Onco-Fetal Antigen P1A in Exocytosis and Tumorigenesis

by

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To Mom and Dad

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Abstract

P1A is the first identified tumor antihgen recognized by cytotoxic T lymphocytes (CTLs). P1A represents a prototype of onco-fetal antigens as it is also expressed in embryonic stem (ES) cells and other fetus derived tissues. We observed that P1A-transgenic mice, which specifically expressed P1A in lymphoid cells, developed various thymic tumors after 7 months of age and had shorter life-span compared to control, especially in Rag2-deficient background. The P1A-transgenic bone marrow cells had higher proliferation ability and more hematopoietic progenitors compared to control. All tumors tested displayed the B-cell lineage marker B220, except for one tumor that expressed T-cell lineage markers. This latter tumor also harbored a Notch1 mutation. In order to understand the oncogenic activity of P1A, we used tandem affinity purification and mass spectrometry to identify P1A-associated proteins. Several vesicle trafficking proteins, such as RalA, AP2, IQGAP1 and Rac1, were identified and confirmed by co-immunoprecipitation. Confocal microscopy revealed co-localization of P1A and RalA in intracellular vesicles. P1A silencing resulted in alteration of RalA distribution and reduced secretion of TNFα, IL-6 and β-hexosaminidase. Ectopic overexpression of P1A increased secretion of mouse bone marrow derived mast cells. In order to determine whether P1A is essential for the function of ES cells, we deleted the gene encoding P1A in mouse ES cells. By cDNA microarray analysis, RapGEF3, Transglutaminase2, Dynein

and RhoBTB1, which are related to membrane trafficking, were decreased in P1A-knockout ES cells. Therefore, our results suggest that the onco-fetal antigen P1A may contribute to tumorigenesis by promoting exocytosis of the pro-inflammatory cytokines TNFα and IL-6. Since priming of tumor-specific CTLs requires cross-presentation of tumor antigen by antigen-presenting cells (APCs), enhancement of exocytosis by the tumor cells may facilitate transfer of tumor antigens to APCs. It is intriguing that the role for P1A in exocytosis may be responsible for its recognition by the immune system. Taken together, our data suggest exocytosis as a potential link between immunobiology and cancer biology of an onco-fetal antigen. Identification of key molecules in vesicle trafficking pathways can help to prevent or minimize the biological dysfunction caused by environmental toxicants which can alter exocytosis or endocytosis.

CHAPTER 1

Introduction

It has been noted that the processes of embryonic development and tumor development share important similarities. For example, the extensive proliferative ability exists both in cancer and embryonic cells. In addition, embryogenesis, similar to oncogenesis, is also involved in invasion and metastasis. This occurs with the migration and invasion of trophoblast cells during placental implantation into maternal endometrium. However, all of these events are well controlled in embryogenesis, but occur in an uncontrolled manner in oncogenesis (Ruddon, 1987b). Thus, understanding tumorigenesis may help to reveal the mechanism of embryogenesis, and vice versa.

1.1 Onco-fetal antigen: shared molecular signature of embryogenesis and tumorigenesis

Firstly, it is of interest to consider the shared molecular signature of tumorigenesis and embryogenesis. During the process of carcinogenesis, a number of the tumor-associated antigens that are originally expressed on embryonic cells are re-expressed on tumor cells. These proteins are called onco-fetal proteins, since they are expressed on both tumors and during the embryonic stage (Ruddon, 1987b). Notable examples include

alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA). AFP is a protein present not only in fetal serum, but also in the serum of both experimental animals and humans with hepatocellular carcinoma and some germ cell tumors (Bader et al., 2004; Calaminus et al., 1991; Kong et al., 2009; Rapellino et al., 1989). CEA is produced during fetal development but the production of CEA decreases after birth (Lindgren, 1980); however, CEA is once again detected in serum from humans with colorectal carcinoma, lung carcinoma, breast carcinoma (Ballesta et al., 1995; Goldstein and Mitchell, 2005). Therefore, these onco-fetal proteins are often used as markers for diagnosis of cancer (Bates, 1991).

Neuwald et al. found that at least one onco-fetal protein was activated in 68% of the 67 malignant cell lines derived from various human cancers (Neuwald et al., 1980). In another study, Rosen et al., observed that onco-fetal proteins were significantly increased in 19 out of 32 cultured human carcinomas, sarcomas, and gliomas (Rosen et al., 1980). The data indicates that the re-expression of embryonic genes is a common event in oncogenesis (Ruddon, 1987a).

Since rapid cellular expansion is a shared feature between embryogenesis and tumorigenesis, it seems plausible to infer that onco-fetal antigens perhaps provide an advantage for transformed cells, and the expression of embryonic genes in cancer gives credence to the claim that they are perhaps one of the driving forces of tumorigenesis. To test this notion, we have produced transgenic mice that express a murine onco-fetal antigen with unknown function and have documented a novel function of this protein in tumorigenesis.

1.2 Immune recognition makes antigens out of molecules

By definition, antigens are defined by their ability to be recognized by either T or B cells. B and T cell antigen receptors recognize distinct forms of antigens. After antigen recognition, B and T cells undergo rapid cell proliferation and further differentiate to effector and memory cells, which are now antigen-specific.

B lymphocytes mediate the humoral immune response because they produce soluble molecules called antibodies. Surface B cell receptors (BCR) on naïve B cells typically recognize macromolecules in their native conformation. After maturation, BCR will be converted into a secreted form which is then called an immunoglobulin or antibody (Armitage and Alderson, 1995). T cells, on the other hand, mediate the cellular immune response. T cell receptors recognize proteolytically processed short peptides bound to major histocompatibility complex (MHC) molecules on the cells. There are two major classes of peptide receptors that the MHC gene complex encodes: MHC class I or II molecules. MHC class I molecules are expressed on all cell types. CD8 cytotoxic T cells recognize 8-11 amino acid antigenic peptides in the context of MHC class I (Konig et al., 1992). MHC class II molecules are expressed on the surface of professional antigen-presenting cells, such as dendritic cells, macrophages and B cells. CD4 helper T cells recognize antigens bound to MHC class II (Cammarota et al., 1992). MHC molecules bind and transport peptide antigen inside the cell to the cell surface.

However, priming of lymphocytes not only requires the signal from antigens bound to MHC molecules, but also a second signal in the form of co-stimulatory molecules from antigen-presenting cells (Janeway and Bottomly, 1994). For naïve B cells, the second signal is presented by an activated effector T cell (helper T cell) (Noelle et al.,

1992). Naïve T cells are usually stimulated by activated dendritic cells (Mellman and Steinman, 2001). When a T cell makes contact with an immature antigen-presenting cell that has not been induced to express co-stimulatory molecules and leads to T cell inactivation or T cell clonal anergy (Janeway and Bottomly, 1994).

For example, in order to prime naïve CD8 T cells, antigen-presenting cells must present the antigen peptide-MHC I complexes and co-stimulatory molecules (Melief, 2003; Mellman and Steinman, 2001). The co-stimulatory receptor expressed on naïve T cells is CD28 and the co-stimulatory molecules on antigen-presenting cells are CD80 and CD86, also called B7.1 and B7.2, respectively (Van Gool et al., 1996). Activation of CD8 T cells occurs when both the T cell receptor and CD28 on the T cells bind to peptide-MHC I complexes and CD80/CD86 on antigen-presenting cells, respectively. As a result of successful priming, effector CD8 T cells and central memory CD8 T cells are then established. The effector CD8 T cells can thus kill target cells after recognizing the antigen.

Even with many over-expressed gene products in tumor cells, the lack of co-stimulatory molecules necessary to elicit a primary T cell response results in a less than effect cytotoxic T cell response to tumors. To induce T cell response, tumor antigens must be acquired, processed and presented to naïve T cells by antigen-presenting cells. The process for antigen-presenting cells to acquire antigens from tumor cells and present these exogenous antigens on MHC class I molecule is called cross priming (Heath and Carbone, 1999; Shen and Rock, 2006).

In direct antigen presentation, endogenous or cytosolic proteins are degraded by the proteasome in cytoplasm. Then, the peptides generated by proteasome are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) proteins (Basta and Alatery, 2007). Peptides are then loaded onto MHC class I molecules in the ER, and the complexes of peptide-MHC class I are transferred through the Golgi apparatus to the cell surface where they can be presented to CD8 T cells (Dick et al., 1994; Gromme and Neefjes, 2002; Shastri et al., 2002). For cross-presentation, exogenous antigens internalized by phagocytosis or pinocytosis are processed via the MHC class I pathway in the ER (Guermonprez et al., 2002), or loaded onto recycling MHC class I molecules in the endosomal system (Gromme and Neefjes, 2002; Gromme et al., 1999; Kleijmeer et al., 2001). Naturally, those proteins that are delivered more effectively to antigen-presenting cells are more likely to induce cross-priming.

Tumor antigens are peptides of tumor cell proteins that are presented to T cells usually by MHC class I molecules (Boon and van der Bruggen, 1996). Since cross-priming is critical for successful activation of CD8 T cells, the processing and presentation of tumor antigens by antigen-presenting cells plays a vital role in eliciting an effective cytotoxic T cell response to a tumor.

Tumor cells have been shown to release vesicular structures defined as exosomes (Wolfers et al., 2001). Exosomes are vesicles with a diameter of 30~100 nm which are secreted when multivesicular endosomes fuse to cell surface (Lakkaraju and Rodriguez-Boulan, 2008). In addition, the release of exosomes is associated with the fusion of lysosome-related vesicles with the plasma membrane in hematopoietic cells (Andrews,

2000). The lysosomes and endosomes are membrane vesicles from endocytic origin; therefore, the release of exosomes is a secretion of the endosomal system (Andrews, 2000).

Several studies showed that tumor-derived exosomes can transfer tumor antigens to dendritic cells and induce tumor-specific cytotoxic T cell responses and subsequent rejection of tumors (Cho et al., 2005; Iero et al., 2008; Wolfers et al., 2001; Zitvogel et al., 1998). Furthermore, exosomes secreted from murine dendritic cells with tumor peptide are able to mediate anti-tumor immunity (Hsu et al., 2003). In addition, several clinical trials are currently under way to evaluate the efficacy of the anti-tumor ability of exosome-based vaccines (Dai et al., 2008; Escudier et al., 2005; Mignot et al., 2006; Morse et al., 2005).

Therefore, exosomes are capable of transferring antigens to dendritic cells. Tumor-derived exosomes loaded onto dendritic cells is one way to trigger T cell cross priming (Schorey and Bhatnagar, 2008). In addition to transferring antigens from tumor cells to dendritic cells, the intracellular routes involved in transferring internalized antigens to the MHC class I loading pathway in ER or endosomal systems in antigen-presenting cells are also critical for successful presentation of antigens to CD8 T cells (Burgdorf and Kurts, 2008). Hence, efficient transport of tumor antigens through exocytosis and endocytosis results in T cell cross priming, which then induces anti-tumor immune responses leading to rejection of tumors. It is intriguing that those proteins that facilitate vesicle trafficking may be recognized as tumor antigens. For this reason, it is of interest to consider the cellular machinery involved in exocytosis and endocytosis.

1.3 Vesicle trafficking

Cells can communicate with their outside environments via two trafficking networks: the endocytic and exocytic system. Endocytosis can respond to the exterior signals by the uptake of molecules from the exterior environment or internalization of receptors into endosomes. Exocytosis can release signaling molecules, like cytokines and growth factors, to the exterior environment. Therefore, the membrane trafficking systems work in response to the extracellular environment.

The compartments that form the secretory and endocytic pathways include the endoplasmic reticulum, the Golgi complex with its different sub-compartments (cis, medial, and trans cisternae, and the trans-Golgi network), endosomes, lysosomes, exosomes, secretory granules and the plasma membrane (Fig1-1).

Adaptor protein

How the vesicle is released from the donor membrane, transported across the cytosol, and is tethered and fused with the target compartment of membrane is well controlled by a number of cytosolic proteins that can mediate the vesicular transport between the organelles of the exocytic and endocytic pathways. Among these cytosolic proteins are adaptor protein (AP) complexes which are heterotetrameric proteins that regulate vesicular transport and play important roles in cargo selection as well as vesicle formation (Edeling et al., 2006; Nakatsu and Ohno, 2003).

AP complexes can control the membrane sorting between the secretory and endocytic pathways (Edeling et al., 2006; Nakatsu and Ohno, 2003). The formation of vesicles requires a protein coat and one of the most common coated vesicles are clathrin-

coated vesicles. The AP complexes are involved in recruiting clathrin and accelerating coat formation (Edeling et al., 2006; Nakatsu and Ohno, 2003). AP complexes also play an important role in sorting cargo proteins in coated pits by recognizing the endosomal/lysosomal sorting signals in the cytosolic domains of transmembrane proteins (Bonifacino and Traub, 2003). There are six AP complexes that have been identified (AP1A, AP1B, AP2, AP3A, AP3B and AP4) (Nakatsu and Ohno, 2003). AP2 is the main clathrin adaptor which controls endocytosis at the plasma membrane. AP1, AP3 and AP4 localized in endosomes and TGN been shown to play a role in endosomal/lysosomal sorting pathways (Edeling et al., 2006; Nakatsu and Ohno, 2003) (Fig1-1).

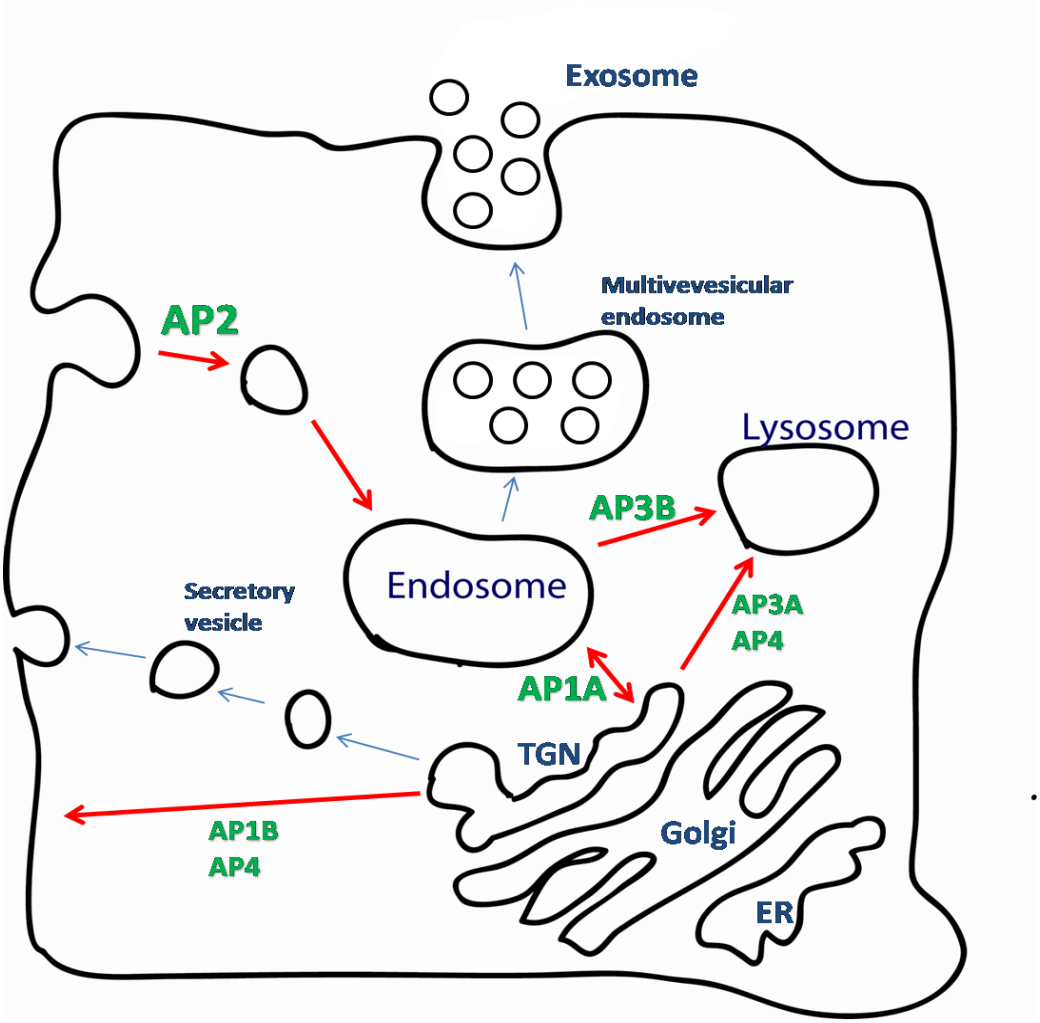

Figure 1-1. Sorting membrane vesicles by AP complex

The secretory and endoctyic pathways include the endoplamic reticulum (ER), the Golgi complex with different sub compartments including trans-Golgi network (TGN), endosomes, lysosomes, secretory vesicles, and the plasma membrane. AP2 mediates endocytosis from the plasma membrane, while AP1A, AP3A/B and AP4 play a major role in endosomal/lysosomal sorting pathways. AP1B and AP4 regulate the sorting to the basolateral plasma membrane in polarized epithelial cells. This figure is modified from Nakatsu and Ohno, 2003.

Ral protein

Other important family of proteins used for vesicle trafficking are Ras-like small G-proteins (Ral) proteins, including RalA and RalB, that show high homology to the Ras family members RasH, RasN and RasK (Colicelli, 2004). Like other small GTPases, the activity of RalA is controlled by GDP/GTP cycling. Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) are responsible for activation and inactivation of RalA, respectively (Colicelli, 2004). The Ral proteins are activated under Ras signaling through RalGEFs (Wolthuis and Bos, 1999; Wolthuis et al., 1998). Recent studies showed that RalA is critical for Ras-induced cell transformation and maintenance of tumor cell proliferation and survival (Chien and White, 2003; Lim et al., 2005; Urano et al., 1996). In addition, Ral has been shown to regulate vesicle trafficking, cell morphology, cytoskeleton dynamics, cell migration, cell proliferation and cell transformation (Feig, 2003).

The first identified binding partner of Ral GTPases is Ral binding protein 1 (RalBP1) which binds GTP bound form of Ral GTPases (Cantor et al., 1995). It has been suggested that Rac 1 and Cdc42 proteins, involved in cytoskeletal rearrangements, are negatively regulated by the GAP domain of RalBP1 (Cantor et al., 1995; Jullien-Flores et al., 1995; Park and Weinberg, 1995). RalBP1 is shown to facilitate the receptor-mediated endocytosis via interaction with the μ2 subunit of the AP2 complex (Jullien-Flores et al., 2000). Mutation of Ral or RalBP1 has been demonstrated to inhibit cell endocytosis of epidermal growth factor (EGF) and insulin (Nakashima et al., 1999). Therefore, Ral and RalBP1 are involved in endocytosis.

On the other hand, Ral is able to modulate the cell exocytosis through binding with the exocyst complex. The exocyst complex controls regulated secretion and is an octameric protein complex consisting of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (Lipschutz and Mostov, 2002). Several small GTPases, including the members of Rab, Rho and Ral families, are involved in proper exocytosis function of the exocyst complex to tether the vesicles from post-Golgi or endocytic region to the plasma membrane (Guo et al., 1999; Guo et al., 2001; Moskalenko et al., 2002; Zhang et al., 2004). The interaction between small GTPases and the subunits of the exocyst might represent regulation of secretory vesicle trafficking.

Ral-GTP binds to two exocyst subunits, Sec5 and Exo84 (Moskalenko et al., 2002; Moskalenko et al., 2003; Sugihara et al., 2002). The mechanism by which Ral regulates the function of exocyst is by promoting assembly of the exocyst subunits to the full octameric exocyst complex (Moskalenko et al., 2002; Moskalenko et al., 2003). Therefore, Ral protein seems to regulate the endocytic or exocytic membrane compartments through binding to the different effector proteins (Fig 1-2).

IQGAP1

IQ-domain GTPase-activating proteins (IQGAPs) are conserved proteins containing several protein-interaction domains (Brown and Sacks, 2006). These domains include: a calponin homology domain, responsible for actin binding, a WW motif, which is necessary for the association of extracellular signal-regulated kinase (ERK), a calmodulin binding IQ domain which interacts calmodulin and a mitogen-activated

protein kinase/extracellular signal-regulated kinase kinase (MEK), Ras GTPase-activating proteins related domain that binds the small GTPases Rac1 and Cdc42 (Hart et al., 1996; Kuroda et al., 1996). Because IQGAP1 interacts with several proteins that have diverse functions, therefore IQGAP1 may function as a scaffolding protein that regulates an broad range of cellular processes mediated by different signaling molecules, such as cell adhesion, migration and metastasis (Brown and Sacks, 2006; Noritake et al., 2005).

The name IQGAP1 originated from its calmodulin-binding IQ domain and Ras GTPase-activating protein-related domain (Weissbach et al., 1994). Traditionally, GTPase-activating proteins (GAPs) switch the guanine nucleotide-binding proteins from the active form (GTP-bound) to the inactive form (GDP-bound) by increase the intrinsic GTPases-activity of GTPase proteins. Thus, GAPs are negative regulators of GTPase proteins. However, instead of hydrolysis of GTP, IQGAP1 stabilizes the active states of Rac1 and Cdc42 by inhibiting their intrinsic GTPase activity and preventing interaction with their negative regulator (ex. RhoGAPs) (Hart et al., 1996). In addition, a study has shown that IQGAP1 could be recruited by Rac1 and Cdc42 during cell polarization and migration (Watanabe et al., 2004).

Several studies show that IQGAP1 is up-regulated in many human cancers such as colon, metastatic melanoma, glioblastoma, ovarian carcinomas and gastric carcinomas (Balenci et al., 2006; Clark et al., 2000; Dong et al., 2006; Nabeshima et al., 2002; Takemoto et al., 2001). In gastric carcinomas, the subcellular localization of IQGAP1 from the cytoplasm to the cell membrane is related to the de-differentiated status of tumors (Takemoto et al., 2001). Moreover, IQGAP1 may be used as a prognostic parameter, since the overexpression and diffuse invasion pattern of IQGAP1 can be

correlated with poor prognosis in ovarian carcinomas (Dong et al., 2006). However, IQGAP1 null mutant mice show no obvious defects in development, but gastric hyperplasia is significantly increased in these mutant mice at old age (Li et al., 2000). Therefore, the mechanisms by which IQGAP1 involved in tumorigenesis remain elusive.

The role for IQGAP1 in exocytosis has emerged fairly recently. Several studies demonstrated that IQGAP1 associates with the exocyst. In Sakurai-Yageta et al.'s study, they showed that the C-terminal region of IQGAP1 interacts with the exocyst subunits Sec3 and Sec8 (Sakurai-Yageta et al., 2008). The invasion of breast tumor cells requires the exocyst complex and IQGAP1, since the exocyst complex plays an important role in transporting and accumulating membrane type 1 matrix metalloproteinase enzyme that degrades extracellular matrix components at invadopoda (Sakurai-Yageta et al., 2008).

Another study reported that N-terminus of IQGAP1 binds EXO70 of the exocyst-septin complex and enhances secretion in pancreatic β-cells (Rittmeyer et al., 2008). Therefore, these data suggests that both the N- and C-terminus of IQGAP1 play specific roles in vesicle trafficking with different exocyst subunits. The proteins that facilitate exocytosis or endocytosis may be recognized as tumor antigens, which we already discussed. Therefore, we hypothesize that tumor antigens may regulate vesicle trafficking by interacting with the key components as described above.

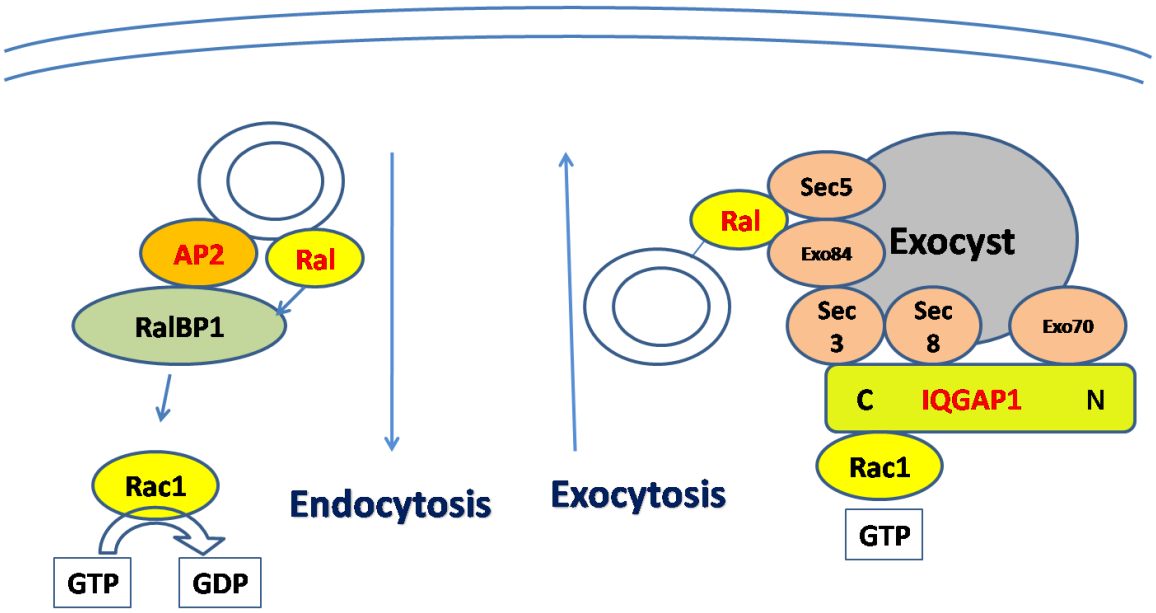

Figure 1-2. Proteins involved in exocytosis and endocytosis

RalA regulates exocytosis through binding with Sec5 and Exo84, and endocytosis through RalBP1. RalBP1 contains a GAP domain that negatively regulates Rac1. RalBP1 has been shown to interact with AP-2 complex and is related to endocytosis. C-terminus of IQGAP1 interacts with active form of Rac1. C-terminus and N-terminus of IQGAP1 mediate exocytosis through Sec3, Sec8, and Exo70, respectively. This figure is modified from Feig, 2003.

