUVEC extracts has revealed four groups of genes that are upregulated by rHuEpo, including those encoding proteins involved in the control of vascular function (e.g. thrombospondin-1), gene transcription (e.g. c-myc), mitochondrial function (e.g. cytochrome C oxidase subunit 1), and regulators of signal transduction (Fodinger et al., 2000).

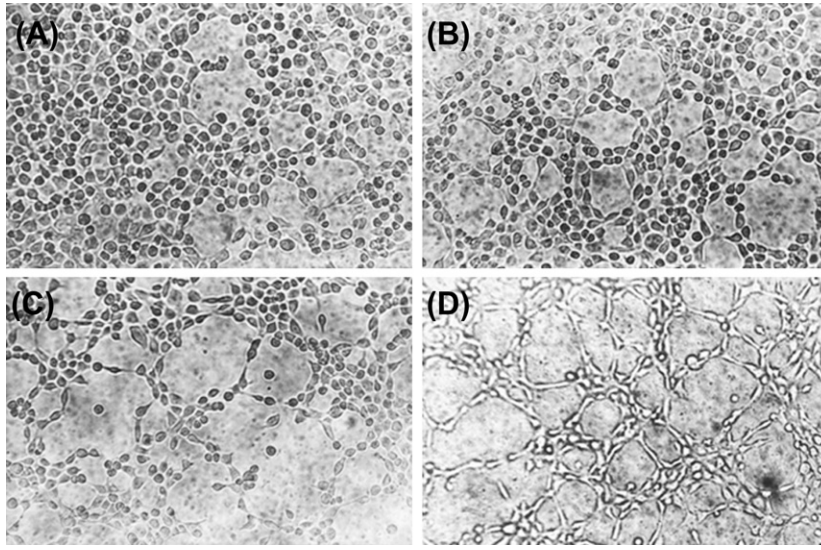

Figure 5.1 Morphogenetic activity of Epo. Endothelial cells were seeded on Matrigel (in A) and incubated with increasing doses of Epo (in B–D). A dose-dependent morphogenetic effect of Epo is appreciable. (Reproduced from Ribatti et al., 1999a).

Kertesz et al. (2004) demonstrated that Epo and EpoR are expressed in the vasculature during embryogenesis and that deletion of Epo and EpoR in null embryos leads to angiogenic defects, whereas de novo vasculogenesis was not affected, consistent with the differential expression of Epo and EpoR during the early stages of embryonic development. Endothelial cells expressed EpoR that bound JAK2 and included its transient activation after rHuEpo exposure (Ribatti et al., 1999). It is interesting to note that JAK2 is involved in the intracellular signaling of receptors for various cytokines, including the angiogenic G-CSF and GM-CSF (Witthuhn et al., 1993). It has been demonstrated that GM-CSF induces JAK2 activation in immortalized EA.hy 926 endothelial cells (Tsukada et al., 1997) and in HUVEC (Soldi et al., 1997). Taken together, these findings suggest a role for JAK2/STAT5 signaling pathway in cytokine-mediated angiogenesis. Moreover, Epo also stimulates JAK2 phosphorylation in cultured muscle cells and neurons (Ogilvie et al., 2000).

3.2.1. Epo and Angiogenesis in the Heart

Ischemic heart disease is characterized by a reduction in blood supply to the myocardium. Angiogenesis is central to cardiac repair after myocardial infarction and impaired angiogenesis may delay cardiac repair and cause

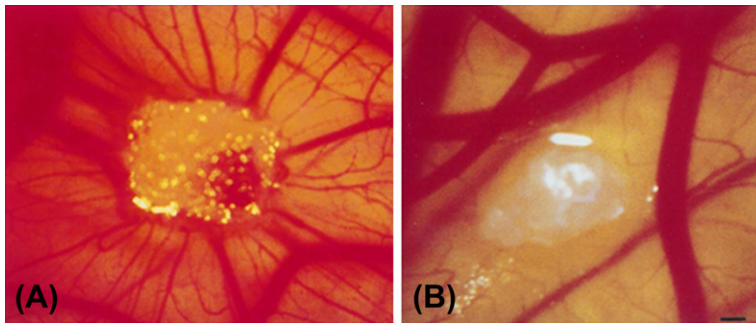

Figure 5.2 Epo stimulates angiogenesis in vivo in the CAM assay (in A). Note numerous vessels with a radially arranged spoked wheel pattern around the gelatin sponge soaked with Epo. In (B), a gelatin sponge soaked with phosphate buffered saline (PBS) used as negative control. (Reproduced from Ribatti et al., 1999a). For color version of this figure, the reader is referred to the online version of this book.

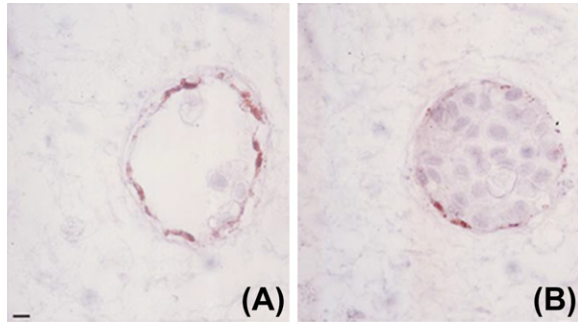

Figure 5.3 Immunohistochemical co-expression of EpoR (in A) and factor VIII-related antigen (in B) in CAM's endothelial cells. (Reproduced from Ribatti et al., 1999a). For color version of this figure, the reader is referred to the online version of this book.

cardiac rupture or immature scar tissue formation (Barandon et al., 2004). Jaquet et al. (2002) investigated the angiogenic potential of rHuEpo on endothelial cells derived from human adult myocardial tissue and compared the angiogenic potential of rHuEpo to that of VEGF. They found that rHuEpo stimulated capillary outgrowth up to 220%, compared to the non-stimulated physiological outgrowth. Epo therefore exhibited the same angiogenic potential as VEGF.

Numerous studies have demonstrated that stimulation of angiogenesis is beneficial to ischemic and infarcted heart (Ahn et al., 2008; Boodhwani et al., 2006; Nelson et al., 2000; Kawachi et al., 2012). Heba et al. (2001) reported that VEGF mRNA was not detectable in the normal rat heart, but was highly expressed in the infarcted myocardium, lasting over the course of

6 weeks. Li et al. (1996) reported that VEGF was expressed in the normal heart and its expression was significantly increased in the infarcted myocardium only in the early stage of myocardium infarcted. Zhao et al. (2011) studied the spatial and temporal expression of VEGF-A (mRNA and protein) and VEGF receptor in the infarcted rat heart. VEGF and VEGFR were found increased at the border zone only in the first day after infarct, but not in the later stages. However, VEGF-A expression was suppressed in the infarcted myocardium, even during the first week when angiogenesis is most active. EpoR activation may regulate VEGF expression during peripheral ischemia (Nakano et al., 2007). Ueda et al. (2010) demonstrated that Epo enhanced the expression of VEGF and angiopoietin-1 in cultured cardiomyocytes and infarcted hearts, which, in turn, stimulated angiogenesis.

In infarcted hearts, expression levels of sonic hedgehog and its downstream target Patched have been reported to be increased and sonic hedgehog has been shown to induce angiogenic effects (Pola et al., 2001). Ueda et al. (2010) have demonstrated that Epo also increases the expression of sonic hedgehog in cardiomyocytes, and inhibition of sonic hedgehog signaling suppresses the Epo-induced increase in angiogenic cytokine expression. Moreover, the beneficial effects of Epo on infarcted hearts are abolished by cardiomyocyte-specific deletion of sonic hedgehog.

Epo stimulates angiogenesis and improves heart function in the ischemic heart after myocardial infarction (Hirata et al., 2006; Van der Meer et al., 2005; Nishiya et al., 2006), associated with decreased after infarct fibrosis (Toma et al., 2007), attenuation of left ventriculum hypertrophy (Van der Meer et al., 2005), and improvement of left ventriculum function (Nishiya et al., 2006; Hirata et al., 2006; Prunier et al., 2007; Toma et al., 2007; Van der Meer et al., 2005; Westenbunk et al., 2007). Moreover, Epo confers protective effects in the myocardium (Calvillo et al., 2003) as it has been suggested by the time-dependent increase of serum Epo in patients with myocardial infarction, after coronary intervention (Nakamura et al., 2009). Another proposed cardioprotective effect after myocardial infarction is the inhibition of apoptosis (Calvillo et al., 2003), through AKT and ERK activation (Parsa et al., 2003), and the phopsphatidylinositol-3 kinase-dependent pathway (Hirata et al., 2005).

Epo decreases the activity of endothelial isoforms of nitric oxide synthase (NOS) in human coronary artery endothelial cells (Wang and Vaziri, 1999). Experimental data indicate that NOS, depending on isoforms, the timing and the degree of activation, displays contradictory effects during physiological and pathological angiogenesis (Donnini and Ziche, 2002). In vascular

smooth muscle cells, Epo stimulates calcium influx, suggesting that calcium mobilization may contribute to the hypertension associated with Epo treatment, and act as a vascular growth factor (Marrero et al., 1998). Moreover, Epo acts as a viability factor during cardiac development and is necessary to prevent apoptosis and expansion or proliferation of myocardial and endocardial progenitor cells (Yu et al., 2002). Van der Meer et al. (2005) demonstrated that over 9 weeks, the Epo analogue darbepoetin alfa increased capillary density and improved heart function.

3.2.2. Epo and Mobilization of Endothelial Progenitor Cells

Extensive data support the existence of endothelial progenitor cells (EPC), their bone marrow origin, and contribution to the formation of new blood vessels in adults. Moreover, their discovery led to the new concept that these cells are able to differentiate when needed into vascular endothelium. The majority of circulating EPC reside in the bone marrow in close association with hematopoietic stem cells and the bone marrow stroma that provides an optimal microenvironment. EPC have the capacity to proliferate, migrate and differentiate into endothelial lineage cells, but have not yet acquired characteristics of mature endothelial cells (Ribatti, 2007).

EPC in the peripheral blood may derive from the bone marrow and be not yet incorporated into the vessel wall. In animal models, EPC home in sites of active neovascularization and mobilization of bone marrow-derived EPC, and differentiate into endothelial cells in response to tissue ischemia (Takakura et al., 1998). EPC mobilized from the bone marrow into the blood stream may be recruited and incorporated into sites of active neovascularization during tissue ischemia, vascular trauma or tumor growth. Moreover, expansion and mobilization of EPC may augment the resident population of endothelial cells competent to respond to exogenous angiogenic cytokines (Isner and Asahara, 1999). Reports on the numeric contribution of EPC to vessel growth are variable, ranging from very low ($<0.1\%$) to high (up to 50%) likely dependent on the type of angiogenesis model used (Orlic et al., 2001; De Palma et al., 2003).

The parenchyma of the systemic vasculature or certain organs may harbor endogenous EPC-like cells. Exposure to organ-specific angiogenic and matrix factors may be necessary to program EPC to home in on and incorporate in a particular tissue. Incubation of EPC with organ-specific growth factors may confer specific instructions for recruitment to a particular organ. Epo mobilizes bone marrow EPC to peripheral circulation (Bahlmann et al., 2004). In an experimental model of femoral artery legation using

EpoR-null mice, blood flow recovery, activation of VEGF/VEGFR system, and mobilization of EPC were all impaired in EpoR-null mice as compared with wild-type mice (Nakano et al., 2007). rHuEpo has also been shown to increase the number of circulating endothelial cells and EPC in preclinical models (Monestiroli et al., 2001) and human breast cancer and lymphoma models (Mancuso et al., 2001). Moreover, Epo stimulates postnatal neovascularization by increasing EPC mobilization from the bone marrow (Heeschen et al., 2003).

EPC mobilization into the ischemic region after myocardial infarction promotes myocardial angiogenesis (Hirata et al., 2006; Prunier et al., 2007; Westenbrink et al., 2007). Hirata et al. (2006) demonstrated that Epo administration after coronary ligation increases $CD34^{+}$ mononuclear cells, which in turn increases both the capillary density and myocardial blood flow in the ischemic region. Epo treatment increases the number of EPC in the bone marrow, spleen, and peripheral blood, and mobilizes EPC from bone marrow (Heeschen et al., 2003), leading to neovascularization in the heart (Bahlmann et al., 2004). Heeschen et al. (2003) examined the effect of Epo on EPC proliferation and mobilization in patients with coronary heart disease and demonstrated that serum Epo levels correlated with EPC number. Hamed et al. (2006) investigated the effect of Epo treatment on EPC derived from rats subjected to doxorubicin-induced cardiomyopathy and demonstrated that Epo treatment increased the number of circulating EPC and in vitro EPC vessel formation in doxorubicin-treated animals. Westenbrink et al. (2007) showed that Epo promotes neovascularization in a chronic model of heart failure post-myocardial infarction in mice. Epo-induced neovascularization is associated with increased mobilization, myocardial homing and vascular incorporation of EPC, which comprise 30% of the newly formed vessels. Kawachi et al. (2012) found a significantly larger number of c-kit-positive cells in the border and infarcted areas of Epo group compared with the corresponding areas of the controls. Epo mobilizes c-kit-positive populations from the bone marrow, which differentiate into endothelial cells and smooth muscle cells (Brunner et al., 2009).

Increased expression of VEGF and of VEGFR with Epo by direct stimulation of endothelial cells has been proposed as mechanism of angiogenesis (Westenbrink et al., 2007; Nakano et al., 2007). Kawachi et al. (2012) demonstrated an acceleration of angiogenesis after the administration of Epo in a porcine myocardial infarction model. The beneficial effects of Epo may be associated with the upregulation of hepatocyte growth factor (HGF), FGF-2, and VEGF. Inhibition of VEGF led to a reduction of Epo-induced

endothelial cell proliferation and abrogates the Epo-mediated increase in capillary density in heart failure (Westenbrink et al., 2010). Animals that simultaneously received Epo and bevacizumab showed a complete abrogation of the Epo-induced angiogenic response without affecting the Epo-mediated tissue protection. This indicates that the formation of new functional capillary networks is VEGF driven as the VEGF antibody bevacizumab prevents from the angiogenic response.

3.3. Epo and Angiogenesis in Female Reproductive Organs

The female reproductive tract of primates undergoes substantial vascular growth and remodeling associated with the menstrual cycle and pregnancy (Reynolds et al., 1992). A paracrine Epo/EpoR system in the uterus may play an important role in uterine angiogenesis through EpoR expressed by endometrial vascular endothelial cells (Yasuda et al., 1998). Moreover, Epo mRNA is expressed in normal human endometrium and ovary, while JAK2, EpoR-phosphotyrosine and STAT5 are expressed at their Epo-responsive sites (Masuda et al., 1993). Epo production in female reproductive organs is estrogen dependent. Administration of 17 β-estradiol leads to a rapid and transient increase in Epo mRNA in the uterus, oviduct and ovaries and injection of Epo into the uterine cavity of ovariectomized mice leads to blood vessel formation in the endometrium (Yasuda et al., 1998).

Yasuda et al. (2001) reported that blockade of Epo signaling with local soluble EpoR or anti-Epo antibody resulted in tumor cell destruction and reduction of vascularity in ovarian and uterine cancer xenografts, associated with an increase in apoptotic death of both tumor cells and vascular endothelial cells.

3.4. Epo and Angiogenesis in the Nervous System

In the brain, there is a paracrine Epo/EpoR system that is independent of the endocrine system in adult erythropoiesis: neurons express EpoR and astrocytes produce Epo (Marrero et al., 1998; Masuda et al., 1993). Both Epo and EpoR become detectable 5 weeks after conception in the brain of human embryos (Juul et al., 1998).

It has been demonstrated in vitro and in vivo that Epo is a potent inhibitor of neuronal apoptosis induced by ischemia and hypoxia and exert a neuroprotective effect in traumatic brain or spinal cord injuries as well as infarction (Siren et al., 2001). The induction of EpoR production in neuronal cultures exposed to low oxygen tension suggests that the Epo

response may involve both an upregulation of Epo production and an increase in neuronal sensitivity to Epo mediated by increasing EpoR production, suggesting that Epo may act as survival factor for neurons and can play a role in stress response to hypoxia or ischemia (Yu et al., 2002). A temporal and spatial cellular expression of Epo and EpoR has been described after focal permanent ischemia in mice (Bernaudin et al., 1999). Moreover, Epo is involved in neural progenitor cell development through the activation of nuclear factor-kB which stimulates the maturation of neural stem cells (Shingo et al., 2001). There are several mechanisms through which Epo induces neuroprotection including normalization of cerebral blood flow, inhibition of neurotransmitter release, antagonism of glutamate cytotoxicity, reduction of apoptosis, anti-inflammatory effects, increased expression of antioxidant enzymes, reduced NO-mediated formation of free radicals, and stimulation of angiogenesis.

Angiogenesis generated in the vascular system may provide indirect neuroprotection in the central nervous system. Furthermore, angiogenesis in the brain may be closely related to neuronal survival in patients with ischemic stroke (Krupinski et al., 1994). A benefit of angiogenesis may result from the restoration of blood flow in the ischemic border through arteriolar growth and capillary formation during cerebral ischemia (Wei et al., 2001). As new vessel formation occurs in the ischemic border of the brain several days following a stroke (Krupinski et al., 1994), its induction by Epo provides indirect protection of the brain tissues and contribute to the functional recovery after Epo treatment in animal models and in human stroke (Bernaudin et al., 1999; Sakanaka et al., 1998; Brines et al., 2000; Wang et al., 2004), and demonstrates that the protective role of Epo is more general and extends beyond hematopoiesis. Morita et al. (2003) have demonstrated that, in addition to VEGF, Epo plays a key role in the development of the neovascularization associated to the retinopathy of prematurity (ROP), suggesting a therapeutic possibility of Epo and VEGF inhibitors in ROP treatment.

3.5. Epo, Hypoxia and Angiogenesis

Epo production is primarily regulated by the oxygen supply to a sensor in the kidney relative to its oxygen requirements and conditions resulting in a decrease in oxygen delivery to the renal sensor will stimulate Epo production. Epo is the best known hypoxia-regulated gene and this regulation occurs mainly at the mRNA level and is mediated by hypoxia-

inducible factor-1 (HIF-1) (Ebert and Bunn, 1999). A deficiency in tissue oxygen results in Epo production in the kidney and liver (Jelkmann, 1992), and also in the brain (Chikuma et al., 2000). Epo production in the kidney appears to be transient, whereas it is more sustained in the brain (Chikuma et al., 2000). Hypoxia induces cells to respond through multiple gene products such as Epo and VEGF that will improve oxygen delivery to the tissues or enzymes of the glycolytic pathway that will adapt the cellular metabolism to decreased oxygen availability.

A specific survival role for Epo and induction of EpoR by hypoxia were demonstrated in cultures of neurons and astrocytes (Yu et al., 2002; Masuda et al., 1994; Chin et al., 2000). The induction of Epo and EpoR by hypoxia suggests that Epo administration has therapeutic potential role for tissue damage from ischemia or hypoxia in the central nervous system (Bernaudin et al., 1999; Brines et al., 2000).

The primary trans-acting factor for Epo and VEGF is HIF-1. This consists of the regulatory subunit HIF-1α and the constitutively expressed HIF-1β subunit (Semenza, 2000) and is overexpressed in the majority of human cancers, even more so in their metastases, and allows cancer cells to better adapt to hypoxia. Hypoxia rescues HIF-1α from proteasomic degradation and leads to its nuclear translocation and heterodimerization a the activation of HIF-1 target genes, including those encoding Epo, VEGF, and other genes involved in erythropoiesis, angiogenesis, vasodilation and glucose metabolism (Semenza, 2000).

4. EPO AND TUMOR

4.1. Epo, Hypoxia and Tumor Growth

Tumor growth and expansion are characterized by the inability of the local vasculature to supply sufficient oxygen and nutrients to the rapidly dividing neoplastic cells. The resulting hypoxic state causes changes in the tumor cells that can lead to cell stasis or apoptosis or, alternatively, to responses aimed at improving tissue oxygenation. Hypoxia-mediated clonal selection is an important biological mechanism of tumor progression and hypoxia mediates the selection of neoplastic cells with diminished apoptotic potential by providing a growth advantage to cells with genetic alterations that impair apoptosis (Acs et al., 2003). Hypoxia also may be involved in the development of a more aggressive phenotype and may contribute to metastasis (Briezel et al., 1996) and treatment resistance (Teicher, 1994). Systemic

anemia, a common phenomenon in cancer (about 32% of cancer patients present with anemia at diagnosis and about 54% of initially non-anemic cancer patients develop anemia during treatment; Groopman and Itri, 1999), significantly reinforces the negative consequences of hypoxia in cancer tissues.

Each of the HIF family members—HIF-1α, HIF-1β, and HIF-3α—has a significant role in regulating the expression of Epo and EpoR. Epo and EpoR expression in tumors improve the hypoxic survival of cancer cells (Acs et al., 2001). Moreover, HIF-1 regulates the expression of several genes known to confer a growth advantage on hypoxic cancers and HIF-1-mediated Epo expression is thus unlikely to be an exclusive mechanism for hypoxic cell survival (Semenza, 2000). Winter et al. (2005) showed a significant correlation between Epo and HIF-1 expression in head and neck squamous cell carcinoma and intratumoral hypoxia plays a key role in the Epo/EpoR autocrine/paracrine loop as both Epo and EpoR are upregulated in hypoxic tumor cell lines (Lester et al., 2005). Leo et al. (2006) analyzed Epo/EpoR expression and their relationship with intratumoral pO_2 levels as well as with survival in patients with cervical cancer and showed that cancers with higher Epo expression showed a significantly reduced overall survival and Epo/EpoR expression correlated significantly with apoptosis. Furthermore, no correlation was observed between Epo/EpoR expression and intratumoral hypoxia, although in well-oxygenated tumors, EpoR localized significantly more often to the invasion front.

Epo stimulates proliferation and inhibits apoptosis of EpoR-bearing tumor cells (Acs et al., 2001); Epo stimulation of tumor cell lines induces signal transduction (Yasuda et al., 2003; Dunlop et al., 2007), but generally has little or no effect on cell proliferation or cell survival following cytotoxic exposure (Gewirtz et al., 2006; Lamontagne et al., 2006). Liu et al. (2004) have reported that Epo may not influence the basal viability of tumor cells but may prevent the cytotoxic effects of chemotherapeutics such as cisplatin and Epo causes resistance to cisplatin in HeLa cells (Acs et al., 2003) and to dacarbazine in melanoma cells (Kumar et al., 2005).

Several tumor cell lines express Epo and EpoR regardless of their origin type, genetic characteristics, and biological properties. They secrete a very small amount of Epo individually and that most of them respond to hypoxic stimuli by enhanced secretion of Epo (Yasuda et al., 2003). EpoR mRNA and/or EpoR protein have been detected in breast (Arcasoy et al., 2002; Acs et al., 2002), lung (Kayser and Gabius, 1992), renal (Westenfelder and Baranowski, 2000), gastric (Ribatti et al., 2003a) and hepatocellular carcinomas (Sugimachi et al., 2003; Nakamatsu et al., 2004), hemangioblastoma

(Krieg et al., 1998), tumors of the cervix and of other organs of the female reproductive tract (Acs et al., 2003; Yasuda et al., 2001), neuroblastoma (Ribatti et al., 2007b), other pediatric tumors (Batra et al., 2003), melanoma (Kumar et al., 2005, 2012), glioma (Nico et al., 2011), and lymphoid malignancies (Kokhaei et al., 2007).