1.4 P1A: the onco-fetal antigen recognized by cytotoxic T cells

P1A is the first described tumor rejection antigen which can be recognized by cytotoxic T cells. Its expression pattern suggests that it is a prototype of onco-fetal antigens.

P1A expression in cells and tissues

P1A is a target antigen found in the rejection response observed in vivo against mouse mastocytoma P815 cells that were derived from DBA/2 mice after exposure to the chemical carcinogen, methylcholanthrene (Van den Eynde et al., 1991). Mouse mastocytoma P815 cells express P1A and present a nonameric peptide of P1A to cytotoxic T lymphocytes by MHC class I molecules (Van den Eynde et al., 1994). In addition to the P815 mastocytoma, P1A is expressed in several tumors such as MethA sarcoma and J558 plasmacytoma (Ramarathinam et al., 1995).

Uyttenhove et al. found that P1A is silent in adult tissues except for the testis and placenta. The levels of P1A mRNA in the testis and placenta are around 10-20% and 150-200% of mastocytoma P815, respectively (Uyttenhove et al., 1997). By using immunohistochemical staining with P1A-specific antiserum, identification of the P1A expression is restricted in spermatogonia and labyrinthine trophoblast cells which are MHC-I negative cells (Haas et al., 1988; Mattsson et al., 1992; Tomita et al., 1993). Since not all spermatogonia in seminiferous tubules contained P1A-positive staining, this suggested that P1A is only expressed in certain stage of differentiation of spermatogonia (Uyttenhove et al., 1997). Therefore, the expression pattern of mouse tumor antigen P1A is reminiscent of the human tumor antigens, MAGE, BAGE and GAGE, which are present in melanomas, breast carcinomas, lung cancer and ovarian cancer (Boel et al.,

1995; Gaugler et al., 1994; Van den Eynde et al., 1995; van der Bruggen et al., 1991). Like P1A, these human antigens are expressed in several tumors but not in normal somatic tissues except testis and placenta.

Using more sensitive methods, P1A mRNA was detectable in spleen, lung, liver and thymus by reverse transcription-polymerase chain reaction (RT-PCR) (Sarma et al., 1999). However, the relative abundance of P1A mRNA found in a normal spleen was about ~100-1000 fold lower than that found in J558 tumor cells. Among these tissues, the mRNA level was highest in spleen followed by decreasing levels in lung, liver and thymus.

Derbinski et al. used RT-PCR to analyze expression of various promiscuous genes including P1A in the subsets of thymic stromal cells, such as thymic cortical epithelial cells ($CD45^{-/lo}CDR1^{+}G8.8^{+}$), thymic medullar epithelial cells ($CD45^{-/lo}CDR1^{-}G8.8^{+}$), dendritic cells ($CD11c^{+}F4/80^{-}$) and macrophages ($CD11c^{-}F4/80^{+}$) (Derbinski et al., 2001). When comparing these four subsets, expression of P1A was high in thymic medullar epithelial cells, medium in thymic cortical epithelial cells, low in dendritic cells and not detectable in macrophages in C57BL/6 mice. Protein expression appeared heterogeneous and restricted to a minor fraction of thymic medullar epithelial cells at a frequency of one in 50–200 cells (Derbinski et al., 2001).

Because P815 is a cell line of mastocytoma, Van den Eynde et al. also tested the P1A expression in a number of mast cell lines (Van den Eynde et al., 1991). The resulting expression of P1A was negative by northern blots both in the MC/9 mast cell line derived from mouse fetal liver and in the mast cells isolated from mouse bone marrow. However,

the P1A message was detectable in mast cell line L138.8A, which was derived from BALB/c bone marrow, and which was IL-3 responsive and secreted IL-6. Taken together, P1A is highly expressed in several tumors, the testis and placenta, but has low or no expression in normal tissues including hematopoietic tissues.

P1A gene structure and subcellular distribution

The P1A gene has been mapped to the X chromosome recently (mouse genome informatics, ID98818) and is composed of three exons and codes for a protein of 224 amino acids (Fig 1-3). The size of the P1A protein is around 40 KDa in SDS-PAGE when detected by the rabbit antiserum against the amino acid sequence at the carboxyl-terminal end of P1A.

The amino acid composition forms one region of hydrophobic amino acids (residues 34-57) and two highly acidic and negatively charged regions (residues 82-132 and 195-215) which are composed richly with glutamic acids (20.1%) and aspartic acids (13.8%) (Amar-Costesec et al., 1994) (Fig 1-3). Although there is one potential transmembrane helical domain, a type I transmembrane protein exposing the C-terminal domain to the cytosol is unlikely because P1A protein does not begin with a secretion sequence or any other targeting signal sequence. A type II transmembrane protein exposing the N-terminal domain to the cytosol is also unlikely, because then a fragment larger than 20KDa should be noticed after protease digestion; however, only a 20KDa fragment was detected after trypsin or chymotrypsin treatment in the absence of detergent (Amar-Costesec et al., 1994). Amar-Costesec et al. showed P1A was also not released by

1 M KCl high salt medium; thus they concluded that P1A may be a peripheral protein binding to other membrane proteins by non-ionic interactions (Amar-Costesec et al., 1994).

The function of P1A is unknown. However, the amino acid sequence of the P1A protein contains several potential phosphorylation sites identified by MyHits database (Pagni et al., 2007). The P1A protein contains several possible serine phosphorylation sites by casein kinase II, protein kinase C, and cAMP/cGMP dependent kinase as illustrated in Fig 1-3. If these sites are phosphorylated, then P1A may function as an intracellular signal molecule and thus may be involved in cellular biological processes. Indeed, Amar-Costesec et al. showed there was a phosphorylated form of P1A which associated with a 62KDa phosphorylated component (Amar-Costesec et al., 1994).

Based on the immunofluorescence staining, P1A signal is particularly intense in small vesicles and not in nucleus. P1A protein is associated with membranes of the secretory pathway, including the endoplasmic reticulum, the Golgi complex and post-Golgi vesicles from the sedimentation patterns (Amar-Costesec et al., 1994). In addition, Altieri et al. using western blot analysis found the presence of P1A in isolated J558 exosomes (Altieri et al., 2004). Exosomes are small membrane vesicles from the multivesicular bodies in the endosomes and are externalized by exocytosis (Lakkaraju and Rodriguez-Boulan, 2008). The fact that P1A showed up in exosomes may explain its ability to be cross-presentated to T cells (Andre et al., 2004). On the other hand, since proteins that facilitate vesicle trafficking may be recognized as tumor antigens, the reason that P1A is recognized by T cells might result from P1A regulating the secretion of exosomes. Therefore, we are interested in a potential role for P1A in the vesicular

transport of the protein products or in membrane trafficking according to the localization of P1A.

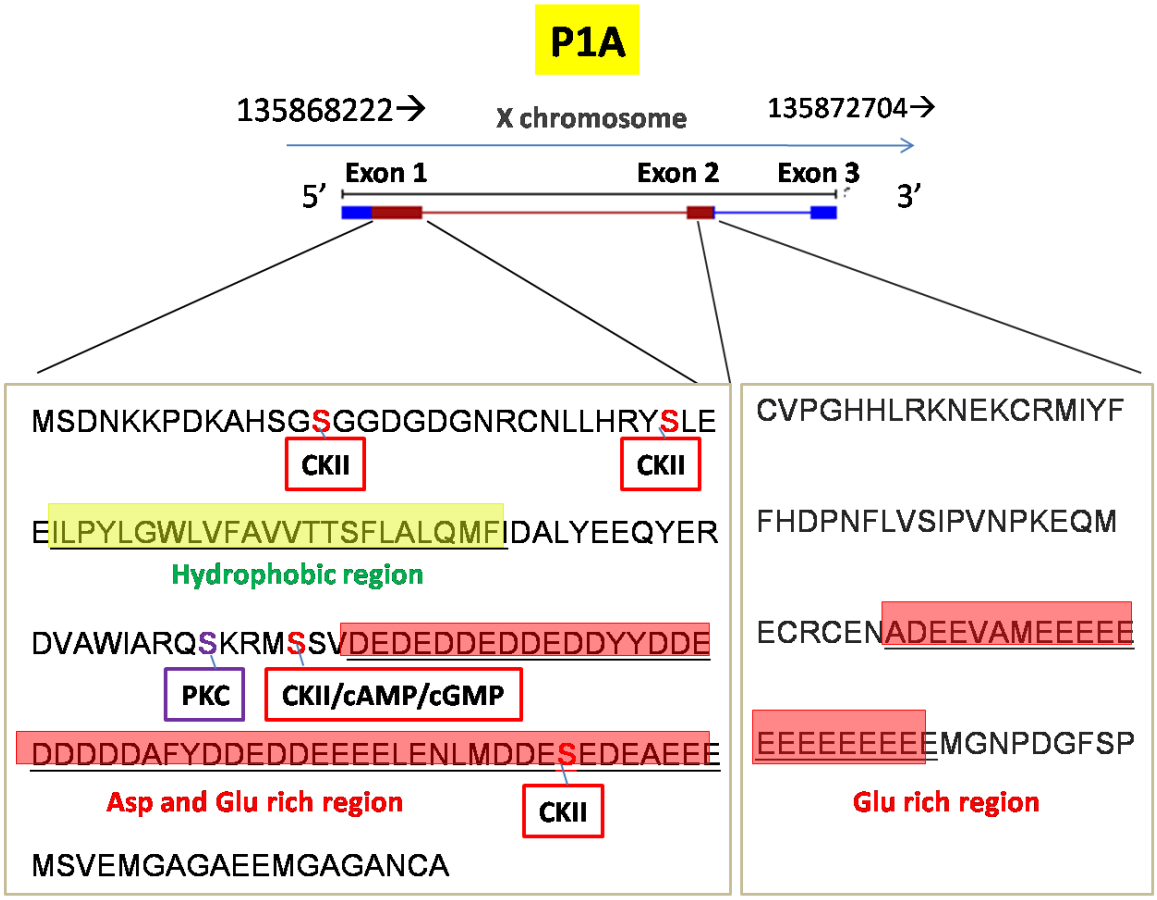

Figure 1-3. Scheme of P1A gene

P1A on X chromosome is composed of three exons and codes for a protein of 224 amino acids. The amino acid sequence has a hydrophobic region and two aspartic acid (Asp) and glutamic acid (Glu) rich regions. Several potential phosphorylation of serine sites by protein kinase C (PKC), casein kinase II (CKII) and cAMP/cGMP dependent protein kinase (cAMP/cGMP) are illustrated.

1.5 Exocytosis/ Endocytosis and tumorigenesis

While over-expression and involvement of vesicle trafficking makes a protein a likely candidate of tumor antigens, its over-expression begs the question of why a tumor would over-express these proteins. A simple explanation is that by facilitating exocytosis and endocytosis, these proteins provide a growth advantage for tumors.

Recently, alterations in proteins of the endocytic pathway have been associated with tumorigenesis. An emerging concept is that the intracellular signal transduction is regulated by the internalization of ligand-receptors into endosomes and the endocytic trafficking pathway (Disanza et al., 2009; Gould and Lippincott-Schwartz, 2009). Endocytosis can modulate the duration, intensity and spatial distribution of cell signaling pathways that affect cell growth, differentiation and survival. Extracellular ligand-induced responses are attenuated by efficient internalization of receptors from the plasma membrane (Disanza et al., 2009). For example, inhibition of epidermal growth factor receptor (EGFR) internalization leads to enhanced EGFR signaling and also enables tumor cells to proliferate and survive (Wang et al., 2009). Huntingtin interacting protein 1 (HIP1) which functions in endocytosis and receptor trafficking has transforming properties and is over-expressed in a variety of human cancers (Hyun and Ross, 2004; Rao et al., 2002). HIP1 interacts with clathrin and AP2. Rao's study showed that EGFR was upregulated in HIP1-transformed fibroblast cells (Rao et al., 2003). Mislocalization of clathrin from the cell periphery to the perinuclear area, as observed in HIP1-transformed fibroblast cells, resulted in attenuation of normal endocytosis and decrease of EGFR degradation (Rao et al., 2003). Therefore, it is plausible that attenuation of

endocytosis might enhance ligand-induced signaling pathways and result in the initiation or progression of cancer.

In addition, recent reports support a role for vesicle trafficking and exocytosis in tumorigenesis. It is clear that the Ral-exocyst is important for the maintenance of epithelial cell polarity, cell motility and cytokinesis (Cascone et al., 2008; Chen et al., 2006; Moskalenko et al., 2002; Rosse et al., 2006). Interruption of these activities would be expected to affect tumor cell proliferation and metastasis. Current findings suggest that the exocyst complex components play an important role in Ras mediated transformation in human embryonic kidney cells (Issaq et al., 2010). Knockdown of the exocyst component of either Sec5 or Exo84, in RalGEF-transformed cells, resulted in decreased cell transformation in anchorage-independent colony formation assay (Issaq et al., 2010). It was previously also observed that in knockdown of RalA cells lacked anchorage-independent growth, which correlated with the decrease of cell transformation (Lim et al., 2005). This suggested that exocyst mediated the downstream transformation signal of RalA.

Rab25 interacts with Sec15 subunit of the exocyst and promotes exocytosis (Zhang et al., 2004). Cheng et al. showed that Rab25 was over-expressed in half of ovarian and breast cancers by analyzing the chromosome amplification regions in these cancers (Cheng et al., 2004). Moreover, another study revealed that Rab25 over-expression increased tumor cell growth and correlated to a poor prognosis, whereas reduced Rab25 expression decreased tumor proliferation (Cheng et al., 2005).

Brain-specific angiogenesis inhibitor 1 associated protein 3 (BAIAP3) contains Ca^{2+}/phospholipid binding domains and Munc13-homology domains which are related to membrane trafficking and exocytosis (Koch et al., 2000; Shiratsuchi et al., 1998). Palmer et al.'s study showed that BAIAP3 is activated in desmoplastic small round cell tumor and colocalized with secretory vesicles (Palmer et al., 2002). Since ectopic expression of BAIAP3 enhances tumor cell growth, BAIAP3 might increase cancer cell proliferation by regulating the exocytotic pathway (Palmer et al., 2002).

IQGAP1 is implicated in the regulation of exocytosis through its binding of Sec3, Sec8 and Exo70 (Rittmeyer et al., 2008; Sakurai-Yageta et al., 2008). IQGAP proteins have been shown to be over-expressed in several tumor types (Jadeski et al., 2008; Nabeshima et al., 2002; Patel et al., 2008; Zhou and Skalli, 2000). In addition, the invasion ability of ovarian carcinoma cells and breast cancer cells is decreased after IQGAP1 silencing (Sakurai-Yageta et al., 2008). It suggests that IQGAP1 mediates cell invasion by coordination with the exocyst complex to facilitate tethering and polarized exocytosis, and then results in release of matrix metalloproteinases (MMPs) and degradation of the extracellular matrix (Sakurai-Yageta et al., 2008).

Exosomes have been found purified from serum, ascites fluids and pleural effusions of cancer patients (Andre et al., 2002; Huber et al., 2005; Valenti et al., 2006). The secreted exosomes from tumor cells not only have the role of the antigenic source for effective T cell cross-priming, but may also have important roles in diverse areas of tumorigenesis including tumor invasion, growth factor release and angiogenesis. For example, developmental endothelial locus-1, which is an angiogenic factor for vascular development, is presented in exosomes in mesothelioma tumor (Hegmans et al., 2004).

Recent studies also show tumor exosomes could transport the pro-angiogenic molecules tetraspanins, and thereby increase expression of MMPs and vascular endothelial growth factor (VEGF) in target cells (Gesierich et al., 2006). Thus, secretion of exosomes could induce neo-angiogenesis even at tumor sites. Since tumor exosomes have been shown to contain several proteins, such as VEGF, MMPs and fibroblast growth factors (Ceccarelli et al., 2007; Fevrier and Raposo, 2004; Hakulinen et al., 2008; Hugel et al., 2005), they may contribute to tumor metastasis process or tumor progression. Together, the studies described above suggest that enhancement of exocytosis or attenuation of endocytosis pathways is an important regulatory event to promote tumorigenesis.

1.6 Effect of Environmental Pollutants on Endocytosis/Exocytosis

Vesicle trafficking is a very important pathway for cells to appropriately respond to and communicate with the exterior environment. Environmental pollutants that alter the functions of endocytosis and exocytosis may result in cell dysfunction and ultimately disease. For example, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the halogenated aromatic hydrocarbons which is a known carcinogen in human and animals. Due to its lipophilic chemical property, TCDD bioaccumulates in food chains and is a persistent organic pollutant (POP). Recently, several epidemiologic studies suggested that TCDD exposure is related to diabetes (Cranmer et al., 2000; Longnecker and Michalek, 2000; Remillard and Bunce, 2002). The association between diabetes and TCDD exposure has been further elucidated in Kim et al.'s study (Kim et al., 2009). The latter study demonstrated that exposure of TCDD induced exocytosis of lysosomal and secretory granules in a rat insulin-secreting beta cell line (Kim et al., 2009). Although TCDD is an environmental endocrine disrupter due to its chemical structure, TCDD-

induced exocytosis is initiated by calcium influx via T-type calcium channel, not by aryl hydrocarbon receptors (AhR) (Kim et al., 2009).

Polychlorinated biphenyls (PCBs), a group of 209 halogenated aromatic compounds, are endocrine disrupters and POPs. Studies have shown that non-coplanar PCB 47 (2,2',4,4'-tetrachlorobiphenyl) stimulates degranulation of rat neutrophils and HL-60, a human promyelocytic leukemia cell line (Bezdecny et al., 2005; Brown and Ganey, 1995). However, coplanar PCB 77 (3,3',4,4'-tetrachlorobiphenyl) did not induce neutrophil degranulation (Bezdecny et al., 2005; Brown and Ganey, 1995). Olivero-Verbel and Ganey also demonstrated that only non-coplanar PCB 8 (2,4'-dichlorobiphenyl) and PCB 128 (2,2',3,3',4,4'-hexachlorobiphenyl) were able to enhance the degranulation of neutrophils, but the coplanar PCB 126 (3,3',4,4',5-pentachlorobiphenyl) has no such effect (Olivero-Verbel and Ganey, 1998). These studies suggested that the results of neutrophil degranulation are highly associated with the structure of PCBs. Coplanar PCBs have dioxin properties with a high affinity to the AhR. Since evidence shows only non-coplanar PCBs can elicit exocytosis, not coplanar PCBs. PCB-induced exocytosis is through an AhR independent pathway (Bezdecny et al., 2005; Brown and Ganey, 1995; Olivero-Verbel and Ganey, 1998).

In addition to affecting the degranulation of neutrophils, several studies also showed that environmental chemicals affect mast cell exocytosis. For example, the chlorinated organic solvents, trichloroethylene and tetrachloroethylene, stimulated release of histamine from antigen-stimulated non-purified rat peritoneal mast cells and rat basophilic leukemia (RBL-2H3) (Seo et al., 2008). Sodium sulfite also induced mast cell degranulation, and released beta-hexosaminidase, serotonin and histamine in RBL-2H3

cells and human peripheral blood basophils (Collaco et al., 2006). In addition, the soluble organic chemicals extracted from diesel exhaust particles can enhance degranulation of a murine mast cell line via FcRI cross-linking stimulation (Diaz-Sanchez et al., 2000). Furthermore, an endocrine-disrupting chemical, atrazine, a widely used herbicide, also increased beta-hexosaminidase release from RBL-2H3 cells and peritoneal mast cells (Mizota and Ueda, 2006). Therefore, environmental pollutants which could enhance the secretion of inflammatory mediator from neutrophils or mast cells may disregulate the whole immune responses that could cause diseases promoted by inflammatory factors.

In addition to influencing exocytosis, several studies also demonstrated that environmental pollutants impair the function of endocytosis in macrophages. Jakab et al. showed that carbon alone or carbon-adsorbate combinations, such as carbon-benzofuran and carbon-acrolein, decreased Fc receptor-mediated phagocytosis of alveolar macrophage in a dose-dependent manner (Jakab et al., 1990). Phagocytosis belongs to one category of endocytosis pathways (Marsh, 2001). This suggested that the pollutants emitted from the incomplete combustion with acrolein and furan which have been found in cigarette smoke and diesel particulate matter, respectively, affect the normal function of alveolar macrophages (Jakab et al., 1990). In addition, lead oxide, a major airborne pollutant in industrial environments, also inhibits the ability to phagocytosis in macrophages lavaged from rabbits exposed to lead oxide (Zelikoff et al., 1993). Thus, disruption of macrophage function may impair the first line of defense against inhaled antigens in human and animals.

Environmental chemicals also can disrupt normal expression of exocytotic-related genes. For instance, Takahashi et al. found the genes associated with exocytosis, such as

VAMP1, VAMP2, SNAP25 and Stx1b2, were upregulated in prenatal exposure to hydroxylated PCBs in rat brains by cDNA microarray and quantitative RT-PCR (Takahashi et al., 2009). This suggested that the endocrine disrupter, hydroxylated PCBs, may affect brain development through abnormal neurotransmission (Takahashi et al., 2009).

Regulation of gene expression can be controlled by epigenetic modification, including DNA methylation, histone modification and small RNA molecule interference (Baccarelli and Bollati, 2009). For example, activation of MAGE gene, a cancer-testis gene like P1A, results from a demethylation and histone acetylation of their promoters (Wischnewski et al., 2006). Since epigenetic modifications are important regulators of normal cellular function, interference of epigenetic mechanisms by environmental chemicals may result in diseases and tumors. Indeed, several studies have shown environmental pollutants could modify epigenetic mechanisms (Baccarelli and Bollati, 2009). For example, Rusiecki et al. showed the association between global DNA hypomethylation and increasing plasma POP concentrations, such as p,p'-DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane), p,p'-DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene), beta-hexachlorocyclohexane, oxychlordane, alpha-chlordane, mirex and polychlorinated biphenyls, from 70 Greenlandic Inuit (Rusiecki et al., 2008). In addition, Bollati et al. showed there was an inverse association between the level of MAGE gene methylation and the level of benzene exposure from human blood DNA samples in Italy (Bollati et al., 2007). The mechanism of benzene exposure with acute myelogeous leukemia risk may be due to demethylation of MAGE, thereby activation of MAGE gene which is highly expressed in malignant tumors. Therefore, in order to

prevent and minimize the biological dysfunction caused by environmental toxicants, understanding the mechanisms and molecules with key roles in vesicle trafficking pathway is an essential step.

1.7 Central hypotheses

Onco-fetal antigens are expressed in highly proliferating cells during embryogenesis and tumorigenesis. Given that the two different roles of exocytosis are involved in the development of cancer and in priming tumor-reactive T cells respectively, I hypothesize: 1) that onco-fetal antigens play an active role in tumorigenesis by enhancing exocytosis in tumor cells; and 2) that the role for onco-fetal proteins in exocytosis may result in immune recognition by cancer-reactive T cells. The data presented in this thesis provides evidence for these hypotheses.

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CHAPTER 2

Transgenic expression of P1A induced thymic tumor: a role for onco-fetal antigens in tumorigenesis

Abstract

P1A is the first known tumor rejection antigen. It is expressed in embryonic stem cells and multiple tumors but is silent in adult tissues except for the testis and placenta. Therefore, P1A represents a prototype for onco-fetal antigens. To test the potential function of P1A in tumorigenesis, we used a transgenic mouse expressing P1A in lymphoid cells. We observed that immunodeficient host P1A transgenic mice developed thymic tumors after 7 months of age and had shorter survival rates compared to control groups. The P1A-transgenic immune competent host also had a shorter life-span, although the difference is not statistically significant. The P1A transgenic bone marrow cells had higher proliferation ability and more potential progenitors compared to control bone marrow cells. Most of the P1A tumors examined displayed B cell lineage markers. Within the 7 examined tumors, only one tumor displayed T cell lineage markers. Interestingly, this latter tumor also harbored a mutation in the *Notch1* gene which is involved in T cell leukemia. Our results suggest that onco-fetal antigen P1A is involved in tumorigenesis and promoted cell transformation.

Introduction

By definition, tumor antigens were identified because cancer-reactive lymphocytes could recognize these proteins (Boon and van der Bruggen, 1996). An interesting but still poorly understood phenomenon is that many tumor antigens are also abundantly expressed at early stages of embryogenesis. These antigens are termed onco-fetal antigens. Theoretically, these antigens might be useful for cancer immune therapy, since they are highly expressed on tumor cells but absent in the adult host with the exception of placenta and testis. In addition, the expression of these types of antigens on tumor cells may reflect the fact that cancer may resemble the biological properties of embryonic cells. One key issue is whether the onco-fetal antigens play a significant role in cell differentiation or proliferation, since tumor and embryonic cells share similar biological properties.

P1A is a prototype of onco-fetal antigens. Only recently, the P1A gene has been mapped to the X-chromosome (Mouse Genome Informatics, ID 98818). Identification of P1A as a tumor rejection antigen marked an important breakthrough in tumor immunology as it was the first tumor antigen known to be recognized by cytotoxic T cells (Van den Eynde et al., 1991). P1A was first identified in the mastocytoma P815 cell line which was derived from DBA/2 mice after exposure to methylcholantrene (Uyttenhove et al., 1983). Later, studies also found that P1A was expressed in several tumors such as Meth A sarcoma and J558 plasmacytoma (Ramarathinam et al., 1995). In Uyttenhove's study, they showed P1A is highly expressed in the testis and placenta (Uyttenhove et al., 1997). The levels of P1A mRNA in the testis and placenta are around 10-20% and 150-200% of mastocytoma P815, respectively (Uyttenhove et al., 1997). In addition, P1A

mRNA was detectable in spleen, lung, liver and thymus by reverse transcription-polymerase chain reaction (RT-PCR), although the relative abundance of P1A mRNA in these tissues was much lower than that found in J558 tumor cells (Sarma et al., 1999). Here, we show that P1A is also expressed in mouse R1 embryonic stem (ES) cells.

The function of P1A is unknown to date. Since tumor cells, germ cells and stem cells have the capacity to differentiate and proliferate, it is possible that P1A may be involved in a particular state of cell differentiation and growth regulation. Therefore, to seek the function of onco-fetal antigens, we used P1A as a model. In this paper, we found that our transgenic mouse line, which over-expresses tumor antigen P1A in lymphoid cells, developed a variety of tumors after 7 months. The results from this study suggest that P1A is involved in tumorigenesis.

Materials and Methods

Cell lines and experimental animals

Mouse plasmacytoma J558 from ATCC were cultured in RPMI 1640 medium containing 5% fetal bovine serum (FBS) and 100 μg/ml penicillin and streptomycin. Murine R1 ES cells which have been manipulated by Dr. Ping Lu in Dr. Yang Liu's lab were originated from the ES cell service center of Ohio State University. Murine R1 ES cells were co-cultured with mouse embryonic fibroblast (MEF) cells in DMEM medium containing 15% FBS, 0.1 mM β-mercaptoethanol, 10^3 u/ml leukemia inhibitory factor, and 4 mM glutamine. $Rag2^{-/-}$ mice were obtained from the Taconic Laboratories (Terrytown, NY). P1A transgenic (P1A Tg) mice were as described previously

(Rosenbaum et al., 1990; Sarma et al., 1999). The P1A Tg mice in $Rag2^{-/-}$ background were bred in Ohio State University. All animal experiments were conducted in accordance with accepted standards of animal care and approved by the Institutional Animal Care and Use Committee of University of Michigan.

Western blot analysis

Samples of mouse tissues or cells were lysed in protein lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40) and protease inhibitor cocktails (Sigma) including 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin, E-64, leupeptin and pepstain A were added. Cell lysates were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride (PVDF) membranes, and incubated with anti-P1A rabbit antibody specific for the peptide sequence, YEMGNPDGFSP (Genemed Synthesis, 1:500 dilution) or anti-actin mouse antibody (Sigma, 1:5000 dilution). Anti-rabbit or anti-mouse IgG horseradish peroxidase–linked antibody at 1:3500 dilutions (GE Healthcare) was used as secondary antibodies. Antibodies were detected with chemiluminescence reaction using the enhanced chemiluminescence kit (Amersham Biosciences) and visualization with exposure to film.

Flow cytometric analysis

P1A transgenic mice were sacrificed when moribund. Thymus and spleen tissue were homogenized and passed through a cell strainer (BD Biosciences) to generate a single-cell suspension. Cells were stained in phosphate-buffered saline plus 2% FBS for 20 minutes on ice with the following monoclonal antibodies at a 1:200 dilution, CD4, CD8, B220, CD11c, Gr1, and CD11b (BD Biosciences). Cells were subsequently

analyzed by BD LSR II Flow Cytometer. The differences in survival rates were analyzed by Kaplan-Meier survival analysis.

Bromodeoxyuridine (BrdU) incorporation assay

Mice were injected intraperitoneally with BrdU (100 mg/kg, Sigma-Aldrich). Then, mice were given BrdU water (1 mg/ml) for 24 hours before sacrifice. BrdU staining kit (BD Biosciences) was used according to the manufacturer's instructions.

Colony-forming cell assay

Bone marrow cells were isolated from the femur and tibia of mice. Bone marrow cells ($1x10^4$) were plated in MethoCult® methylcellulose-based media (StemCell Technologies) per 35mm dish and incubated at 37°C, 5% CO_2. After 12 days culture, colony number and morphology was counted using inverted microscope.

DNA sequencing of Notch1

Notch1 mutations occur in the most of T-cell acute lymphoblastic leukemias (O'Neil et al., 2006; Weng et al., 2004). To determine whether Notch1 mutations were acquired in our P1A tumor models, genomic DNA was isolated from tumor tissues using the genomic DNA purification kit (Invitrogen). Exons 26 and 27 of Notch1 were amplified using the following primers: exon 26 forward, 5'-ACGGGAGGACCTAACCAAAC-3'; exon 26 reverse, 5'-CAGCTTGGTCTCCAACACCT-3'; exon 27 forward, 5'-CGCTGAGTGCTAAACACTGG-3'; and exon 27 reverse, 5'-GTTTTGCCTGCATGTACGTC-3'. Exon 34 was amplified in 2 fragments using the following primers: forward 1, 5'-GCTCCCTCATGTACCTCCTG-3'; reverse 1, 5'-

TAGTGGCCCCATCATGCTAT-3'; forward 2, 5'-ATAGCATGATGGGGCCACTA-3'; reverse 2, 5'-CTTCACCCTGACCAGGAAAA-3'. Amplification products were sequenced in both directions at the DNA Sequencing Core Facility in University of Michigan.

Results

P1A express in embryonic stem cells and P1A transgenic (P1A Tg) thymus

Our P1A transgene contained the enhancer of the immunoglobulin heavy chain gene (Eμ), the mb-1 promoter, followed by P1A open reading frame, an ID2 gene, and an SV40 polyadenylation signal (Fig 2-1A). P1A was under the control of the mb-1 promoter, which is expressed specifically in B cells from the progenitor to mature stages (Sakaguchi et al., 1988). In addition, the enhancer of Eμ has been shown to promote transgene expression in B and T lymphoid cells (Adams et al., 1985; Rosenbaum et al., 1990; Rosenbaum et al., 1989; Sun, 1994; van Lohuizen et al., 1989). Thus, our P1A Tg mice can highly express P1A in lymphoid cells. P1A protein was able to be detected in P1A Tg thymus but not in P1A Tg spleen by the anti-P1A antibody which against the C-terminal of P1A protein. As shown in Fig 2-1B, P1A was highly expressed in the testis, mouse R1 embryonic stem cells and J558 plasmacytoma cells.

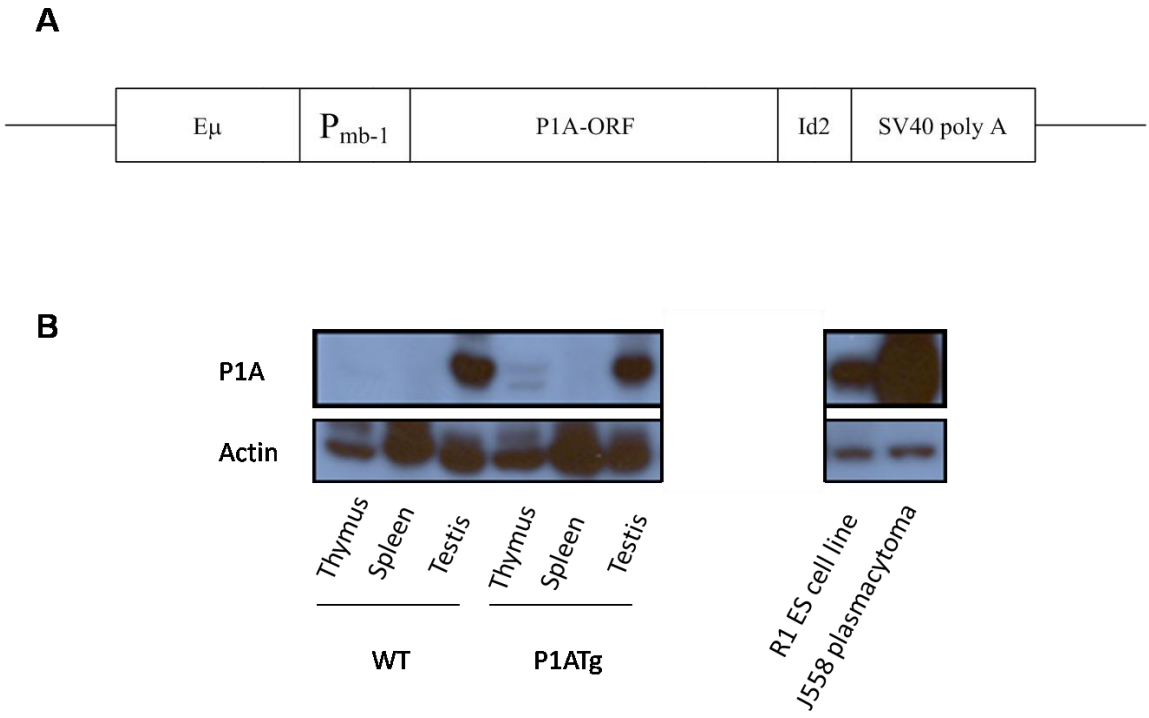

Figure 2-1. P1A transgenic (P1A Tg) mice

A. Structure of Eμmb-P1A transgene. P1A open reading frame (P1A-ORF) is under controlled by enhancer of immunoglobulin heavy chain (Eμ) and mb-1 promoter (P_{mb-1}), followed by ID2 gene and an SV40 polyadenylation signal sequence. B. Western blot analysis of P1A protein expression in J558 plasmacytoma cells, R1 murine ES cells, thymus, spleen, testis from P1A Tg mice and BALB/c WT mice; actin as internal control.

P1A Tg mice developed thymic tumors

We observed that 19 out of 49 P1A Tg mice in Rag2 deficient background developed thymic lymphoma after 7 months of age. The $Rag2^{-/-}$P1A Tg mice had enlarged thymuses compared to control mice (Fig 2-2A). In addition to the thymus, the spleen was also enlarged (data not shown). Moreover, tumor infiltration into target organs, including liver and lung, were also widespread (data not shown). In addition, when using tumor incidence as a survival end point, P1A Tg mice in immunodeficient background ($Rag2^{-}$) had significant shorter survival rates due to the tumor formation compared to control groups (p=0.0004, Fig 2-2B) although not in immunocompetent mice ($Rag2^{+}$) (p=0.1157, Fig 2-2C).

In order to determine the lineages of the tumors, we analyzed the cell surface markers of the thymic tumors. As shown in Fig 2-3, only 1 of the 7 tumors analyzed expressed significant levels of CD4 and CD8. In contrast the majority of tumors expression markers of non-T lineages, including that of B cells ($B220^{+}$), plasmacytoid dendritic cells ($B220^{+}CD11c^{+}$) , myeloid cells ($B220^{+}Gr1^{+}CD11b^{+}$) (Fig 2-3). The result is consistent with the fact that Rag2 deficiency results in the thymocyte differentiation being blocked before they can reach the $CD4^{+}CD8^{+}$ stage (Shinkai et al., 1992). It is noteworthy that all but one of tumors express B220, a marker for the B cell lineage (Table 2-1).

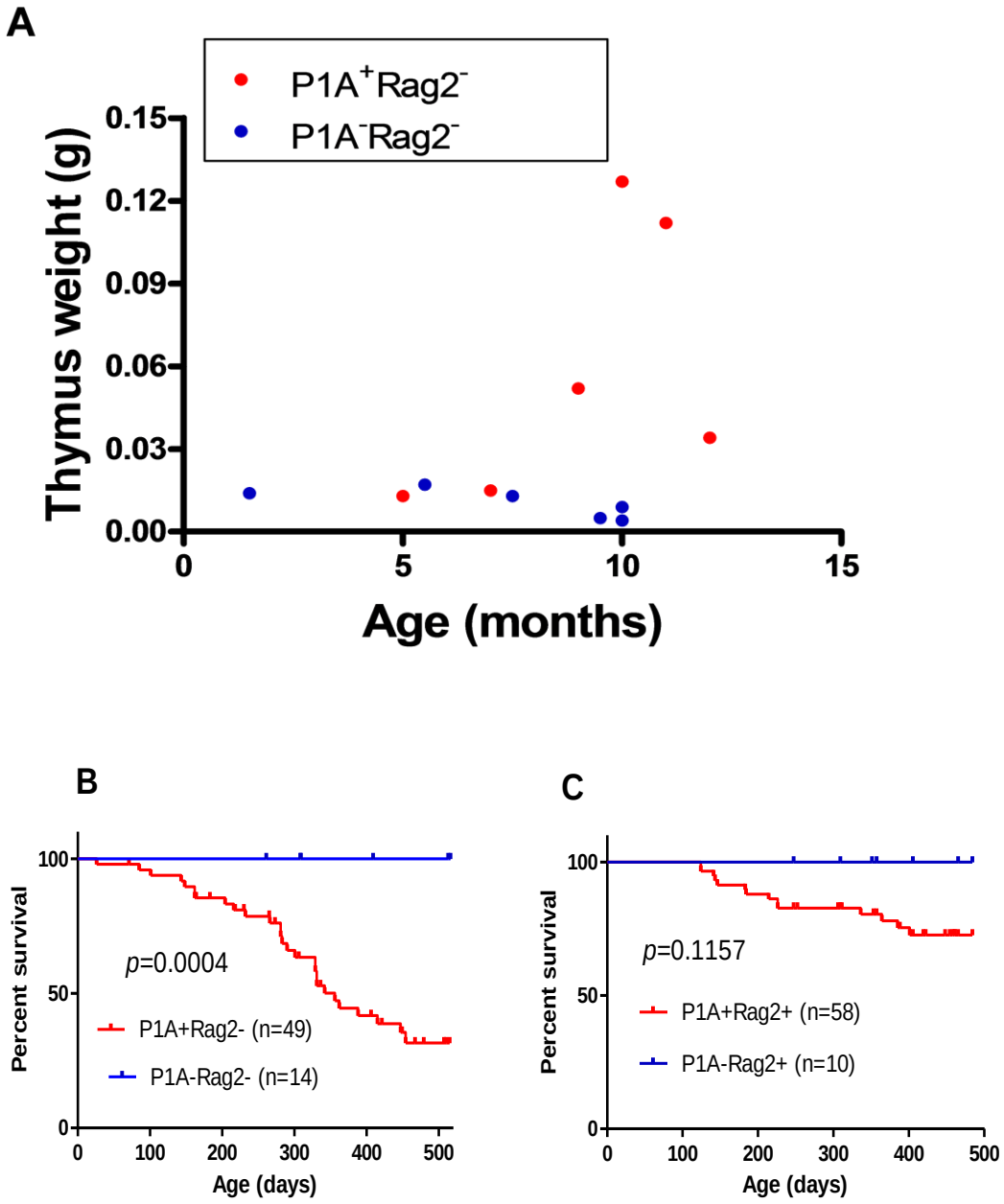

Figure 2-2. P1A Tg mice developed tumors

A. Thymus weights of $P1A^{+}Rag2^{-}$ mice and $Rag2^{-}$ mice. Enlarged thymus in $P1A^{+}Rag2^{-}$ mice after 7 months of age compared to $P1A^{+}Rag2^{-}$ mice. B. $P1A^{+}Rag2^{-}$ mice had shorter survival rates compared to $P1A^{-}Rag2^{-}$ groups. Only mice that died due to tumor formation were recorded. p=0.0004 (Kaplan-Meier analysis). C. Although several $P1A^{+}Rag2^{+}$ mice died due to tumor formation, differences in the tumor incidences were not statistical significant. p=0.1157 (Kaplan-Meier analysis).

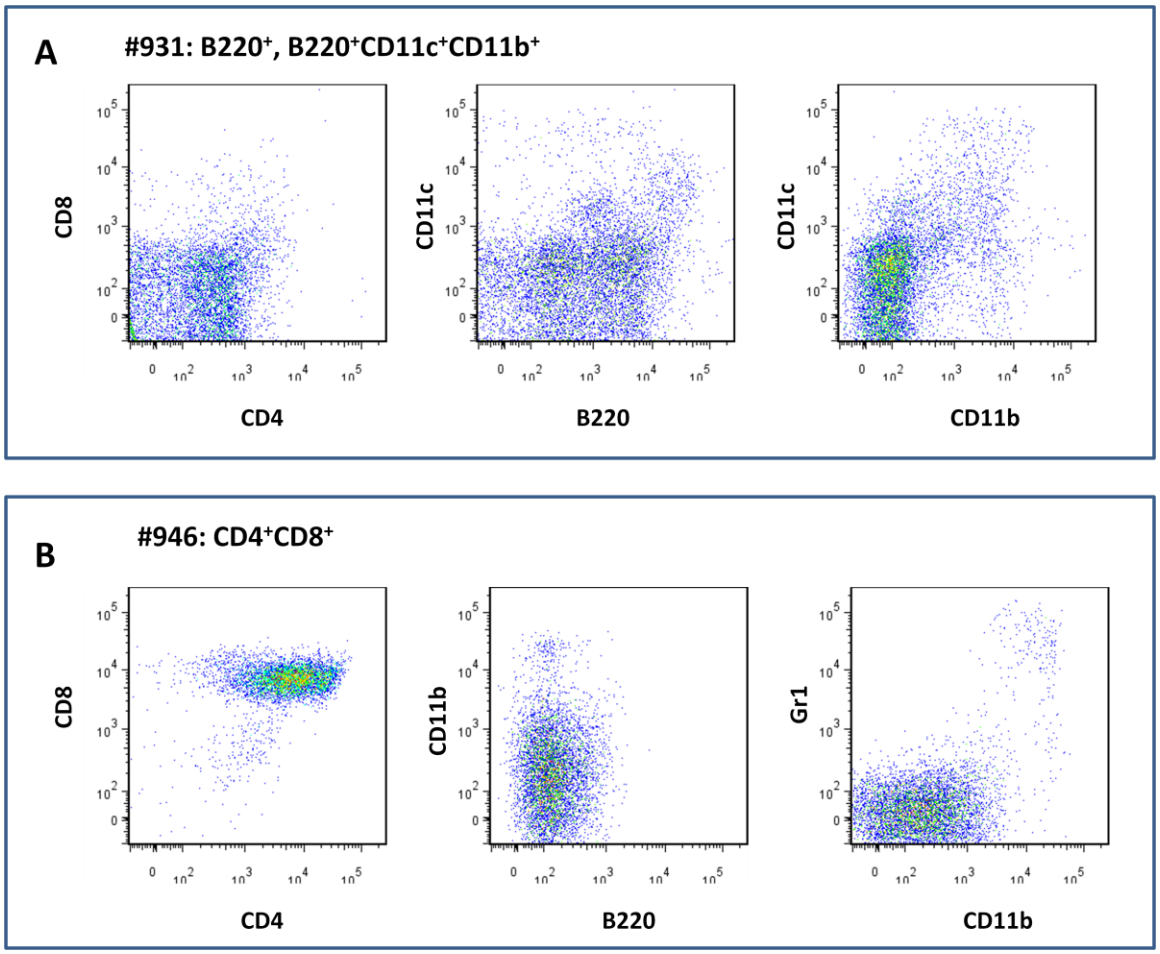

Figure 2-3. Heterogeneity of tumors in individual mouse

The thymic tumor from different mice was stained by the following monoclonal antibodies, CD4, CD8, B220, CD11c, Gr1, and CD11b. After antibody staining, thymic tumor cells were subsequently analyzed by flow cytometry. A. Mouse #931 showed $B220^+$ and $B220^+CD11c^+CD11b^+$ cell populations. B. Mouse #946 showed $CD4^+CD8^+$ cell population.

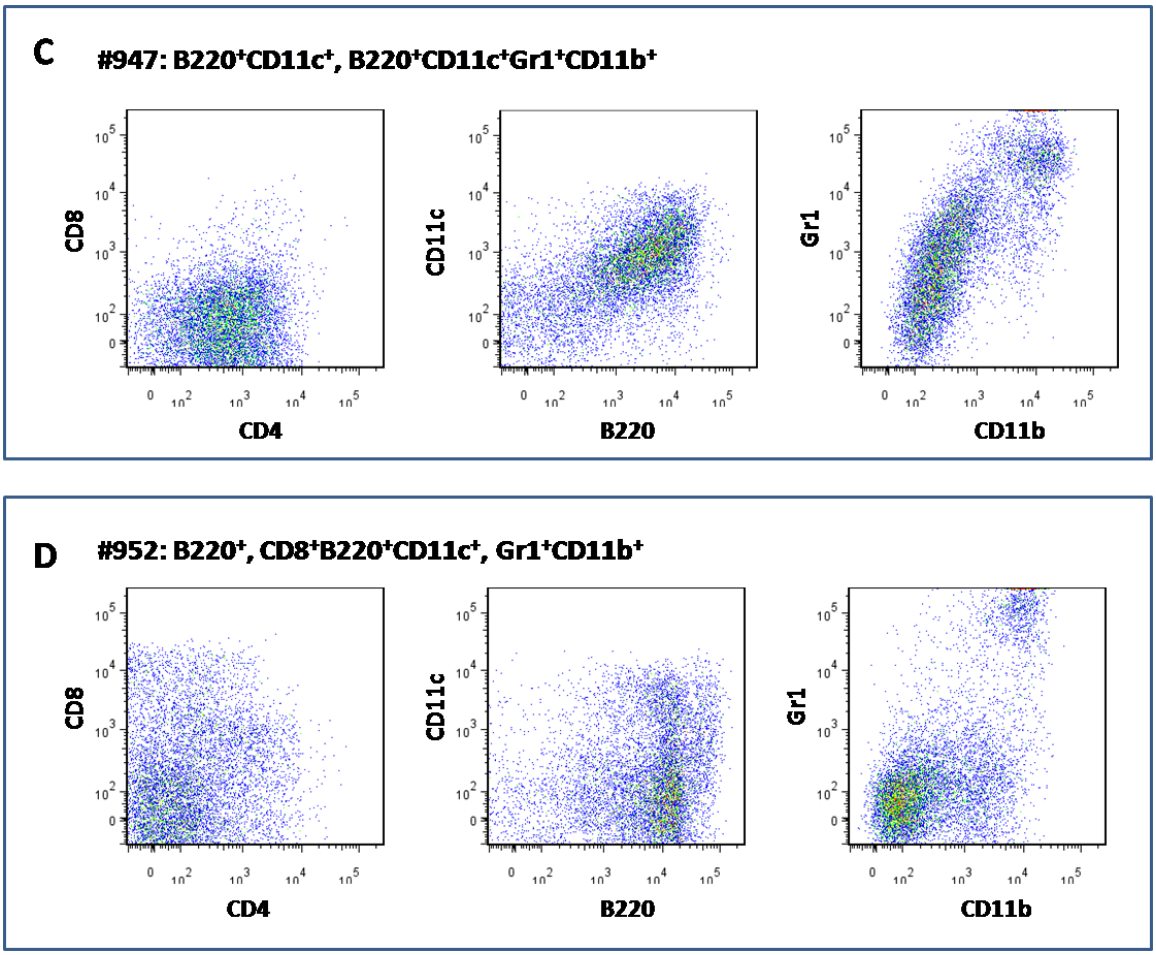

Figure 2-3. (Continue) Heterogeneity of tumors in individual mouse

C. Mouse #947 showed $B220^{+}CD11c^{+}$ and $B220^{+}CD11c^{+}Gr1^{+}CD11b^{+}$ cell populations.
D. Mouse #954 showed $B220^{+}$, $CD8^{+}B220^{+}CD11c^{+}$, $Gr1^{+}CD11b^{+}$ cell populations.

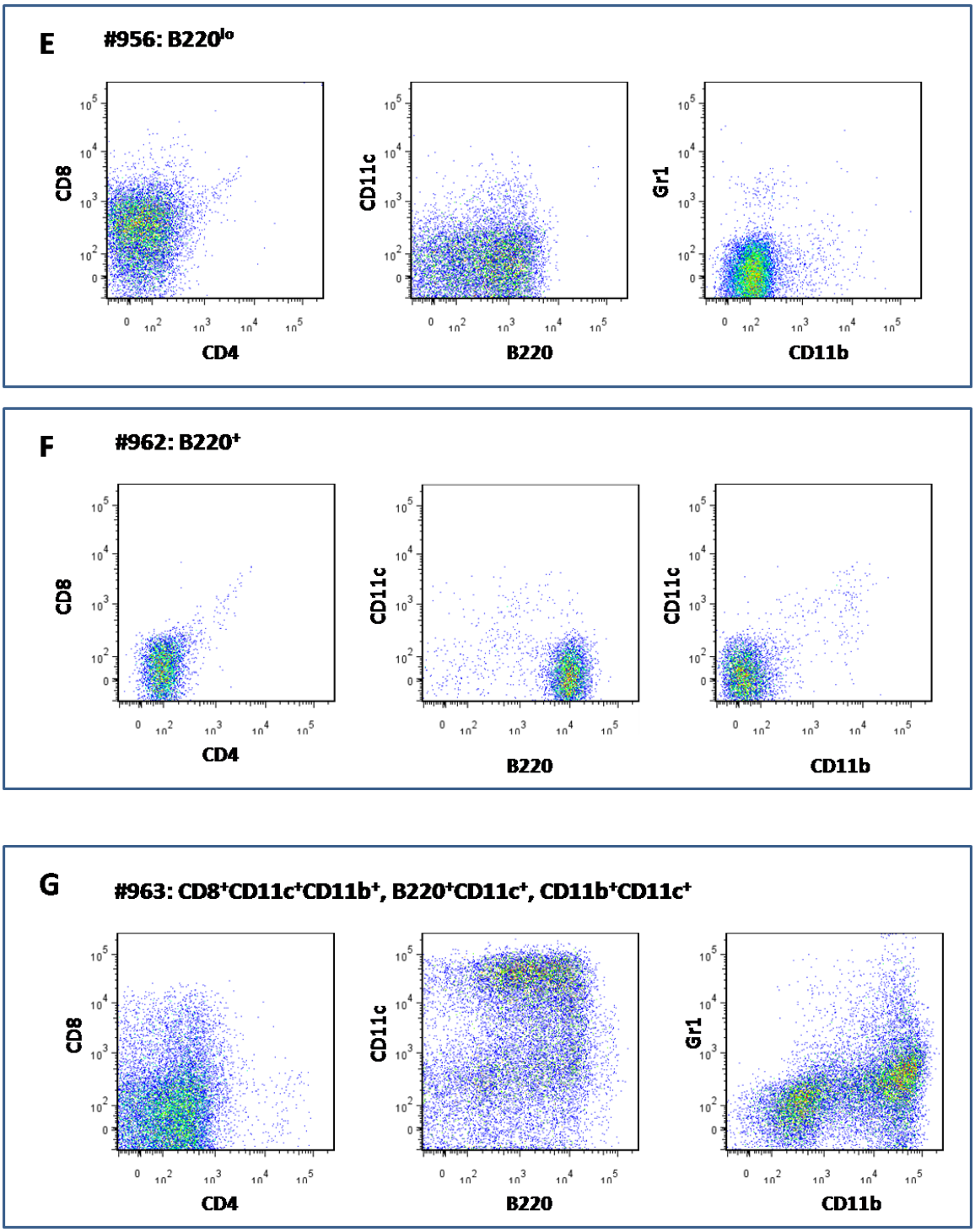

Figure 2-3. (Continue) Heterogeneity of tumors in individual mouse

E. Mouse #956 showed $B220^{lo}$ cell population. F. Mouse #962 showed $B220^{+}$ cell population. G. Mouse #963 showed $CD8^{+}CD11c^{+}CD11b^{+}$, $B220^{+}CD11c^{+}$ and $CD11b^{+}CD11c^{+}$ cell population.