4.2. Epo and Tumor Angiogenesis

Angiogenesis and the production of angiogenic factors are fundamental for tumor progression in the form of growth, invasion and metastasis (Folkman, 1971; 1995). New vessels promote growth by conveying oxygen and nutrients and removing catabolites. These requirements vary, however, among tumor types, and change over the course of tumor progression. Endothelial cells secrete growth factors for tumor cells and a variety of matrix-degrading proteinases that facilitate tumor invasion and an expanding endothelial surface also gives tumor cells more opportunities to enter the circulation and metastasize.

Solid tumor growth occurs by means of an avascular phase followed by a vascular phase (Ribatti et al., 1999b). Assuming that such growth is dependent on angiogenesis and that this depends on the release of angiogenic factors, the acquisition of angiogenic ability can be seen as an expression of progression from neoplastic transformation to tumor growth and metastasis. Practically all solid tumors, including those of the colon, lung, breast, cervix, bladder, prostate and pancreas, progress through these two phases. The role of angiogenesis in the growth and survival of leukemias and other hematological malignancies has only become evident since 1994 thanks to a series of studies demonstrating that progression in several forms is clearly related to their degree of angiogenesis (Ribatti and Vacca, 2008).

Epo favors tumor progression through effects on angiogenesis and it may be considered as an endogenous stimulator of vessel growth during tumor progression through an autocrine/paracrine loop. The vascular effects of Epo would be relevant in tumor angiogenesis and the negative effect of Epo on tumor growth may be further aggravated by its known angiogenic activity (Acs et al., 2003). Tumor cell can directly release increasing amounts of VEGF and placental growth factor in response to Epo (Perelman et al., 2003). Exogenous Epo decreased both the host- and tumor-derived VEGF expression suggesting that the proliferation-promoting effect of rHuEpo on tumoral endothelial cells is independent of VEGF production (Blackwell et al., 2003).

Okazaki et al. (2008) showed in a Lewis lung carcinoma xenograft model in mice that administration of subcutaneous Epo promotes tumor growth through enhancement of angiogenesis even if these tumor cells do not express EpoR and Epo treatment in vitro did not affect their proliferation. Hardee et al. (2007) demonstrated by using the dorsal skin-fold window chambers that co-injection of Epo with labeled rodent mammary carcinoma cells or expression of EpoR in tumor cells stimulated tumor neovascularization and growth. Co-injection of Epo antagonist proteins with tumor cells or stable expression of Epo antagonist inhibited angiogenesis and impaired tumor growth. These data have been confirmed by using orthotopic tumor xenograft model, where EpoR expression promoted tumor growth, increased Ki67 proliferation antigen expression, enhanced microvessel density and decreased tumor hypoxia. On the contrary, Epo antagonist expression in tumor cells was associated with disruption of primary tumors.

More recently, Xue et al. (2012) have demonstrated in mouse tumor models that platelet-derived growth factor BB (PDGF-BB) induces Epo mRNA and protein expression by targeting perivascular cells that express PDGF receptor-β. These authors suggested that PDGF-BB-induced Epo expression promotes tumor growth through two mechanisms: a paracrine stimulation of tumor angiogenesis and an endocrine stimulation of extramedullary hematopoiesis leading to increased oxygen perfusion and protection against tumor-associated anemia.

A correlation between Epo/EpoR expression, angiogenesis, and tumor progression has been established in human gastric cancer, hepatocellular carcinoma, neuroblastoma, melanoma, glioma (Ribatti et al., 2003a, 2007a,b, 2010; Nico et al., 2011), and pituitary adenoma (Yang et al., 2012). In neuroblastoma, EpoR is diffusely and strongly expressed on endothelial cells within neoplastic nodules (Fig. 5.4), whereas in most cases Epo shows a weak and focal cytoplasmic granular pattern in a few neuroblastoma cells in stages I and III (Fig. 5.5A and B), while this immunoreactivity increases in stage IV (Fig. 5.5C). In human primary melanoma, Epo shows a weak and focal cytoplasmic pattern of expression in tumor tissue, where positive tumor cells are frequently associated in clusters (Fig. 5.6). EpoR is diffusely and strongly expressed on endothelial cells within neoplastic nodules (Fig. 5.6). Epo/EpoR expression correlates with angiogenesis and tumor thickness. In human glioma, Epo and EpoR immunoreactivity of tumor cells and, respectively, endothelial cells increases in IV malignancy grade tumor specimens (Fig. 5.7A and C), when compared with II malignancy

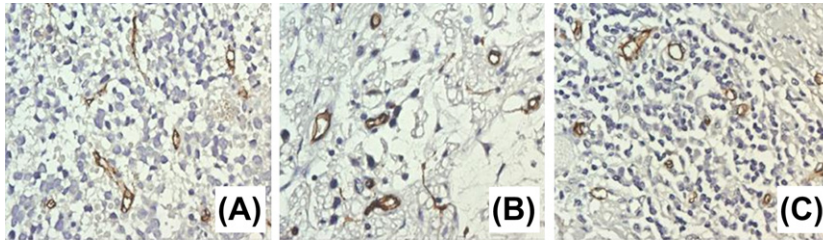

Figure 5.4 Immunohistochemical staining for EpoR in human neuroblastoma. The immunoreactivity for EpoR is higher in stage IV neuroblastoma (C), as compared with stages III (B) and I (A). (Reproduced from Ribatti et al., 2007a). For color version of this figure, the reader is referred to the online version of this book.

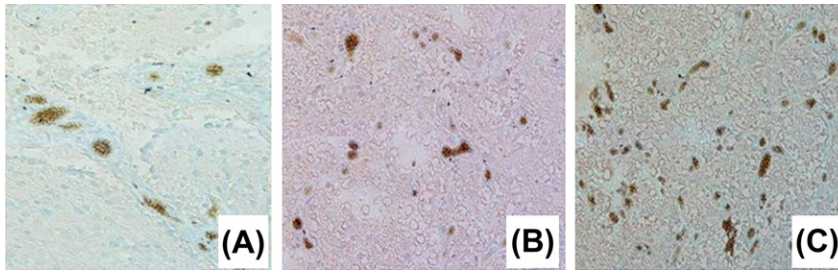

Figure 5.5 Immunohistochemical staining for Epo in human neuroblastoma. Few tumor cells are immunoreactive for Epo in stages I and IIII (A, B), while immunoreactivity is increased in stage IV (C). (Reproduced from Ribatti et al., 2007a). For color version of this figure, the reader is referred to the online version of this book.

grade (Fig. 5.7B and D). Moreover, when tested in the CAM assay, a grade IV glioma bioptic specimens induce an angiogenic response comparable to that induced by VEGF (Fig. 5.8), which is significantly inhibited by the coadministration of an anti-Epo blocking antibody (Fig. 5.8).

Tumor-derived Epo may influence also tumoral vascular lymphatic biology. Acs et al. (2003) demonstrated that cells of squamous dysplasia and cell carcinoma of the uterine cervix show an increased expression of EpoR, detectable also in the endothelial cells of lymphatic vessels.

5. THERAPEUTIC USE OF EPO

In 1990, Miller et al. (1990) showed that the concentration of circulating Epo is relatively low in patients in many cancer patients. The primary goals of Epo therapy in these patients are to maintain the hemoglobin values above the transfusion requirement, prevent fatigue and improve quality of life parameters.

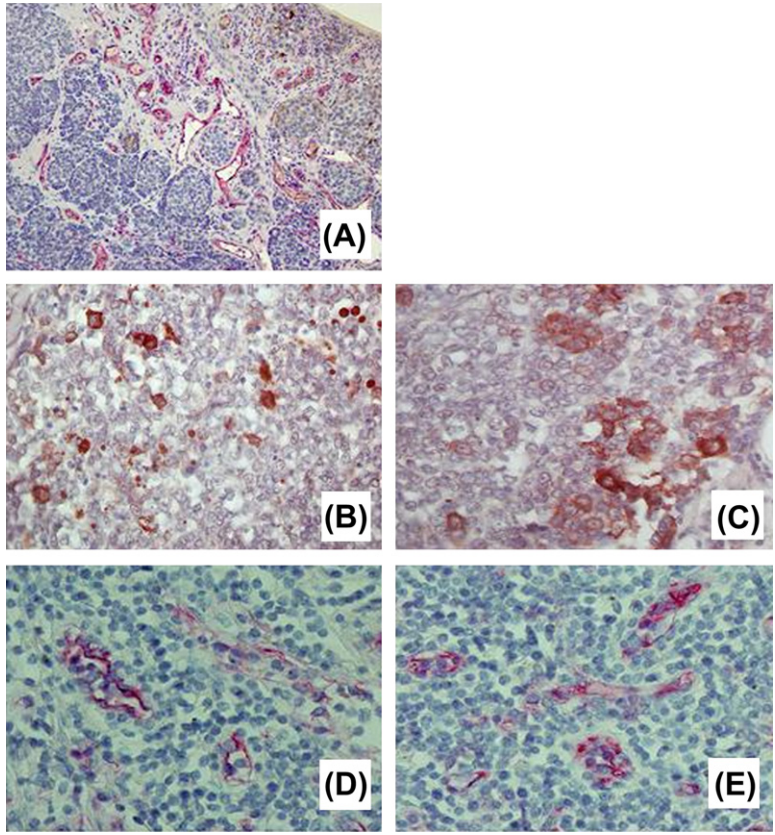

Figure 5.6 Bioptic samples of human primary melanoma with a thickness >3.6 mm. In (A) numerous blood vessels stained with an antibody anti-CD31 are recognizable in the tumor stroma. In (B, C) melanoma tumor cells positive to Epo are present isolated or associated in clusters. In (D, E) numerous vessels positive to EpoR are detectable intermingled between tumor cells. (Reproduced from Ribatti et al., 2010). For color version of this figure, the reader is referred to the online version of this book.

The effect of Epo on the survival rate of cancer patients is variable. A meta-analysis of 60 relevant studies found that anemia increased the relative risk of death by 19% in lung cancer, 75% in head and neck cancer, 47% in prostate cancer, and 67% in lymphoma, and was associated with an overall estimated increased risk for death of 65% (Caro et al., 2001). On the contrary, two phase III trials showed a statistically significant Epo-associated worsening of survival (Henke et al., 2003; Leyland-Jones et al., 2005).

Epo administration to patients with multiple myeloma and myelodysplastic syndrome induced bone marrow angiogenesis and further malignant transformation in plasma cell leukemia and acute monoblastic leukemia,

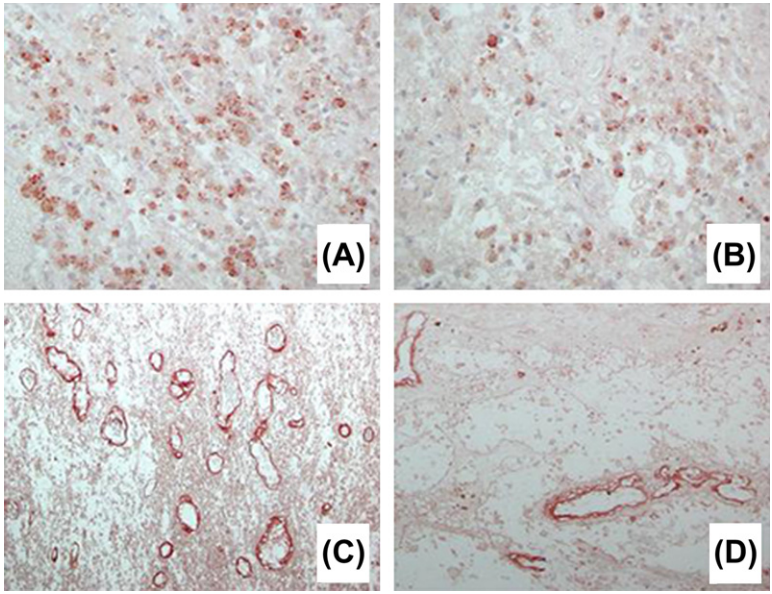

Figure 5.7 Immunohistochemical staining of tumor cells positive to Epo (A, B) and endothelial cells positive to EpoR (C, D) in human glioma. Immunoreactivity increases in IV malignancy grade tumor specimens (in A, C) as compared to stage II (in B, D). (Reproduced from Nico et al., 2011). For color version of this figure, the reader is referred to the online version of this book.

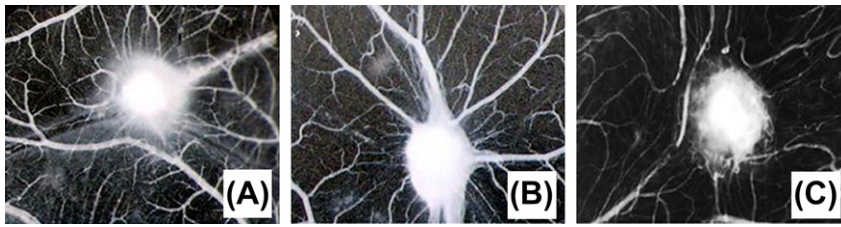

Figure 5.8 A 12-day-old chick embryo CAM incubated for 4 days with a gelatin sponge loaded with 50 ng of VEGF used as positive control (in A), with a grade IV human glioma bioptic specimen (in B) or with an anti-Epo blocking antibody coadministered with glioma bioptic specimens (in C). Note that in (C) the number of vessels converging toward the graft is significantly reduced and compared to (B). (Reproduced from Nico et al., 2011).

respectively (Olujohungbe et al., 1997; Bunworasate et al., 2001; Ribatti, 2002). On the contrary, Mittelman et al. (2001) demonstrated that rHuEpo treatment induced complete tumor regression in 30–60% of mice with a syngeneic multiple myeloma.

Inhibition of Epo signaling might be suggested as a new antiangiogenic therapeutic approach in the treatment of cancer. The administration of anti-Epo antibody, soluble EpoR or an inhibitor of JAK2 resulted in a delay in tumor growth with 45% reduction in maximal tumor depth in a tumor Z chambers model with rat mammary adenocarcinoma cells (Arcasoy et al., 2002). Yasuda et al. (2002) reported that the injection of an anti-Epo monoclonal antibody or the soluble form of EpoR into malignant tumors of the female reproductive organs reduces capillaries and causes tumor destruction, and blockade of Epo signaling on xenografts of uterine and ovarian cancer cell lines leads to the destruction of tumors in nude mice.

The systemic administration of putative antiangiogenic agents targeting Epo and EpoR may be limited by the development of anemia due to the suppression of erythropoiesis. Otherwise, alleviation of anemia by systemic rHuEpo treatment has different effects on tumors: it can decrease hypoxia, enhances proliferation or survival of cancer cells, increases endothelial cell proliferation causing enhanced radiosensitivity of the vessels and tumor perfusion by oxygen and chemotherapeutic agents, favoring their delivery (Tovari et al., 2005). However, improved systemic perfusion may not translate in improved intratumoral blood flow, as tumors often have aberrant vasculature.

Epo-stimulating agents (ESA), including epoetin alfa, epoetin beta and darbepoetin, have been approved for the treatment of anemia in patients with nonmyeloid malignancies whose anemia is due to the effect of concomitantly administered chemotherapy and the risk of thromboembolic events, decreased survival and poorer tumor control as a consequence of treatment with ESA has been recognized (Juneja et al., 2008). In head and neck cancer, metastatic breast cancer, lymphoproliferative malignancies and advanced non-small-cell lung cancer, Epo has an unfavorable effect on survival rate (Henke et al., 2003; Leyland-Jones et al., 2005; Wright et al., 2007). However, in Epo-treated patients the bulk of increased deaths was attributable to accelerated cancer progression, independently of the increased cardiovascular and thromboembolic events.

6. CONCLUDING REMARKS

Recent findings demonstrated tissue-protective properties of Epo independent of its hematopoietic properties. Epo activates a multitude of

EpoR-dependent and independent cellular pathways, including the induction of anti-apoptotic genes and anti-inflammatory molecules, the increase of VEGF as well as the production of NO.

Angiogenesis is regulated, under both physiological and pathological conditions, by numerous "classic" factors (Table 5.2) (Ribatti et al., 2007a). In the recent years, evidence has been accumulated that, in addition to the "classic" factors, many other endogenous "non-classic" peptides, including Epo, play an important regulatory role in angiogenesis, especially under pathological conditions (Table 5.3) (Ribatti et al., 2007a). It is well established that the angiogenic phenotype results from the imbalance between positive and negative regulator factors, so that the contribution of each "classic" and/or "non-classic" angiogenic factor may play a different role in the definition of the angiogenic phenotype. Increased production of angiogenic stimuli and/or reduced production of "classic" and/or "non-classic" angiogenic inhibitors may lead to abnormal neovascularization.

Much research effort has been concentrated on the role of angiogenesis in cancer and inhibition of angiogenesis is a major area of therapeutic development for the treatment of this disease. New pathophysiological concepts generated in the last decades have given way to the development of a large variety of new drugs to interfere with angiogenesis. Preclinical and clinical studies have made it increasingly clear that strategies that target tumor blood vessel networks ultimately will be most effective if used in conjunction with, or adjuvant to, conventional anticancer therapies.

The mechanism of tumor growth in the context of Epo is not completely clarified, and it is not still clear whether there is a direct effect of

Table 5.2 "Classic" pro-angiogenic and antiangiogenic factors

Pro-angiogenic
Vascular endothelial growth factor (VEGF)
Fibroblast growth factor-2 (FGF-2)
Transforming growth factor beta (TGF-β)
Platelet-derived growth factor (PDGF)
Angiopoietin-1 (Ang-1)
Antiangiogenic
Thrombospondin-1
Angiostatin
Endostatin

Table 5.3 Non-classic pro-angiogenic and antiangiogenic factors

Pro-angiogenic
Erythropoietin (Epo)
Angiotensin-II (Ang-II) (interacting with receptor AT1)
Endothelin (ET)
Adrenomedullin (AM)
Urotensin-II (UT-II)
Leptin
Neuropeptide Y (NPY)
Vasointestinal peptide (VIP)
Substance P
Antiangiogenic
Angiotensin-II (Ang-II) (interacting with receptor AT2)
Adiponectin
Somatostatin
Ghrelin
Natriuretic peptides

Epo in tumor cells as opposed to exogenous effect on angiogenesis. It is also possible that the effect of Epo is multifactorial depending on the type of tumor and level or functionality of EpoR expression in tumor cells as well as other variables such as hypoxic stress, degree of anemia, chemotherapy, radiotherapy or surgical intervention.

It should be mentioned that commercial available EpoR antibodies are not specific and there is not compelling evidence for functional EpoR expression in tumor cells (Elliott et al., 2006). Recently, a monoclonal antibody specific for EpoR has been developed (Elliott et al., 2010) and studies using this antibody have indicated that many tumor cell lines express low-to-undetectable levels of EpoR and that EpoR is nor functional (Swift et al., 2010).

A detailed knowledge of the mechanism of action and expression as well as the interactions of the new "non-classic" endogenous regulators of angiogenesis with their receptors will provide new insights that are essential for the future development of chemical compounds that can modulate the activity of these new "non-classic" endogenous regulators and may have potential for antitumor activity. In fact, tumors and other angiogenic pathologies exploit redundant mechanisms to induce angiogenesis, and neutralization of multiple factors, including both "classic" and "non-classic" regulators, may be required to suppress tumor growth.

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CHAPTER SIX

Radiation Responses and Resistance

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Contents

Abstract

The cytotoxic effect of ionizing radiation makes it a popular therapeutic tool against cancer. It is, however, a double-edged sword. Radiation exposure unleashes a plethora of protective and survival mechanisms that include increased proliferation, remodeling of the vasculature and altered cell motility. These mechanisms can play a pro-survival role in remaining cells, contributing to repopulation of tumors after radiation treatment. Understanding these mechanisms and finding ways to minimize their impact would improve the outcome of radiotherapy.

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1. INTRODUCTION

A tissue after exposure to radiation is not the same as what was there before irradiation (Valerie et al., 2007; Kargiotis et al., 2010). In this review, radiation refers to ionizing radiation (IR) such as X-rays and γ-rays. The difference in tissues before and after radiation exposure is due to the obvious reasons, which include elimination of some cells through cell death and arrested proliferation of others through checkpoint activation. Cell cycle arrest by checkpoint activation and induction of cell death could be considered "short-term" responses that initiate almost immediately after radiation exposure and last several hours, after which cells resume proliferation and are no longer prone to death. The irradiated tissue is different from naive tissue, also because of "long-term" responses that occur through more lasting changes in cellular RNA and protein composition over a much longer time course, typically for several days. The overall result of these changes is a group of cells that proliferate faster, enjoy increased blood supply through angiogenesis, produce more survival factors for both cell-autonomous (autocrine) and non-autonomous (paracrine) use, and are more motile and invasive. Basically, the old adjunct "what does not kill you makes you stronger" applies here and can have important implications in the clinical use of radiation. In this review, we will briefly discuss short-term responses before focusing on the longer term responses in more detail. The latter responses will be discussed in terms of their genetic basis, molecular mechanism, and clinical implications.

2. MODULATING THE DAMAGING EFFECT OF RADIATION; HYPOXIA

Before discussing cell and tissue responses to radiation, we will review the relationship between hypoxia and radiation resistance. This relationship has been most frequently investigated in cancer models, as opposed to normal tissue, where intra-tumoral hypoxia is found to reduce the efficacy of radiation treatment (Harrison et al., 2002). Since regions of solid tumors are hypoxic, this is a clinically relevant issue.

The mechanism by which IR kills cells can shed some light on why hypoxia reduces the killing effect of radiation (Hall and Giaccia, 2005). IR liberates electrons from atoms and molecules to generate ions. Within a biological specimen, frequent targets are water molecules and oxygen

molecules, and frequent products are reactive oxygen species such as superoxide, peroxide and hydroxyl radicals. These can attack macromolecules such as nucleic acids, lipids and proteins. The effect of reactive oxygen species can lead to oxidative damage to bases and breakage of the DNA strand. Breaks that are close to each other and occur on opposing strands can result in DNA double-strand breaks, the most lethal type of DNA damage if un-repaired. IR can also directly break the DNA strand but the incidence of this is likely to be lower than damage through oxygen radicals given the abundance of intracellular water and oxygen. It follows then that under hypoxic conditions when oxygen concentrations are lower, production of reactive oxygen species by IR would be less efficient. This would lead to less effective radiation treatment.

In reality, reduced production of reactive oxygen species may not be the only effect of hypoxia. Several studies have found that hypoxia itself is mutagenic. Cells grown in hypoxia have been found to harbor more than three fold higher mutations than those grown in normoxia (Reynolds et al., 1996). Furthermore, the pattern of mutations found in cells cultured in hypoxia resembles those found in tumors, suggesting that a hypoxic tumor microenvironment itself is mutagenic. Mutagenicity of tumors, coupled with cell killing by ionization radiation, would remove less resistant cells and "select" for cells with mutations that allow for radiation survival. Repeated rounds of "selection", for example through fractionated radiation therapy, would produce a tumor with the most "fit" cells, thereby reducing the efficacy of radiation therapy.