P1A Tg bone marrow (BM) cells had high proliferation ability and more potential progenitors

We determined P1A Tg BM cell proliferation ability by BrdU incorporation assay. BrdU is an analog of the DNA precursor thymidine. The proliferating cells will incorporate BrdU into their DNA. The amount of BrdU in the DNA of cells can be detected with specific anti-BrdU fluorescent antibodies followed by flow cytometry. After 24 hours of labeling BrdU in vivo, we analyzed the BrdU incorporation rate by flow cytometry. Forty-six percent of BM cells from wild-type (WT) control mice were BrdU positive. However, the P1A Tg mice had a significant increase in the proportion of BrdU-positive BM cells both in immunocompromised (61%) and immunodeficient backgrounds (59%) (Fig 2-4AB).

We also cultured P1A Tg BM cells and control cells in methylcellulose based medium to quantify potential progenitors in vitro. The hematopoietic progenitors were able to proliferate and differentiate, resulting in the generation of mature cells. Colony-forming cells (CFCs) from individual progenitors can proliferate in semi-solid media with appropriate cytokines. We found P1A Tg BM had a higher CFC number compared to control BM and the CFC number was much higher in the second round culture which was from the first round culture (Fig 2-4C). The types of colonies can be characterized according to their morphology. The increases of colony number in all colony types, including BFU-E (burst-forming unit-erythroid), CFU-G (colony-forming unit granulocyte), CFU-M (colony-forming unit macrophage) and CFU-GM (colony-forming unit granulocyte macrophage), were seen in P1A Tg BM by inverted microscopy (Fig 2-4D).

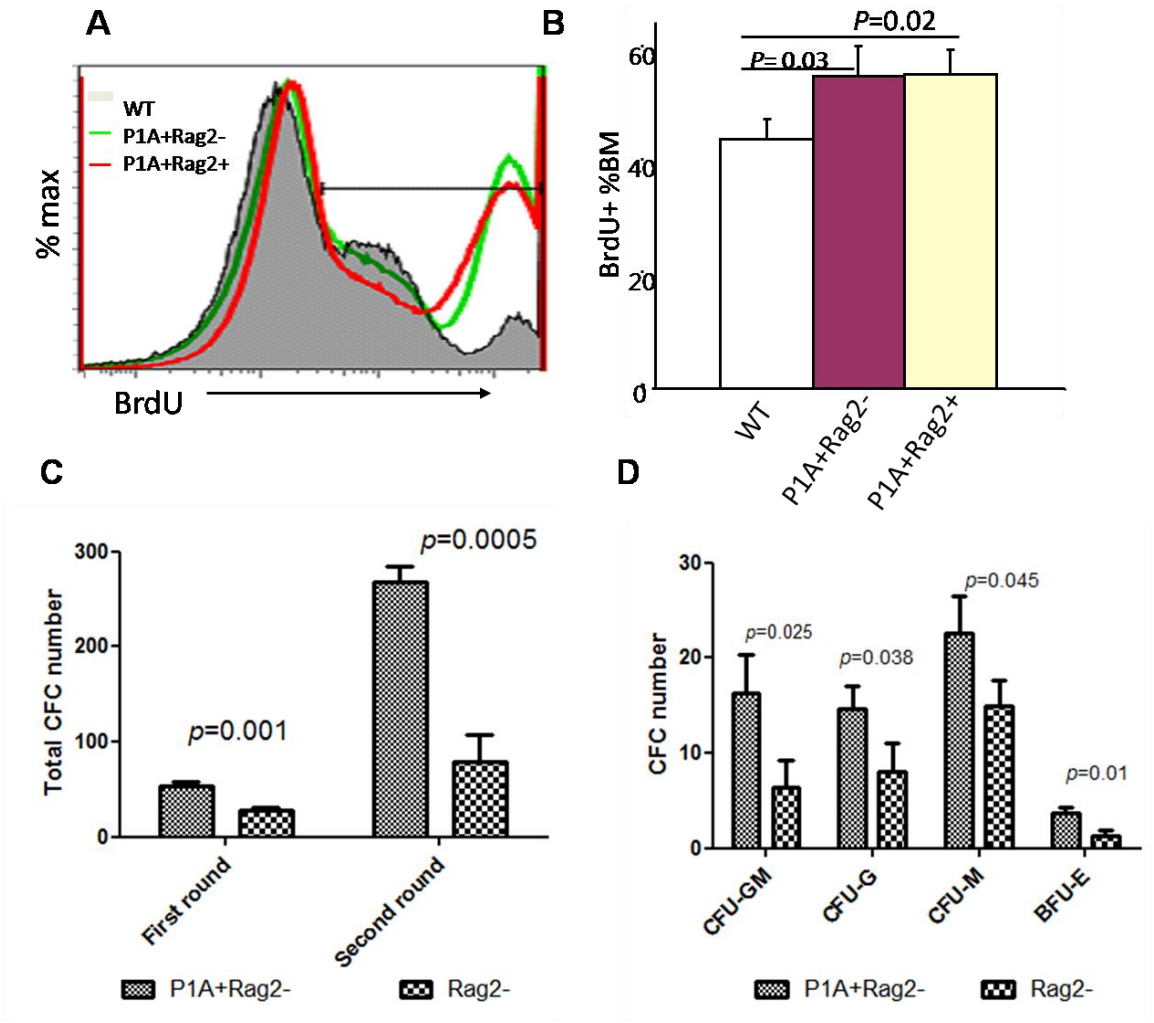

Figure 2-4. Proliferation ability of P1A Tg bone marrow

A. BrdU incorporation by flow cytometry; representative profiles of BrdU incorporation in bone marrow cells. B. The proportion of BrdU positive cells among bone marrow cells, showing mean ± SD of BrdU positive cells (n = 3). C. Colony-forming cell (CFC) assay. There was a higher colony number in $P1A^{+}Rag2^{-}$ compared to $Rag2^{-}$ bone marrow cells. The cells cultured in second round were taken from the first round culture (n=3, Student's t-test). D. Higher colony number in all colony types, including burst-forming unit-erythroid (BFU-E), colony-forming unit granulocyte (CFU-G), colony-forming unit macrophage (CFU-M) and colony-forming unit granulocyte macrophage (CFU-GM), were seen in $P1A^{+}Rag2^{-}$ than in $Rag2^{-}$ by inverted microscopy. (n=3, Student's t-test)

The majority of thymic tumors did not harbor Notch1 mutation

Notch1 mutations occur in the most of human T-cell acute lymphoblastic leukemia subtypes and many T-cell acute lymphoblastic leukemia genetic backgrounds in mice (O'Neil et al., 2006; Weng et al., 2004). This suggests an important role of aberrant Notch1 signaling in T-cell acute lymphoblastic leukemia.

To determine whether Notch1 mutations are acquired in our P1A tumor models, we next sequenced the Notch1 gene for mutations in the heterodimerization or PEST domains that have been previously observed to occur at a high frequency in both human and murine models of T-cell acute lymphoblastic leukemia. No mutations were detected at the heterodimerization domains and only one tumor harbored PEST domain mutations. Notch1 cDNA nucleotides 7339-7347 (AACACACGG) were deleted and a cytosine base was inserted into 7335 nucleotide position which resulted in the consequence of a frame shift and premature stop in PEST domain (Table 2-1).

Table 2-1. Tumor types and Notch1 mutation in P1A tumors from Rag2$^{-/-}$ background

Mouse #	Phenotype	Nucleotide change	Consequence
931	$B220^+$, $B220^+CD11c^+CD11b^+$	WT	NA
946	$CD4^+CD8^+$	Insertion C 7335, deletion 7339-7347 (AACACACGG)	Frame shift and premature stop in PEST domain
947	$B220^+CD11c^+$, $B220^+CD11c^+Gr1^+CD11b^+$	WT	NA
952	$B220^+$, $CD8^+B220^+CD11c^+$, $Gr1^+CD11b^+$	WT	NA
956	$B220^{lo}$	WT	NA
962	$B220^+$	WT	NA
963	$CD8^+CD11c^+CD11b^+$, $B220^+CD11c^+$, $CD11b^+CD11c^+$	WT	NA

Discussion

In this study, we first reported that the expression of P1A in embryonic stems cell and its presence in tumor cell lines was consistent with the characteristics of onco-fetal genes. Thus, constitutive expression of the prototype onco-fetal gene, P1A, in immortalized cells might thereby contribute to their capacity for cell transformation. The phenotypes of P1A Tg mice suggested that P1A was indeed involved in tumorigenesis. First, we observed that P1A Tg mice developed thymic leukemia after 7 months of age in immunocompromised mice (Fig 2-2). Second, we demonstrated higher BrdU incorporation in P1A Tg BM cells compared to WT cells in vivo. Third, there was more colony-forming cell growth from P1A Tg BM progenitors compared to control in vitro.

Rag2 deficiency increased tumor formation and incidence in our P1A Tg mice. Although we observed tumor formation in P1A $Rag2^{+}$ mice, the tumor incidence was not statistical significant (Fig 2-2C).

B and T lymphocyte development require assembling the germline variable (V), diversity (D) and joining (J) gene segments by a process called V(D)J recombination to form the variable regions of antigen receptors (Sleckman, 2005). Rag1 and Rag2 are the two important genes for initiation of the V(D)J recombination (Oettinger et al., 1990; Schatz et al., 1989). Therefore, mice had mutations of Rag1 or Rag2 cannot rearrange lymphocyte antigen receptors and therefore lack peripheral T cells and B cells (Mombaerts et al., 1992; Shinkai et al., 1992).

In thymus, after TCR-β gene rearrangement, the $CD4^{-}CD8^{-}$ double negative cells differentiate into $CD4^{+}CD8^{+}$ double positive thymocytes which are selected to single-

positive cells (CD4 or CD8) following TCR-α rearrangement. In bone marrow, after partial heavy-chain gene rearrangement, B cell precursors differentiate into pro-B cells; following complete heavy-chain rearrangement, pro-B cells become to pre-B cells and pre-B cells become to immature B cell after light chain gene rearrangement. However, in Rag1 or Rag2 deficient mice, T cell development is arrested at the $CD4^{-}CD8^{-}$ double negative stage; therefore, immature $CD4^{+}CD8^{+}$ double positive and mature CD4 or CD8 single positive thymocytes are absent (Mombaerts et al., 1992; Shinkai et al., 1992). In addition, B cell development is blocked at the pro-B cell stage which could express cell surface marker, B220 (Mombaerts et al., 1992; Shinkai et al., 1992).

Several clinical studies have indicated that the frequency of cancer in immunodeficient background is more frequent (Dunn et al., 2004; Hadden, 2003; Penn, 1994). For example, children with immunodeficiency are at a higher risk of malignancy, such as lymphomas and leukemias (Mueller and Pizzo, 1995). Therefore, the immune system, including innate and adaptive immunity, is able to inhibit the tumor development and eradicate the tumors already established (Dunn et al., 2004). A similar phenomenon was also shown in animal models. For example, after subcutaneous injection of the chemical carcinogen methylcholanthrene, $Rag2^{-/-}$ background mice developed higher frequency of sarcomas compared to the strain-matched controls (Shankaran et al., 2001). This suggested that the immunodeficient mice were more susceptible and vulnerable to tumor development compared to immunocompromised mice.

The tumor development is a consequence of high P1A expression in hematopoietic cell lineages. Our P1A expression was driven by the regulatory elements, immunoglobulin heavy chain (Eμ) enhancer and mb-1 promoter. The transgene promoted

by Eμ enhancer has been demonstrated in several transgenic mouse lines (Adams et al., 1985; Rosenbaum et al., 1990; Rosenbaum et al., 1989; Sun, 1994; van Lohuizen et al., 1989). Although transcriptional studies of the μ constant-region locus suggested that Eμ can activate in B cells, T cells and myeloid cells (Kemp et al., 1980). Most of tumors found in these transgenic mice driven by Eμ enhancer were B-lymphoid lineage. In the combination of mb1 promoter, P1A presumably highly expressed in the B lymphoid lineage, since transcripts of the mb1 gene are detected in the early stages of the B cell lineage, including Pro-B, Pre-B and surface IgM-positive B cells (Sakaguchi et al., 1988). Therefore, it is predictable that most of our tumor phenotype is of B cell lineage marker, B220. Since B cell development is blocked at Pro-B cell stage in Rag2 mutant mice, these tumors derived from B cell lineage at least were Pro-B cell type; whether these B cell tumors differentiated into further stage is unknown.

A high frequency of $CD11c^{+}B220^{+}$ tumor phenotype was also observed in our P1A transgenic mice. It is generally agreed that $CD11c^{+}$ and $B220^{+}$ are the markers of plasmacytoid dendritic cells (pDCs) (Nakano et al., 2001). Because pDCs carry parts of rearrangements of μ gene and express mb1 gene (Corcoran et al., 2003; Pelayo et al., 2005; Shigematsu et al., 2004), this suggests that P1A, driven by Eμ enhancer and mb1 promoter, should be activated in pDCs. In addition, although the total cellularity of nucleated cells were reduced in $Rag2^{-/-}$ BM, but the number of pDCs was within the normal range indicating the existence of pDC progenitors in $Rag2^{-/-}$ mice (Pelayo et al., 2005). Therefore, pDC tumor type was also observed in our P1A Tg mice.

Since Eμ enhancer is also active in other hematopoietic cells such as myeloid cells, the malignancies of myeloid cells are also possible (Kemp et al., 1980). In addition, the

development of myeloid/monocyte cell lineage is not affected by the lack of Rag2 function (Shinkai et al., 1992); therefore, it was not surprising to observe the myeloid cell tumor phenotype ($Gr1^+CD11b^+$). Moreover, it has been known that $CD4^-CD8^-$ double negative thymocytes contain a mixture of lineage-committed progenitors which can reconstitute T, B, NK and dendritic cell lineages (Kawamoto et al., 1998; Shortman and Wu, 1996; Wu et al., 1991). Our transgene P1A was expressed during hematopoietic progenitor development; therefore, any stage of cells may be the targets of the transformation or immortalization process by P1A activation. The heterogeneity was probably the result of tumor initiation at different stage of progenitor cell development. Hence, P1A induced tumorigenesis is not stage-specific and resulted in polyclonal and various thymic tumor types in mice, such as B cells ($B220^+$), plasmacytoid dendritic cells ($B220^+CD11c^+$) and myeloid cells ($Gr1^+CD11b^+$).

The long latency of more than 7 months to develop thymic leukemia in P1A Tg mice suggests that high expression of P1A itself is not sufficient to induce the fully malignant phenotype, but additional events might be required for full malignant transformation. Therefore, the P1A Tg mice expression of P1A might only reflect a single step in the multiple stages of tumorigenesis. The occurrence of secondary mutation might be elevated by the increase of cell proliferation in which the genetic mutation is happened more frequently.

In Kindler's study, when bone marrow cells which overexpresses oncogenic K-Ras were transplanted into wild-type mice. These mice developed $CD4^+CD8^+$ double-positive T-cell leukemias (Kindler et al., 2008). Interestingly, there were about 50% of these mice also bearing Notch1 mutations.

The Notch1 gene encodes a transmembrane receptor expressed on T cells (Felli et al., 1999). After binding to its ligands Delta1 or Jagged1, γ-secretase proteolytic cleavage is activated and then the intracellular Notch domain is released. Next, the intracellular Notch domain translocates to the nucleus and, where it regulates gene transcription (Fortini, 2002). The PEST domain is the negative regulator of intracellular Notch1 (Chiang et al., 2006). Therefore, loss of the PEST domain will increase the stabilization of intracellular Notch1 domain. It has been shown that more than 50% of human and mouse T-cell acute leukemias harbor Notch1 mutations in the heterodimerization domain and/or the PEST domain (O'Neil et al., 2006; Weng et al., 2004). In addition, development of double-positive T-cell leukemia in bone marrow has been demonstrated after expression of active Notch1 in hematopoietic stem cells (Allman et al., 2001).

We sought to determine whether Notch1 mutation also occurred in our P1A mouse tumors. We sequenced Notch1 in heterodimerization and PEST domains in seven P1A frozen tumor samples that were collected from 7 different P1A Tg mice in $Rag2^{-/-}$ background. No mutation was detected in heterodimerization domain in our tumor samples; however, we detected one tumor that harbored mutations in the PEST domain of Notch1. The phenotype of this Notch1 mutation P1A tumor is $CD4^{+}CD8^{+}$ double positive in $Rag2^{-/-}$ background. However, Allman et al. have demonstrated that expression of active Notch1 in hematopoietic stem cells from $Rag2^{-/-}$ failed to develop double positive T cells in the bone marrow (Allman et al., 2001). Therefore, the development of the $CD4^{+}CD8^{+}$ double positive population through Notch1 is dependent on T cell signaling. Thus, it is possible that, in this tumor sample, another mutation is required or P1A is able to compensate for the lack of T cell signaling.

To study the development of lymphocyte in vivo, one method is transfecting the constructs into $Rag1^{-/-}$ embryonic stem cells which are consequently injected into the $Rag2^{-/-}$ blastocyst and then the blastocyst is implantated into foster mothers (Chen et al., 1993). The lymphocytes in the somatic chimeras are all derived from $Rag1^{-/-}$ ES cells. As expected, lymphocyte development in the $Rag^{-/-}$ somatic chimeras is still blocked at the $CD4^{-}CD8^{-}$ stage in T cell and pro-B cell stage in B cells. Using this system, Swat et al. demonstrated that expression of activated Ras (Ha-Ras^{v12}) driven by the Lck promoter in T cell lineage is able to differentiate $CD4^{-}CD8^{-}$ thymocytes into $CD4^{+}CD8^{+}$ thymocytes in vivo (Swat et al., 1996). On the other hand, expression of Ha-Ras^{V12} driven by Eμ enhancer in B cell lineage cells showed some pre-B cells in $Rag^{-/-}$ chimera mice (Shaw et al., 1999). Therefore, in Rag deficient mice, the block in T and B cell development can be overcome by the introduction of activated Ras. Hence, it is possible that activation of Ras may be involved in our P1A tumors. In addition to Ras activation, other mutations that have been demonstrated to develop double positive T cells in $Rag2^{-/-}$ mice in vivo were deficiency of P53, Atm and the histone H2A variant (H2AX) gene (Liao et al., 1998; O'Neil et al., 2006; Petiniot et al., 2002).

In Luo's study, mouse survival rate was reduced and the intestinal tumor incidence was increased when conditional expression of mutated K-ras^{v12} in conjunction with DNA mismatch repair deficiency (Luo et al., 2007a). By RT-qPCR analysis, the expression of P1A was increased by 7.3 fold in large intestinal adenomas from K-ras^{v12} overexpressed mice compared to control (Luo et al., 2007a). In another study, Luo et al. studied the Ras gene by using ES cell lines. From their gene expression profiles by cDNA microarray, the results showed P1A was 4.3 fold up-regulated in the K-ras^{v12} expressing

ES cells without endogenous K-ras expression relative to WT ES cells (Luo et al., 2007b). The K-ras^{v12} expressing ES cells showed an increase of undifferentiated, alkaline phosphatase-postive, stem cells (Luo et al., 2007b). According to their data, it seems that P1A was a downstream effector of K-ras and activated by active K-ras^{v12}.

However, after we ectopically expressed K-ras^{v12} in NIH3T3 cells, a mouse fibroblast cell line, P1A was not induced, or it was undetectable by RT-qPCR. Although the results seem to contradict Luo's studies, this might be explained by the use of different cell types. Luo detected P1A mRNA in ES cells and adenoma tumor tissue which has already shown P1A expression (ES cells) or P1A expression could be predicted (tumor tissue). Since NIH3T3 is just a normal mouse fibroblast cell line, it is possible that activated K-ras alone in normal cells is not enough to activate P1A gene. Therefore, the activation of P1A gene may require other factors. It is well known that gene expression could be controlled by chromatin structure which can be affected by the modifications of the histones and of CpG methylation. For example, Wischnewski et al. demonstrated that activation of MAGE genes results from a demethylation and histone acetylation of their promoters (Wischnewski et al., 2006). The expression of human MAGE gene, like mouse P1A gene, is restricted to the testicular germ cells and different human tumor types (Itoh et al., 1996). Thus, to study the promoter region of P1A is a one way to address how P1A gene is regulated and controlled.

Taken together, P1A induced tumorigenesis and resulted in polyclonal and various thymic tumor types in mice. Most of the P1A tumors examined displayed the B cell lineage marker, B220, because P1A transgene expression was driven by the regulatory elements Eμ enhancer and mb-1 promoter, which were activated in B cell

lineage. One tumor displayed T cell lineage markers harbored a mutation in the Notch1 gene which is involved in T cell leukemia. Our present work demonstrated that onco-fetal antigen P1A was associated with the transformed phenotype in P1A transgenic mice. Further studies are required to dissect the mechanisms of the process of oncogenesis by P1A.

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CHAPTER 3

Interaction between P1A and vesicle trafficking proteins

Abstract

The tumor rejection antigen P1A is normally expressed in fetal tissue and is aberrantly expressed in multiple lineages of tumors. The data presented in chapter 2 indicate a significant role for P1A in promoting tumorigenesis. However, how P1A mediates tumorigenesis is not understood. In order to address this question, we investigated possible protein partners of P1A in cells. Mass spectrometry analysis of P1A interacting proteins identified several vesicle trafficking proteins, including RalA, IQGAP1, Rac1 and AP2. Sucrose gradient ultracentrifugation indicated that P1A and IQGAP1 had similar sediment properties and thus were likely associated with each other. In addition, deletion analysis indicated that N-terminal P1A was the major site to bind C-terminus of IQGAP1. Confocal microscopy revealed co-localization of P1A and RalA in intracellular vesicles. Interestingly, shRNA silencing of P1A in the murine mastocytoma cell line P815 dramatically altered the distribution of RalA without affecting its expression and GTP binding. Furthermore, secretion of TNFα, IL-6 and β-hexosaminidase was reduced after P1A knockdown in P815 cells. The functional

association between P1A and vesicle trafficking machinery may explain efficient priming of P1A-reactive T cells and the role for P1A in the tumorigenesis.

Introduction

P1A is a prototype for onco-fetal proteins that is expressed in several neoplastic and embryonic cells, whereas it is down regulated in adult tissues except for the testis and placenta (Ramarathinam et al., 1995; Uyttenhove et al., 1997). P1A was identified as a tumor antigen from the mouse mastocytoma P815 cell line which is derived from a tumor induced by methylcholanthrene in DBA/2 mice (Van den Eynde et al., 1991). Since P1A is re-expressed in tumors, it may contribute to malignant transformation. Consistent with this notion, I have presented data in Chapter 2 that demonstrated increased tumorigenesis in P1A transgenic mice. However, the mechanisms by which P1A is promoting or sustaining the transformed cell phenotype remain unknown.

As reported by Amar-Costesec et al., P1A is associated with the membranes of secretory pathways, and P1A expression is particularly intense in small vesicles including the endoplasmic reticulum, Golgi complex and post-Golgi vesicles (Amar-Costesec et al., 1994). In addition, Altieri et al. documented P1A in the isolated exosomes from J558 plasmacytoma (Altieri et al., 2004). Exosomes are small vesicles externalized by exocytosis (Lakkaraju and Rodriguez-Boulan, 2008). A tumor antigen must be transferred from a tumor into professional antigen-presenting cells, like dendritic cells, in order to activate tumor-specific T cells (Heath and Carbone, 1999; Shen and Rock, 2006). In addition, activation of vesicle trafficking machinery is associated with tumorigenesis

(Camonis and White, 2005; Huber et al., 2005; Issaq et al., 2010; Palmer et al., 2002; Sakurai-Yageta et al., 2008). Based on these considerations and the subcelluar localization of P1A, we hypothesize that the P1A protein regulates the secretory pathway or membrane trafficking by interaction with vesicle trafficking proteins.