3. CELLULAR RESPONSES TO IR

Once a cell sustains a break in DNA, there are three key ways in which it responds: activation of cell cycle checkpoints, induction of DNA repair, and induction of apoptosis (for recent reviews, see Smith et al., 2010; Smits et al., 2010; Su, 2011). Cell cycle checkpoint activation is thought to provide the necessary delay to allow for repair. In fact, in classical experiments in budding yeast that first identified the genetic basis for checkpoints, mutants in Rad9 show reduced viability after exposure to IR (Weinert and Hartwell, 1990, 1993; Weinert et al., 1994). Reduced viability of irradiated Rad9 mutants, however, can be rescued by artificially inducing a cell cycle delay with a microtubule-depolymerizing drug. The rescue was abolished in double mutants of Rad9 and DNA repair genes. These data led to the understanding

that DNA repair is essential for surviving DNA damage induced by IR and that the role of checkpoint functions is to allow time for repair to occur. Cells with un-repaired DNA damage ultimately succumb to cell death.

A key protein in cellular responses to radiation is the tumor suppressor p53. The role of p53 in induction of cell death is better understood in the so-called "intrinsic" death pathway in which damage within the cell results in autonomous cell death. The signaling cascade that can ultimately lead to cell death begins with the recognition of the DNA double-strand break and the activation of proteins such as the Mre11/Rad50/NBS1 (MRN) complex and ATM/ATR kinases (Su, 2006). The signal from a single DNA double-strand break can be amplified to halt the cell cycle. Mechanisms for signal amplification include the spreading of damage-responsive phosphorylation of nucleosomal histone H2AX from the site of damage. This modification, the so-called "γ-H2AX", is induced by the activity of ATM kinase but results in further recruitment of MRN complexes and activation of additional ATM kinases through phosphorylation. Repetition of this process results in the spreading of γ-H2AX tens of kilobases away from the damage. Another mechanism for signal amplification is through the recruitment of soluble Checkpoint Kinase 1 (Chk1) and Chk2 kinases to sites of damage, where these become phosphorylated and activated. Active Chk1 and Chk2 then dissociate and disperse into the cytoplasm to act on their effectors. Additional naive Chk1 and Chk2 are then recruited, further amplifying the signal. Budding yeast RAD9 participates in this process (Gilber et al., 2001).

Active ATM/ATR and Chk1/Chk2 kinases phosphorylate and activate downstream effectors that include p53. These and other modification on p53 increase their stability and activity as a transcriptional factor to activate genes involved in DNA repair, cell cycle checkpoints and apoptosis. p53 targets also include anti-apoptotic genes, thereby establishing a negative feedback loop to limit apoptosis. The balance of pro-survival and pro-apoptotic activities of p53 is dependent on cell type, presence or absence of co-activators and on the type of DNA damage itself (Donner et al., 2007; Gomes and Espinosa, 2010). Activation of the intrinsic cell death pathway ultimately results in mitochondria-dependent caspase activation and apoptosis.

Caspase activation and cell death can also be brought about by an extrinsic cell death pathway that occurs through cell surface "Death Receptors" upon activation by extracellular ligands. Known transcriptional targets of p53 include the Death Receptor Fas (Bouvard et al., 2000). Signaling through the extrinsic pathway also contributes to life–death decisions. Upon experimental induction of p53 in human colorectal cancer

cells, both pro-survival genes (cell cycle inhibitors) and pro-death genes (encoding PUMA, BAX) become activated. It is the activation of Death Receptor 4 (DR4) at the cell surface, in a p53-dependent manner but by an unknown mechanism, that activates the pro-apoptotic protein BAX at the mitochondria and flips the balance toward death and against cell cycle arrest in this system (Henry et al., 2012).

IR causes damage within the cell, but there is good evidence that it activates both intrinsic and extrinsic cell death pathways. This is not surprising since p53, which plays a role in both mechanisms, is activated by radiation. In osteosarcoma cells, IR exposure results in upregulation of DR4 and DR5 (Hori et al., 2010). In colorectal cancer cells, exposure to sublethal doses of IR results in upregulation of DR4 (Ifeadi and Garnett-Benson, 2012). These findings have led to the understanding that combining IR with death ligands could be a viable therapeutic modality because the combination will result in activation of both intrinsic and extrinsic death pathways (Marini and Belka, 2003; Marini et al., 2006; Niemoller and Belka, 2009). Consistently, irradiated colorectal cancer cells that have upregulated DR4 are found to be susceptible to exogenously added death-inducing ligand TRAIL and an activating antibody against Fas receptor (Ifeadi and Garnett-Benson, 2012).

4. PTEN AND SURVIVAL PATHWAYS

PTEN (phosphatase and tensin homolog) is a tumor suppressor that is frequently mutated in human cancers. Through its phosphatase activity, PTEN antagonizes phosphorylation by phosphatidylinositol-3 Kinase (PI3K). PI3K signaling results in activation of the pro-survival Akt/PKB pathway (for review, Cantley and Neel, 1999; Populo et al., 2012). Therefore, loss of PTEN by mutations is postulated to upregulate Akt and thereby confers resistance to killing by IR. Consistent with this idea, PTEN mutations are correlated with shorter survival after radiation treatment in cervical cancer patients (Harima et al., 2001). Conversely, forced expression of PTEN in glioma cells or transfer of the PTEN gene into PTEN mutant glioma cells renders the cells more sensitive to radiation (Wick et al., 1999; Inaba et al., 2011).

While the above-cited studies paint a simple picture, other reports point to a more complex situation. In one study in a head and neck cancer model, PTEN was found to confer radioresistance, which is the opposite of what was found in studies in glioma and cervical cancer models (Pattje et al., 2010). In this study, PTEN expression in patient tissues correlated with poor response to

radiation treatment. PTEN expression also correlated with elevated phospho (active) Akt staining. While this finding was a surprise, because PTEN normally inhibits the signaling pathway that activates Akt, it can explain why PTEN expression correlated with radioresistance. Thus, the effect of PTEN may be tumor type specific. Furthermore, the mechanism of action of PTEN may be more complex than antagonizing PI3K signaling. There are reports that PTEN has a role in DNA repair (Ming and He, 2012) and DNA damage signaling through Chk1 (Puc et al., 2005), while others found that PTEN-deficient cells are proficient for the DNA damage signaling and DNA repair, but show an inability to activate the spindle checkpoint (Gupta et al., 2009). We postulate that whether PTEN is radioprotective or radiosensitizing may depend on its primary role in a given cell type. If PTEN's primary role in a cell is DNA damage checkpoint activation, loss of PTEN would increase the effect of radiation. If PTEN's primary role in a cell is inhibiting PI3K/Akt and pro-survival signaling, loss of PTEN would decrease the killing effect of radiation.

5. TISSUE-LEVEL MECHANISMS FOR RADIATION RESISTANCE

Once cells within a tissue have been killed by radiation, the survivors proliferate to repopulate the tissue. In the context of radiotherapy of tumors, the ability of survivors to repopulate would determine "tumor-free survival", an important clinical end point. Mechanisms that allow surviving cells to repopulate could be grouped into two: those that preexist in cells prior to radiation exposure and those that are acquired by cells upon or following radiation exposure. Within a tumor, there may be a subpopulation of cells that is intrinsically radiation resistant. Their survival of radiation exposure and subsequent proliferation would represent a preexisting mechanism. The concept of cancer stem cells would be in line with this scenario. Mechanisms that are induced by radiation exposure itself would include signaling pathways that operate through vascular endothelial growth factor (VEGF), MET (encoded by *c-Met* or MNNG HOS transforming gene), and angiogenins, to name a few, that are activated by radiation and act to confer survival, growth and proliferation. These are discussed in detail below.

5.1. Acquired Resistance: Compensatory Proliferation

In wing imaginal discs of *Drosophila melanogaster* larvae, dying cells release mitogenic signals (Grusche et al., 2011; Ryoo et al., 2004; Sun and Irvine,

2011). The resulting proliferation of the neighbors compensates for cell loss and helps regenerate the disc. Signaling from dying cells operates through activation of Wingless (*Drosophila* Wnt) and c-Jun N-terminal kinase (JNK) and through repression of the tumor suppressor Salvador/Warts/Hippo pathway. Cross talk between JNK activation and Hpo has also been reported (Sun and Irvine, 2011). The mitogenic effect of undead cells is seen 3–5 cells away from the source. Consequences on the neighbors include an increased number of cells in S phase and activation of targets of Yki, a transcription factor that is normally repressed by Hpo signaling, such as the anti-apoptotic protein *Drosophila* inhibitor of apoptosis protein 1 (DIAP1) (Grusche et al., 2011).

The finding in Drosophila that dying cells promote compensatory proliferation presaged by several years the identification of a similar but mechanistically different phenomenon in mammals. A response called "Phoenix Rising" occurs in mice after cell killing by IR. Here, the activity of executioner caspases, Caspase 3 and Caspase 7, is required in dying cells and mediates the activation of phospholipase A2 and the subsequent production and release of prostaglandin E2, a stimulator of cell proliferation (Li et al., 2010). These signals act non-autonomously in a paracrine fashion to stimulate compensatory proliferation and tissue regeneration. In a follow-up study in murine models, Caspase 3 was also found to be necessary for tumor regeneration after radiation treatment (Huang et al., 2011).

We note that not all consequences on neighboring cells are protective or mitogenic. In the classical "radiation bystander effect", the effect of irradiated cells on the neighbors is destructive, making the latter more prone to death by cytotoxic agents. Bystander effect has been described in mammalian cell culture and in mice (Mothersill and Seymour, 2006a, 2006b; Singh et al., 2011). There is evidence that the signal is soluble; media from irradiated cells can induce bystander effect on naive cells. Inhibitors of bystander effect include the antioxidants L-deprenyl and lactate (Mothersill et al., 2000), suggesting that energy metabolism may be involved in the radiation bystander effect.

5.2. Acquired Resistance: Survival, Growth, Proliferation and Angiogenesis

Most studies of IR have been on tumors or tumor-derived cells and very few use intact healthy tissue. We will begin by noting that available data indicate differences in radiation responses between normal and tumor-derived cells

and tissue. For instance IR results in decreased angiogenesis in normal rat brains (Lee et al., 2011), but increased angiogenesis in brain tumors xenografted in mice (e.g. Kargiotis et al., 2008). Irradiation of whole rat brains in intact animals led to increased apoptosis and decreased proliferation of endothelial cells (Lee et al., 2011). These responses are accompanied by decreased messenger RNA (mRNA) and protein levels of VEGF, Angiopoietin-1 and its target, endothelial-specific receptor tyrosine kinase (Tie-2). Irradiation led to an overall decline in the CD31-positive endothelial cells. This effect is seen also in endothelial cell culture where radiation induces senescence (Igarashi et al., 2007).

The above-described effects are the opposite of what is seen in disease models. Here, irradiation promotes endothelial cell survival and angiogenesis. For instance, irradiation increased CD31+ (endothelial) cell population in irradiated xenografts of meningioma cells (Kargiotis et al., 2008), and xenografts made from irradiated carcinoma cells (Pueyo et al., 2010). The key difference here is the cell-autonomous versus non-autonomous responses. In the case of cancer models, irradiated cancer cells recruit CD31+ cells, presumably from the soma. In the intact brain or endothelial cell models, it is the endothelial cells that decline in number or enter senescence after their own exposure to radiation. This suggests that in a clinical setting, irradiation of tumors may result in initial depletion of endothelial cells, only to be followed by induction and compensation of these through increased growth factor release from transformed cells of the tumor (figure 6.1). There is evidence that such paracrine signaling acts through the VEGF and Angiopoietin family of ligands.

Three receptor tyrosine kinase pathways function in mammalian angiogenesis. VEGF and its receptors establish a vascular network; Angiopoietins and their receptors, Tie-1 and Tie-2, remodel and stabilize the vasculature; and Ephrins and their receptors mediate steps such as cell–cell contact, cell–matrix interaction, and cell migration (for review, Kwak et al., 2000; Jones et al., 2001; Cheng et al., 2002). Ang1, a ligand for mammalian Tie receptors, is a pro-survival factor for endothelial cells during serum deprivation and after irradiation in cell culture models (Holash et al., 1999; Kwak et al., 1999; Papapetropoulos et al., 1999). Exogenous addition of Ang1 decreased IR-induced death of HUVEC cells in culture (Kwak et al., 2000). This effect can be reversed with an extracellular domain of Tie-2 but not Tie-1. These results suggest that Ang1/Tie-2 signaling promotes HUVEC survival after IR. The protective effect of Ang1 on irradiated HUVEC cells was blocked by two drugs that inhibit PI3K/Akt pro-survival

signaling. These data led the authors to postulate that PI3K/Akt signaling acts downstream of Tie-2 to promote the survival of HUVEC cells after irradiation.

These findings in cells were extended to an in vivo mouse model where Ang1 was shown to protect the endothelium from radiation (Cho et al., 2004). COMP-Ang1, an engineered soluble and stable Ang1 variant, was more potent than native Ang1 in phosphorylating the Tie-2 receptor in lung endothelial cells in culture. In irradiated mice, COMP-Ang1 administered intravenously protected against radiation-induced apoptosis in microcapillary endothelial cells of the intestinal villi and prolonged survival of the animals.

Experiments described above used exogenously added Ang1. Interestingly, Ang1 is produced not by endothelial cells but by their neighbors, at least in cell culture experiments (Kim et al., 2000). Therefore, it is possible that radiation exposure results in Ang1 production by dead/dying cells, which then activates Tie-2 in endothelial cells through non-autonomous paracrine signaling to promote survival of the latter. The effect of Tie-2 signaling may not be limited to endothelial cells. Blocking Tie-2 signaling by exogenous addition of only the extracellular domain of Tie-2 (to soak up any ligand) results in a reduced number of implanted tumor cells (murine mammary carcinoma and murine melanoma) into skin folds (Shan et al., 2004). These results suggest that Tie-2 activation may promote tumor growth directly (Tie-2 activation in tumor cells) as well as indirectly (Tie activation in nearby endothelium for vasculature maintenance and angiogenesis).

In addition to Angiopoietins, radiation induces VEGF. Irradiation of U251 or LN18 glioma cells results in the elevation of VEGF levels in the growth media (Kil et al., 2012). Irradiation increased the expression of epidermal growth factor receptor (EGFR), secretion of VEGF and levels of phosphorylated (active) extracellular signal-regulated kinases 1 and 2 (ERK1/2)1/2 and Akt in A431 human epidermoid squamous carcinoma cells (Pueyo et al., 2010). Irradiated or mock-treated controls were implanted subcutaneously in nude mice to mimic residual disease after radiotherapy. Tumors derived from irradiated cells were more angiogenic, showed greater mitotic index and grew faster than control. These features were attenuated by treatment of mice with Cetuximab, a humanized monoclonal antibody against EGFR, suggesting that upregulation of EGFR signaling is responsible for increased growth and angiogenesis after irradiation. Increased EGFR expression and phospho-ERK1/2 levels, but not increased phospho-Akt levels, were also seen in irradiated head and neck

cancer cells (FaDu). In fact, the level of EGFR expression correlates with resistance to radiation therapy in head and neck cancers (Zimmermann et al., 2006).

In sum, irradiation elevates the abundance of soluble growth and angiogenic factors that can lead to tumor growth and survival both through autocrine signaling and through paracrine signaling to the endothelium to promote vasculature survival and function (figure 6.1). These responses appear to be attenuated in healthy animals.

5.3. Preexisting Resistance: Tumor Stem Cells

Tumor stem cells have been an attractive idea to explain repopulation of tumors after treatment. In this model, only a subset of cells in a tumor is tumorigenic. Indeed, when transplanted into immune-compromised mice, less than 0.1% of human cancer cells are computed to be able to produce tumors. In melanoma, this number is about 0.0001% (Quintana et al., 2008). In analysis of tumor cells for molecular differences, a small subset is found to display different cell surface markers and/or amenability to stain with different dyes, allowing their isolation as a "side population" by fluorescence-activated cell sorting. Typically, these are markers of stem cells that are lost upon tissue differentiation during normal development. For instance, CD90, CD133 and NG2 have been proposed as markers for brain cancer stem cells (Singh et al., 2003; He et al., 2010, 2011). All are markers of stem cells or progenitor cells during normal development; NG2 and CD133 are found on neural and glial progenitors, CD133 and CD90 are found on hematopoietic stem cells, and CD133 is found on different types of developmental stem cells (Uchida et al., 2000; Diers-Fenger et al., 2001; Gaipa et al., 2002; Shmelkov et al., 2005). In glioblastomas isolated from patients, a subpopulation of cells that co-express CD133 and CD90 is more clonogenic in the formation of neurospheres in culture than cells with just one or no marker (He et al., 2011). CD133+ fractions of human brain tumors are found to be more tumorigenic than CD133− fractions upon transplantation into mice (Singh et al., 2004). These and other results led to the idea that not all cells within a tumor are equal and that some are more tumorigenic than others, the so-called "tumor stem cells".

In some cancer models, cells with cancer stem cell markers are found to be more resistant to chemo and radiotherapy than cells without these markers (for example, Svendsen et al., 2011; Lopez et al., 2012; Piao et al., 2012). Collectively, these data led to the current thinking that treatment

with cytotoxic agents preferentially select for the survival of tumor stem cells, which then proliferate to repopulate the tumor after treatment. As such much effort is being used to identify agents that can kill cancer stem cells.

While the tumor stem cell hypothesis remains attractive, additional data call into question the significance of cell surface markers. Glioblastoma cells isolated from human patients are found to be tumorigenic in nude mice regardless of CD133 status (Gambelli et al., 2012). More recent data call into question the very existence of tumor stem cells. The selective tumorigenicity of a small population of cells, for example, is found to depend more on the mouse host used for transplantation than the human tumor cells themselves. For example up to 27% of melanoma cells from human patients can produce tumors when transplanted as single cells if the NOD/SCID mouse strain with mutated interleukin 2 receptor is used instead of NOD/SCID mice. In other words, tumorigenicity of so-called cancer stem cells is more because they are better at invading the mouse immune system than otherwise (Quintana et al., 2008). Since the "side population" in melanoma is <1%, clearly cells without "cancer stem cell" markers can produce tumors. A similar result has since been found in leukemia (Agliano et al., 2008).

6. UNINTENDED CONSEQUENCES

6.1. Radiation-Induced Cell Motility

Besides increasing angiogenesis in tumor models, IR also results in increased migration and invasiveness of mammalian cancer cells. This means that, if the dose of radiation is such that the effect is sublethal, i.e. cell kill is less than 100%, the remaining cells could not only repopulate but also result in a more aggressive cancer. This is a real problem because radiation treatment schemes are often fractionated, that is a small fraction of radiation is delivered at regular intervals over a large time frame. The schedule is calculated to counter accelerated repopulation, a phenomenon in which after irradiation the surviving cells proliferate at a rate faster than what was before irradiation. The idea is to irradiate most cells of the tumor and get at the survivors with subsequent fractions. However, sublethal doses also means there is potential to increase the aggressiveness of the cancer with each fraction because each exposure may make cells more invasive, more migratory and could further escalate angiogenesis. Mechanisms for increased migration include the activation of proteases to help remodel the microenvironment and

activation of the protooncogene MET that activates the epithelial–mesenchymal transition as discussed in the following paragraphs.

In a tissue culture model of IOMM-Lee cells of meningioma, a tumor of the central nervous system, IR resulted in changes in the expression of urokinase plasminogen activator (uPA) mRNA, uPA activity and the level of secreted uPA (Kargiotis et al., 2008). Though secreted, the activity of uPA may be anchored to the cell by the binding of uPA to its receptor, uPAR, which is a membrane-bound glycoprotein. uPA is a serine protease whose primary substrate is plasminogen. Plasminogen is a proenzyme, which, upon cleavage, becomes plasmin, another serine protease. The activation of the protease cascade is thought to help remodel the extracellular matrix and aid in cell migration and invasion (Kwaan and McMahon, 2009). In the same study, irradiation also increased the level of phosphorylated (active) forms of ERK and p38 mitogen-activated protein kinase. Treatment with pharmacological inhibitors of ERK, p38 and EGFR attenuated both the basal (nonirradiated) level of uPA activity as well as the increase after irradiation. Inhibitors of JNK and Akt had little effect whereas a PI3K inhibitor partially reduced basal and radiation-induced uPA activity. Small interfering RNA (siRNA) RNA-mediated knockdown of uPA and uPAR reduced basal as well as radiation-induced increase in (i) cell migration in spheroid models of IOMM-Lee cells and (ii) invasion of the same cells through matrigel. siRNA-mediated knockdown of uPA and uPAR reduced capillary-like network formation by human microvascular endothelial cells (HMEC-1) and attenuated the increase in the same process after irradiation. Most relevant to a clinical setting, treating tumor xenografts with radiation alone or siRNA against uPA/receptor alone had little effect but the combination of two treatments showed the most significant tumor control.

6.2. Unintended Consequences; Increased Motility and Invasiveness of Irradiated Cells

Irradiation of U251 or LN18 glioma cells produces media with increased VEGF that causes phosphorylation of the VEGF receptor and increased cell motility in terms of migration and invasion when added to unirradiated U251 cells (Kil et al., 2012). In this experiment, no change in mitotic index of recipient cells was observed, suggesting that the effect of VEGF was not mitogenic on glioma cells. Increased motility and receptor phosphorylation are blocked by an antibody to VEGF.

c-Met is a proto-oncogene that encodes a receptor tyrosine kinase. The ligand for MET is hepatocyte growth factor (HGF), which is also called the "scatter factor" for its ability to induce epithelial cells to take on mesenchymal characteristics and to migrate. Activating mutations in MET are found in human hereditary renal cancers (Gherardi et al., 2012). MET signaling has been implicated in tumor growth, survival, and metastasis, based on numerous studies in vitro and in vivo cancer models. Clinically, MET signaling is implicated in tumor angiogenesis, metastases and in the resistance of non-small cell lung cancers to EGFR inhibitors. Exogenously added HGF protected human and murine mammary tumor cells from apoptosis induced by X-rays and other DNA-damaging agents whereas knockdown of HGF/MET signaling enhanced the death of glioblastoma cells from exposure to IR (Fan et al., 1998; Lal et al., 2005). Interestingly, in a number of human cancer cell lines, IR itself induces a fast (within 10 min) activation of MET and slower (>1 h) activation of MET mRNA and protein expression (De Bacco et al., 2011). In the same study, primary human fibroblasts are found to induce not MET but its ligand HGF. The induction of MET in cancer cells requires nuclear factor kappaB (NF-kB), which is found to bind the MET promoter. MET induction was found to be p53 independent, which could mean that in p53-mutant cells, the pro-survival activity of MET may be un-opposed by the pro-apoptotic activity of p53. This is of clinical relevance because about half of solid tumors are p53 deficient. NF-kB also activates tumor necrosis factor-alpha (TNF-α), which then activates NF-kB in an autocrine loop to result in further expression of MET. Thus, irradiation results in both cell-autonomous and non-cell-autonomous activation of MET. Pharmacological inhibition of MET with a small molecule inhibitor, PHA665752, resulted in reduced viability, clonogenicity, and invasiveness of irradiated breast carcinoma and glioma cells. Thus, radiation-induced MET activation may serve to make cancer cells harder to kill and render then better at metastasis.