The key to understanding P1A function is to identify its binding partners. One strategy for the identification of interacting partners is co-immunoprecipitation of P1A-associated proteins in combination with mass spectrometry. In this chapter, I present evidence that P1A interacts with several vesicle trafficking proteins, including RalA, AP2, IQGAP1 and Rac1. In addition, cytokine and lysosomal enzyme secretion is decreased when the P1A gene is silenced by shRNA in P815 mastocytoma. The data suggest that one of the biological functions of P1A protein is to regulate exocytosis pathway by interaction with vesicle trafficking proteins.

Materials and Methods

Cell culture and transfection

Murine mastocytoma P815 cells from ATCC were cultured in RPMI1640 medium with 10% fetal bovine serum (FBS), 100 U penicillin and 100ug/ml streptomycin. Human embryonic kidney 293T cells from ATCC were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units of penicillin, and 100 μg/ml of streptomycin and grown at 37 °C in 5% CO_2. Lipofectamine 2000 (Invitrogen) was used to transfect 293T cells.

Mass spectrometry

P1A protein was fused to two affinity tags in pCTAP-A vector (Stratagene): a streptavidin-binding peptide and a calmodulin-binding peptide, using Mammalian Tandem Purification Systems (Stratagene), following the manufacturer's instructions. The P1A interacting proteins were co-purified in a two-step purification process with wash and elution. After gel electrophoresis, the P1A protein complexes were submitted to Taplin Spectrometry Facility at Harvard Medical School for high throughput analysis.

Plasmid Construction

Full length P1A, N-terminal P1A (1-122 a.a.) or C-terminal P1A (123-224 a.a.) was inserted into pcDNA6 vector between BamHI and XhoI sites. All P1A peptides were Myc-tagged. IQGAP1-N1 (1-570 a.a.), IQGAP1-N2 (1-722 a.a.), IQGAP1-N3 (1-863 a.a) or IQGAP1-C (864-1657 a.a.) was inserted between SalI and ApaI sites in pCMV-Tag2B vector. All of IQGAP1 peptides were flag tagged. The selected regions of IQGAP1 and P1A were produced by PCR. The sequence of all constructs was confirmed by both restriction mapping and DNA sequencing. Plasmids were purified with a QIAprep Spin miniprep kit according to the manufacturer's instructions.

Immunoprecipitation

293T cells were transfected with full length P1A, N-terminal P1A, C-terminal P1A, IQGAP1-N1, IQGAP1-N2, IQGAP1-N3, or IQGAP1-C. After 48 hours, cells were washed twice with ice-cold phosphate-buffered saline, lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% n-octyl-d-galactopyranoside) containing a protease inhibitor mixture (Sigma), including 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin, E-64, leupeptin and pepstain A, and subjected to

centrifugation at 15,000 × g for 10 min at 4 °C to remove the debris. Supernatants were pre-cleared with protein G-Sepharose (GE Healthcare) for 30 min at 4 °C. Anti-myc antibody was incubated in lyaste overnight at 4°C, then with protein G-Sepharose beads for 2 hr. The complexes were sediment by centrifugation, washed five times with buffer A, and heated for 5 min at 95°C in 2X sample buffer. Sample was then analyzed by Western blotting.

Active RalA pull down assay

The active form of RalA protein was studied by active RalA pull-down and detection kit (Thermo Scientific), following the manufacturer's instructions. Lysates containing 400 μg of protein were incubated for 1 hr at 4°C with glutathione resin and 200 μg recombinant glutathione *S*-transferase (GST) or GST-RalBP1-PBD. The resin was then washed 3 times with lysis buffer, and the samples were eluted by 2X reducing sample buffer. Samples were heated and eluted for 5 min at 95°C and then analyzed by Western blotting.

Western blot analysis

Samples were lysed in protein lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% n-octyl-d-galactopyranoside) and protease inhibitor cocktails (Sigma) including 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin, E-64, leupeptin and pepstain A were added. Cell lysates or immunoprecipitates were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and incubated with the corresponding antibodies including anti-P1A rabbit antibody (Genemed Synthesis, 1:500 dilution), anti-actin mouse antibody (Sigma, 1:5000 dilution), anti-RalA mouse antibody (Thermo Scientific,

1:1000 dilution), anti-IQGAP1 rabbit antibody (Santa Cruz Biotechnology, 1:1000), anti-Rac1 mouse antibody (Thermo Scientific, 1:1000) and anti-α-AP2 mouse antibody (Santa Cruz Biotechnology, 1:500). Anti-rabbit or anti-mouse IgG horseradish peroxidase–linked antibody at 1:3500 dilution (GE Healthcare) was used as a secondary antibody. Antibodies were detected with chemiluminescence reaction using the enhanced chemiluminescence kit (Amersham Biosciences) and visualization with exposure to film.

Sucrose gradient assay

The following sucrose solutions in 50 mM Tris-HCl buffer (pH 7.5) were prepared: 45% (0.25 ml), 40% (0. 5 ml), 37.5% (0.75 ml), 35% (1 ml), 30% (1ml) and 25% (1ml). First, the 45% sucrose solution was loaded into a Beckman polyallomer tube, and then the procedure was continued with the 40 %, 37.5 %, 35 %, 30% and 25 %, respectively. Finally, 0.5 ml P815 cell lysate was added on the top of the prepared sucrose gradient. The tube was centrifuged for 17 hr at 170000 g (37500 rpm) at 4℃ in Centrifuge SW55.1. A tiny hole was introduced into the very bottom of the tube using a fine needle. A total of 22 fractions of equal volume were then collected and the fractions were electrophorized and analyzed by Western blotting.

Immunofluorescence

P815 cells were placed on glass microslides the previous day, then fixed and permeabilized by cold acetone/methanol at -20°C for 15 min. After washing, the slides were incubated with rabbit anti-P1A (Genemed Synthesis, 1:200) and goat anti-RalA (Santa Cruz Biotechnology, 1:200) antibody overnight, washed, followed by anti-rabbit Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 568 donkey anti-goat IgG

secondary antibody (Invitrogen, 1:2000) for 1 hr at room temperature. The protein co-localization was imaged by the Zeiss LSM 510 confocal microscope.

P1A silencing

P1A shRNA (sense: GATTACACTTGTACCTGTT; antisense: AACAGGTACAAGTGTAATC) and a scrambled-sequence-insert non-silencing negative control of mouse GIPZ lentiviral shRNAmir vectors were purchased from Open Biosystems. Lentivirus was made by transfecting 293T cells with the pGIPZ vector and the second generation packaging plasmids, pMD2.G and psPAX2. Forty-eight hours after transfection, lentiviral supernatant was collected and filtered through a 0.45-μm filter. The filtered supernatant was used at 1 to 1 ratio with fresh media to infect P815 cells with 10 μg/ml polybrene. After 8 hours incubation, the media containing lentiviral particles were removed and fresh media were added to the P815 cells. The stable single clone was selected with 10 μg/ml puromycin in culture medium.

Cell adhesion and migration assay

The laminin–coated and fibronectin-coated 48 well CytoSlelect cell adhesion assay (Cell Biolabs) was used according to manufacturer's instructions. Briefly, P815 cells were seeded onto the coated substrate, where the adherent cells were attached. Next, unbound cells were washed away, and the adherent cells were fixed and stained. Finally, the stain was quantified at OD at 560 nm after extraction by the extraction buffer. For the migration assay, the QCM 5 μm Chemotaxis assay (Millipore) was used. Cells were seeded into a migration chamber and medium with 10 % FBS were added to the lower chambers of the plate. The chamber was incubated for 24 h at 37°C in a CO_2 incubator, to allow cell migration through the membrane. Next, the insert was placed into the stain

buffer for 20 min at room temperature. After washing the inserts with water several times, cells remaining in the upper chamber were removed. The inserts were transferred to a new well containing extraction buffer at room temperature for 15 min. The extraction was quantified at OD 560 nm.

Cytokine and β-hexosaminidase measurement in P815 cells

In a 96 well plate, 2 x10^5 of P815 or P1A knocked down P815 cells were seeded. After 4 hr culture, the cell supernatant and cell lysates (cell pellet in 0.2% Triton X-100) were collected. The levels of TNFα and IL-6 in cell supernatant were measured by BD Cytometric Bead Array- Mouse Inflammation Kit according to the manufacturer's instructions. Briefly, cell supernatant was mixed with mouse inflammation capture bead suspension and PE detection reagent. The mixture was incubated for 2 hr at room temperature. After washing and centrifugation, wash buffer was added into each sample. The cytokine level was analyzed by BD LSR II Flow Cytometry. β-hexosaminidase is a marker of lysosomal. For β-hexosaminidase measurement, 50 ul of sample supernatant or cell lysates were transferred to a 96-well plate and 1 mM p-nitrophenyl acetyl-D glucosamine (pNAG) substrate (Sigma-Aldrich) in 0.1 M citrate buffer (pH 4.5) was added. pNAG was hydrolyzed by β-hexosaminidase and then p-nitrophenol was released. The amount of p-nitrophenol released is equal to the amount of β-hexosaminidase in the supernatant or cell lysates. After incubation for 5 hr at 37°C, 0.2 M glycine (pH 10.4) was added to stop the reaction. The absorbance was read at 405 nm.

Isolation and culture of mast cell

Bone marrow cells isolated from the femurs of P1A Tg and control mice were cultured in complete RPMI medium containing 10% FBS, 25 mM HEPES (pH 7.4), 1

mM sodium pyruvate, 100 μM nonessential amino acids (glycine, alanine, asparagines, aspartic acid, glutamic acid, proline and serine), 50 μM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin with 30 ng/ml IL-3 (Peprotech) at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was changed every 3 days. The bone marrow derived mast cells (BMMCs) were developed after culture for 4 week.

Measurement of Degranulation

Release of granules was determined by measuring the release of β-hexosaminidase. Briefly, 4-week-old BMMCs (5x 10^6/ml) were sensitized with 0.5 μg/ml anti-DNP IgE (Sigma-Aldrich) in culture medium for 4 hr at 37°C. Next, sensitized cells were washed 2 times with Tyrode's buffer and stimulated with 50 ng/ml of DNP-HSA (Sigma-Aldrich) for 50 min at 37°C. After stimulation, cells were placed on ice for 10 minutes and centrifuged. The cell pellet was solubilized with 0.2 % Triton X-100. The enzymatic activities of β-hexosaminidase were determined as previous described and calculated according to the equation: % specific release = ($release_{stimulated}$ -$relese_{spontaneous}$)/ ($content_{total}$) x 100.

Real-time reverse transcription quantified PCR

Total RNA was isolated from the P1A Tg BMMCs and control BMMCs using the QIAamp RNA minikit (Qiagen). All RNA samples were treated with DNase I (Invitrogen) to remove any genomic DNA and the cDNA was synthesized using the SuperScript first-strand synthesis system kit (Invitrogen). Next, the amount of cDNA was analyzed by Applied Biosystems 7900HT fast real-time PCR using SYBR green PCR master mix kits (Qiagen). The relative expression of P1A (forward: 5'-AGAGATGAGCGTGGAAATGG-3'; reverse: 5'-CAGGAAATTAGGGTCGTGGA-3')

was calculated and HPRT gene as an internal control (forward: 5'-CAGGCCAGACTTTGTTGGAT-3'; reverse: 5'- GCGCTCATCTTAGGCTTTGT-3').

Statistics

P-value was calculated using Student's t-test. The data are presented as mean ± standard deviation (SD).

Results

Tandem affinity purification-based identification of P1A-associated proteins

In order to identify P1A-associated proteins, we fused P1A protein to two tandem affinity tags, a streptavidin binding peptide and a calmodulin binding peptide, and then introduced this construct into 293T cells. Next, cell extracts were prepared and the fusion P1A protein as well as associated partners were recovered by two specific affinity purification and elution steps. Finally, the P1A binding partners were defined by the mass spectrometry peptide analysis. There were around 300 proteins identified in the mass spectrometric results. The chosen criteria were based on the peptide sequence that was matched to an already known protein related to exocytosis or endocytosis, and the number of peptide matches to that protein should be more than 3. Mass spectrometric results revealed that there were several peptides matching with membrane trafficking related protein sequences, such as RalA, several subunits of AP2, β subunit of AP1, and IQGAP1. The number of peptide matches and the corresponding name of protein were indicated in Table 3-1.

Table 3-1. The number of matched peptide sequences from mass spectrometry

Protein	Peptide match
RalA	10
AP2A1	11
AP2M1	10
AP2A2	8
AP2B1	8
AP1B1	7
IQGAP1	4

P1A interacted with RalA, AP2, IQGAP1 and Rac1 were confirmed by co-immunoprecipitation

Next, co-immunoprecipitation was used to confirm the results from mass spectrometry. First, AP2 was detected in the immunoprecipitates from P815 cells (endogenous P1A) and 293T cells (ectopic P1A), after co-immunoprecipitated with anti-P1A antibody but not with IgG control antibody (Fig 3-1AB). Second, we also showed that Rac1 was detected in p815 lysates after co-immunoprecipitated with anti-P1A antibody but not with control IgG antibody (Fig 3-1C). Moreover, examination of the lysate from p815 and $p815^{P1A-}$ revealed that IQGAP1 were immunoprecipitated with anti-P1A antibody and P1A were immunoprecipitated with anti-IQGAP1 antibody (Fig 3-2). Furthermore, P1A, AP2, IQGAP1 were all pulled down together with the active RalA by the GST-Ral binding protein 1 protein-binding domain (GST-RalBP1-PBD) protein which specifically pull-down active RalA (Cantor et al., 1995), but not by the control GST protein in P1A Tg thymus (Fig 3-3). Collectively, these data indicate that P1A interacted with AP2, IQGAP1, Rac1 and active form of RalA.

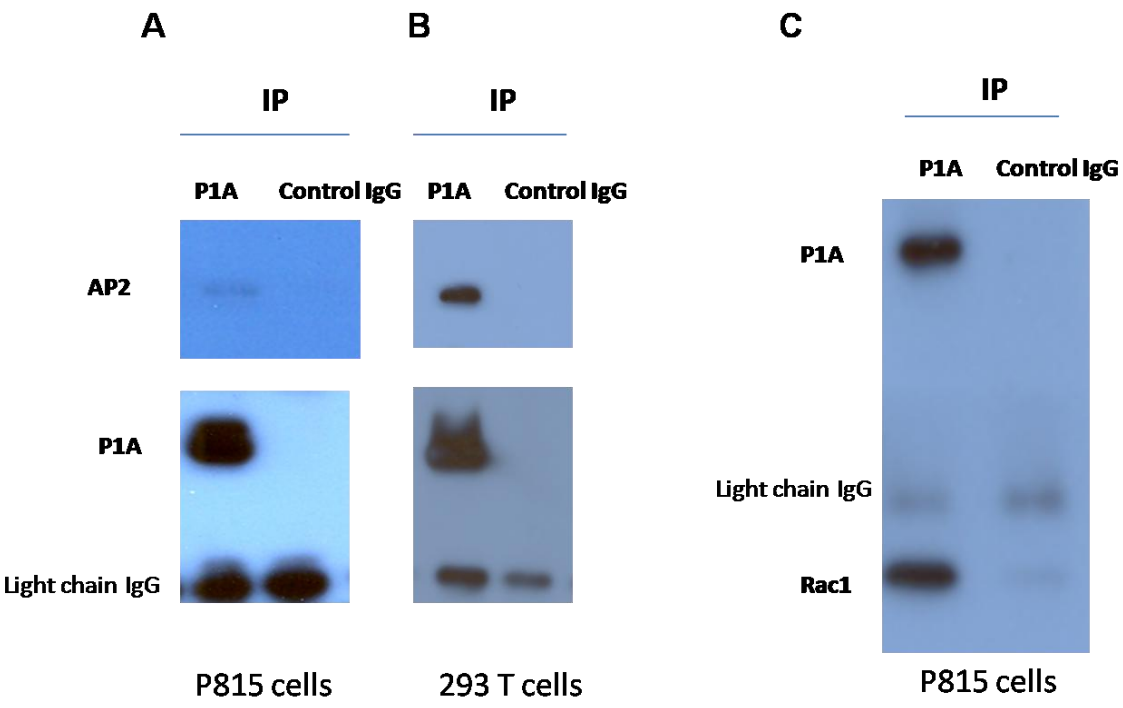


Figure 3-1. AP-2 and Rac 1 co-immunoprecipitated with P1A

A. AP-2 protein was co-immunoprecipitated with anti-P1A antibody in P815 cell lysates but not in IgG control antibody. B. 293T cells were transiently transfected with Myc-tagged P1A. After 48 hrs, cells were lysed and the protein lysates were immunoprecipitated with anti-P1A antibody or control IgG. AP-2 protein was only detected after P1A precipitated. C. Rac-1 protein was co-immunoprecipitated with anti-P1A antibody in P815 cell lysates but not in control IgG antibody.

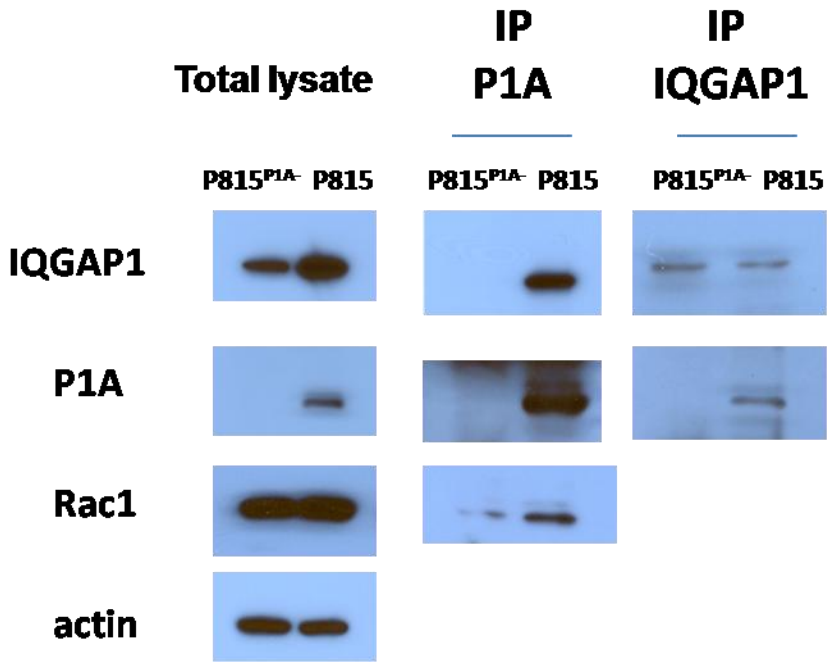


Figure 3-2. IQGAP1 co-immunoprecipitated with P1A in P815 cells

IQGAP1 immunoprecipitated with anti-P1A antibody in P815 cells but not in $P815^{P1A-}$ cells. In addition, P1A co-immunoprecipitated with anti-IQGAP1 antibody in P815 cells but not in $P815^{P1A-}$ cells. Rac1 was also co-immunoprecipitated with anti-P1A antibody in P815 cell lysates.

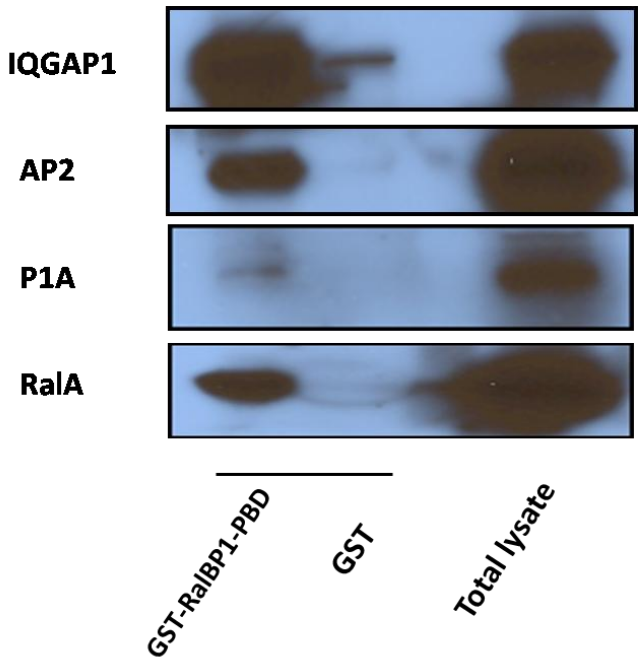


Figure 3-3. Active RalA pull down assay

GST-RalBP1-PBD protein precipitated the active form of RalA. GST protein was used as control. P1A, AP-2, IQGAP1 proteins were pulled down together in the active RalA protein complex from P1A Tg thymus lysates.

Co-sedimentation of P1A and IQGAP1 during sucrose gradient centrifugation

Sucrose gradient ultracentrifugation was used to determine whether P1A and IQGAP1 had similar co-sedimentation properties. We layered the P815 cell lysate on a sucrose gradient from 25% to 45%, and the proteins of different sedimentation properties separated from each other during the ultracentrifugation. After ultracentrifugation, a hole was punched in the bottom of the centrifuge tube, fractions were collected, and the amount of P1A and IQGAP1 was analyzed in each fraction by western blotting (Fig 3-4A). As shown in Fig 3-4B, P1A and IQGAP1 have peaks in the same gradient fraction. Therefore, P1A and IQGAP1 must have concentrated on the same complex.

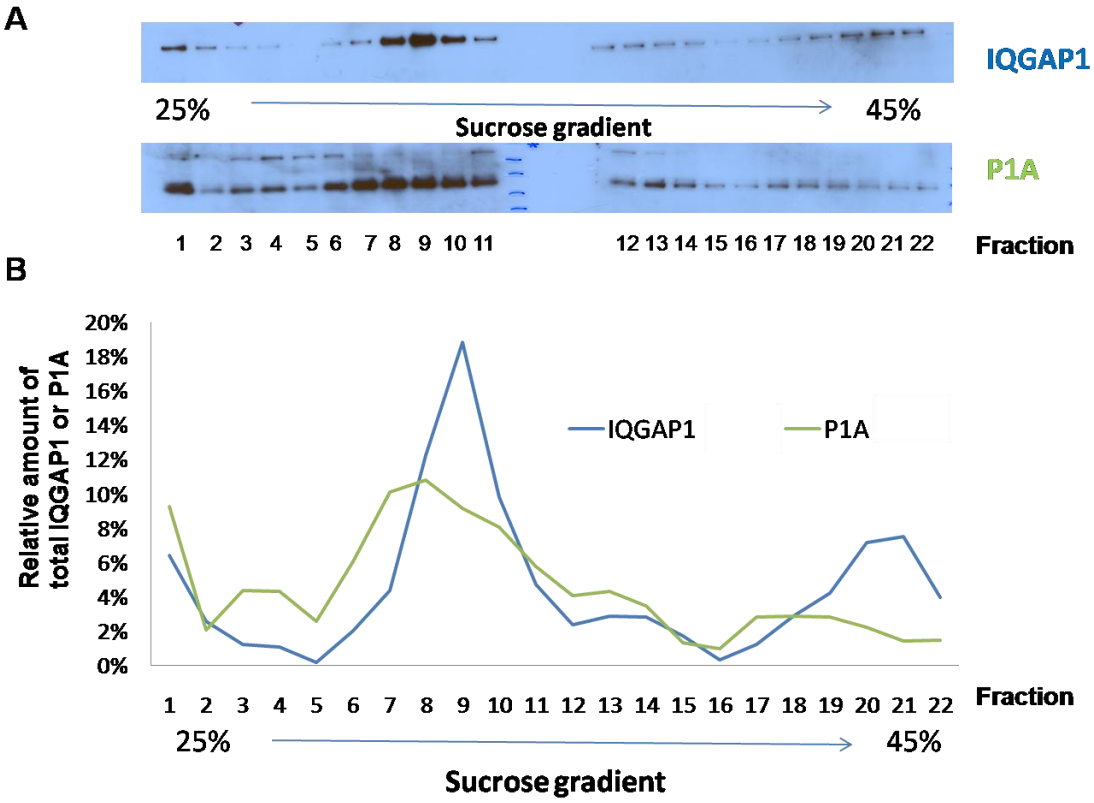


Figure 3-4. Sucrose gradient assay

P815 cell lysates were layered on a sucrose gradient from 25% to 45%. After ultracentrifugation, 22 fractions were collected. A. The amount of P1A and IQGAP1 in each fraction was analyzed by western blotting and each lane represented one fraction. B. The relative amounts of total IQGAP1 and total P1A in each fraction (in A) were determined by densitometry.

N-terminus P1A bound C-terminus IQGAP1

Immunoprecipitation was used to examine whether N-terminus P1A (P1A-N) and C-terminus P1A (P1A-C) bind endogenous IQGAP1 in cells. Myc tagged P1A-N or P1A-C or full length P1A (P1A-F) was transfected into 293T cells, and lysates were immunoprecipitated with anti-Myc antibody. Probing immunoprecipitates with anti-IQGAP1 antibody revealed that P1A-N and P1A-F co-immunoprecipitated with endogenous IQGAP1. However, a very small amount of IQGAP1 protein was co-immunoprecipitated with P1A-C (Fig 3-5). The data suggested that P1A bound to IQGAP1 though the N-terminal site.

Further analysis was performed to narrow the binding portion of IQGAP1 to P1A. Different regions of IQGAP1 were tagged with Flag peptides, termed IQGAP1-N1 (1-570 a.a.), -N2 (1-722 a.a.), -N3 (1-863 a.a.), -C (864-1657 a.a.) as noted in Fig 3-6. Myc tagged P1A-F and different portion of Flag-tagged IQGAP1 were co-transfected into 293T cells. After the lysates pulled down with anti-Myc antibody, the immunoprecipitates were probed with anti-Flag antibody. The results showed IQGAP1-N3 and-C, but not –N1 and-N2, bound to full-length P1A (Fig 3-7). These data revealed that C-terminus of IQGAP1 (733-1657 a.a.) was necessary for the interaction of P1A.