7. CONCLUDING REMARKS

Radiation therapy is a popular tool in combating cancer due to its ability to kill cells and shrink tumors. The studies reviewed here show, however, that both individual cancer cells and the overall tumor have or can develop the ability to overcome the effects of radiation therapy and, in some cases, potentially become a more aggressive tumor. Understanding these

responses is crucial in finding ways to prevent tumor resistance and regrowth. There are many examples of such efforts and even successes. A particularly clinically relevant example involves inhibition of EGFR signaling which increases upon irradiation and causes more aggressive tumor behavior. Attenuation of EGFR with a humanized monoclonal antibody against EGFR, Cetuximab, enhances the therapeutic effect of radiation in both preclinical and clinical settings (Raben et al., 2005; Bonner et al., 2006). At the whole tumor or tissue level, another method of resistance exists, called compensatory proliferation, described above. Identifying methods to prevent compensatory proliferation will likely lead to increased

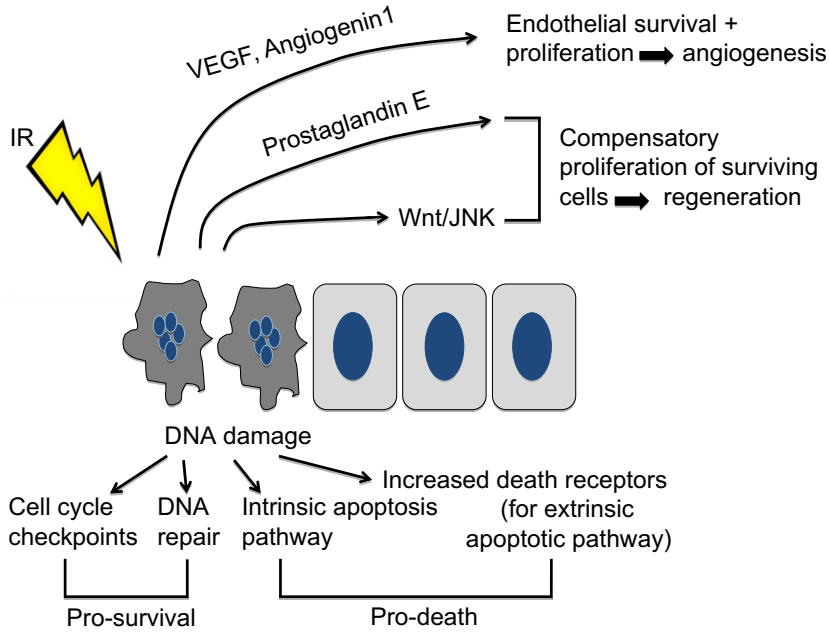

Figure 6.1 ***Summary of mechanisms that are induced by radiation and play a role in cellular or tissue-level resistance to radiation.*** In response to DNA damage induced by IR, cells activate both pro-survival pathways (cell cycle checkpoints and DNA repair) as well as pro-death pathways (intrinsic cell death pathway and expression of Death Receptors). The balance of these determines the life–death fate of the cell. Cells that die in response to IR (depicted with fragmented nuclei) then activate non-autonomous pathways in near-by cells through released factors (Wnt/prostaglandin E). The result is the increased proliferation of survivors to allow for regeneration. In some cases, secreted factors include angiogenic factors that allow survival and proliferation of endothelial cells. Only representative mechanisms are shown here. Please refer to the text for a more comprehensive discussion of pro- and anti-survival signaling in response to IR. For color version of this figure, the reader is referred to the online version of this book.

tumor sensitivity to radiation. In a screen designed to identify inhibitors of regeneration after irradiation in a model organism, we identified inhibitors of protein translation. One of these, Bouvardin, has since been shown to enhance the effects of radiation in both human cancer cells and tumor xenografts (Gladstone et al., 2012). We anticipate that as we reach a better understanding of novel mechanisms that are induced by radiation and contribute to radiation resistance, we will generate better therapeutic tools to enhance the efficacy of a popular standard therapy against cancer.

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CHAPTER SEVEN

Effects of Ethanol Exposure on Nervous System Development in Zebrafish

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Contents

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Abstract

Alcohol (ethanol) is a teratogen that adversely affects nervous system development in a wide range of animal species. In humans numerous congenital abnormalities arise as a result of fetal alcohol exposure, leading to a spectrum of disorders referred to as fetal alcohol spectrum disorder (FASD). These abnormalities include craniofacial defects as well as neurological defects that affect a variety of behaviors. These human FASD phenotypes are reproduced in the rodent central nervous system (CNS) following prenatal ethanol exposure. While the study of ethanol effects on zebrafish development has been more limited, several studies have shown that different strains of zebrafish exhibit differential susceptibility to ethanol-induced cyclopia, as well as behavioral deficits. Molecular mechanisms underlying the effects of ethanol on CNS development also appear to be shared between rodent and zebrafish. Thus, zebrafish appear to recapitulate the observed effects of ethanol on human and mouse CNS development, indicating that zebrafish can serve as a complimentary developmental model system to study the molecular basis of FASD. Recent studies examining the effect of ethanol exposure on zebrafish nervous system development are reviewed, with an emphasis on attempts to elucidate possible molecular pathways that may be impacted by developmental ethanol exposure. Recent work from our laboratories supports a role for perturbed extracellular matrix function in the pathology of ethanol exposure during zebrafish CNS development. The use of the zebrafish model to assess the effects of ethanol exposure on adult nervous system function as manifested by changes in zebrafish behavior is also discussed.

1. INTRODUCTION

An important and all-too-common behavior contributing not only to poor newborn health but also to long-term disability and adverse social consequences is maternal alcohol use (Kelly et al., 2000; Streissguth et al., 2004). It is estimated that prenatal ethanol exposure may be the leading cause of mental retardation in the Western world (Abel and Sokol, 1986). Fetal alcohol spectrum disorder (FASD) is more prevalent in African-Americans and socioeconomically disadvantaged populations, making this an important health disparities disease in the U.S. and worldwide. Fetal alcohol syndrome (FAS) represents the morphologically severe end of the FASD spectrum and is estimated to occur in 1–3 per 1000 live births (Barr and Streissguth, 2001). For all prenatal ethanol exposure-induced birth defects (i.e. all morphological and behavioral abnormalities that fall within FASD), approximately 1 in 100 live births are affected (Barr and Streissguth, 2001). The importance of a better understanding of the genesis of ethanol-induced developmental abnormalities and reducing the incidence of FASD is obvious, as FASD is a serious healthcare problem.

Understanding the mechanisms that lead to FASD is a critical emphasis of FASD research, and a wealth of data have been obtained to support the use of rodent models for the study of FASD. Rodent studies have provided important insight into our understanding the pathologies of ethanol exposure during fetal development, and likely molecular targets of ethanol exposure. While rodent models have furthered our understanding of a possible molecular basis of FASD, the use of zebrafish as a complimentary animal model for FASD research is now being realized. This review discusses recent advances in FASD research, and the effects of ethanol exposure in general on nervous system function, that have been made using the zebrafish model system.

2. ZEBRAFISH AS A MODEL SYSTEM TO STUDY HUMAN DISEASE

It is well recognized that understanding the causative factors underlying human disease is aided by the use of animal models, especially mammalian models that more closely resemble humans. The mouse model is the gold standard for modeling human disease, in part owing to the high degree of homology between the mouse and human genomes. Transgenic

mouse models in particular have allowed biomedical researchers to elucidate molecular pathways involved in human disease and to identify potential therapeutic targets and strategies for the treatment of human disease. For developmental disorders such as FASD, mammalian modeling of the disease is possible, including examining the effects of ethanol during the three trimester equivalents of human fetal development. The fact that the mouse model can faithfully recapitulate many of the aspects of a variety of human disease has helped solidify this animal model as the gold standard for biomedical research.

Despite the advantages of the mouse model for the study of human disease, biomedical research has also benefited from simpler animal models that can mimic the molecular pathways associated with specific human disease. Simple invertebrate animal models, such as *Caenorhabditis elegans*, can faithfully recapitulate the molecular pathways associated with a variety of human diseases. More complex invertebrate systems, such as *Drosophila melanogaster*, can also mimic many of the molecular pathways that may be altered and therefore lead to human disease. A major advantage of these simple animal model systems is the ability to readily identify genetic pathways and the function of genes that may then be linked to human disease. Studies from these simple animal models can then be extrapolated to mammalian models to confirm the role of specific molecular targets in human disease.

While the advantages of invertebrate animal model systems are recognized, especially with regard to the study of nervous system development owing to simpler nervous systems containing defined numbers of neurons, models such as *C. elegans* and *Drosophila* have the disadvantage of being invertebrate animals that lack the organ structure of vertebrate animals. This is particularly true for the central nervous system (CNS), with invertebrate animals lacking a true CNS and CNS structures that may be the target of a disease such as FASD. The zebrafish model system is therefore able to complement the use of invertebrate models for the study of human disease, in that zebrafish is a genetically tractable vertebrate animal model system that can serve as a bridge to the study of human disease in mammalian models (Lieschke and Currie, 2007). Zebrafish have long been recognized as a formidable animal model for the study of development and embryogenesis, in particular nervous system development. A major advantage of the zebrafish model is the optical transparency of embryos, allowing organogenesis to be readily observed in living embryos. The high fecundity of female zebrafish provides the biomedical researcher with access to an

enormous number of embryos for modeling human disease. Combined with the ability to experimentally manipulate embryos, as well as introduce a host of molecules (DNA, RNA) into early embryos, zebrafish therefore offer numerous advantages for the study of embryogenesis in a vertebrate model. External development of embryos also affords advantages over mammalian models, especially in toxicological studies such as ethanol exposure of embryos (Linney et al., 2004). The introduction of toxicants directly into the fish water bathing embryos allows easy dosing of toxicants to embryos. The high degree of homology between the zebrafish and human genomes, combined with the genetic tractability of zebrafish, helps to illustrate the enormous potential this simple vertebrate model affords for the elucidation of disease mechanisms in diseases such as FASD. In addition, a goal of biomedical research on animal models of human disease is to identify potential therapeutics for the treatment of human disease. Zebrafish offer a significant advantage over mammalian animal models in terms of high-throughput screening of small chemical modifiers of the disease pathways (Lessman, 2011). Zebrafish embryos can be raised in 96-well or 384-well plates, allowing high-throughput screening of chemical modifiers. Screening of drug efficacy can be based on changes in expression of fluorescent reporter genes, which are readily observable in transparent embryos, as well as rescue of morphological phenotypes in embryos. The successful application of high-throughput screening using zebrafish has been documented in disease models ranging from cancer to cardiovascular disease (Zon and Peterson, 2005).

We will discuss how the use of zebrafish as a model for studying ethanol pathologies has been progressing rapidly, especially with regard to using zebrafish to assess the effects of ethanol on nervous system development or function as measured by altered behavior in juvenile and adult zebrafish. Much like the number of published articles utilizing the zebrafish model has grown exponentially over the last two decades, the use of zebrafish for the study of ethanol effects on the nervous system is likewise rapidly expanding. As we will discuss, the genetic tractability of zebrafish is especially important for the eventual molecular dissection of the effects of ethanol on nervous system development and behavior. From studies in our laboratories, we will discuss how using subthreshold doses of ethanol, that alone will not affect nervous system development, can be combined with partial disruption of genetic pathways to begin to identify potential molecular targets of ethanol exposure during development. In view of the marked similarity in the zebrafish and human genomes, these types of analyses may allow biomedical

researchers and clinicians to begin to understand what factors may contribute to the susceptibility of specific groups of individuals to ethanol exposure during pregnancy, ultimately leading to potential mechanisms to reduce the incidence of FASD in at-risk groups.

3. FETAL ALCOHOL SPECTRUM DISORDER

FASD represents a broad spectrum of disorders that result from fetal exposure to alcohol (ethanol). The most severe form of the disorder is FAS, which presents as a collection of congenital abnormalities that include craniofacial defects, a failure to thrive (growth retardation), and a variety of behavioral deficits as juveniles and adults. FAS as a disease has been recognized for centuries, with children born to alcoholic mothers in the 1700s being described as "weak, feeble and distempered" (Manning and Hoyme, 2007). It was not until 1973 that the studies of Jones and Smith recognized FAS as a major health concern in the Western world and that the disease was finely given a name, FAS (Jones and Smith, 1973). It is now recognized to be a serious health issue, as 1–3 per 1000 births are estimated to be FAS children (Barr and Streissguth, 2001). FAS is considered to be the leading cause of mental retardation in the Western world (Abel and Sokol, 1986).

FASD is also comprised of other alcohol-related conditions, such as fetal alcohol effects (FAE), alcohol-related neurodevelopmental disorder (ARND) and alcohol-related birth defects. For all prenatal ethanol exposure-induced birth defects (i.e. all morphological and behavioral abnormalities that fall within FASD), approximately 1 in 100 live births are affected (Barr and Streissguth, 2001). FAE presents as a less severe form of FAS, while ARND is characterized by functional and behavioral deficits in the absence of the facial features of FAS.

3.1. Effects of Ethanol on Mouse and Human Nervous System Development and Behavior

Relevant to this review, many brain abnormalities are observed in the brains of children with FASD. Early studies used autopsy findings to identify numerous neuropathologies, which ranged from microencephaly and holoprosencephaly to severe CNS disorganization that included cerebral dysgenesis, agenesis of the corpus callosum, and hypoplasia of the cerebellar hemispheres (Clarren et al., 1978; Pfeiffer et al., 1979; Clarren, 1981). However, many of these observed defects are incompatible with life. More

recent neuroimaging studies using modalities such as magnetic resonance imaging (MRI) have identified a range of changes in brain structure that may underlie the behavioral and social deficits that are observed in FASD children. MRI analyses do demonstrate conditions such as microencephaly, holoprosencephaly and brain region-specific hypoplasia in FAS (Mattson and Riley, 1996; Roebuck et al., 1998; Lebel et al., 2011). Also observed is reduced brain volume, particularly in prefrontal cortex (PFC), parietal and temporal cortices (Archibald et al., 2001; Spadoni et al., 2007), consistent with observed deficits in executive function, spatial memory, and language processing (Mattson et al., 1996; Guerri et al., 2009). Deep gray matter structures are also affected by fetal alcohol exposure, with reduced volume observed in basal ganglia, again being consistent with observed cognitive and learning deficits in FASD children (Mattson et al., 1996; Archibald et al., 2001; Lebel et al., 2011). A reduction in cerebellar volume is also consistently observed in FASD children, with deficits in motor coordination, attention and motor learning being a hallmark of FASD and consistent with cerebellar function abnormalities in FASD (Riikonen et al., 1999; Archibald et al., 2001; Autti-Ramo et al., 2002). Other congenital abnormalities that are consistently observed in FASD include effects on the eyes (microphthalmia; optic nerve hypoplasia) (Stromland, 1985; Chan et al., 1991; Stromland and Pinazo-Duran, 1994; Dangata and Kaufman, 1997) and auditory structures (Church and Gerkin, 1988; Church and Kaltenbach, 1997). Also noteworthy is that in some children and adults with FASD, the corpus callosum is abnormal in structure and may even be absent (Clarren et al., 1978; Mattson and Riley, 1996; Bookstein et al., 2002).

In rodents, ethanol also causes developmental stage-dependent dysmorphology. Ethanol exposure during the period equivalent to the third week of human gestation produces craniofacial and midline forebrain defects, which include holoprosencephaly (Sulik et al., 1981, 1984; Sulik and Johnston, 1982). Ethanol exposure equivalent to the fourth week of human gestation produces craniofacial malformations, forebrain and midbrain hypoplasia, and hindbrain dysmorphogenesis characterized by perturbed development of cranial nerves (Sulik et al., 1986; Van Maele-Fabry et al., 1995; Dunty et al., 2002). Later developmental ethanol exposure in rodents, corresponding to the last trimester of human gestation, produces cerebellar dysmorphology (Cragg and Phillips, 1985; Goodlett et al., 1990). Comparable to human FASD defects, midline neural tube development is disrupted in rodents (Zhou et al., 2003), providing evidence that axon commissures may not form properly as a result of prenatal ethanol exposure.

Studies in rats have been used to demonstrate that behavioral deficits similar to those observed in humans are produced following prenatal ethanol exposure. These behavioral defects include hyperactivity and deficiencies in response inhibition (Randall, 1987). FAE mice assessed for open-field exploration and fear-conditioned learning exhibit altered behavior as a consequence of prenatal ethanol exposure, with behaviors typical of reduced anxiety being exhibited, such as reduced contextual fear and increased exploration of novel objects (Allan et al., 2003). Rodent studies of behavior as a result of prenatal ethanol exposure also demonstrate a constellation of behavioral defects that are remarkably similar to behaviors observed in FASD children and include impaired attention, learning, executive function, and motor activity (Schneider et al., 2011). Thus, studies in rodents and humans indicate that prenatal ethanol exposure produces similar behavioral deficits in adults, that as described below are also detected in zebrafish exposed to ethanol.

3.2. Effects of Ethanol on Zebrafish Development and Behavior

Recent studies have begun to employ zebrafish as FASD models and demonstrate that embryonic zebrafish ethanol exposure results in phenotypes comparable to those observed in other vertebrate models. Exposure of 0–72 hours postfertilization (hpf) embryos to ethanol results in eye defects including microphthalmia and/or cyclopia (Bilotta et al., 2004; Arenzana et al., 2006; Dlugos and Rabin, 2007; Kashyap et al., 2007; Santos-Ledo et al., 2011), as observed in rodents and humans. Zebrafish embryos exposed to ethanol also exhibit increased cell death in hindbrain (Loucks and Carvan, 2004; Carvan et al., 2004), with ethanol also leading to augmented cell death in mice following prenatal ethanol exposure (Dunty et al., 2001, 2002). This review will discuss in greater detail in Sections 7.4–7.7 the pronounced effects ethanol has on the development of specific CNS regions in zebrafish, and possible underlying molecular pathways that may be impacted by ethanol exposure in zebrafish.

Zebrafish have been gaining favor as a behavioral model, both using embryos and larvae as well as adult zebrafish. In the last decade an increasing number of studies have shown that both acute and chronic ethanol exposure will affect zebrafish behavior. These studies show that ethanol has a dose-dependent effect on zebrafish behavior and that the effects of ethanol on zebrafish behavior bear similarities to those seen in rodent models and even

in humans. Numerous behavioral defects are observed following brief exposure of zebrafish embryos and larvae to ethanol (Carvan et al., 2004; Lockwood et al., 2004; Fernandes and Gerlai, 2009). These behavioral deficits may, in part, correspond to altered social behavior in humans, with zebrafish exhibiting a reduction in shoaling (group preference) following embryonic ethanol exposure (Fernandes and Gerlai, 2009). In a startle reflex assay, zebrafish embryos exposed to ethanol and tested as 7 days post-fertilization (dpf) larvae exhibited a significantly diminished startle response (Carvan et al., 2004). In this same study zebrafish embryos exposed to ethanol and tested as adults in a learning/memory paradigm demonstrated significantly impaired learning and memory (Carvan et al., 2004), again exhibiting similarity to behavioral effects of ethanol in humans. In a study using 7 dpf larval zebrafish exposed for 20 min to ethanol, it was found that these zebrafish exhibited dose-dependent responses to ethanol that are remarkably similar to those observed in humans. At low doses of ethanol the larvae exhibited hyperactivity, while at high ethanol doses the larvae exhibited hypoactivity (Lockwood et al., 2004). Similar behavioral responses to different doses of ethanol are observed in humans as well as other animal models for alcoholism (Schumann et al., 2003). In a thigmotaxis (wall seeking) behavioral assay that is measure of anxiety and stress, zebrafish embryos exposed to ethanol exhibited thigmotaxis behavior (Lockwood et al., 2004). This response can be interpreted as a reduction in anxiety and stress with ethanol exposures, again mimicking human behavior.

Adult zebrafish exposed to ethanol exhibit a plethora of behavioral effects, such as reduced anxiety/fear, decreased shoaling, and altered locomotor activity (Dlugos and Rabin, 2003; Gerlai et al., 2006, 2008; Wong et al., 2010; Dlugos et al., 2011; Echevarria et al., 2011; Mathur and Guo, 2011). These studies have used both acute and chronic ethanol exposure of adult zebrafish to assess the effects of ethanol exposure on zebrafish behaviors. Much like studies on zebrafish larvae, adult zebrafish exposed to ethanol exhibit dose-dependent differences in locomotor behavior. At low doses of ethanol adult zebrafish exhibit hyperactivity and at high ethanol doses the adult zebrafish exhibit hypoactivity (Gerlai et al., 2000; Gerlai, 2003). Again, the high degree of similarity between zebrafish responses to ethanol and human responses lends credence to utilizing zebrafish as a model to study the mechanisms underlying ethanol effects on zebrafish development and behavior.

In terms of specific behaviors that are modified as a consequence of ethanol exposure in adult zebrafish, anxiety and fear have been analyzed in

zebrafish, since it is well recognized that ethanol will decrease anxiety and fear responses in humans. In adult zebrafish anxiety and fear have been assessed using predator responses as well as fish moving away from the side of the tank. Both acute and chronic ethanol exposure have an anxiolytic effect in adult zebrafish, although the zebrafish will also show adaptation to this response (Gerlai et al., 2000, 2006; Dlugos and Rabin, 2003; Wong et al., 2010). While many of the studies on adult zebrafish have employed chronic ethanol exposure, a recent study by Mathur and Guo (2011) employed repeated acute ethanol exposure to adult zebrafish to mimic more closely the typical human pattern of ethanol use. This study demonstrated that adult zebrafish acutely exposed to ethanol also exhibited an anxiolytic effect in a novel tank diving test or a light/dark choice test (Mathur and Guo, 2011). With repeated intermittent acute ethanol exposure followed by withdrawal, the treated adult zebrafish exhibited enhanced anxiety in these assays, mimicking behavior observed with alcohol withdrawal in humans (Mathur and Guo, 2011).

Alcohol also affects aggression behavior and social behavior in humans (Bushman and Cooper, 1990; Hellemans et al., 2010), and these behaviors can likewise be studied in the zebrafish model. In a mirrored aggression assay it was demonstrated that in response to ethanol exposure adult zebrafish exhibited increased aggression (Gerlai et al., 2000; Gerlai, 2003). Conversely, studies by Echevarria et al. (2011) observed an opposite effect of ethanol exposure on aggression in zebrafish, with aggressive behavior significantly decreased. Social behavior can be studied in zebrafish by assessing shoaling behavior, which as described above has been shown to be affected in zebrafish embryos exposed to alcohol and then tested as adults (Fernandes and Gerlai, 2009). Adult zebrafish exposed to ethanol also exhibit reduced shoaling behavior (Gerlai et al., 2000; Dlugos and Rabin, 2003), indicating that social behavior is modified in zebrafish as a consequence of ethanol exposure. This effect of ethanol on shoaling behavior interestingly displays a gender effect, with female wild-type zebrafish exhibiting enhanced sensitivity to ethanol in a shoaling behavior paradigm (Dlugos et al., 2011).

Recent studies have examined the ability of zebrafish to display an ethanol preference, that was assayed by exposing adult zebrafish to ethanol in a compartment of a two-compartment tank, and then later assessing whether the exposed fish would display an increased preference for the compartment that had contained the ethanol. After a single 20-min ethanol exposure the exposed zebrafish displayed this ethanol preference (Mathur

et al., 2011), again illustrating the similarity between zebrafish and human behavior as a consequence of ethanol exposure.

A critical question that has been raised in zebrafish studies on FASD is whether the ethanol concentrations used to induce FASD phenotypes are not physiological, since ethanol exposures greater than 1.0% are typically required to observe development or behavioral abnormalities. Several analyses of tissue levels of ethanol in fish exposed to ethanol have been conducted, and suggest that 1.5% alcohol exposure in zebrafish is similar to human blood alcohol levels associated with chronic ethanol abuse or acute alcohol intoxication. For example, zebrafish exposed to ethanol maintained steady-state levels of ethanol over a 24-h period following exposure, with ethanol readily diffusing across the chorionic membrane (Dlugos and Rabin, 2003). In zebrafish embryos, 1.5% ethanol exposure produced tissue levels of 0.12% w/v (Lockwood et al., 2004) and embryos exposed to alcohol for 2 h at 24 hpf displayed tissue levels of 0.015–0.04% for 0.5 and 1% ethanol, respectively (Fernandes and Gerlai, 2009). A 30-min dose of 1.0% ethanol in zebrafish embryos has been shown to produce tissue levels of 0.117% (Echevarria et al., 2011). In our studies, exposure of zebrafish embryos to 1.5% ethanol from 6 to 24 hpf results in tissue ethanol levels of 0.12% when measured at 24 hpf, while exposure to 2.0% ethanol results in tissue ethanol levels of 0.17%. When measured at 27 hpf, or 3 h after removal of ethanol, the tissue ethanol levels are 0% (Zhang C., Cole G.J., unpublished data). Thus, it appears that tissue levels of ethanol in zebrafish displaying phenotypes characteristic of FASD are comparable to blood alcohol levels that result from social drinking in humans.

3.3. Studies on Possible Molecular Basis of FASD During CNS Development

Since a wide range of developmental processes appear to be perturbed as a consequence of ethanol exposure during CNS development, a focus has been placed on elucidating the molecular mechanisms that may underlie these disrupted developmental processes. One aspect of ethanol-induced pathogenesis is a marked neuronal cell death that occurs in embryos exposed to ethanol (Ikonomidou et al., 2000; Dunty et al., 2001, 2002; Tenkova et al., 2003; Carvan et al., 2004; Olney, 2004; Dikranian et al., 2005). Even blood alcohol levels that are typical of social drinking can lead to cell death in the developing rodent CNS (Young and Olney, 2006). One possible mechanism to account for the observed ethanol-mediated cell death in the

developing CNS is loss of neurotrophic support, which may then lead to activation of apoptotic cell death programs. Nerve growth factor (NGF) receptor levels are reduced in cerebellum in association with ethanol-induced cell death (Jaatinen and Rintala, 2008). NGF also can reduce the effect of ethanol on Purkinje cell death in mouse cerebellum (Heaton et al., 2000). In cerebellum ethanol induces apoptosis in granule neurons (Luo, 2010), with this effect possibly being retinoic acid (RA) dependent. In addition, Fgf2 could prevent the ethanol-mediated depletion of granule cells in cerebellum (Bonthius et al., 2003; Luo, 2010). This is consistent with an earlier demonstration that NGF and Fgf2 decreased the ethanol-induced loss of cerebellar granule cells (Luo et al., 1997). Previous studies have shown that ethanol decreases brain-derived neurotrophic factor (BDNF)-mediated stimulation of extracellular-signal-regulated kinase (ERK) signaling in cerebellar granule cells (Ohrtman et al., 2006), lending support to a mechanism whereby disrupted signaling by growth and/or neurotrophic factors leads to augmented cell death as a consequence of ethanol exposure. The ethanol-induced cell death is mediated via apoptosis, as overexpression of bcl-2 in mouse brain protects against ethanol-induced cell death (Heaton et al., 1999).

Neurogenesis and gliogenesis may also be targets of prenatal ethanol exposure, as neural stem cells exhibit decreased proliferation and diminished Notch and Fgf2 expression (Hao et al., 2003; He et al., 2005; Rubert et al., 2006). Radial glia exhibit abnormal morphology as a result of ethanol exposure, with decreased *Pax6* expression suggesting loss of radial glia in ethanol-exposed brain (Aronne et al., 2008, 2011). In cerebellum Bergmann glia appear immature morphologically as a consequence of ethanol exposure (Gonzalez-Burgos and Alejandre-Gomez, 2005). Accordingly, microarray studies have identified in mouse brain ethanol-induced decreases in gene expression, with the identified genes regulating neuronal proliferation and differentiation (Hard et al., 2005). However, despite its severe consequences, the molecular basis of these ethanol-induced pathological changes still remain poorly understood.

Growing experimental evidence indicates that ethanol may exert its effects on brain development via disruption of extracellular matrix (ECM) function. Sulfation of heparan sulfate chains of heparan sulfate proteoglycans (HSPGs) is diminished, with concomitant loss of axon promoting function, in response to ethanol (Dow and Riopelle, 1990). Ethanol affects L1 function (Bearer et al., 1999) and diminishes L1-mediated cell adhesion and neurite outgrowth (Gubitosi-Klug et al., 2007). Genes encoding ECM proteins that are essential to ECM function and CNS development, such as

Timp4, BMP15, Fgf2, and Fgf8, are targets of ethanol exposure in prenatal mice (Hard et al., 2005; Rubert et al., 2006, Aoto et al., 2008). Ethanol also perturbs neuronal interactions with laminin and laminin-mediated neurite outgrowth and cell migration (Liesi, 1997). In limb patterning ethanol also appears to act via disruption of Fgf8, as well as Sonic hedgehog (Shh), signaling (Chrisman et al., 2004).

Shh signaling is an apparent key target of prenatal ethanol exposure. Its perturbation may at least in part be responsible for the craniofacial abnormalities of FAS (Ahlgren et al., 2002; Arenzana et al., 2006; Li et al., 2007; Aoto et al., 2008; Loucks and Ahlgren, 2009; Fan et al., 2009). Prenatal ethanol exposure during the period of gastrulation and early neurulation leads to reduced *Shh* gene expression in mouse and chick embryos, as well as phenotypes characteristic of disruption of Shh signaling (Ahlgren et al., 2002; Loucks et al., 2007; Aoto et al., 2008). However, a recent study by Kashyap et al. (2011) suggested ethanol-mediated effects on zebrafish eye development were not Shh dependent, although these studies exposed embryos to ethanol at later developmental stages than those of Loucks et al. (2007) that showed Shh dependence. Ali et al. (2011) have recently shown that zebrafish eye development is sensitive to ethanol exposure at all stages of development analyzed in their study, raising the possibility that Shh-dependent and Shh-independent effects on eye development may occur based on the timing of ethanol exposure. However, with the abundance of evidence that Shh signaling is a major target of ethanol during development, it is tempting to speculate that molecules that are capable of interacting with Shh, or that are dependent on Shh for function, might exhibit impaired function as a consequence of prenatal ethanol exposure. This possibility will be discussed in further detail in the following sections of this review.

Collectively, these above studies using zebrafish indicate that zebrafish will be a powerful complimentary animal model system to assess both molecular and behavioral consequences of prenatal ethanol exposure. These cumulative data also indicate that the zebrafish model can serve as an excellent vertebrate and developmental model for the study of FASD, with the accessibility of zebrafish embryos allowing a detailed analysis of the underlying molecular mechanisms associated with FASD. In particular, as will be reviewed in the following sections, the ability to readily knockdown developmental expression of genes in signaling and/or genetic pathways considered to be critical to the etiology of FASD will allow biomedical researchers to model possible FASD defects in both the absence and presence of ethanol, in order to unequivocally establish the role of specific candidate

genes in ethanol-mediated teratogenesis. Thus, the zebrafish model has the potential to serve as a strong complimentary vertebrate animal model for the study of the molecular basis of FASD.

4. VISUAL SYSTEM DEVELOPMENTAL ABNORMALITIES ASSOCIATED WITH ETHANOL EXPOSURE IN ZEBRAFISH

4.1. Morphological Abnormalities Resulting From Ethanol Exposure During Embryogenesis

Ocular defects are a common finding in FASD and include microphthalmia and optic nerve hypoplasia (Stromland, 1985; Cook et al., 1987; Chan et al., 1991; Stromland and Pinazo-Duran, 1994; Dangata and Kaufman, 1997). The ocular defects observed in rodent and human as a consequence of fetal alcohol exposure are manifested in the facial defects observed in FASD, such as short palpebral fissures. A major focus of the developmental consequences of ethanol exposure during zebrafish embryogenesis has been on eye development, with considerable understanding of the effects of ethanol on eye development resulting from zebrafish studies. Different strains of zebrafish exhibit differential susceptibility to ethanol-induced cyclopia (Bilotta et al., 2004; Arenzana et al., 2006), making it critical that comparative studies between different laboratories employ the same zebrafish strain and comparable ethanol concentrations for exposure. The commonly used AB zebrafish strain is particularly sensitive to 2.4% ethanol exposure for the generation of cyclopia, while at a lower 1.5% ethanol dose the Ekkwill strain exhibits the highest degree of cyclopia (Arenzana et al., 2006).

A common phenotype observed in zebrafish as a result of ethanol exposure during embryogenesis is microphthalmia (Bilotta et al., 2004; Reimers et al., 2004; Dlugos and Rabin, 2007; Kashyap et al., 2007; Loucks et al., 2007; Ali et al., 2011; Zhang et al., 2011). Microphthalmia is observed when zebrafish embryos are acutely exposed to 10% ethanol for 1 h over a wide range of developmental stages, from 5.25 to 48 hpf (Ali et al., 2011). The microphthalmia persists through development after early exposures to ethanol (Kashyap et al., 2007; Zhang et al., 2011). The induction of microphthalmia does appear to be dose dependent and dependent on the timing of ethanol exposure, as studies in our laboratory have shown that exposure to 1.5% ethanol from 6 to 10 hpf does not induce microphthalmia in AB strain zebrafish embryos, while exposure to 1.5% ethanol from 10 to

18 hpf induces microphthalmia (Zhang C., Ojiaku P., Cole G.J., unpublished data). However, a 3-h acute ethanol exposure of zebrafish embryos to 2.4% ethanol beginning at 4.3 hpf produces cyclopia (Blader and Strahle, 1998). These data indicate that zebrafish ocular development is sensitive to ethanol in a concentration-dependent manner at these early stages of development, during gastrulation.

Retinal lamination is delayed and/or disrupted in zebrafish embryos following ethanol exposure (Matsui et al., 2006; Arenzana et al., 2006; Dlugos and Rabin, 2007; Kashyap et al., 2007; Zhang et al., 2011). Using markers to photoreceptors or histological analysis, a reduced volume of the photoreceptor layer as well as marked loss of immunostaining for photoreceptors were observed in zebrafish embryo eyes following ethanol exposure (Matsui et al., 2006; Dlugos and Rabin, 2007; Kashyap et al., 2007). Reduction in volume of the retinal ganglion cell layer was also observed in zebrafish embryos exposed to ethanol (Dlugos and Rabin, 2007; Zhang et al., 2011), and a concomitant optic nerve hypoplasia was observed in ethanol-exposed embryos (Matsui et al., 2006). The projection of retinal ganglion cell axons to the optic tectum in zebrafish is also altered by ethanol exposure (Cowden et al., 2012). The expression of markers of retinal ganglion cells, such as islet1, demonstrated a reduction in ganglion cell differentiation during the period of ethanol exposure, but 24 h after ethanol exposure ended the pattern of islet1 expression was fairly normal (Kashyap et al., 2007). These data suggest that developmental delays induced by developmental ethanol exposure can be corrected subsequent to the ethanol exposure. Collectively, these studies indicate that ocular development in zebrafish exhibits remarkable similarity to mammalian models of FASD in sensitivity of eye development to ethanol exposure during development. Zebrafish and mammalian models of FASD display a microphthalmia phenotype characterized by optic nerve hypoplasia.

4.2. Molecular Mechanisms Underlying Ocular Defects in Response to Ethanol Exposure

While zebrafish studies have demonstrated a similarity with mammals in morphological and histological phenotypes as a consequence of ethanol exposure during embryogenesis, it is unclear if shared molecular mechanisms exist for ethanol's effects on eye development. An advantage of the zebrafish model system as a FASD model is that genetic pathways can be analyzed in ethanol-exposed embryos, and the injection of exogenous messenger RNAs

(mRNAs) into zebrafish embryos prior to ethanol exposure can be utilized to "rescue" ethanol-treated embryo phenotypes. Furthermore, low doses of ethanol that produce no ocular phenotypes can be combined with partial gene knockdown studies to identify potential molecular targets of ethanol. In this case, if partial gene knockdown and subthreshold ethanol exposure generate the typical FASD phenotypes, one can postulate that the function of the gene that has been partially knocked down is perturbed following ethanol exposure, suggesting that ethanol is inducing ocular and other phenotypes via the genetic pathway targeted by partial gene knockdown.

4.2.1. Gene Expression Analysis in the Developing Zebrafish Ocular System

Using in situ hybridization to analyze the expression of genes that are known to be critical regulators of eye development, insight has been gained regarding possible molecular mechanisms that underlie the effects of ethanol on ocular development. It is well recognized that *Pax6* mutations produce microphthalmia, with *Pax6*, a paired-box gene, being considered as a master regulator of eye development (Glaser et al., 1992). *Pax6* also has been shown to play a critical role in ethanol-induced microcephaly in *Xenopus* (Peng et al., 2004), suggesting that it may be an important target of ethanol during development. A number of studies in zebrafish have analyzed *Pax6* expression during eye development, obtaining disparate results. *Pax6* was reported to be unaffected by a 3-h ethanol exposure beginning at approximately 5 hpf, when analyzed in 24 hpf zebrafish embryos (Blader and Strahle, 1998). *Pax6* gene expression is altered in zebrafish retina following ethanol exposure (Kashyap et al., 2007; Loucks et al., 2007; Santos-Ledo et al., 2011), including in 48 hpf zebrafish retina (Kashyap et al., 2007), and an altered expression pattern for *Pax6* has been observed in zebrafish retina following ethanol exposure (Kashyap et al., 2007; Loucks et al., 2007; Zhang et al., 2011). Studies from our laboratory have also examined the effects of ethanol exposure on *Pax6* expression, analyzing *Pax6* expression at 30 and 48 hpf, following ethanol exposure from 6 to 24 hpf (Zhang et al., 2011). We observed a more marked diminution in *Pax6a* mRNA expression in 48 hpf retina than reported previously (Kashyap et al., 2007). Using a similar time course for ethanol exposure as used in our laboratory, Santos-Ledo et al. (2011) also observed a pronounced diminution in the number of *Pax6*-positive cells in 5 dpf zebrafish retina, suggesting the loss of *Pax6*-positive cells is maintained throughout much of retinal development. We therefore speculate that these differences reported by different laboratories in

Pax6a expression in retina may be attributable to differences in the developmental stages exposed to ethanol, as we exposed embryos to ethanol from 6 to 24 hpf and Santos-Ledo et al. (2011) exposed embryos from approximately 4.3 to 24 hpf, whereas Kashyap et al. (2007) treated zebrafish embryos with ethanol from 24 to 48 hpf. Importantly, it can be concluded from these studies that *Pax6* gene expression is a critical target of ethanol exposure during embryogenesis, with the developmental timing of ethanol exposure likely determining the sensitivity of *Pax6* gene expression to ethanol.

Studies from our laboratory also examined the effects of ethanol exposure on *Mbx* gene expression, since this paired-type homeobox gene is also required for ocular development and is thought to act upstream of *Pax6* during retinal development (Kawahara et al., 2002). Our studies showed that *Mbx* gene expression is markedly reduced as a result of ethanol exposure, which was the first evidence that *Mbx* expression/function is a potential target of ethanol during ocular development (Zhang et al., 2011).

Gene function can be readily studied in zebrafish using morpholino (MO) antisense oligonucleotides to knockdown protein expression either by inhibition of mRNA translation or by perturbing mRNA splicing. Our laboratory has been studying the function of agrin, an abundant HSPG, in zebrafish brain (Tsen et al., 1995; Kim et al., 2007; Liu et al., 2008). Using agrin MOs a number of prominent CNS morphological phenotypes are produced, with a prominent phenotype being microphthalmia (Kim et al., 2007; Liu et al. 2008). Agrin morphant embryos also exhibit optic nerve hypoplasia, loss of photoreceptors, disrupted retinal lamination, and microcephaly (Liu et al., 2008). Since these phenotypes are also observed following ethanol exposure in zebrafish embryos, this suggested that loss of agrin function, due to reduced protein expression, may produce ocular and brain phenotypes that are commonly present in FASD.

Our recent studies demonstrated that agrin gene expression is diminished in zebrafish eye as a consequence of ethanol exposure (Zhang et al., 2011). We also employed subthreshold doses of ethanol (0.5%) combined with a partial knockdown of agrin gene expression (using low-dose agrin MO injection) to demonstrate that agrin function is a likely target of ethanol exposure in the developing zebrafish eye (Zhang et al., 2011). Microphthalmia can be induced in zebrafish following subthreshold ethanol exposure combined with low-dose agrin MO (Zhang et al., 2011). *Mbx* gene expression as a consequence of combined subthreshold treatment with agrin MO and 0.5% ethanol is markedly reduced, indicating that its

expression is regulated by agrin function via a molecular pathway that is a target of prenatal ethanol exposure (Zhang et al., 2011). Interestingly, we also showed that *Pax6a* gene expression is diminished in agrin morphant eyes (Liu et al., 2008), and with combined subthreshold treatment with agrin MO and ethanol (Zhang et al., 2011). In light of the demonstration that *Mbx* function regulates *Pax6* expression (Kawahara et al., 2002), which suggests that *Mbx* acts upstream of *Pax6* (Kawahara et al., 2002), our data suggest that ethanol-induced diminution in *Pax6* expression is a consequence of perturbed *Mbx* expression in response to ethanol exposure.

4.2.2. Shh and Fgf Function Following Ethanol Exposure in the Zebrafish Ocular System

Another important putative target of ethanol during CNS development is Shh. Shh interacts with the Fgf signaling pathway to modulate multiple aspects of nervous system development, including eye development (Vinothkumar et al., 2008). Shh signaling regulates expression of Fgf3, Fgf8 and Fgf19 in brain (Miyake et al., 2005), and Fgf8 signaling regulates Shh expression in eye (Vinothkumar et al., 2008). There is a wealth of evidence that Shh may be a key target of prenatal ethanol exposure, including in zebrafish (Ahlgren et al., 2002; Arenzana et al., 2006; Li et al., 2007; Aoto et al., 2008; Fan et al., 2009; Loucks and Ahlgren, 2009). Shh is a critical morphogen during development of the CNS, with disruption of Shh signaling leading to a variety of well-documented developmental morphogenetic defects. Loss of Shh signaling leads to eye defects that range from microphthalmia to cyclopia (Chiang et al., 1996). Loss of Shh function in zebrafish also leads to retinal abnormalities that include loss of photoreceptors (Stenkamp et al., 2008), a phenotype observed in zebrafish exposed to ethanol and zebrafish treated with agrin MO (Liu et al., 2008). A major cause of holoprosencephaly is perturbation of Shh function (Roessler et al., 1996; Ming et al., 2002), and again this phenotype is observed following ethanol exposure. Pertinent to this review is evidence that Shh function is regulated by interactions with HSPGs such as glypicans and perlecan (Park et al., 2003; Bornemann et al., 2004; Takeo et al., 2005; Datta et al., 2006; Giros et al., 2007), and that the establishment of functional Shh gradients is mediated by binding and transport of Shh following binding to HSPGs (Han et al., 2004a, 2004b). Since our recent studies have shown that the HSPG agrin is a likely target of ethanol during zebrafish eye development (Zhang et al., 2011), this raised the interesting possibility that the observed disruption of agrin function following ethanol exposure may

lead to a perturbation in Shh function. Importantly, overexpression of agrin in transgenic mice produces alterations in Shh expression in the optic stalk and ocular defects similar to agrin loss of function in zebrafish (Fuerst et al., 2007). A second basement membrane HSPG, perlecan, when mutated results in diminished Shh signaling and brain morphological defects common to FAS (hypoplasia, ectopias) (Giros et al., 2007). In addition, mutant mice lacking a heparan sulfate-modifying enzyme (*Ndst1*) exhibit developmental defects, such as cerebral hypoplasia and craniofacial defects, that are due to impaired Shh signaling and that phenocopy some FAS phenotypes (Grobe et al., 2005). Collectively, these different studies on HSPG function strongly implicate HSPGs as key regulators of Shh function that may in turn be targets of ethanol during CNS development.

Our laboratory has recently analyzed the role of Shh signaling in the induction of FASD ocular phenotypes in zebrafish, in particular as a result of disruption of agrin function. We have shown that microphthalmia following ethanol exposure in zebrafish can be induced with a combined low-dose ethanol exposure (0.5% ethanol) and low-dose agrin MO (Zhang et al., 2011). The microphthalmia phenotype can be rescued by injection of Shh mRNA prior to exposure to ethanol and agrin MO treatment (Zhang et al., 2011). Interestingly, the effects of combined ethanol exposure and agrin MO treatment on *Mbx* gene expression, in other words, the markedly reduced *Mbx* gene expression in retina, can also be rescued by Shh mRNA overexpression (Zhang et al., 2011). Our ability to rescue the agrin/ethanol microphthalmia phenotype and *Mbx* gene expression pattern extends previous studies from our laboratory that showed that Fgf8-coated bead implantation in agrin morphant embryos rescued microphthalmia and retinal lamination phenotypes (Liu et al., 2008). We have therefore proposed a model (Fig. 7.1) where agrin function is postulated to act upstream of Shh, via interactions with Fgf, to modulate ocular development in zebrafish (Zhang et al., 2011). In this proposed model, ethanol perturbation of agrin function/expression leads to diminished Fgf and Shh signaling. In support of this model, Shh function in eye development depends on Fgf8 signaling (Vinothkumar et al., 2008). In addition, loss of Shh signaling produces microphthalmia (Chiang et al., 1996), and Shh overexpression rescues ocular phenotypes in zebrafish, such as cyclopia (Loucks and Ahlgren, 2009). Collectively, these data from numerous laboratories suggest a molecular pathway whereby Fgf signaling is required for Shh expression and function, with loss of either Fgf or Shh function

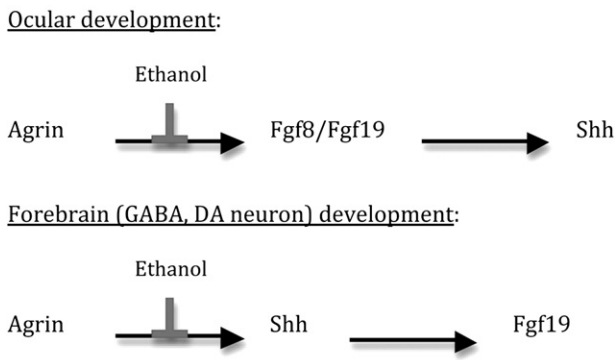

Figure 7.1 Model depicting proposed site of action for agrin modulation of Shh and Fgf signaling in ocular and forebrain development. Fgf signaling is known to act upstream of Shh in ocular development, and Fgf8 bead implantation rescues agrin loss-of-function eye defects in zebra fish. GABAergic neuron development is regulated by Shh, which also regulates Fgf gene expression that is required for development of GABAergic neurons.

leading to impaired eye development that can manifest as microphthalmia. We have postulated that agrin may function upstream of Shh and Fgf signaling to modulate ocular development in zebrafish, with ethanol perturbation of agrin function and/or expression leading to downstream disruption of Fgf and Shh signaling. Since our studies have also shown that combined low-dose ethanol and agrin MO treatments perturb *Pax6a* and *Mbx* gene expression in retina, our studies suggest a molecular pathway that is sensitive to ethanol exposure and involves agrin modulation of Fgf and Shh signaling, which is required for expression of critical transcription factors that are needed for proper eye development.