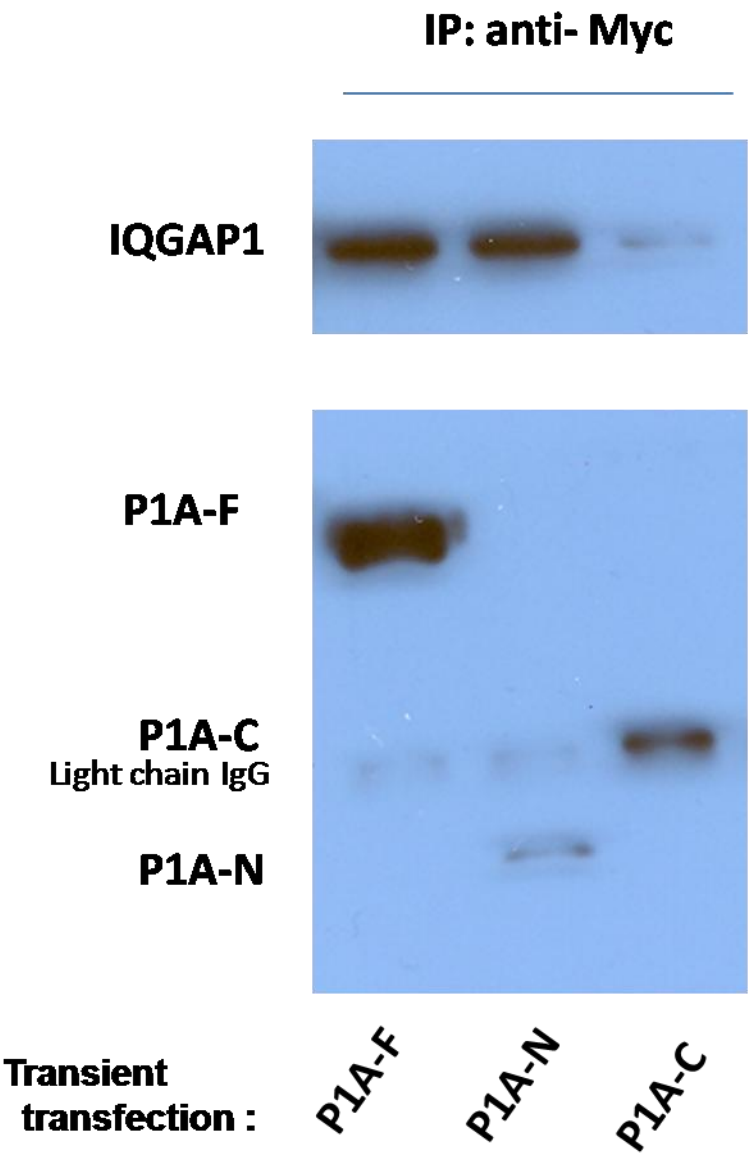


Figure 3-5. Endogenous IQGAP1 co-immunoprecipitated with N-terminal P1A

293T cells were transiently transfected with Myc-tagged full length P1A (P1A-F), Myc-tagged N-terminal of P1A (P1A-N), or Myc-tagged C-terminal of P1A (P1A-C). After 48 hrs, cells were lysed, and lysates were co-immunoprecipitated with anti-Myc antibody, followed by separation on SDS-PAGE. After transfer to PVDF, blots were probed with anti-IQGAP1 antibody and anti-Myc antibody.

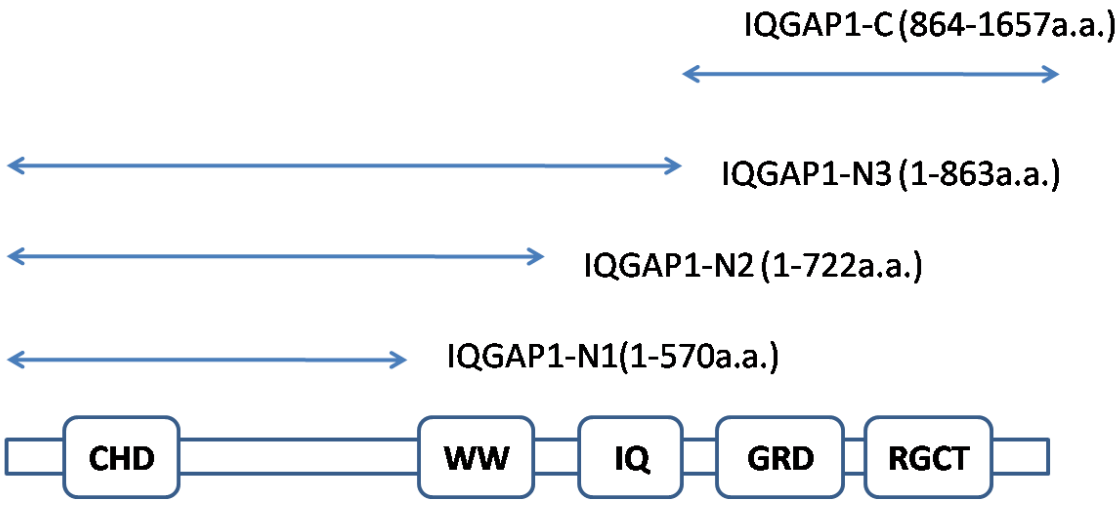


Figure 3-6. A schematic representation of IQGAP1 constructs

Different regions of IQGAP1 construct are illustrated. CHD: calponin homology domain. WW: poly-proline protein-protein binding domain. IQ: calmodulin binding IQ domain. GRD: Ras GTPase-activating proteins-related domain. RGCT: RasGAP C-terminus. IQGAP1-N1 included CHD domain; IQGAP-N2 included CHD and WW domain; IQGAP-N3 included CHD, WW and IQ domain; IQGAP-C included GRD and RGCT domain.

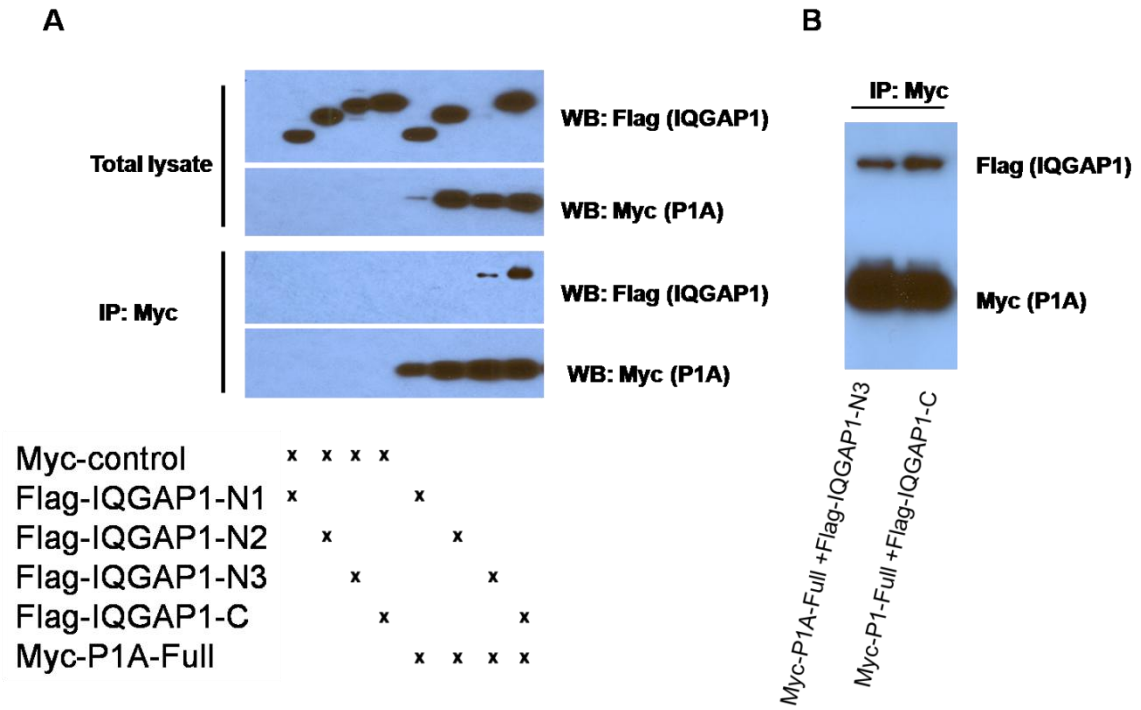


Figure 3-7. IQGAP-C and IQGAP-N3 co-immunoprecipitated with P1A

293T cells were transiently transfected with different Flag-tagged IQGAP1 constructs (Flag-IQGAP1-N1, Flag-IQGAP1-N2, Flag-IQGAP1-N3 or Flag-IQGAP1-C) and Myc-tagged full length P1A (Myc-P1A-Full) or Myc-control. After 48 hrs, cells were lysed and the lysates were co-immunoprecipitated with anti-Myc antibody, followed by separation on SDS-PAGE. After transfer to PVDF, blots were probed with anti-Flag antibody and anti-Myc antibody. A and B were two independent experiments.

P1A was co-localized with RalA and required for RalA proper localization

We used confocal microscopy to determine the co-localization of P1A and RalA. Fluorescence microscopic analysis of p815 cells showed that the distribution of P1A fully coincided with RalA and they localized on a vesicular-like network in cytoplasm (Fig 3-8A). However, after knock-down of P1A in P815 cells by P1A-shRNA lentivirus, the vesicular localization of RalA was absent (Fig 3-8B). Western blotting and active RalA pull-down assay revealed that the absence of RalA in the vesicular localization was neither due to decrease of total RalA expression nor reduction of GTP-bound of RalA (Fig 3-9A). In addition, there was also no difference of GTP-bound RalA between P1A Tg and WT mouse thymus (Fig 3-9B). Therefore, P1A knock-down caused redistribution of RalA away from the vesicular network. The data revealed that P1A was responsible for the proper localization of RalA in P815 cells.

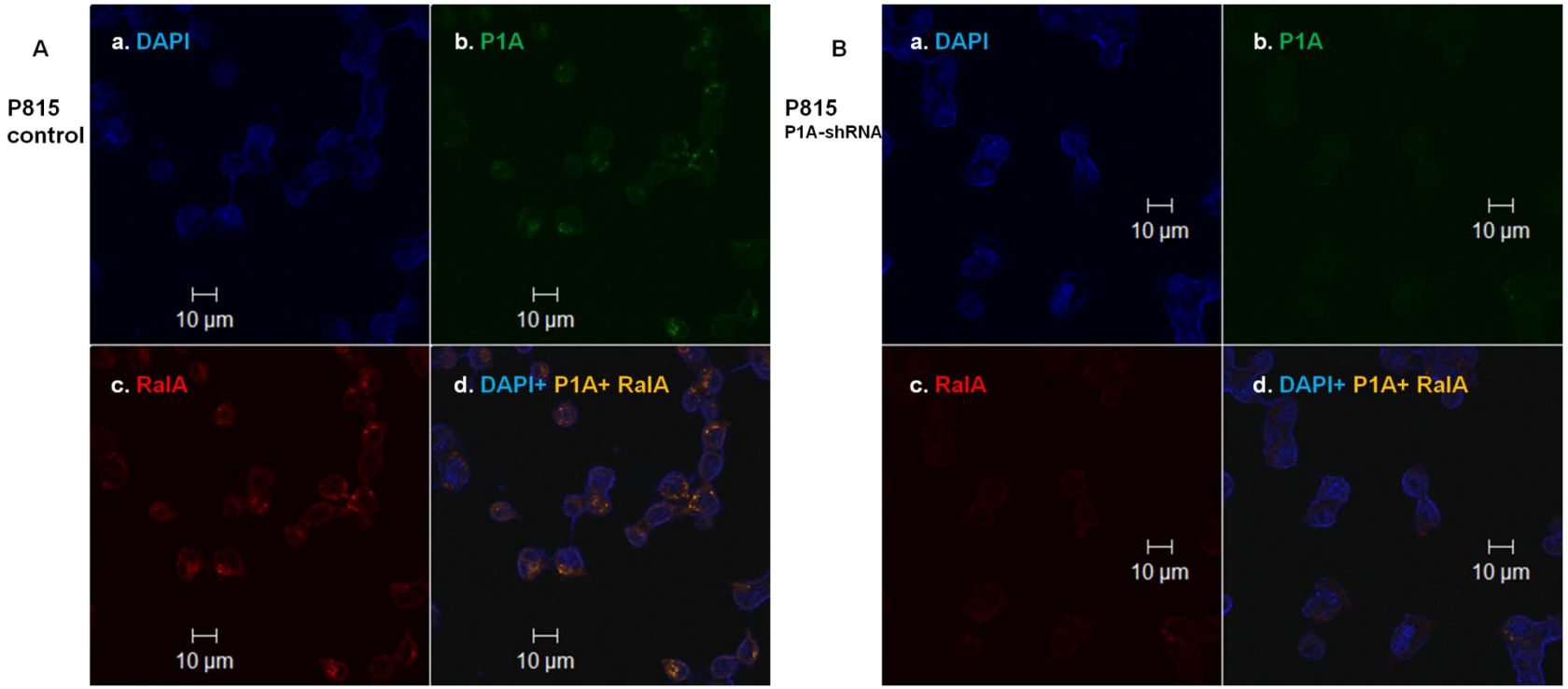
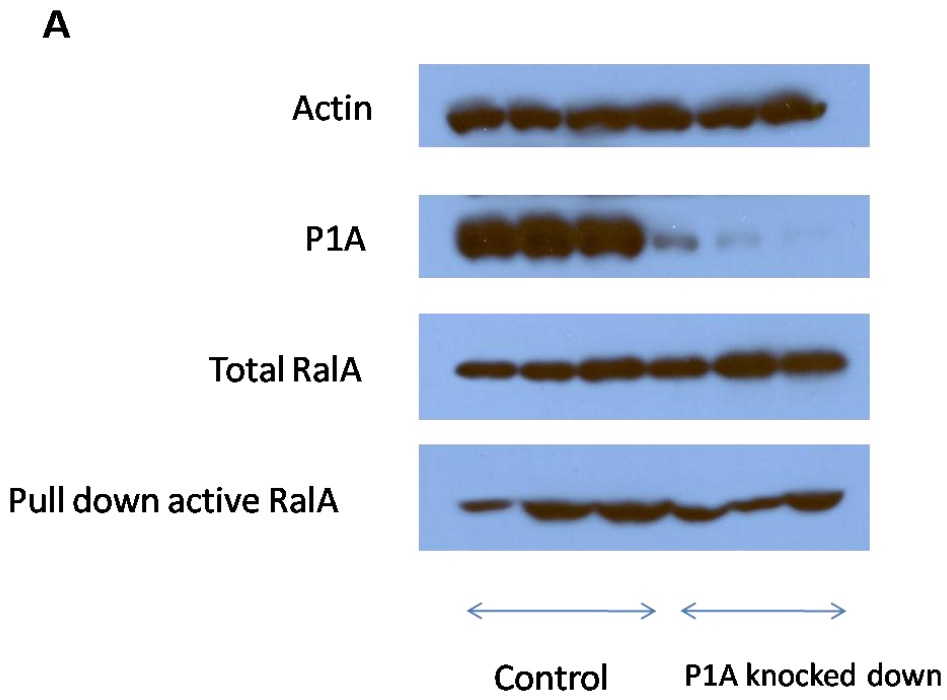


Figure 3-8. P1A co-localized with RalA and was required for proper localization of RalA

P815 control and P815 P1A-shRNA cells were placed on glass microslides the previous day, then fixed and permeabilized. After washing, cells were treated with rabbit anti-P1A antibody and goat anti-RalA antibody, and stained by Alexa Fluor® 488 goat anti-rabbit IgG and Alexa Fluor® 568 donkey anti-goat IgG. A. The distribution of P1A coincided with RalA and they localized on a vesicular-like network in cytoplasm. B. the vesicular localization of RalA were absent after knock-down P1A in p815 cells by P1A-shRNA lentivirus. a. showing DAPI signals, b. showing P1A protein signals, c. showing RalA protein signals, d. showing overlay of DAPI, P1A and RalA signal.



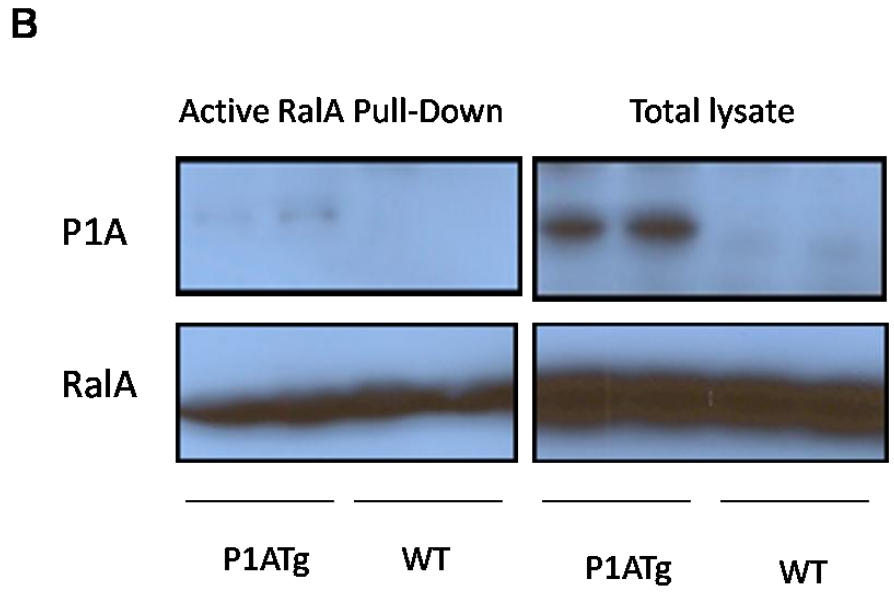


Figure 3-9. P1A not changed RalA expression and activity

A. Total RalA and active form of RalA pulled down by GST-RalBP1-PBD from control P815 and P1A knocked down P815 cells. B. Total RalA and active RalA from WT or P1A Tg thymus; western blotting showed P1A was co-precipitated from the active RalA complex.

Knock down P1A did not affect cell adhesion

Since it is well known that IQGAP1 is able to regulate cell adhesion through interactions with Rac1 (Kuroda et al., 1999), thus we used shRNA lentivirus to knock down expression of P1A in p815 cells to explore the function of P1A. We found the P1A-shRNA sequences were able to specifically inhibit expression of P1A, and more than 90% reduction of P1A was detected by western blotting (Fig 3-10A). In P815 cells with P1A expression silenced, the ability of cell attachment to laminin and fibronectin was similar between P1A knock down cells and control cells (Fig 3-10B). The data showed that P1A did not affect cell adhesion of P815 cells.

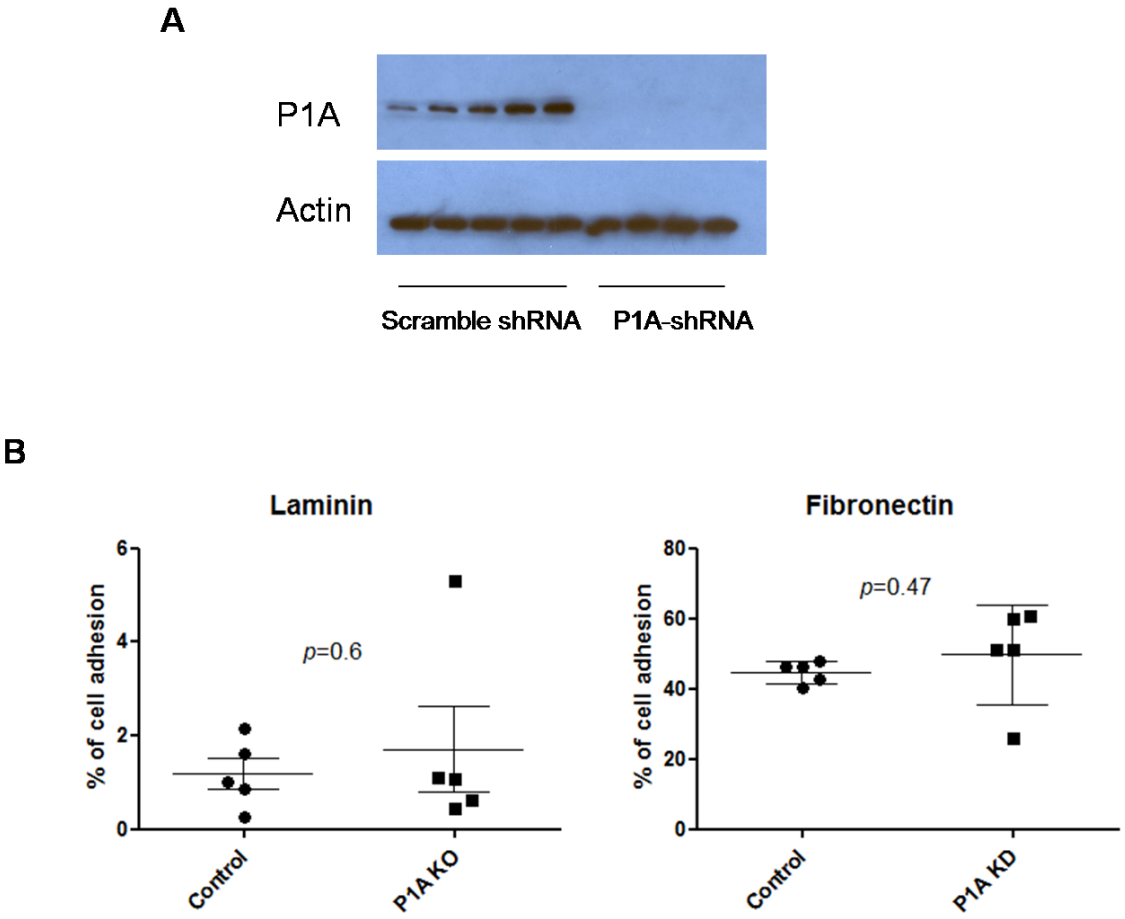


Figure 3-10. Knock down P1A had no effect on cell adhesion

A. Western blot showed P1A protein was knocked down by P1A-shRNA, not scramble shRNA in P815 single clones; actin was included as internal control. B. Five clones of control cells and 5 clones of P1A KO cells were seeded on laminin–coated and fibronectin-coated plates. Then, the adherent cells were captured and unbound cells were washed away. The adherent cells were fixed and stained. The stain was quantified at OD 560nm after extraction. Each point represents one individual clone, showing mean ± SD (Student's t-test).

P1A affected the secretory pathway

To directly examine the effect of P1A knock-down on the secretory pathway in cells, we analyzed the levels of cytokine and β-hexosaminidase in P815 cell supernatant. β-hexosaminidase is a marker of lysosomal enzyme, and there is a high fraction of lysosomal compartments in the secretory granules in hematopoietic lineage cells, such as mast cells, macrophages and cytotoxic T cells (Andrews, 2000). Relative quantification revealed that knock-down of P1A decreased the granular secretion as well as retaining more enzyme within cells compared to control (Fig 3-11A). We observed a marked decrease in TNF and IL6 levels in supernatant of P1A knock down cells (Fig 3-11B). Together these data indicate that P1A affected cell secretary pathways in P815 cells.

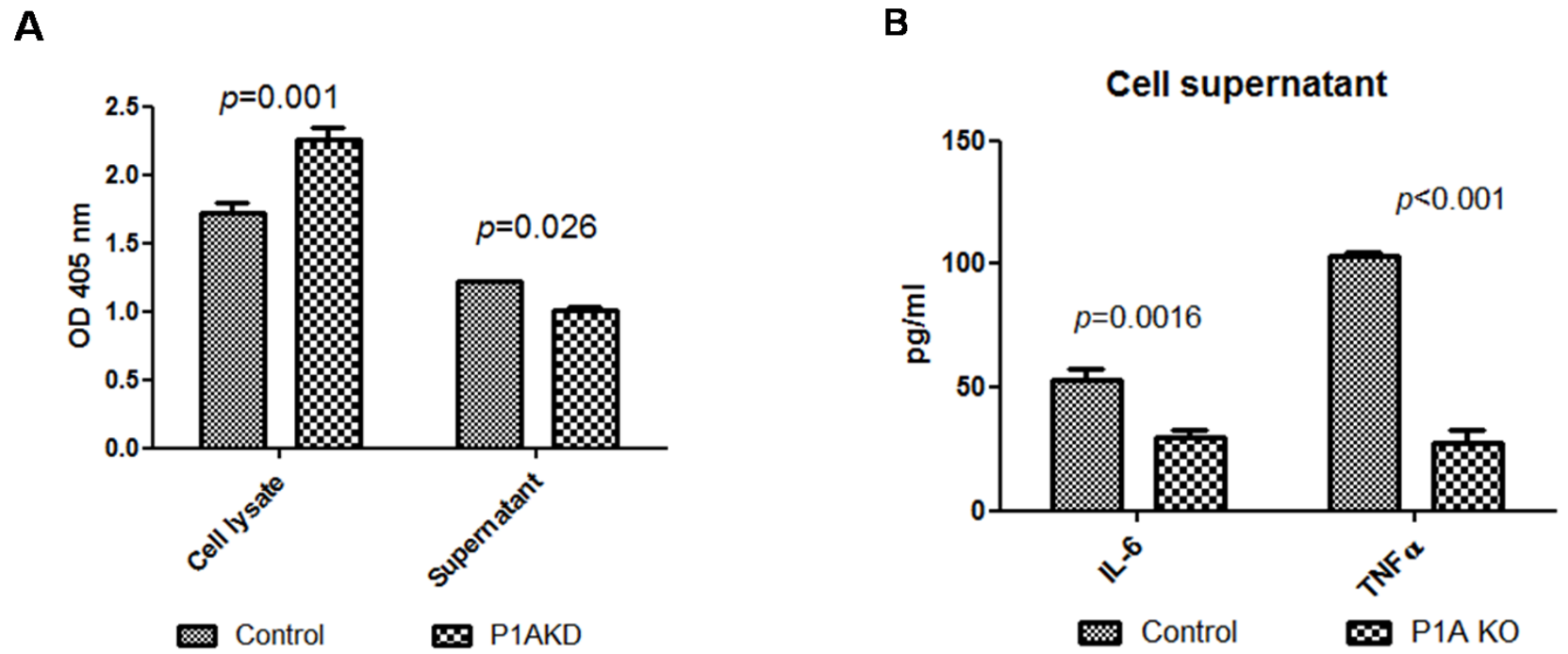


Figure 3-11. Decreased cell secretion when P1A silencing

A. β-hexosaminidase measurement. 10^6 of control or P1A knocked down P815 cells were seeded into a 96 well plate. After 4hr culture, 1 mM pNAG substrate was added into sample supernatant or cell lysates (cell pellet in 0.2% Triton X-100). After incubation for 5 hr at 37°C, 0.2 M glycine was added to stop the reaction. The absorbance was read at 405 nm. B. 2 x10^5 of control or P1A knocked down P815 cells were seeded into a 96 well plate. After 4hr culture, TNFα and IL-6 in cell supernatant were measured by BD Cytometric Bead Array. (n=3, Student's t-test).