While these above studies clearly show an effect of ethanol exposure on Shh signaling and function, a recent study by Kashyap et al. (2011) contradict these conclusions, showing that neither Shh nor RA signaling/function is perturbed as a consequence of ethanol exposure. Like Shh signaling, numerous studies have implicated RA function as a target of prenatal ethanol exposure (Sulik et al., 1981; Duester, 1991; Pillarkat, 1991; Zachman and Grummer 1998; Leo and Lieber, 1999; McCaffery et al., 2004; Yelin et al., 2005). Especially relevant to this review, following embryonic exposure to ethanol from 3 to 24 hpf, it was shown that RA could rescue FASD phenotypes in zebrafish (Marrs et al., 2010). In these experiments the phenotypes evaluated and rescued included craniofacial cartilage formation and ear development. The studies by Kashyap et al. (2007), while not

demonstrating a rescue of phenotypes by RA supplementation of ethanol-exposed embryos, were significantly different in the timing of ethanol exposure and the morphological phenotype being assessed. In these latter studies ethanol exposure was from 24 to 48 hpf, and microphthalmia was the phenotype that was assessed for rescue (Kashyap et al., 2011). Thus, from these two studies in zebrafish that have examined the effect of ethanol on RA function, it can be concluded that the timing of ethanol exposure, and the developmental defects elicited by ethanol exposure, may play a critical role in whether RA function is ethanol sensitive during zebrafish development.

Likewise, the study by Kashyap et al. (2011) indicated that Shh was not a target of ethanol in ethanol-mediated microphthalmia, as assessed by cholesterol injection of embryos. This study again suggests that the timing of ethanol exposure appears to be a critical factor. Studies from our laboratory (Zhang et al., 2011) and Loucks and Ahlgren (2009) have demonstrated that Shh mRNA overexpression will rescue ocular phenotypes induced by ethanol exposure, such as microphthalmia and cyclopia. In the case of microphthalmia, ethanol exposure from 6 to 24 hpf that leads to microphthalmia could be rescued by Shh overexpression (Zhang et al., 2011). In the case of the more severe ocular phenotype cyclopia, which was induced by ethanol exposure from approximately 4.3 to 24 hpf, this phenotype also could be rescued by Shh overexpression (Loucks and Ahlgren, 2009). Thus, it appears that while ethanol exposure over a broad range of zebrafish developmental stages will induce microphthalmia (for example, Ali et al., 2011), it is likely that different signaling mechanisms may be impacted by ethanol at different developmental stages to induce abnormal phenotypes such as microphthalmia.

5. FOREBRAIN AND CEREBELLAR DEVELOPMENTAL ABNORMALITIES RESULTING FROM ETHANOL EXPOSURE IN ZEBRAFISH

5.1. Forebrain Defects Resulting From Developmental Ethanol Exposure and Possible Mechanisms

A prominent feature of prenatal ethanol exposure during CNS development is holoprosencephaly. Ethanol exposure at embryonic day 7 (E7) leads to holoprosencephaly in mouse (Sulik et al., 1981; Aoto et al., 2008). An acute ethanol exposure of 0.25 days at E7 is sufficient to induce the holoprosencephaly (Aoto et al., 2008). Severe impairment of telencephalic

development was also noted, with an almost complete absence of the telencephalon and eyes being observed in embryos with the most severe holoprosencephaly (Aoto et al., 2008). A characteristic feature of prenatal ethanol exposure in mice is marked apoptosis throughout the brain, including the forebrain (Ikonomidou et al., 2000; Dunty et al., 2001; Aoto et al., 2008). In zebrafish a similar loss of neurons via apoptosis has been observed following exposure of zebrafish embryos to ethanol (Carvan et al., 2004). Thus, the reduced forebrain volume that has been observed on autopsy, as well as following MRI, may be a consequence of augmented apoptosis as a result of prenatal ethanol exposure (Clarren et al., 1978; Clarren, 1981; Pfeiffer et al., 1979; Mattson and Riley, 1996; Roebuck et al., 1998).

The timing of ethanol exposure in mouse embryos appears to determine which brain structures will display abnormal development and morphologies. As noted above, exposure at E7 leads to midline forebrain defects and holoprosencephaly (Sulik et al., 1981; Aoto et al., 2008). Exposure of mouse embryos at E8.5 leads to diffuse brain hypoplasia (Sulik et al., 1986). Finally, exposure during rodent postnatal development leads to hindbrain defects, which are discussed in the following section.

Prenatal ethanol exposure impairs neuron migration in the telencephalon, with some of the morphological abnormalities observed in rodent brain likely due to abnormal migration of neurons. These structural defects include heterotopias, which occur as a result of neuron migration beyond the pial surface (Pilz et al., 2002; Mooney et al., 2004). Abnormal radial migration of cortical neurons is also observed following ethanol exposure (Miller, 1986, 1997), which may occur as a result of ethanol perturbing radial glia development (Miller and Robertson, 1993; Aronne et al., 2008, 2011). Inhibitory gamma amino butyric acid or GABAergic interneurons in the cerebral cortex do not exhibit radial migration, rather these neurons undergo tangential migration from the medial ganglionic eminence to the cerebral cortical layers (Anderson et al., 1997; Tamamaki et al., 1997; Lavdas et al., 1999). Interestingly, prenatal ethanol exposure alters tangential migration of GABAergic interneurons in mouse telencephalon (Cuzon et al., 2008).

The abnormal telencephalic development in mice exposed to ethanol also leads to altered gene expression, especially genes that are considered critical targets of prenatal ethanol exposure. *Pax6* gene expression is altered in cortical radial glia as a result of ethanol exposure, with decreased *Pax6* expression observed in E14 rat brain (Aronne et al., 2008) and a decrease in

Pax6-positive radial glia observed in cerebral cortex (Aronne et al., 2011). Consistent with these data, 12-h exposure of *Xenopus* embryos to ethanol induces a marked decrease in *Pax6* expression that is accompanied by microcephaly (Peng et al., 2004). The direct role of this diminished *Pax6* gene expression in the generation of microcephaly was demonstrated by the ability of overexpression of *Pax6* to rescue the microcephaly phenotype (Peng et al., 2004).

In whole mouse embryo cultures, ethanol exposure at E7 leads to loss of *Shh* expression in embryos exhibiting holoprosencephaly (Aoto et al., 2008). These embryos also exhibited diminished *fgf8* expression in the telencephalon. It is likely this reduced *fgf8* expression results from the dependence of telencephalic *fgf8* gene expression on *Shh* expression in the medial ganglionic eminence (Ohkubo et al., 2002). These data are noteworthy as an abundance of experimental evidence exists that strongly supports a key role for Shh and Fgfs in the generation of GABAergic neurons from the lateral and medial ganglionic eminences (Yung et al., 2002; Gulasci and Lillien, 2003). In cortical monolayer cultures ventral forebrain stem cells exposed to Shh give rise to GABAergic interneurons (Yung et al., 2002). These cortical GABAergic interneurons are generated from stem cells located within the lateral and medial ganglionic eminences, after tangential migration. The ventral forebrain Shh-responsive progenitor cells are derived from Fgf-responsive stem cells (Marin and Rubenstein, 2001). Fgf2 increases proliferation of stem cells in the dorsal telencephalon, but does not cause an increase in GABAergic interneurons (Gulasci and Lillien, 2003). However, treatment of dorsal telencephalon with Shh results in enhanced production of GABAergic interneurons (Gulasci and Lillien, 2003). Yung et al. (2002) also demonstrated that dorsal forebrain progenitors exposed to Fgf2 and Shh generated GABAergic interneurons at the expense of glutamatergic neurons. It appears that dorsal telencephalon expresses suboptimal levels of Shh (Gulasci and Lillien, 2003), and thus exposure of dorsal telencephalon to Shh can lead to ventralization of this cortical region, with augmented production of GABAergic interneurons. In basal telencephalon exposure to Shh also leads to expression of ventral markers and generation of GABAergic neurons (Watanabe et al., 2005). In summary, these data demonstrate that Shh plays a key role in regulating the generation of GABAergic interneurons in the mammalian cerebral cortex.

Evidence exists for the dependence of forebrain GABAergic neuron development on Shh and Fgf cross talk. In zebrafish disruption of Shh function results in impaired differentiation of GABAergic neurons, based on

diminished expression of *GAD1* (*GAD67*; Miyake et al., 2005). As discussed above GABAergic neuron specification requires Shh signaling in ventral telencephalon (Varga et al., 2001; Yung et al., 2002). Perturbation of Fgf19 function in zebrafish produces the identical phenotype with regard to *GAD1* (*GAD67*) expression and GABAergic neuron differentiation, and is due to Shh signaling regulating *Fgf19* gene expression (Miyake et al., 2005). In addition, *fgf3* and *fgf8* gene expression was shown to be under control of Shh signaling (Miyake et al., 2005), supporting the possibility that reduced *fgf8* gene expression following prenatal ethanol exposure (Aoto et al., 2008) may be due to impaired Shh function. Interestingly, Shh and Fgf8 signaling are also required for specification and development of populations of dopaminergic neurons during development in chicken, mouse and zebrafish (Hynes et al., 1995a, 1995b; Ye et al., 1998; Holzschuh et al., 2003). These data raise the intriguing possibility that the development of multiple specific classes of neurons may be a target of prenatal ethanol exposure as a result of disruption of Shh or Fgf function.

In support of this hypothesis, recent studies from our laboratory have examined the effect of ethanol exposure on the development of forebrain GABAergic neurons in zebrafish. Zebrafish embryos had chronic exposure to ethanol from 6 to 24 hpf, with forebrain GABAergic neuron differentiation assessed by *GAD1* expression. Our studies showed that exposure of zebrafish embryos to 1.5 or 2% ethanol resulted in a marked diminution in *GAD1* gene expression. The timing of ethanol exposure was also limited to defined developmental windows, such as 6–10 hpf (gastrulation) and 10–18 hpf (neurulation). Under these conditions, forebrain *GAD1* expression by GABAergic neurons was perturbed at both time courses of ethanol exposure, although a higher percentage of embryos exhibited decreased forebrain *GAD1* expression following ethanol exposure from 10 to 18 hpf. We also examined whether ethanol was exerting its actions via disruption of Shh signaling by combining a subthreshold exposure to ethanol (0.5% ethanol) with a low-dose Shh MO injection. These experiments demonstrated that forebrain *GAD1* gene expression was markedly reduced and was similar to *GAD1* gene expression observed following exposure of zebrafish embryos to higher doses of ethanol (such as 2% ethanol). We extended these studies to ascertain whether, as previously observed with the zebrafish ocular development, agrin function was disrupted by ethanol exposure, leading to downstream effects on Shh and/or Fgf signaling. These experiments showed that agrin MO treatment reduces forebrain *GAD1* gene expression in 24 hpf embryos, and likewise combined subthreshold ethanol exposure and agrin

MO injection markedly decreased *GAD1* gene expression in 24 hpf embryos. Finally, we examined whether the decreased forebrain *GAD1* gene expression could be rescued by Shh or Fgf mRNA overexpression, which would be predicted from the earlier studies of Miyake et al. (2005). Our experiments clearly show an ability of Shh, Fgf8 or Fgf19 mRNA overexpression to rescue the reduced *GAD1* gene expression phenotype in 24 hpf zebrafish forebrain. These studies therefore begin to provide an understanding of a possible molecular basis for the disruptive effect of ethanol on forebrain GABAergic neuron development, with ethanol perturbing a signaling pathway involving Fgf8 and Fgf19, Shh and modulatory HSPGs such as agrin.

5.2. Effects of Ethanol Exposure on Cerebellum Development

In humans it is well documented that prenatal alcohol exposure leads to deficits in cerebellar function. That the cerebellum appears to be particularly sensitive to ethanol exposure is exemplified by the motor behaviors of chronic alcoholics, who display ataxia and gait disturbances (Mattson and Riley, 1998). As a result of prenatal alcohol exposure FASD children may exhibit cerebellar motor dysfunction, such as impaired balance and other cerebellar motor skills (Mattson and Riley, 1998). Analysis of cerebellar neuroanatomy in humans exposed to ethanol during fetal development indicates the occurrence of cerebellar dysgenesis (Pfeiffer et al., 1979; Clarren, 1981; Sowell et al., 1996). Histological analysis of the brains of chronic alcoholics indicates cerebellar atrophy characterized by loss of Purkinje cells and reduced volume of the molecular layer of the cerebellum (Allsop and Turner, 1966; Ferrer et al., 1984; Phillips et al., 1987; Anderson, 2004). Using animal models, previous studies have demonstrated that prenatal ethanol exposure in mice produces augmented cell death in the hindbrain as assayed by vital dye staining (Dunty et al., 2001), as well as cranial nerve dysmorphogenesis that in particular affects cranial nerves V, VII, IX and X (Van-Maele-Fabry et al., 1995; Dunty et al., 2002). Similarly, embryonic ethanol exposure in zebrafish produces excessive hindbrain cell death (Carvan et al., 2004). Thus, all vertebrate animal models employed in FASD studies appear to indicate a uniform sensitivity of the cerebellum to ethanol exposure during embryogenesis.

Numerous laboratories have examined the effect of ethanol exposure on the survival and differentiation of specific classes of neurons in the mammalian cerebellum, which provides support for observations made from

the study of the chronic alcoholic human brain. Ethanol is known to affect neurotransmitter receptor function, including GABA receptors (Weiner and Valenzuela, 2006; Harris et al., 2008), consistent with the hypothesis that GABAergic neurons may be a target of ethanol exposure. Accordingly, prenatal ethanol exposure in mice or rats reduces the number of GABAergic Purkinje cells in cerebellum (Bauer-Moffett and Altman, 1977; Cragg and Phillips, 1985; Goodlett et al., 1990; West, 1993; Hamre and West, 1993; Napper and West, 1995; Goodlett and Eilers, 1997; Maier et al., 1999; Maier and West, 2001; Karacay et al., 2008). The loss of Purkinje cells from rodent cerebellum as a result of alcohol exposure was dependent on the timing of the alcohol exposure, with the greatest Purkinje cell loss occurring with ethanol exposure at postnatal day (PD) 4-5, which is equivalent to the third trimester of human gestation (West, 1993; Hamre and West, 1993). Rat pups exposed to ethanol after PD7 were shown to be resistant to an ethanol-induced Purkinje cell loss (West, 1993; Hamre and West, 1993), confirming the importance of the timing of ethanol exposure on Purkinje cell survival. Rats exposed to ethanol at all three trimester equivalents exhibited the greatest Purkinje cell loss, when compared to the extent of cell loss with a third-trimester equivalent ethanol exposure (Maier et al., 1999). Rats exposed to ethanol from E1 to E20 also exhibit Purkinje cell loss (Maier and West, 2001). Significant apoptosis is induced in the brains of rats exposed prenatally to alcohol, suggesting that ethanol exposure induces apoptotic pathways that lead to neurodegeneration (Olney et al., 2000, 2002). Accordingly, overexpression of bcl-2 protects against ethanol-induced cell death (Heaton et al., 1999).

Pronounced granule cell loss is also observed in cerebellum following prenatal ethanol exposure (Jaatinen and Rintala, 2008). There are many similarities between granule cell and Purkinje cell loss in rodent cerebellum, with granule cell loss occurring during all three trimester equivalents of human gestation (Maier et al., 1999), and greater granule cell loss observed with ethanol exposure at PD4–5 than when ethanol exposure occurs after PD7 (Hamre and West, 1993; Napper and West, 1995; Karacay et al., 2008). There is a strong correlation between granule cell loss and Purkinje cell loss, leading to the suggestion that granule cell loss is dependent on Purkinje cell loss in the cerebellum after ethanol exposure (Bonthius and West, 1991; Hamre and West, 1993; Luo, 2010). However, granule cell loss in the absence of substantial Purkinje cell loss has been demonstrated, thus questioning this dependence of granule cell loss on Purkinje cell loss (Heaton et al., 2006). Interestingly, granule cell migration from the external granule

cell layer (EGL) appears to be perturbed by ethanol exposure (Gonzalez-Burgos and Alejandre-Gomez, 2005; Kumada et al., 2010; Luo, 2010) and granule cell precursors in the EGL appear reduced as a result of ethanol exposure (Luo, 2010).

5.3. Possible Molecular Pathways Impacted by Ethanol Exposure During Cerebellar Development

While a wealth of evidence has been provided to support a significant loss of Purkinje cells and granule cells during cerebellar development, as a consequence of ethanol exposure, the molecular mechanisms accounting for this cell loss are less well understood. It is clear that apoptotic neurodegeneration is contributing to this cell loss (Heaton et al., 1999; Olney et al., 2000, 2002). It is likely that effects on neuronal survival, as regulated by neurotrophic factor support, are also contributing to the cell loss as NGF decreases the effects of ethanol on Purkinje cell loss in mouse cerebellum (Luo et al., 1997; Heaton et al., 2000). NGF receptors are decreased in cerebellum following ethanol exposure, supporting the ability of NGF to prevent the cell loss (Dohrman et al., 1997; Heaton et al., 1999). Ethanol exposure also diminishes BDNF stimulation of ERK signaling in cerebellar granule cell (Ohrtman et al., 2006).

Growth factor function may also be a target of ethanol exposure during cerebellar development. Proliferation of neural progenitors is regulated by Fgfs such as Fgf2 (Yung et al., 2002). Decreases in neuron number are accompanied by reduced *fgf2* expression (Rubert et al., 2006) and Fgf2 treatment diminishes ethanol-induced loss of cerebellar granule cells (Luo et al., 1997; Bonthius et al., 2003). Since Fgf signaling modulates Shh signaling in the generation of GABAergic interneurons from the lateral and medial ganglionic eminences (Marin and Rubenstein, 2001; Yung et al., 2002), this raises the interesting possibility that impaired Fgf2 function in the cerebellum following ethanol exposure may lead to perturbed Shh signaling/function. Of interest in this regard, previous studies from our laboratory have shown that agrin modulates Fgf2 signaling (Kim et al., 2003), and agrin function is disrupted by ethanol exposure in zebrafish in an Shh-dependent manner (Zhang et al., 2011). Shh signaling does play an important role in cerebellar development, with *Shh* expressed by Purkinje cells, Shh is required for Purkinje cell development, and Shh regulates granule cell proliferation (Dahmane et al., 1999). Thus, if Fgf and Shh signaling are sensitive to ethanol exposure during cerebellar development, ethanol may act to decrease proliferation of progenitor cells for Purkinje cell and granule cells.

5.3.1. Molecular Mechanisms Underlying Purkinje Cell Defects in Zebrafish Cerebellum

Recent studies from our laboratory have been examining Purkinje cell and granule cell development in zebrafish following ethanol exposure of zebrafish embryos. The expression of *GAD1* (*GAD67*) was used as a marker of Purkinje cell differentiation, with ethanol exposure from 6 to 24 hpf resulting in a diminution of *GAD1* gene expression in 3 dpf zebrafish hindbrain. A more acute exposure to ethanol, from 10 to 18 hpf, also led to the reduction in *GAD1* expression in 3 dpf zebrafish hindbrain. Analysis of *GAD1* gene expression in 5 dpf zebrafish cerebellum also indicated a detrimental effect of ethanol exposure on GABAergic neuron development, with exposures at either 24–48 or 48–72 hpf leading to diminished *GAD1* expression in 5 dpf zebrafish cerebellum. Interestingly, exposure to ethanol from 6 to 24 hpf appeared to produce only a marginal decrease in *GAD1* gene expression in 5 dpf cerebellum, suggesting that as observed in the zebrafish retina the developmental delay produced by ethanol exposure may be recovered during later development. To begin to elucidate potential molecular mechanisms underlying the induction by ethanol of diminished *GAD1* gene expression in zebrafish hindbrain, we treated zebrafish embryos with either high doses of agrin or Shh MO or a low dose of the agrin or Shh MO combined with a low dose of ethanol. In these experiments, however, we observed that exposure to 0.5% ethanol combined with low-dose agrin or Shh MO produced barely discernible decreases in *GAD1* expression, while using 1% ethanol led to more pronounced *GAD1* expression phenotypes. These studies showed that both agrin and Shh MO treatment, that produces severe morphological phenotypes in zebrafish embryos, markedly reduced *GAD1* gene expression in 3 dpf zebrafish hindbrain. When 1% ethanol exposure was combined with either low-dose agrin MO or low-dose Shh MO, we again observed a marked diminution in *GAD1* gene expression in 3 dpf zebrafish hindbrain. Importantly, overexpression of Shh mRNA could rescue the *GAD1* expression phenotype in the ethanol/agrin MO- and ethanol/Shh MO-treated embryos. In addition, Fgf19 mRNA overexpression could rescue these *GAD1* phenotypes in the ethanol/agrin MO- and ethanol/Shh MO-treated embryos. Thus, these studies demonstrate that Shh, which is required for Purkinje cell development (Dahmane et al., 1999), appears to be a target of ethanol exposure during development of the cerebellum. In view of studies showing that Fgf2 could prevent the effects of ethanol exposure on Purkinje cell loss in rodent cerebellum, our data provide support for these rodent studies and predict

a signaling mechanism involving Fgfs and Shh, possibly modulated by HSPGs such as agrin, which is sensitive to ethanol exposure during Purkinje cell differentiation.

5.3.2. Granule Cell Development Following Ethanol Exposure

Granule cell death in the cerebellum appears to occur as a result of apoptosis, and it has been suggested that cerebellar granule cells are more sensitive to ethanol than Purkinje cells (Luo, 2010). Granule cell death may result from the neuroprotective and neurotrophic effects of *N*-methyl-D-aspartate (NMDA) receptors being inhibited by ethanol (Pantazis et al., 1995; Zhang and Rubin, 1998). NMDA receptor activation induces the expression of BDNF (Zhang and Rubin, 1998), acute ethanol exposure induces a diminution in BDNF mRNA and TrkB receptor expression in PD4 rat cerebellum (Light et al., 2001; Ge et al., 2004), and ethanol decreases BDNF stimulation of the ERK pathway in cerebellar granule cells (Ohrtman et al., 2006). As described previously, PD4 is a particularly vulnerable period of cerebellum development for ethanol exposure. Additional evidence for impaired neurotrophic factor function as a consequence of ethanol exposure is that NGF treatment can prevent ethanol-induced cerebellar granule cell death (Luo et al., 1997; Heaton et al., 2000; Bonthius et al., 2003).

RA has been shown to be a possible target of ethanol exposure during nervous system development (Sulik et al., 1981; Duester, 1991; Pillarkat, 1991; Leo and Lieber, 1999; McCaffery et al., 2004; Yelin et al., 2005) and in zebrafish RA can rescue abnormal phenotypes induced by ethanol exposure (Marrs et al., 2010). RA is synthesized in cerebellum (Yamamoto et al., 1999) and RA receptor expression is decreased in rat cerebellum following ethanol exposure (Kumar et al., 2010). Fgf signaling may also be a target of ethanol during granule cell development, with Fgf2 treatment preventing cerebellar granule cell death in vitro (Luo et al., 1997). This Fgf2 neuroprotective effect on cerebellar granule cells may be mediated via an NO-cGMP-PKG signaling pathway (Bonthius et al., 2003).

5.3.3. Molecular Mechanisms Underlying Granule Cell Defects in the Zebrafish Hindbrain

Since granule cells exhibit a pronounced sensitivity to acute ethanol exposure in mammalian cerebellum, our laboratory was interested in ascertaining whether granule cell development would likewise be sensitive to ethanol

exposure in zebrafish cerebellum. Presumptive granule cells are derived from the upper rhombic lip, which in mouse brain is characterized by *atoh1/atonal1* gene expression (Alder et al., 1996; Ben-Arie et al., 1997; Wang et al., 2005). In zebrafish presumptive glutamatergic granule cells are also derived from the upper rhombic lip and express the *atonal1a* transcription factor (Volkmann et al., 2008). Neuronal migration from the upper rhombic lip is initiated at 28 hpf in zebrafish (Koster and Fraser, 2001), with *NeuroD* expression marking the migrating granule cell (Volkmann et al., 2008). To examine granule cell differentiation we used in situ hybridization analysis using *vglut1* riboprobes, as this gene is a marker of cerebellar granule cells in zebrafish (Bae et al., 2009). Beginning at 4 dpf the cerebellar granule cells in zebrafish begin to express *vglut1*, and possess functional cerebellar circuits, as directed swimming behavior is present (Volkmann et al., 2008). At 6 dpf the cerebellar granule cells express *Pax6a* (Volkmann et al., 2008). Expression of $GABA_AR\alpha 6$ is seen by 4 dpf and is a marker of cerebellar granule cells (Volkmann et al., 2008).

Our laboratory has examined *atonal1a* gene expression beginning at 18 hpf in zebrafish, and do not observe *atonal1a* expression until 20/22 hpf. Expression of *atonal1a* mRNA in the zebrafish upper rhombic lip is markedly reduced by either 1.5 or 2.0% ethanol exposure in 24 and 48 hpf embryos, with an apparent developmental delay in *atonal1a* expression occurring as a result of ethanol exposure. Interestingly, both agrin and Shh MO treatment also reduce *atonal1a* gene expression in the upper rhombic lip, and combined low-dose agrin or Shh MO and low-dose ethanol exposure (0.5% ethanol) reduces *atonal1a* expression in the upper rhombic lip. We also performed an Shh or Fgf19 mRNA rescue of the reduced *atonal1a* phenotype, and can demonstrate that either Shh or Fgf19 mRNA overexpression can rescue the reduced *atonal1a* gene expression in the upper rhombic lip of 24 hpf embryos. We also examined *NeuroD* gene expression in 1 and 3 dpf zebrafish embryos, and find that *NeuroD* expression is diminished in 1 dpf brain but not 3 dpf brain, as a result of ethanol exposure from 6 to 24 hpf. Taken together, the *atonal1a* and *NeuroD* expression data suggest that development of presumptive granule cells in the rhombic lip is perturbed as a consequence of ethanol exposure in zebrafish, and that migration of these cells may be perturbed and/or delayed, at least during early stages of zebrafish hindbrain development.

To specifically assess the effects of ethanol exposure on granule cell development and/or survival, we used markers of differentiated cerebellar granule cells (*vglut1* and $GABA_AR\alpha 6$) to visualize granule cell development. In both 3 and 7 dpf zebrafish embryos we observed a marked decrease in

vglut1 gene expression as a result of ethanol exposure or agrin or Shh MO treatment. $GABA_AR\alpha 6$ gene expression was also markedly diminished in 7 dpf zebrafish cerebellum. Thus, two markers of mature granule cells in zebrafish cerebellum exhibit a pronounced downregulation as a consequence of ethanol exposure. These data suggest that ethanol exposure in zebrafish cerebellum results in a marked loss of granule cells similar to that observed in mammalian brain.

6. EFFECT OF ETHANOL EXPOSURE ON NEUROGENESIS IN THE DEVELOPING AND ADULT CNS

6.1. Ethanol and Neurogenesis

6.1.1. Neurogenesis in Adult CNS

Neurogenesis or the ability of the brain to generate new neurons was originally thought to occur only during brain development in the fetus and younghood. The observation that neurogenesis continued through juvenile stages and beyond was highly speculative and controversial. Since the first report by Altman (1962) until the 1990s, few studies were conducted to define if new neurons are formed in the brains of adult mammals (Kaplan and Hinds, 1977; Kaplan and Bell, 1984). In 1992 in vitro studies confirmed the presence of neuronal precursors in the adult mouse brain (Reynolds and Weiss, 1992; Richards et al., 1992) and in vivo results provided evidence of cell division in the adult rat brain (Gould et al., 1992) and avian brain (Alvarez-Buylla, 1990). These ground-breaking findings were soon followed by many reports that confirmed the presence of adult neurogenesis in many different types of vertebrate animals, including fish, reptiles, birds, and rodents (Alvarez-Buylla, 1992; Cameron et al., 1993; Kuhn et al., 1996; Kempermann et al., 1997a, 1997b, 2004; Eriksson et al., 1998; Lavenex et al., 2000; Delgado-Gonzalez et al., 2008; Zhang et al., 2009; Amrein and Slomianka, 2010). The discovery of neural stem cells in the adult human brain (Eriksson et al., 1998) finally proved that the neurogenesis in the adult brain is as important as that in the developing brain.

The most active progenitor cells that divide throughout life exist in two brain regions of adult mammalian brain: a) the subgranular zone (SGZ) of the hippocampal dentate gyrus and b) the forebrain subventricular zone (SVZ) of the lateral ventricle (Gage, 2000; Alvarez-Buylla and Garcia-Verdugo, 2002). Besides these two active regions, adult neurogenesis is also known to occur in other brain areas, including the cerebral cortex, the

striatum, the substantia nigra, the hypothalamus, and the amygdala (Magavi et al., 2000; Cossette et al., 2003; Zhao et al., 2003; Banasr et al., 2007; Perez-Martin et al., 2010; Vessal and Darian-Smith, 2010). In the SVZ, neuroblasts migrate to the olfactory bulbs via the rostral migratory stream (Doetsch et al., 1999). The neurons generated along this path/region are primarily involved in olfactory function (Ninkovic et al., 2007). An equivalent site has only recently been identified in humans (Curtis et al., 2007). Adult hippocampal progenitor cells are located in the SGZ between the dentate hilus and the dentate granule cell layer. Newly formed cells in the hippocampus migrate into the dentate granule cell layer, where most of the cells differentiate into neurons that have a granule cell phenotype (Cameron et al., 1993; Gage, 2000; Choi et al., 2003).

It is now well established that neurogenesis occurs throughout the lifetime of an organism and postnatal neurogenesis events are now referred as adult neurogenesis to distinguish this phenomenon from embryonic or developmental neurogenesis. There are four major processes in the process of neurogenesis that are essential for the successful production of new neurons: a) neural stem cell proliferation, b) cell differentiation, c) cell migration, and d) cell survival and/or integration. Each of these processes is regulated independently by complex signaling events. In the following few segments we will summarize the published research literature regarding the effect of acute and chronic alcohol (ethanol) exposure on the changes in hippocampal neurogenesis and neurodegeneration in developing (adolescent) and adult brain.

6.1.2. Ethanol Effect in Developing Adolescent Brain

During adolescence brain undergoes dynamic transition and this may result in the way adolescent brain responds distinctly to ethanol from adults (Nixon and McClain, 2010; Spear and Varlinskaya, 2005). Usually adolescent brains are less sensitive to negative effects of alcohol (for example rewarding cues for self-intake), but adolescents are more sensitive to positive reinforcing effects of ethanol (Spear and Varlinskaya, 2005). Two hypotheses have been postulated to explain why adolescence ethanol exposure led to addiction and alcohol use disorder (AUD): a) the adolescent brain responds to alcohol, involves fewer cues for self-intake (Spear et al., 2005) and b) the adolescent brains exhibit enhanced sensitivity to neurodegenerative effects of alcohol that in turn dysregulates behavioral control systems and leads to addiction and AUD (Crews et al., 2007; Spear, 2007). The distinct response to alcohol by adolescent brain also can be explained by the distinct pharmacodynamic and pharmacokinetic components that regulate alcohol (ethanol) absorption and

metabolism (Walker and Ehlers, 2009). Human studies showed that it takes a higher dose of alcohol for an adolescent to achieve same blood alcohol concentrations compared to an adult (Spear, 2006; Spear, 2007).

Adolescents showed decreased sensitivity to alcohol-induced sedation and motor impairment and alcohol withdrawal-induced depression and anxiety (Little et al., 1996; Doremus et al., 2005). Several studies reported that adolescents exhibit less severity in alcohol withdrawal symptoms including withdrawal-induced seizures (Chung et al., 2008; Acheson et al., 1999), but others reported that when blood alcohol concentrations are matched no differences are observed between adult and adolescent rats in withdrawal-induced anxiety (Morris et al., 2010b). In contrast to their response to alcohol's aversive effects, adolescents are more sensitive to the rewarding effects of alcohol. The enhanced rewarding effects of alcohol in adolescents have been shown to augment future alcohol-seeking behavior and terminal addiction (Pautassi et al., 2008; Ristuccia et al., 2008). Self-administration of alcohol in adolescence may reach a dose that will induce tachycardia (an effect linked to alcohol's hedonic value) but an adult will never self-consume that amount of alcohol (Ristuccia et al., 2008; Ristuccia and Spear, 2008; Doremus-Fitzwater et al., 2010).

Adolescents exhibit increased sensitivity to alcohol-induced neurodegeneration (Spear et al., 2007; Nixon et al., 2010). Alcohol-induced neurodegeneration occurs in the brain regions that control drug-seeking behaviors. The repeated exposure to the high blood alcohol levels characteristic of binge drinking (common in adolescent during development of brain) is one of the critical steps that finally lead to an AUD (Crews and Boettiger 2009; Nixon et al., 2010). Adolescents with AUD characteristics exhibit cognitive deficiency along with a decrease in PFC and hippocampal volume (De Bellis et al., 2005; Medina et al., 2008; Nagel et al., 2005). PFC and hippocampus play an important role in behavioral inhibition and decision-making processes and alcohol-induced damage to these regions lead to poor decision making, resulting in excessive alcohol consumption (Crews et al., 2009b). Susceptibility of adolescent brain to neurodegeneration may be due to multiple factors including impaired integrity or decreased brain volume. Alcohol may also exert teratogenic effects in adolescent brain (that is still being developed) that includes degeneration in the PFC and hippocampus (Evrard et al., 2006; Spear et al., 2007; Crews and Boettiger 2009; Nixon et al., 2010a). Recent studies revealed that the deleterious effect of alcohol on neural stem cells may be due to higher susceptibility of the adolescent hippocampus to alcohol-induced degeneration (Morris et al., 2010a; Nixon et al., 2010b). In animal models of an AUD, alcohol impairs neural stem cell

proliferation and also decreases survival of newborn neurons (Morris et al., 2010a). The number of new neurons produced in an adolescent brain is much higher than in adults (Hasin et al., 2007). The higher rate of neurogenesis in adolescents has been proposed to result in a greater number alcohol-induced cell loss compared to adults (Morris et al., 2010a; Nixon et al., 2010a, 2010b).

6.1.3. Ethanol and Adult Neurogenesis

In the last 15 years considerable evidence from cellular, ex vivo organotypic culture, animal models and alcoholic human brain studies have proved beyond doubt that ethanol adversely affects different aspects of adult neurogenesis (Nixon et al., 2010b). Interestingly, brain regions (namely frontal cortex and the hippocampus) of neural progenitor cell proliferation and neurogenesis are the same areas of brain that are adversely affected by ethanol. Alcohol's effect on adult neurogenesis in the research literature varies depending on the dose and duration of alcohol treatment, and pattern of exposure, as well as the brain regions examined. For example, acute doses of alcohol dose dependently reduce cell proliferation in the SGZ of the dentate gyrus (Crews et al., 2006a; Jang et al., 2002), and chronic exposures reduce both cell proliferation and new cell survival (He et al., 2005; Nixon and Crews, 2002; Richardson et al., 2009). In contrary to these findings other studies reported that chronic exposures do not inhibit cell proliferation (Aberg et al., 2005; Herrera et al., 2003; Rice et al., 2004). The differences in the treatment paradigm, time of measurement following last administration of ethanol, blood alcohol concentrations, and analysis of cell proliferation markers may explain some of these anomalies in the research literature.

Interestingly, during abstinence (following alcohol dependence) the rate of cell proliferation increased in adult rats (Nixon and Crews, 2004). This response is similar to that observed in other kinds of cerebrovascular injury model, including traumatic brain injury and ischemic stroke (Liu et al., 1998; Dash et al., 2001). Aberg and colleagues reported that low, anxiolytic doses of ethanol promote proliferation by lowering stress-induced decreases in neurogenesis (Aberg et al., 2005). Collectively, these findings highlight the importance of the blood alcohol concentration and time of measurement of cell proliferation in assessing effects of alcohol on neurogenesis.

6.1.4. Comparisons of the Ethanol Effect on Developing Adolescent Brain Neurogenesis and Adult Brain Neurogenesis

When the various stages of neurogenesis are examined independently, studies have consistently shown that alcohol dose dependently decreases cell proliferation in the SGZ of the dentate gyrus of adolescent rats (Crews et al.,

2006a; Jang et al., 2002). In adult brain alcohol inhibits cell proliferation at a consistent rate of 40–50% but in adolescent brain the rate is much more variable (20–45%; Nixon, 2010a, 2010b). Analysis of markers of neurogenesis showed a similar decrease in neurogenesis in adolescent and adult neurogenesis (Crews et al., 2006a; Morris et al., 2010a; Nixon, 2010b). Alcohol-induced decreases in bromodeoxyuridine (BrdU) labeling of cell proliferation vary between 20 and 50% versus control (Crews et al., 2006a; Jang et al., 2002; Morris et al., 2010a). With regard to cell differentiation, in both adult and adolescent models most of the cells became neurons independent of the nature of alcohol exposure (He et al., 2005; Morris et al., 2010a). However, in adolescents newborn cells differentiate into neurons at a slightly higher rate (90%) than in adults (75%) (Nixon et al., 2010a; Nixon, 2010b).

Alcohol treatment differentially affects cell cycle markers in adolescent brain compared with adult brain. Four-day alcohol binge results in a significant decrease in BrdU labeling of proliferating cells in adolescent rats without any change in the number of Ki-67-positive actively dividing cells compared to control (McClain et al., 2011; Morris et al., 2010a). In contrast to this finding, alcohol treatment significantly decreases Ki-67 labeling in adult rats with the identical exposure model (Crews et al., 2006a; Leasure and Nixon, 2010). The lack of effect in Ki-67-positive cells concomitant with a significant decrease in BrdU labeling of cells in S-phase suggests that in adolescents alcohol inhibits cell proliferation by altering or arresting the cell cycle. Specifically, alcohol may arrest the cells in the G1-phase of the cell cycle, preventing cells from entering S-phase (Morris et al., in press). More comparative studies under similar experimental conditions are required to accurately define the differential effect of alcohol on adolescent and adult neurogenesis.

6.1.5. Hippocampal Integrity in Alcohol-Mediated Neurogenesis and Abstinence Effects

It is now well accepted that impairment or inhibition of adult neurogenesis results in a decrease in the number of cells in the dentate gyrus and a smaller or degenerated dentate gyrus compared to controls (Imayoshi et al., 2008). In both adolescent and adult rodent models, alcohol-induced cell death rate (estimated in the 100s) is much lower compared to the rate of alcohol-induced inhibition of neurogenesis (estimated in 1000s). Alcohol reduces neurogenesis at a rate similar to alcohol-induced cell loss in the dentate gyrus (Walker et al., 1980; Nixon and Crews, 2002). This suggests that hippocampal integrity is critical for regulating effect of alcohol on different aspects of neurogenesis. The cells that are generated during alcohol intoxication have been shown to have trimmed

dendritic trees (He et al., 2005) and shorter processes and/or shrinking nuclei (Ibanez et al., 1995), which may explain the loss in brain volume during AUD (Sullivan et al., 1995; De Bellis et al., 2000). The "reactive neurogenesis" has been reported in the adult binge model suggesting that during abstinence hippocampal recovery takes place (Nixon and Crews, 2004). Others reported permanent depletion of stem cells following chronic alcohol dependence (Richardson et al., 2009). It is not clear if this "reactive neurogenesis"-induced increase in the number of new neurons is beneficial or is further deleterious to hippocampal function (Kempermann et al., 2004). It has been shown that during abstinence neurogenesis returns to control levels in adolescents (Morris et al., 2010b), but if these cells become incorporated into the granule cell layer and function appropriately is not clear at this point. More studies need to investigate the long-term effects of alcohol on newborn neurons and the neurogenic niche in both adolescent and adult brain.

6.2. Possible Molecular Mechanisms Underlying Ethanol Effects on Neurogenesis

The mechanisms of alcohol-induced brain damage and subsequent abstinence-induced regeneration are a complex process. The extent of neurodegeneration and subsequent regeneration and recovery varies between brain region and depends on alcohol dose, intake pattern, genetics and age. Early studies in in vitro culture models suggest that chronic ethanol inhibits glutamatergic NMDA receptors that leads to progressive NMDA supersensitivity (Chandler et al., 1993a, 1993b). Other in vitro studies also suggested that ethanol-mediated neurotoxicity occurs during withdrawal via NMDA receptor (Prendergast et al., 2000, 2004). However, NMDA receptor antagonists failed to reduce binge-induced brain damage in several animal models (Collins et al., 1998; Crews et al., 2004; Hamelink et al., 2005). Time course studies of binge-induced brain damage also suggest that alcohol-induced brain degeneration increases during ethanol treatment and then reduces with abstinence (Crews and Nixon, 2009). Collectively these findings suggest that ethanol-induced neurodegeneration is not due to glutamate excitotoxicity but occurs during ethanol intoxication (Crews and Nixon, 2009).

Human data also support that alcohol-induced neurodegeneration occurs during intoxication. The frequency of heavy drinking has been shown to be the best indictor of alcoholic brain damage corresponding with frontal lobe gray matter loss (Parsons, 1987; Sullivan and Pfefferbaum, 2005). During abstinence white matter recovers rapidly (Shear et al., 1995;

O'Neill et al., 2001). Since there is no apparent correlation with lifetime alcohol consumption and neurodegeneration, the frequency of alcohol intoxication is still the best predictor of alcoholic neurodegeneration.

Many findings suggest that ethanol-induced brain damage is related to oxidative stress from pro-inflammatory enzymes activated during ethanol intoxication. Studies in organotypic hippocampal–entorhinal cortex slice (HEC) cultures (that maintains all the cellular components of the brain and maintains interconnections of brain) showed that ethanol treatment alters protein transcription via increasing and decreasing transcription factor nuclear factor-kappaB NF-κB and cAMP response element-binding (CREB) activity respectively (Crews and Nixon, 2009). CREB family transcription factors promote neuronal survival through regulating the transcription of pro-survival factors (Lonze and Ginty, 2002; Mantamadiotis et al., 2002). On the contrary, NF-κB is activated by stimuli of oxidative stress, cytokines and glutamate (O'Neill and Kaltschmidt, 1997) and is the key player in the induction of pro-inflammatory cytokines and enzymes that triggers pro-inflammatory cascade by further activating NF-κB transcription (Yakovleva et al., 2011). The balance in the activation of these transcription factors regulates pro-survival versus pro-inflammatory cues (Yakovleva et al., 2011). The alteration in CREB and NF-κB expression and function following alcohol treatment suggests a mechanism of alcohol-mediated inhibition of neurogenesis.

In vivo, binge ethanol treatment results in a decrease in pCREB immunoreactivity in brain during ethanol intoxication (Bison and Crews, 2003) and coincides with the peak of neurodegeneration. In HEC cultures, ethanol treatment reduces CREB-regulated BDNF expression (Zou and Crews, 2006). Thus, ethanol-mediated reduction of CREB transcription and loss of trophic signals with concomitant increase in the induction of oxidative stress results in the alcohol-mediated neurodegeneration and inhibition of neurogenesis (Crews and Nixon, 2009). Both in vivo and in vitro studies suggest the involvement of a pro-inflammatory cascade including increased NF-κB-driven induction of oxidative stress enzymes as a key factor in alcohol-induced brain damage (Crews and Nixon, 2009). Crews and colleagues showed that ethanol induces cyclooxygenase-2 (COX2) (Knapp and Crews, 1999), inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase gp91 and increases reactive oxygen species-producing enzymes that are downstream of NF-κB. Furthermore, ethanol treatment significantly increases the brain expression of NADPH oxidase subunits, gp91phox and p67phox (Qin et al., 2008). Thus, ethanol promotes a pro-inflammatory and anti-survival

environment through the activation of pro-inflammatory transcription factors and the inhibition of pro-survival transcription factors. Further support for a pro-inflammatory mechanism of alcohol-mediated inhibition of neurogenesis came from studies showing antioxidant-mediated protection against alcohol-induced brain damage (Collins et al., 1998; Hamelink et al., 2005). Crews and colleagues showed that administration of the antioxidant butylated hydroxytoluene (BHT), during binge ethanol treatment, blocked COX2 induction and neuronal cell death as well as reversing ethanol inhibition of neurogenesis (Crews et al., 2006b). In HEC slices BHT treatment reduced NF-κB activation (DNA binding) and reduced ethanol-mediated increase in tumor necrosis factor-α TNFα, glutamate and/or $H_2O_2^-$ and ultimately reduced TNFα, glutamate and/or H_2O_2-induced neuronal cell death (Zou and Crews, 2004). Alcohol treatment reduces adult neurogenesis in mice (Crews et al., 2004; Qin et al., 2008) and rats (Nixon and Crews, 2002) and these responses are correlated with increased expression of cytokines and innate immune genes in brain (Crews et al., 2006a; Qin et al., 2008; Crews et al., 2011). Several studies have found that antioxidants also blunt ethanol inhibition of neurogenesis (Herrera et al., 2003; Crews et al., 2006b).

Collectively these findings suggest that ethanol-induced oxidative stress and increase in pro-inflammatory cytokines, with concomitant decreases in pro-survival cues, are the mechanism of alcohol-mediated neurodegeneration and inhibition of neurogenesis. Additional studies are required to fully understand the signaling processes that contribute to alcohol-mediated degeneration and inhibition of different aspects of neurogenesis in developing and adult brain during alcohol dependence and the abstinence stage.