The P815 cell line was originally established from a mastocytoma in a DBA/2 mouse (Dunn and Potter, 1957). Van den Eynde et al. showed that P1A mRNA is detectable in the mast cell line L138.8A, which was established from BALB/c bone marrow (Van den Eynde et al., 1991). The L138.8A cells are IL-3 responsive and secrete IL-6. To test whether P1A was expressed in bone marrow derived mast cells (BMMCs), we isolated bone marrow cells from control and P1A Tg mice and then cultured them in medium containing IL-3 for 4 weeks to obtain BMMCs. P1A was expressed in P1A Tg BMMCs in whole culture period (Fig 3-12A). In addition, P1A expression was detectable in control BMMCs after the first week and fourth week in culture (Fig 3-12B). This suggested that the P1A gene is at least expressed by mast cell precursors located in the bone marrow. Furthermore, 4 week old BMMCs were sensitized with anti-2,4 dinitrophenol (anti-DNP) IgE and then stimulated with DNP-human serum albumin (DNP-HAS). The release of secretory granules was measured by the release of β-hexosaminidase. After relative quantification of β-hexosaminidase, P1A Tg BMMCs released more enzyme compared to control BMMCs (Fig 3-12C). However, the difference is small; this slight difference in degranulation response may be restricted by the low P1A expression in mature P1A Tg BMMCs.

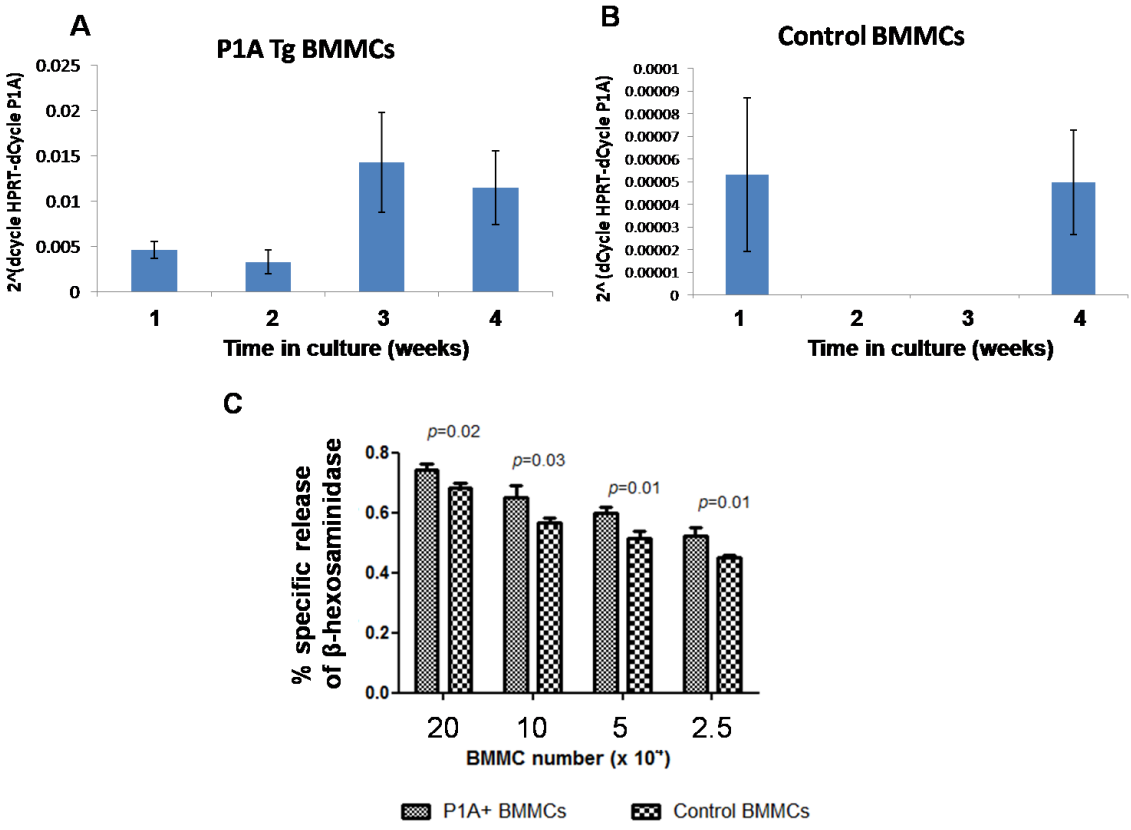


Figure 3-12 P1A increased degranulation in bone marrow derived mast cells (BMMCs)

A. P1A mRNA in P1ATg BMMCs was measured weekly through culture period by RT-qPCR, using HPRT as internal control. B. P1A mRNA in Control BMMCs was measured weekly through culture period by RT-qPCR, using HPRT as internal control C. Release of β-hexosaminidase was measured in $P1A^+$ BMMCs and control BMMCs. 4-week-old BMMCs were sensitized with anti-DNP IgE, and then stimulated with DNP-HSA or medium only. The enzymatic activities of β-hexosaminidase were determined and calculated according to the equation: % specific release = ($release_{stimulated}$ -$relese_{spontaneous}$)/ ($content_{total}$) x 100.

Discussion

One method to study protein function is by elucidating the protein-protein interaction. Using tandem affinity purification systems, we found that P1A interacted with several vesicular trafficking related proteins, such as IQGAP1, RalA and AP2 protein. Moreover, all the results from mass spectrometry were confirmed by co-immunoprecipitation. In addition, sucrose gradient fractionation further supported that P1A and IQGAP1 interacted with each other since they each had a peak in the same subcellular fraction.

Here we showed that the C-terminus of IQGAP1 was required for interaction with N-terminal P1A. IQGAP1 is an important scaffolding protein which interacts with various structural and signaling proteins (Brown and Sacks, 2006); for example, it is well known that active Rac1 binds to the Ras GTPase-activating proteins-related domain in C-terminal IQGAP1 (Kuroda et al., 1996). When activated Rac1 binds to IQGAP1 which prevents the interaction of IQGAP1 and β-catenin, thus also inhibits the dissociation of α-catenin from the cadherin-catenins complex (Noritake et al., 2005). Therefore, the cadherin-catenins complex is stabilized and results in strong cell-cell adhesion (Fukata et al., 1999; Kuroda et al., 1999).

Thus, we wanted to investigate whether P1A is involved in cell adhesion and migration, since we revealed that P1A not only interacted with C-terminal of IQGAP1 but also with Rac1. However, cell adhesion was not affected when P1A was silenced. This suggested that P1A was not related to adhesion signaling pathways, even though P1A interacted with IQGAP1 and Rac1.

Another ability of IQGAP1 is to modulate exocytosis through binding with the exocyst complex. The exocyst complex can mediate the transport of vesicles from post-Golgi or endocytic region to the plasma membrane (Lipschutz and Mostov, 2002). A previous study demonstrated that the exocyst subunits Sec3 and Sec8 interact with the C-terminal region of IQGAP1, resulting in exocytosis of transport vesicles in breast carcinoma human cells (Sakurai-Yageta et al., 2008). Another study reported that N-terminal of IQGAP1 binds to Exo70 of the exocyst complex and enhances secretion in pancreatic β-cells (Rittmeyer et al., 2008).

In this study, we found that P1A was pulled down together with active RalA, which has been found mostly in vesicles associated with exocytosis (van Dam and Robinson, 2006). RalA binds to two exocyst subunits, Sec5 and Exo84 (Moskalenko et al., 2002; Moskalenko et al., 2003; Sugihara et al., 2002). Moreover, the assembly or stability of the exocyst complex was significantly reduced after RalA silencing, demonstrating that Ral regulates exocyst function (Moskalenko et al., 2002; Moskalenko et al., 2003). In addition, fluorescence microscopic analysis of P815 cells showed that the distribution of P1A fully coincided with RalA and they localized on a vesicular-like network in cytoplasm. However, after knock-down of P1A in P815 cells by P1A-shRNA lentivirus, the vesicular localization of RalA was absent. These results provide support for the hypothesis that P1A can regulate the exocytosis pathway. Indeed, after we silenced P1A in P815 cells, beta-hexosaminidase, TNF-α and IL6 secretion were reduced. The data revealed that P1A played an important role in secretory pathways.

The P815 cells used in the present study were mastocytoma cells originally derived from a male DBA/2 mouse whose skin had been exposed to methylcholanthrene

(Dunn and Potter, 1957). Mast cells can secrete a variety of inflammatory mediators through the regulated exocytosis (Grimbaldeston et al., 2006). Physiologically, secretion from mast cells can be induced by aggregation of high-affinity immunoglobulin E (IgE) receptors via IgE molecules (Blank and Rivera, 2004). The granules of mast cells contain β-hexosaminidase, histamine and cytokines (Gordon and Galli, 1990; Schwartz and Austen, 1980; Suarez-Quian, 1987).

We demonstrated that the P1A is required for the proper exocytosis in P815 mastocytoma cells. Next, we wanted to see whether P1A was expressed in normal mast cells and whether P1A expression affected the secretion capacity. We found that P1A was expressed at very low levels in mast cells in wild type BMMCs. There was a slight increase of β-hexosaminidase release from P1A Tg BMMCs compared with control BMMCs.

The data suggest that one of the biological functions of P1A involves exocytosis. Based on the data in Chapter 2 that P1A transgenic mice increased tumor formation, it is possible that P1A is beneficial to the transformed cell by increasing secretion of cytokines, such as TNFα and IL6. Several studies have shown that cytokines secreted by cancer cells can stimulate tumor progression and promote tumor development and tumor cell survival. For example, in Moore's study, they showed higher skin tumor incidence after exposure to the 7,12-di-methylbenz[a]anthracene (DMBA) carcinogen in TNFα sufficient mice than TNFα deficient mice (Moore et al., 1999). In addition, Gado et al's study showed that IL-6 deficient mice were resistant to plasmacytoma which was induced by the pristane oil (2,6,10,12-tetramethylpentadecane) (Gado et al., 2001).

In addition, the roles of exocytosis in tumorigenesis of the P1A partners, IQGAP1 and RalA, are also been revealed. It has been suggested that IQGAP1 mediates cell invasion by regulating exocytosis which releases matrix metalloproteinases which results in degradation of the extracellular matrix (Sakurai-Yageta et al., 2008). Moreover, knockdown of the exocyst component, either Sec5 or Exo84, in RalGEF-transformed cells resulted in decreasing the ability of cell transformation (Issaq et al., 2010). Since RalA is activated by RalGEF which is one of effectors of Ras, this suggested that exocyst mediated the downstream transformation signal of RalA (Wolthuis and Bos, 1999; Wolthuis et al., 1998). Therefore, besides increase of cytokine secretion, P1A may also promote tumorigenesis by secretion of other proteins involved in tumor development or regulation of the exocytotic-related proteins.

The present of P1A in the isolated exosomes from J558 plasmacytoma (Altieri et al., 2004), also suggested that P1A may regulate exosome secretion. The secreted exosomes by tumor cells not only have the role of the antigenic source for effective T cell cross-priming but may also promote tumorigenesis (Fevrier and Raposo, 2004; Hugel et al., 2005; Thery et al., 2009). Several studies have been shown that tumor exosomes contain several proteins, such as VEGF, MMPs and fibroblast growth factors which contribute to tumor invasion, metastasis process and angiogenesis (Fevrier and Raposo, 2004; Hugel et al., 2005). Probably, the reason why P1A was originally identified as a tumor antigen, the primary target of immune recognition, may be because P1A facilitates the secretion of exosome or vesicle trafficking.

Here, we revealed that P1A interacted with AP2 protein as well. When we used the GST-RalBP1-PBD protein to specifically pull-down active RalA, AP2 and P1A were

also present in the immunoprecipitates. AP2 is involved in the formation of clathrin-coated endocysts from the plasma membrane (Sorkina et al., 1999). Moreover, RalBP1 is also involved in the receptor-mediated endocytosis through the interaction with μ subunit of AP2 complex which plays an important role of recruitment integral membrane proteins into clathrin-coated pits (Jullien-Flores et al., 2000). Mutation of Ral or RalBP1 has shown to inhibit cell endocytosis (Nakashima et al., 1999). Furthermore, RalBP1 contains a GAP domain that has the potential to interact with Rac1 (Jullien-Flores et al., 1995; Park and Weinberg, 1995). According to this evidence, it seems that P1A may possibly be involved in the endocytosis pathway through interaction with these endocytic-related proteins. Future studies should investigate the function of the P1A protein in the vesicular trafficking in endocytosis as well. The diagram showed in Fig 3-13 illustrated how these proteins related to each other.

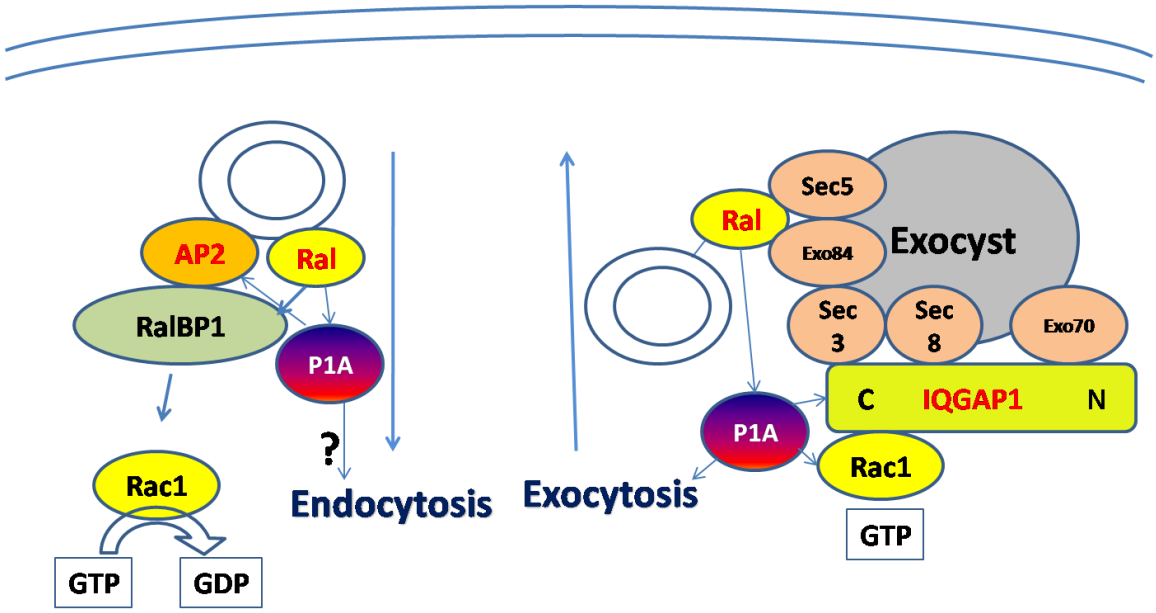


Figure 3-13. P1A involved in vesicle trafficking proteins

RalA regulates exocytosis through binding with Sec5 and Exo84, and endocytosis through RalBP1. RalBP1 contains a GAP domain that negatively regulates Rac1. RalBP1 has been shown to interact with AP-2 complex and is related to endocytosis. C-terminus of IQGAP1 interacts with active form of Rac1. C-terminus and N-terminus of IQGAP1 mediate exocytosis through Sec3, Sec8, and Exo70, respectively. In this study, we showed P1A interacted with C-terminus of IQGAP1, Rac1, AP2 and RalA. In addition, we also showed P1A promotes exocytosis, but whether P1A affects on the function of endocytosis needs to be determined.

Overall, this study showed that P1A interacted with several vesicle trafficking proteins, such as RalA, AP2, IQGAP1 and Rac1. The biological function of P1A was involved in exocytic systems as well as highly possible in endocytic pathway; therefore through vesicle trafficking, P1A regulate cells to communicate with their outside environments. It is probable that P1A is beneficial to tumor cells by increasing of cytokine secretion, such as TNFα and IL6 which can stimulate tumor progression and promote tumor development and tumor cell survival.

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CHAPTER 4

Preliminary approach to study the function of P1A in embryonic stem cells

Abstract

P1A, identified as the first known tumor rejection antigen, is expressed in embryonic stem cells and various tumors but is mostly silent in adult tissues. The biological function of the P1A gene is unknown to date; thus, we propose to test whether P1A plays an essential role in embryonic stem cell development. First, we knocked out P1A by Cre-loxP recombinase technique in ES cells and compared the global RNA expression profile between control and P1A-null cells. Our results indicated that ES cell morphology, growth rates and genes responsible for maintenance of ES cells, such as Nanog, Oct3/4 and Sox2, are normal in P1A-null ES cells. Furthermore, there was no significant difference of teratoma size between control and P1A-null cells. In addition, P1A-null ES cells were still able to differentiate into all three embryonic germ layers: endoderm, mesoderm and ectoderm. It seems that P1A is not critical for the early stage of ES cell growth and differentiation. However, Rap guanine nucleotide exchange factor 3 (RapGEF3), Rho-related BTB domain containing 1 (RhoBTB1), transglutaminase 2 (TGase2) and dynein, which have been shown to be involved in exocytosis or endocytosis, had decreased expression when P1A was knocked out. Thus, it is possible that P1A may interfere in vesicle trafficking in ES cells.

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of mammalian blastocysts (Evans and Kaufman, 1981). ES cells have two distinct features: one is the ability of self-renewal, where even after extensive proliferation, ES cells still maintain an undifferentiated state; another is the capacity of pluripotency which means that ES cells are capable to differentiate into multiple cell lineages (Evans and Kaufman, 1981; Martin, 1981; Suda et al., 1987). Several transcription factors are essential for maintaining the characteristics in ES cells, such as Nanog (Chambers et al., 2003; Mitsui et al., 2003), Oct3/4 (Nichols et al., 1998; Niwa et al., 2000), Sox2 (Avilion et al., 2003), and zfp296 (Mitsui et al., 2003).

Moreover, recent studies have hypothesized that the characteristics of ES cells may be connected to oncogenesis and malignancy (Al-Hajj and Clarke, 2004; Pardal et al., 2003). For example, several genes which are usually overexpressed in tumors have also been shown to have important functions in the maintenance of the ES cell features, such as c-myc (Cartwright et al., 2005), Stat3 (Matsuda et al., 1999), E-Ras (Takahashi et al., 2003), Klf4 (Li et al., 2005), and β-catenin (Kielman et al., 2002).

The P1A gene has recently been mapped in X chromosome (mouse genome informatics, ID98818) and is composed of three exons which codes for a protein of 224 amino acids. The P1A gene is up regulated in several tumors and also highly activated in ES cells, but it is down regulated in most adult tissues except the testis and placenta (Ramarathinam et al., 1995; Uyttenhove et al., 1997). However, the function of P1A in ES cells has not been investigated until now. One method to study the function of a specific gene in ES cell is to knock out the gene by gene targeting through homologous

recombination (Thomas and Capecchi, 1987; Thomas et al., 1986). Thus, in this study we first knocked out the P1A gene in ES cells by gene targeting combined with Cre-loxP recombination technique (Brault et al., 2007). Next, we compared the transcriptional profiles of the P1A-null ES and control ES cells by cDNA microarray in order to reveal how P1A impacts the global gene expression in ES cells.

Materials and Methods

Generation of P1A mutant ES cells

Mouse P1A genomic clones were obtained by screening a 129/SvJ BAC library. A neomycin/TK cassette flanked by two loxP sites was cloned between P1A exon1 and exon 2, introducing a new BamHI site for genotyping purposes. A third loxP site was located before exon 1. This construct was electroporated into murine R1 ES cells and homologous recombination was confirmed by Southern blot analysis after BamHI digestion. Positive clones were then electroporated with a Cre vector to eliminate the neomycin/TK cassette and P1A exon 1 (Fig 4-1A). ES cells were co-cultured with mouse embryonic fibroblast (MEF) feeder cells in DMEM medium containing 15% FCS, 0.1 mM β-mercaptoethanol, 10^3 u/ml leukemia inhibitory factor, 4 mM glutamine.

Western blot analysis

Samples were lysed in protein lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40) and protease inhibitor cocktails (Sigma) including 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, Aprotinin, Bestatin, E-64, Leupeptin and Pepstain A were added. Cell lysates were separated by 10% sodium dodecyl sulphate-

polyacrylamide gel electrophoresis, transferred to PVDF membranes, and incubated with the corresponding antibodies including anti-P1A rabbit antibody (Genemed Synthesis, 1:500 dilution), anti-actin mouse antibody (Sigma, 1:5000 dilution). Anti-rabbit or anti-mouse IgG horseradish peroxidase–linked antibody at 1:3500 dilutions (GE Healthcare) was used as secondary antibodies. Antibodies were detected with chemiluminescence reaction using the enhanced chemiluminescence kit (Amersham Biosciences) and visualization with exposure to film.

RNA isolation and cDNA microarray

The total RNA was isolated by Trizol (Invitrogen). Stem cells were washed with cold PBS, 0.5 ml Trizol reagent was added, and the cells were vortexed. Next, chloroform was added and the phases were separated. Then, isopropanol-precipitated RNA was recovered from the aqueous phase. The RNA pellet was resuspended in RNase-free water and further purified by the RNeasy Mini Kit (Qiagen). RNA was quantified using spectrophotometric measurement of the ratio at 260/280 nm. The A_{260}/A_{280} ratio was at least 1.9. The qualified RNA samples were sent to the Affymetrix and Microarray Core Facility in University of Michigan for full service sample preparation and data analysis.

Teratoma formation

Five of 129S/v mice were injected subcutaneously into the flanks with 10^7 ES control cells or ES P1A knockout cells from each clone. After 21 days, the mice were sacrificed, and the teratomas were removed and weighed. Teratomas were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Sections were stained with haematoxylin and eosin dyes.

Results

Targeted deletion of the *Trap1a* gene by loxP/Cre method

Cre recombinase of the bacteriophage P1 can recognize a specific 34-bp nucleotide sequence motif called a loxP site (Abremski and Hoess, 1984). After recognition of the loxP sites, Cre excises the DNA fragment in between and then bring the two loxP sites together (Hoess and Abremski, 1985). Dr. Ping Lu in our laboratory already built the ES cell contained a P1A targeting construct which included a selectable neomycin/TK cassette (flanked by loxP sites) and regions of identify with mouse X chromosome and loxP sties flanking P1A exon 1 that resulted in a recombination even near to the region of identify. The neomycin-TK cassette and P1A exon 1 was removed by transfection with a Cre recombinase expression vector into ES cells. Cre excised the intervening DNA segment and resulted in a single remaining loxP site (Fig 4-1). Several single P1A-null clones were selected and cultured for further analysis.

As shown in Fig 4-2A, we successfully knocked out P1A in ES cells after removed of exon 1 of P1A on X chromosome. After P1A removal, the morphology of ES cells was maintained with a compact and round shape, as seen in control cells (Fig 4-2B), and the cell growth rate not changed as well (Fig 4-2C).