6.3. Ethanol–Cannabinoid Interaction in the Regulation of Neurogenesis

The roles of the endogenous cannabinoid system [endogenous cannabinoid receptor ligands (eCB), their synthesizing and degrading enzymes and G_i-coupled CB1R and CB2R] in the regulation of neurogenesis is poorly understood. To date most of the studies are carried out in cultured cell and/or rodent models. The relative expression of CB1R on neuroprogenitor cells (NPC) is during proliferation and appears to increase with maturation of differentiation to a neuron (Mulder et al., 2008). CB2R expression on NPC has been reported to be downregulated upon commitment to a neuronal lineage (Molina-Holgado et al., 2007). Agonist activation of CB1R and CB2R has been shown to increase NPC proliferation, increase

neurosphere formation and promote neurogenesis, and these responses are blocked by respective receptor antagonists (Aguado et al., 2005, 2007; Molina-Holgado et al., 2007). CB1R antagonist (SR141716A) and CB2R antagonist (SR144528) decrease neuronal progenitor cell proliferation and increase apoptosis (Molina-Holgado et al., 2007). In vivo, CB1R agonists increase neurogenesis through induction of trophic factors Fgf2 and EGF in adjacent cells (Aguado et al., 2007). Genetic deletion of the endocannabinoid anandamide-degrading enzyme fatty acid amide hydrolase has been shown to increase neurogenesis in neurogenic proliferation zones in the developing and adult brain by increasing endogenous cannabinoid levels (Mulder et al., 2008; Aguado et al., 2005). Deletion of CB1R or pharmacological inhibition of its function inhibits proliferation in neurospheres in vitro (Goncalves et al., 2008; Aguado et al., 2005; Molina-Holgado et al., 2007), and in proliferative zones under in vivo conditions in both the developing and adult brain (Goncalves et al., 2008).

Ethanol–cannabinoid interaction for the development of alcoholism and tolerance to alcohol intake has been known for many years (Basavarajappa and Hungund, 2002, 2005; Erdozain et al., 2011; Rettori et al., 2007). However, the role of the endocannabinoid system in the regulation of alcohol's effect on neurogenesis is not known. Ethanol increases the endogenous cannabinoid anandamide (Basavarajappa, 2007). Ethanol-induced anandamide is expected to increase neurogenesis (acting on CB1R), but ethanol decreases neurogenesis.

In our laboratories we have recently used zebrafish as the model to investigate a) if zebrafish cannabinoid receptor activation regulates developmental neurogenesis, and b) what role (if any) zebrafish CB1R (zCB1R) plays in the regulation of ethanol's effect on developmental neurogenesis. As shown in Fig. 7.2, activation of zCB1R by the endocannabinoid analog methanandamide significantly increases neurogenesis in zebrafish embryos, and this effect was significantly blocked when embryos were co-treated with CB1R antagonist SR141716 (SR1). Furthermore, we found that CB1R mRNA expression is also increased under similar conditions and was also blocked by SR1. Interestingly, CB1R antagonist SR1 treatment alone significantly reduced basal CB1R mRNA expression and basal neurogenesis. This suggests that endogenous cannabinoid is functional in zebrafish embryonic development and contributes to the regulation of basal neurogenesis. When tested for ethanol's effect on developmental neurogenesis, we found that chronic ethanol (0.5–1.5% V/V) treatment for 30 h (starting at 6 hpf) significantly reduced basal neurogenesis and CB1R mRNA

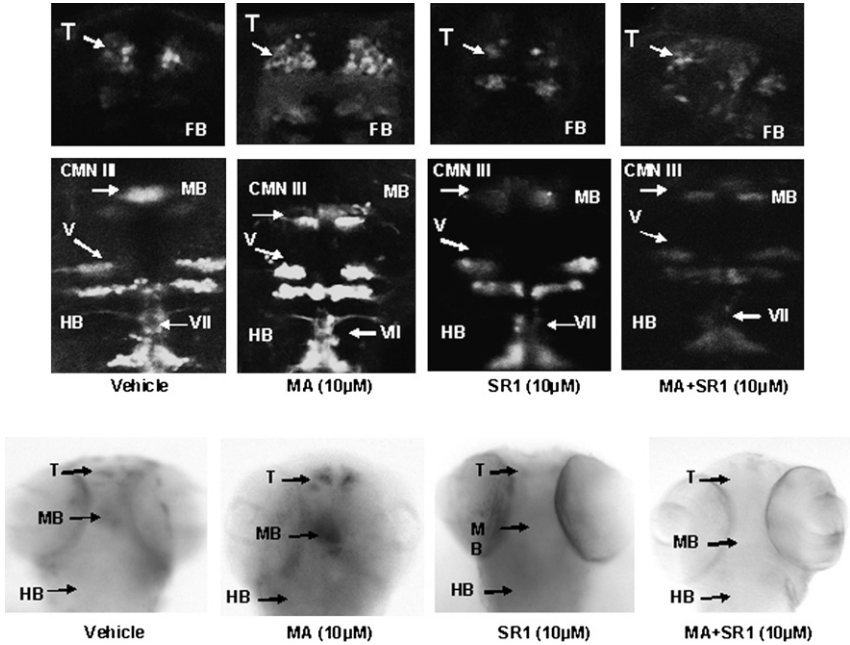

Figure 7.2 Activation of zebrafish CB1R (CB1R) on neurogenesis (upper panel) and CB1R expression (lower panel) in developing zebrafish. Motor neuron-specific (under *islet1* promoter) GFP expressing transgenic zebrafish (*islet1*-GFP) embryos were treated (6–72 hpf) with CB1 receptor agonists methanandamide (MA) in the absence and presence of CB1R antagonist (SR141716; SR1). Upper panel: Neurogenesis is assessed by GFP expression. The representative confocal images ($n = 26$ from three separate experiments with similar results) show the dorsal view of GFP expression in zebrafish embryos at 72 hpf. Lower panel: The representative images of in situ hybridization of CB1R ($n = 30$ from four separate experiments with similar results). Activation of CB1R stimulates neurogenesis (as measured by increase in GFP expression) and CB1R expression. These responses are attenuated by CB1R antagonist SR1, confirming the involvement of CB1R in the processes.

expression (Devkota, S., Mukhopadhyay, S., unpublished data). Furthermore, we found that concomitant activation of zCB1R with ethanol treatment partially reversed the ethanol-mediated inhibition of neurogenesis (Devkota, S., Mukhopadhyay, S., unpublished data). To further validate the involvement of zCB1R in these processes, we knocked down zCB1R (using MO against zCB1R) and tested the effect on basal neurogenesis, and ethanol's effect on neurogenesis in zCB1R-knockdown embryos. zCB1R knockdown significantly reduced basal neurogenesis and augmented ethanol's effect on neurogenesis (Devkota, S., Mukhopadhyay, S., unpublished

data). Collectively, these data clearly suggest that zCB1R indeed plays a role in the regulation of neurogenesis and eCB–EtOH interaction is important in ethanol's effect on neurogenesis. However, the molecular mechanisms of this interaction are yet not clear. We are currently testing the following hypotheses to define the molecular mechanisms of these processes: a) ethanol treatment may downregulate production of eCB or stimulate the breakdown of eCB by inhibiting and activating eCB-synthesizing and degrading enzymes respectively, b) ethanol treatment affects CB1R-mediated activation of trophic factor signaling (such as BDNF or vascular endothelial growth factor (VEGF)), and c) does ethanol affect coupling of CB1R with its partners in the signalosome, and thereby inhibit generic CB1R signaling?

7. EFFECT OF ETHANOL EXPOSURE ON DEVELOPMENT OF MOTOR NEURONS AND THE NEUROMUSCULAR JUNCTION

7.1. Effects of Ethanol on Spinal Cord Motor Axon Guidance in Zebrafish

Motor deficits are a common behavioral phenotype of prenatal ethanol exposure (Kalberg et al., 2006) and even in zebrafish ethanol exposure during embryogenesis can produce behavioral changes associated with motor deficits (Carvan et al., 2004). These changes in motor behavior could result from effects of ethanol on the development of either upper motor neurons or lower motor neurons. Zebrafish is a particularly attractive model for assessing the role of ethanol on the development and function of motor neurons, since transgenic lines exist with green fluorescent protein (GFP) expression driven by the *islet1* promoter in motor neurons (Higashijima et al., 2000), and simple swimming behaviors can be assessed using zebrafish embryos (Sylvain et al., 2010). Relevant to this review, Shh signaling has been shown to induce *islet1* expression in mouse brain, and thus in view of the sensitivity of Shh signaling to ethanol exposure, it raises the possibility that the differentiation of *islet1*-positive motor neurons may be sensitive to ethanol exposure during development. Accordingly, we have used *islet1-GFP* transgenic zebrafish and *islet1* gene expression analysis to assess the development of cranial motor neurons in zebrafish following ethanol exposure. As shown in Fig. 7.3, increasing concentrations of ethanol lead to more marked decreases in *islet1* gene

expression in zebrafish brain, and interestingly both agrin MO and Shh MO treatment also result in diminished *islet1* gene expression. These data suggest that cranial motor neuron development in zebrafish is sensitive to ethanol exposure, and that motor function abnormalities that are present in FASD children could be a consequence of perturbed motor neuron development as a result of prenatal ethanol exposure.

To further address the effects of ethanol on motor neuron development and motor behavior in zebrafish, Sylvain et al. (2010) exposed zebrafish embryos to ethanol concentrations ranging from 1 to 3% and from 8 to 24 hpf, and then analyzed swimming behavior and motor neuron properties, such as axonal outgrowth, in 3 dpf embryos. These studies showed that ethanol exposure disrupted swimming behavior in 3 dpf zebrafish embryo with the most marked disruption of swimming behavior occurring following exposure to 2.5% ethanol (Sylvain et al., 2010). The pattern of spinal cord motor neuron axonal growth in zebrafish embryos was also analyzed using anti-acetylated tubulin immuno-staining of motor neurons, with numerous morphological abnormalities observed for spinal cord motor neuron axons. These abnormalities included curved axons, aberrant branching patterns, and defasciculation of axon bundles (Sylvain et al., 2010). Interestingly, previous studies from our laboratory demonstrated that agrin knockdown by agrin MO produced similar abnormalities in zebrafish spinal cord

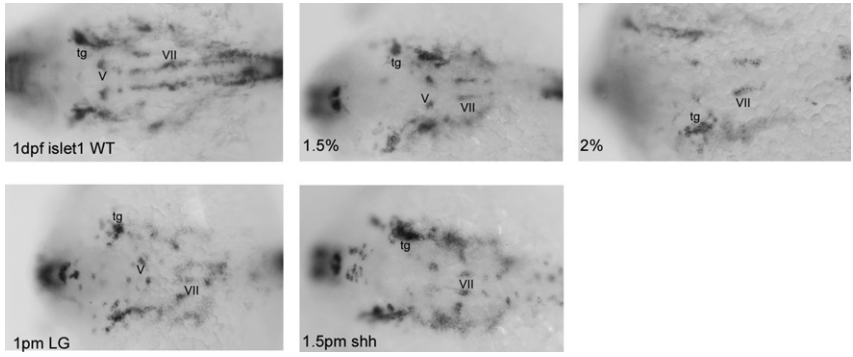

Figure 7.3 Ethanol exposure from 6 to 24 hpf of zebrafish development leads to a pronounced diminution in *islet1* gene expression. High doses of ethanol such as 2.0%, in particular, disrupts *islet1* expression. Injection of one-cell zebrafish embryos with either agrin MO (LG) or Shh MO also results in diminished *islet1* gene expression. A disrupted pattern of *islet1* mRNA expression is observed, particularly in the trigeminal ganglion neurons (tg, V) and neurons of the facial nucleus (VII), with ethanol exposure or agrin and Shh MO treatment.

motor axons (Kim et al., 2007). We therefore undertook studies to begin to address possible molecular mechanisms that might account for the observed effects of ethanol on spinal cord motor neuron development as noted by Sylvain et al. (2010). We examined spinal cord motor neuron axon growth using znp-1 immunostaining and examined axon morphologies and outgrowth at either 1 or 2 dpf (Figs 7.4–7.6). Our data confirm those of Sylvain et al. (2010), with truncation of motor neuron axon growth being observed at 24 hpf and defasciculation as well as reduced outgrowth being observed at 48 hpf, as a consequence of ethanol exposure from 6 to 24 hpf. When axonal outgrowth at 24 hpf is examined following either high-dose agrin or Shh MO treatment, or combined low-dose MO and low-dose ethanol treatment, we observe a similar disruption of spinal cord motor neuron axon outgrowth as observed following high-dose ethanol exposure (Fig. 7.5). Upon examination of 2 dpf embryos and the pattern of spinal cord motor neuron axon outgrowth, again combined ethanol and agrin or Shh MO treatment reproduced the ethanol-induced abnormalities, and interestingly Shh mRNA overexpression could rescue the abnormal phenotype (Fig. 7.6). Thus, these data suggest that ethanol exposure also perturbs development of spinal cord motor neurons, in particular axon outgrowth to target muscle, in an agrin- and Shh-dependent mechanism. These data also suggest that ethanol may exert its teratological effects on the development of a multitude of neuron classes by a similar molecular signaling pathway being disrupted, a pathway that requires functional Shh signaling.

7.2. Neuromuscular Junction Formation and Synaptic Function in Response to Ethanol Exposure

Although the above studies indicate that ethanol exposure during zebrafish development disrupts spinal cord motor neuron differentiation, as assessed by the pattern of motor neuron axons and swimming behavior in embryos, these studies did not provide evidence that neuromuscular function was also disrupted due to ethanol exposure. Skeletal muscle in zebrafish embryos exposed to ethanol displays abnormalities, such as lack of segment division, with fibers extending over two segments rather than one, narrower muscle fibers, and altered angles of muscle fibers between dorsal and ventral hemi-segments (Sylvain et al., 2010). Recent studies by Sylvain et al. (2011) have examined the synaptic properties of the neuromuscular

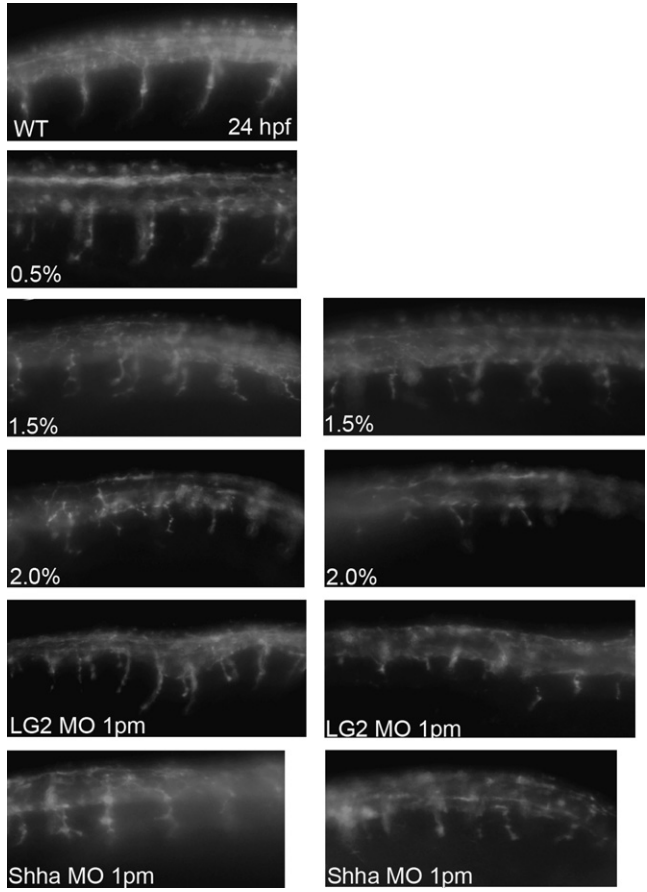

Figure 7.4 Ethanol treatment disrupts spinal cord motor neuron axon growth in 24 hpf zebrafish embryos, as assessed by znp-1 immunostaining, with truncated and defasciculated axons observed. A similar phenotype is observed when agrin or Shh function is disrupted by MO treatment. These data indicate that ethanol disrupts the growth of spinal cord motor neuron axons, in a manner similar to that regulated by agrin and Shh.

junction in zebrafish, following ethanol exposure. Their studies suggest that normal formation of the neuromuscular junction occurs following ethanol exposure. However, synaptic function as a result of ethanol exposure appears to be impaired. Miniature end plate currents were recorded from synapses of untreated and ethanol-exposed zebrafish embryos, with ethanol-exposed embryos exhibiting significant differences in the frequencies of fast and slow miniature end plate currents (Sylvain et al., 2011). These ethanol-induced abnormalities included altered fast

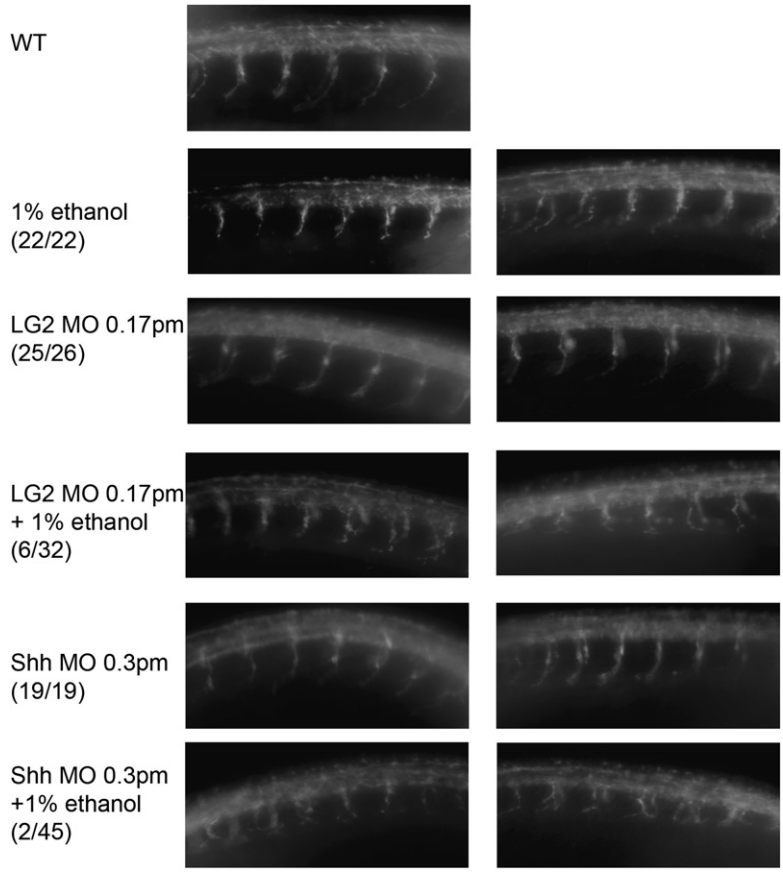

Figure 7.5 Combined low dose of ethanol (1% ethanol) and low-dose agrin or Shh MO perturb spinal cord motor axon growth in 24 hpf zebrafish embryos. The number of embryos displaying the wild-type phenotype is indicated for each treatment. These data suggest a pathway where agrin modulation of Shh function is sensitive to ethanol exposure, during spinal cord motor neuron axon growth and guidance.

miniature end plate currents in white muscle and altered slow miniature end plate currents in red muscle.

8. SUMMARY

The studies summarized in this review indicate that zebrafish can be used to assess both molecular and behavioral consequences of developmental (prenatal) ethanol exposure. The zebrafish studies that have been reviewed

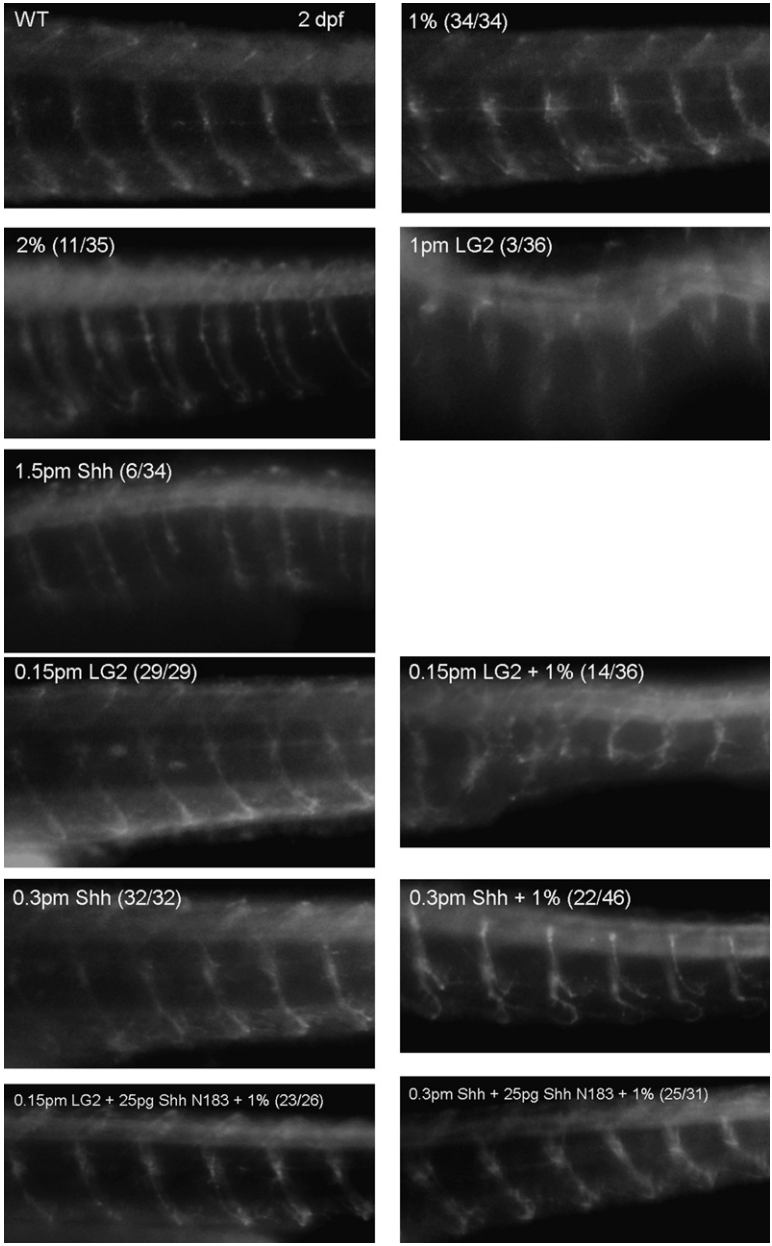

Figure 7.6 In 2 dpf zebrafish embryos, spinal cord motor neuron axon growth is disrupted following ethanol exposure and combined ethanol exposure and agrin or Shh MO treatment. The abnormal axon phenotype is rescued by Shh mRNA overexpression, indicating that the effects of ethanol on spinal cord motor neuron development, as measured by axon growth, are mediated by disrupting a signaling pathway involving agrin and Shh. The number of embryos displaying the wild-type phenotype is indicated for each treatment.

also demonstrate that the zebrafish model system can serve as a powerful vertebrate and developmental model for the study of FASD, with the accessibility of zebrafish embryos permitting detailed analyses of the underlying molecular mechanisms associated with FASD. In particular, as described from studies in our laboratories, the ability to readily knockdown developmental expression of genes in signaling and/or genetic pathways considered to be critical to the etiology of FASD, such as Shh or Fgfs, will allow the modeling of FASD defects in both the absence and presence of ethanol, in order to unequivocally establish the role of specific candidate genes in ethanol-mediated teratogenesis. Thus, the zebrafish model has the potential to serve as a powerful complimentary vertebrate animal model for the study of the molecular basis of FASD, and will likely allow detailed analyses of candidate genes that may be associated with ethanol-induced behavioral changes as a consequence of prenatal alcohol exposure.

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