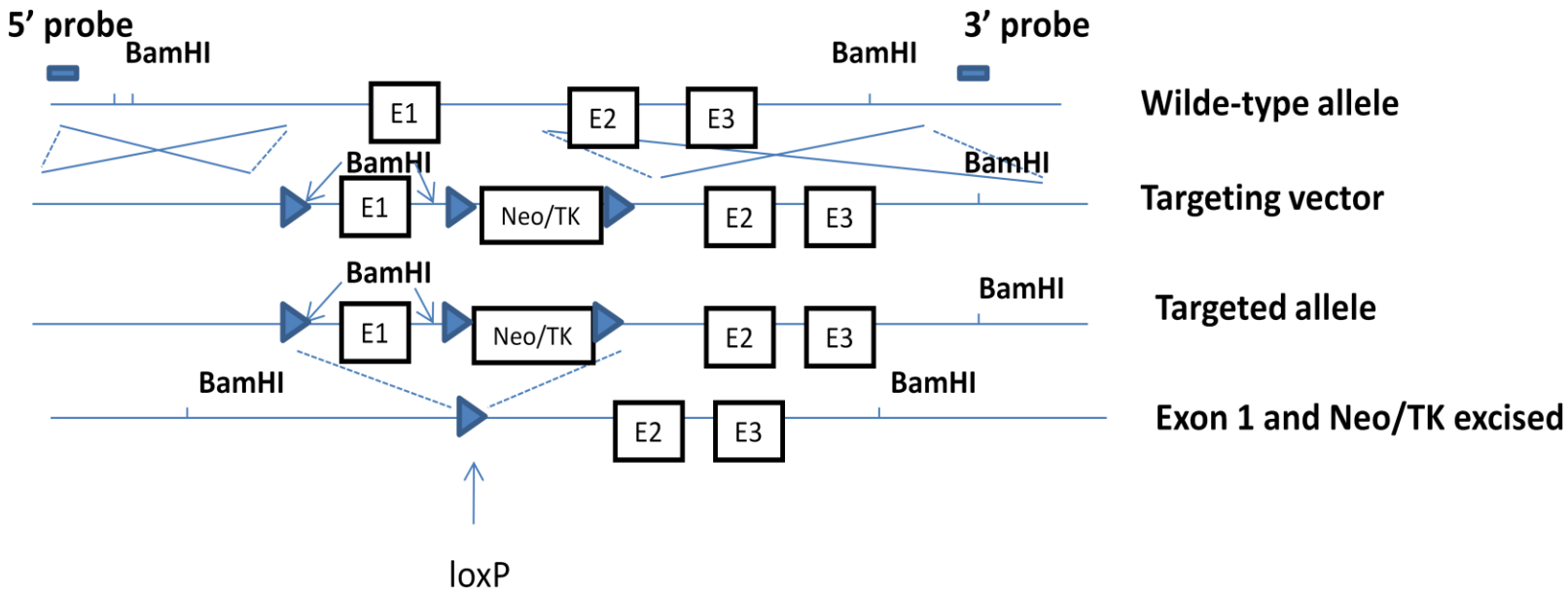

Figure 4-1. Scheme of the targeting construct

A neomycin/TK cassette flanked by two loxP sites was cloned between P1A exon1 and exon 2, introducing a new BamHI site for genotyping purposes. A third loxP site was located before exon 1. This construct was electroporated into murine R1 ES cells and resulted in a homologous recombination. Triangles represent loxP sites. After Cre recombination, P1A Exon 1(E1) gene was deleted in X chromosome.

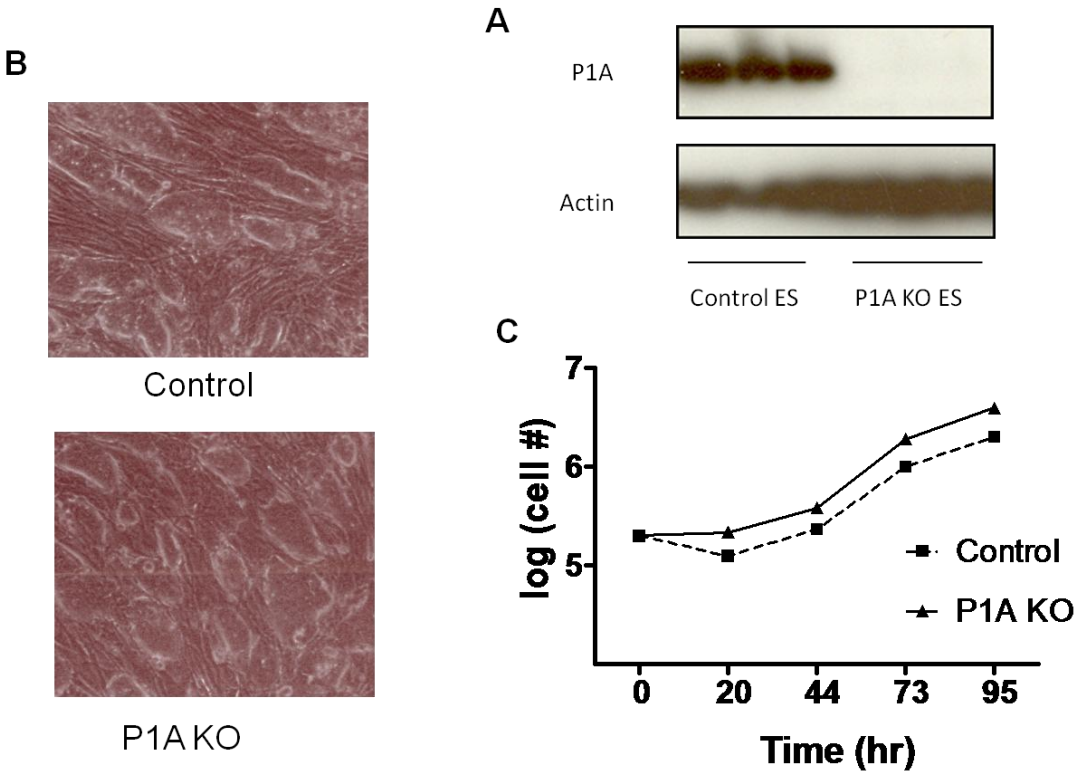

Figure 4-2. Morphology and growth rate of ES not changed after P1A knocked out

A. P1A protein was undetectable by western blotting in P1A knock out (P1A KO) ES cells; actin served as internal control. B. Morphology of ES cells after P1A was knocked out (P1A KO) maintained a compact and round shape similar to controls; ES cells were co-cultured with MEF feeder cells, 100X. C. Cell proliferation rates were similar between control and P1A knocked out (P1A KO) ES cells; Mean values ± S.D. were shown (n = 3).

Global gene expression profile

Three of P1A-null clones and three of control clones were cultured on gelatin-coated plates with leukemia inhibitory factor (LIF) for two passages after mouse embryonic fibroblast (MEF) removal. Then, the total RNA was isolated and purified by the commercial kit and submitted to analysis using the Affymetrix GeneChip Mouse 430 2.0 array containing approximately 34,000 genes. The data were then normalized and analyzed (Benjamini and Hochberg, 1995; Irizarry et al., 2003; Ritchie et al., 2006; Smyth, 2004). The probe sets were selected based on an adjusted p-value of 0.05, at least a two-fold difference, and consistent results present in all three replicates. Thirty-three different expressed genes in the comparison of P1A-null versus control cells are shown in Table 4-1. Clustering analysis of redundant annotation terms was performed by DAVID (The Database for Annotation, Visualization and Integrated Discovery), which is a set of functional annotation tools for analyzing the biological meaning from a list of genes of interest (Dennis et al., 2003; Huang da et al., 2009). The display of related many-genes-to-many-terms on 2-D view is shown in Fig 4-3. Most differentially expressed genes were involved in morphogenesis, development and metabolic process. The results revealed that mRNAs of genes responsible for maintenance of ES cells, such as Nanog, Oct3/4, Sox2, Fgf4, E-Ras, Zfp296, Stat3 and Myc were normal in P1AKO ES cells.

Table 4-1. Different expressed genes in the comparison of P1A-null versus control cells

Symbol	Description	Fold Change (log 2)
Trap1a	tumor rejection antigen P1A	-3.39
Rapgef3	Rap guanine nucleotide exchange factor (GEF) 3	-1.75
Dnaic2	dynein, axonemal, intermediate chain 2	-1.7
TGase2	transglutaminase 2, C polypeptide	-1.66
Rhobtb1	Rho-related BTB domain containing 1	-1.6
3632451O06Rik	RIKEN cDNA 3632451O06 gene	-1.51
Vegfc	vascular endothelial growth factor C	-1.47
Igsf21	immunoglobin superfamily, member 21	-1.27
Ppp1r14d	protein phosphatase 1, regulatory (inhibitor) subunit 14D	-1.26
Satb1	special AT-rich sequence binding protein 1	-1.25
Rnf17	ring finger protein 17	-1.24
Sfrp1	secreted frizzled-related sequence protein 1	-1.21
8430427H17Rik	RIKEN cDNA 8430427H17 gene	-1.2
Baz2b	bromodomain adjacent to zinc finger domain, 2B	-1.2
Tgfbi	transforming growth factor, beta induced	-1.19
1700010H23Rik	RIKEN cDNA 1700010H23 gene	-1.14
Fbxo2	F-box protein 2	-1.13
Cbln1	cerebellin 1 precursor protein	-1.13
Slc15a1	solute carrier family 15 (oligopeptide transporter), member 1	-1.09
Itpka	inositol 1,4,5-trisphosphate 3-kinase A	-1.04
Tbx3	T-box 3	-1.04
Inhbb	inhibin beta-B	-1.03
Smarcd3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3	-1.02
Fgd5	FYVE, RhoGEF and PH domain containing 5	-1.01
She	src homology 2 domain-containing transforming protein E	-1.01
Ankrd44	ankyrin repeat domain 44	-1.01
BB176347	expressed sequence BB176347	-1
Atp10a	ATPase, class V, type 10A	1.15
Trh	thyrotropin releasing hormone	1.18
Rhox5	reproductive homeobox 5	1.19
Lefty1	left right determination factor 1	1.4
Gm784	gene model 784, (NCBI)	1.41
Klhl13	kelch-like 13 (Drosophila)	1.81
Anxa11	annexin A11	2.12

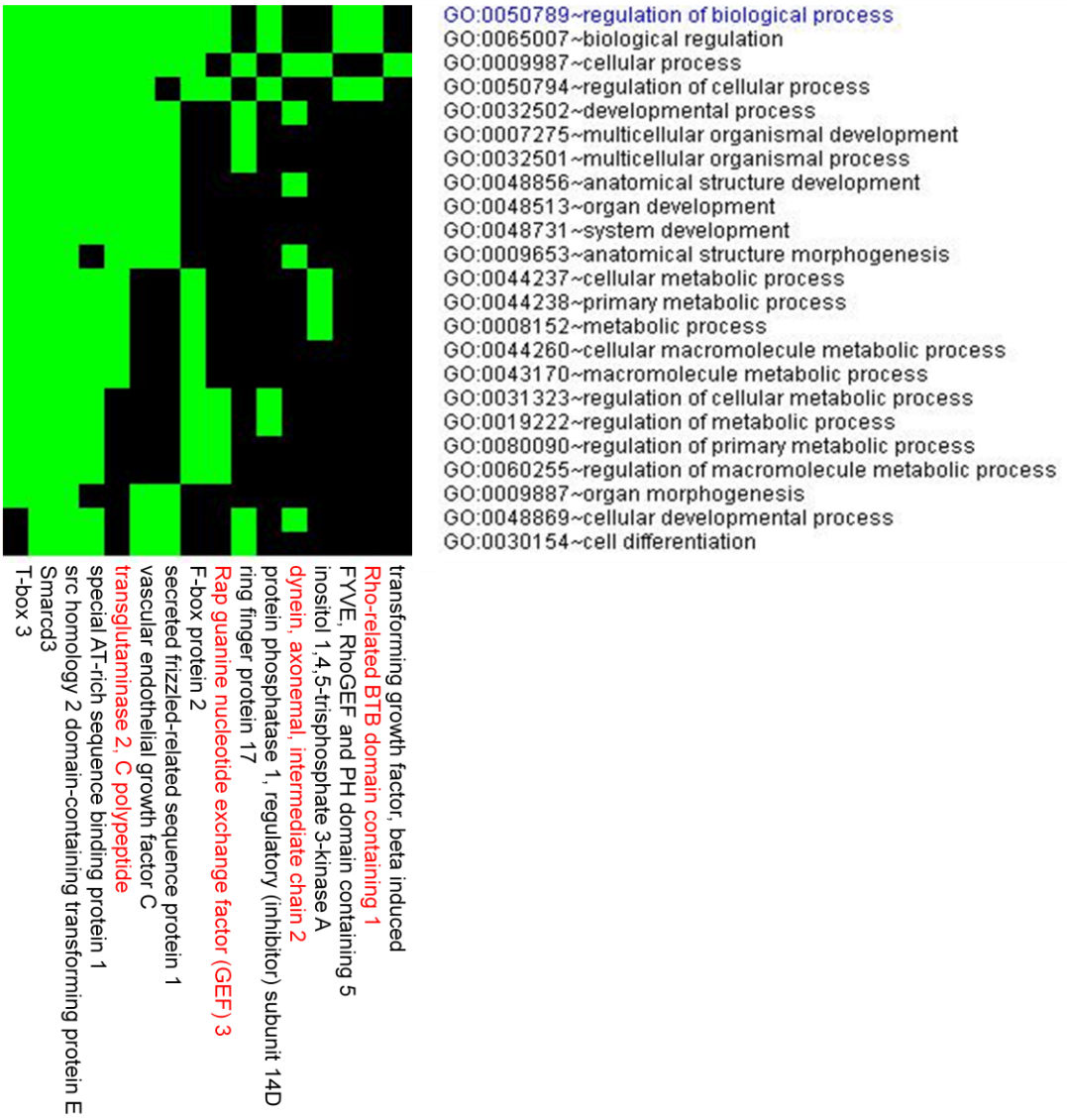

Figure 4-3. The display of related genes to terms on 2-D view

Corresponding gene-term association which has been positively reported is shown in green color. In contrast, the black color indicates the association not yet reported. The top four decreased genes are shown in red color.

Teratoma formation

Teratoma is a useful model to study the potential of ES cells to differentiate into specific germ layers after ectopic injection of ES cells into mice (Solter, 2006). To test whether the lack of P1A affects the development of teratomas, three of normal and three of P1A-null clones were injected subcutaneously into 5 of syngeneic 129/SV mice, respectively. After 21 day of incubation, no difference in tumor growth was observed between P1A-null tumor and normal tumor (Fig 4-4A). Hematoxylin/eosin staining showed that both control teratomas and P1A-null teratomas still retained the ability to differentiate to all three embryonic germ layers: endoderm (epithelium), mesoderm (cartilage, muscle) and ectoderm (neuron) (Fig 4-4B).

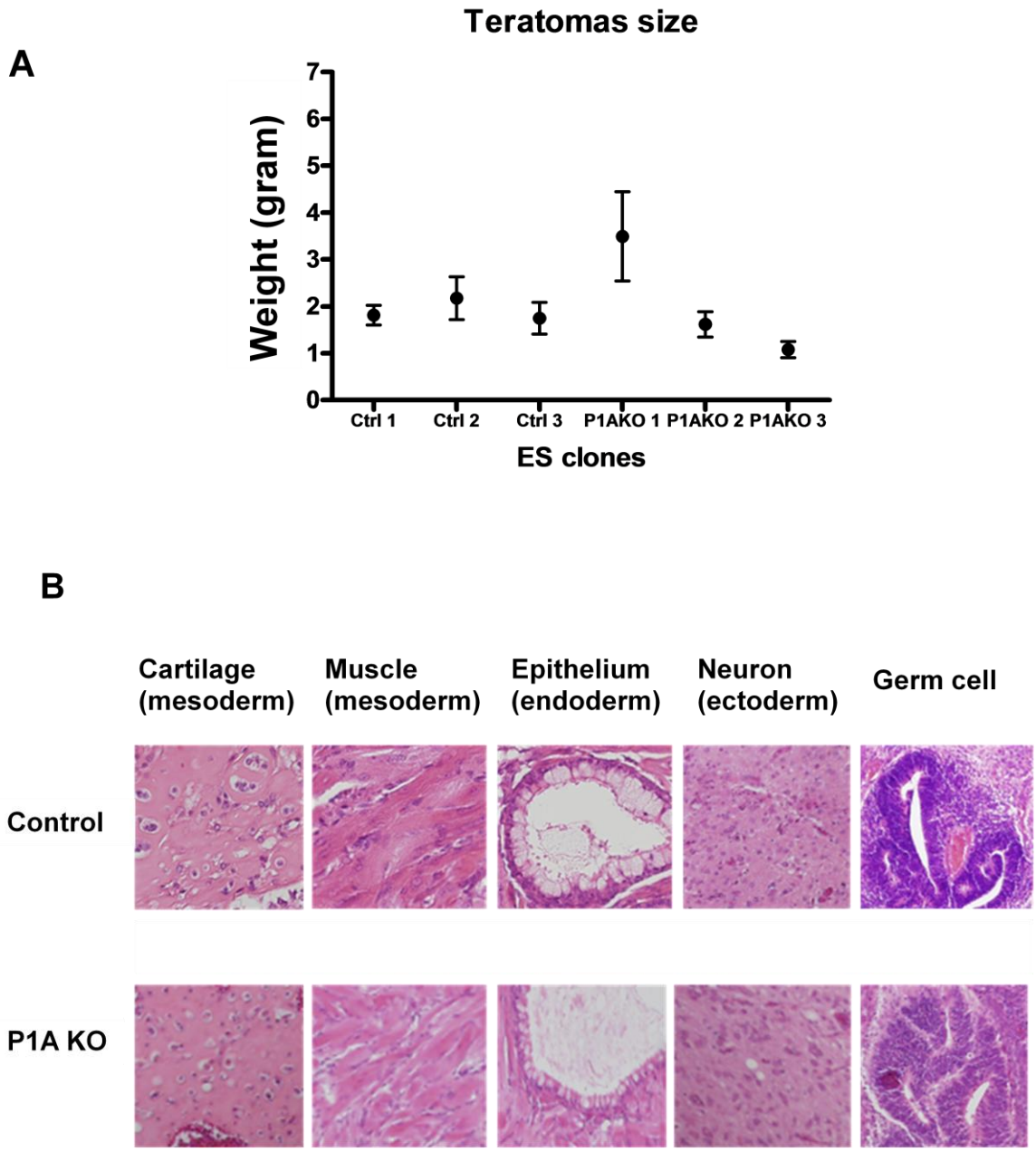

Figure 4-4. Teratoma formation

A. No significant difference of teratoma size between control and P1AKO cells; each point which represents the average weight ± S.D. of 5 tumors derived from a single clone is shown. B. Control ES and P1A KO ES cells were able to differentiate to all three embryonic germ layers, endoderm, mesoderm and ectoderm (H&E staining; 100X).

Discussion

Fetus and adult organisms all develop from embryonic stem cells located in the inner cell mass in blastocysts. The unique features of ES cells are self-renewal and pluripotency, thus allowing ES cells to produce identical pluripotent progenitors and to differentiate into the three germ layers, ectoderm, endoderm and mesoderm. To elucidate the mechanism by which stem cell gene expression is controlled, we chose to analyze the transcriptional profiles of the P1A-null ES and control ES cells to investigate whether P1A play a specific role in ES cell gene regulation. In total, 33 genes were identified as significantly differentially expressed. However, several factors previously identified as important for, or markers of, ES cell pluripotency and proliferation, including Nanog, Oct3/4, Sox2, Fgf4, E-Ras, Dax1, Zfp296, Stat3 and Myc were unchanged.

The absence of change of ES cell markers was consistent with the results that P1A-null ES cells still possessed normal ES cell morphology and proliferation rate. In addition, when examining the pluripotency of ES cells by teratoma formation, P1A-null ES cells were still able to differentiate to all three embryonic germ layers: endoderm, mesoderm and ectoderm.

Even so, there were 7 genes up-regulated and 26 genes down regulated in P1A-null cells compared to control cells. Functional classification of the differentially expressed genes into appropriate biological processes was thus performed by DAVID Functional Annotation Bioinformatics Microarray Analysis. Among the 26 decreased genes, these genes were clustered by the associated terms of gene ontology /biological process/ all level (GOTERM BP ALL) under medium stringency. Most differentially expressed genes were involved in morphogenesis, development and metabolic process.

Furthermore, Rap guanine nucleotide exchange factor 3 (RapGEF3), Rho-related BTB domain containing 1 (RhoBTB1), transglutaminase 2 (TGase2) and dynein, showed the most significant decrease in the group of 26 genes. These genes have been shown to be related to membrane trafficking, thus supporting the data presented in chapter 3 that P1A is involved in vesicle trafficking, specifically regulating exocytosis.

RapGEF3 is also known as exchange protein directly activated by cAMP (Epac) (Roscioni et al., 2008). Epac can bind to Rap GTPases to active Rap1 and Rap2 (de Rooij et al., 1998; Kawasaki et al., 1998). In addition, Epac can interact with the cytoskeleton network and is involved in calcium-induced exocytosis (Gupta and Yarwood, 2005; Hashiguchi et al., 2006; Mei and Cheng, 2005). Furthermore, Epac seems to regulate exocytosis through the interaction with Rim (Rab3-interacting molecule) and Rim2, which are secretory associated proteins (Ozaki et al., 2000).

RhoBTB1, which is an atypical Rho GTPases, is comprised of a Rho-related domain followed by two BTB (Bric-a-brac, Tramtrack, Broad-complex) domains (Berthold et al., 2008; Ramos et al., 2002). BTB domains are found in about 5 to 10% of the zinc finger proteins and are thought to mediate protein protein interactions (Collins et al., 2001). In the cytoplasm, RhoBTB1 is present in vesicular structures like endosomes and lysosomes in the cytoplasm (Aspenstrom et al., 2004). The function of RhoBTB1 is not well known. However, the localization of RhoBTB1 in vesicles suggests a role in vesicle trafficking.

Small GTPase Rab6 directs targeting of secretary vesicles to plasma-membrane sites and controls the organization of exocytosis in the cytoplasm (Grigoriev et al., 2007).

Strikingly, the number of immotile Rab6 vesicles was increased in dynein knockdown cells (Grigoriev et al., 2007). This suggests that cytoplasmic dynein plays an important role in the Rab6-bound motor complex and is necessary for vesicle movement.

The function of TGase 2 is to form the covalent bonds between primary amines (ex. lysine) and the glutamine residues in proteins (Facchiano et al., 2006). In addition, TGase 2 is also a GTP-binding protein with GTPase activity (Achyuthan and Greenberg, 1987; Lee et al., 1989). Moreover, TGase2 can transamidate RhoA (Singh et al., 2001). It has been shown that RhoA transamidation is involved in exocytosis and cytoskeleton rearrangement (Schoenwaelder et al., 2002; Steffan et al., 2009). In addition, TGase2 has been shown to involve in receptor-mediated endocytosis and phagocytosis (Abe et al., 2000; Davies et al., 1980; Szondy et al., 2003).

Taken together, P1A is highly expressed in ES cells and tumor cells; however, P1A knocked out did not affect cell proliferation in ES cells. In addition, embryonic stem cells grew normally and were able to differentiate into three germ layers even with P1A knocked out. P1A-null ES cells were also able to differentiate into three germ layers: endoderm, mesoderm and ectoderm. Despite this, whether P1A influences the later stages of embryogenesis and development is still unexplored and unknown. Unfortunately, the ES cells containing a floxed P1A gene were incapable of germline transmission, which makes it difficult to determine the function of the P1A protein in embryogenesis. Since knock out of P1A in ES cells indeed changed 33 genes expression, especially decreasing genes related to vesicle trafficking, it is possible that P1A may interfere in the cell function by affecting exocytosis or endocytosis.

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CHAPTER 5

Conclusion

The main purpose of this study was to use P1A as a model to study the biological function of onco-fetal proteins. The mouse tumor antigen P1A is a good model found in mice for human tumor antigens, MAGE, BAGE and GAGE. Like these cancer/testis human genes, expression of P1A has been found to be markedly elevated in tumors such as P815 mastocytoma, J558 plasmacytoma and MethA sarcoma; however, low or no P1A is detected in normal adult tissues except testis and placenta. Because the P1A protein highly expresses in embryonic stem cells, we could reliably characterize P1A as an onco-fetal protein. Thus, P1A expression in tumor cells might represent the reactivation of the features of fetal phenotype, as well as induction of oncogenesis events.

First of all, I elucidated the involvement of P1A in tumor formation. I showed that P1A transgenic mice, which specifically expressed P1A in hematopoietic cells, developed thymic leukemia after 7 months of age in immunocompromised mice. In addition, P1A transgenic bone marrow cells had higher proliferation ability and more potential progenitors compared to control bone marrow cells. Moreover, the majority of P1A tumors were not contributed by a mutation of Notch1. I also found a variety of tumor types formed in P1A Tg mice, suggesting that P1A may promote tumor initiation at a

different stage of progenitor cell development. Rag2 deficiency increased tumor formation and incidence in our P1A Tg mice. However, although several $P1A^{+}Rag2^{+}$ mice died due to the tumors, the tumor incidence was not statistical significant. This suggests that for a normal immune-competent individual the immune system might be able to eradicate the tumor and protect the host from tumor formation.

One mechanism of P1A for its role in tumor initiation or tumor promotion might be due to the increase of secretion of tumor-promoting cytokines, such as IL-6 and TNFα, after P1A activation. Newly synthesized cytokines first travel to the ER, the Golgi, and finally to the trans Golgi network (TGN), where these cytokines can be released through different secretory pathways (Stow et al., 2009). In haematopoietic cells, cytokines have been shown to be released from constitutive secretory vesicles, regulated secretory vesicles, secretory lysosomes, and exosomes (Blott and Griffiths, 2002; Skokos et al., 2002; Stow et al., 2009) (Fig 5A). I have demonstrated that P1A-silenced P815 cells have reduced cytokine secretion. Intracellular distribution of P1A illustrated that P1A might be related to the membranes of secretory pathways, and indeed, we demonstrated that P1A interacts with the exocytic-related proteins, RalA, IQGAP1 and Rac1 (Fig 5A). In addition, the distribution of P1A fully coincided with RalA and they both localized on a vesicular-like network in the cytoplasm. I also revealed that P1A was responsible for the proper localization of RalA in P815 cells rather than to regulate RalA activity. Moreover, I found there was a slight increase of β-hexosaminidase release from P1A Tg bone marrow derived mast cells (BMMCs) compared from control BMMCs, although the expression level of P1A in mature P1A Tg BMMCs was very low.

It has been established that cancer can be abetted by inflammation and cytokines secreted by cancer cells, which then can stimulate tumor progression, promote tumor development and tumor cell survival (Lin and Karin, 2007). The correlation between inflammation and cancer has been shown in epidemiologic studies; for example, alcohol abuse causes inflammation which leads to cancers of the liver and pancreas (Berasain et al., 2009; Gupta et al., 2010; McKillop and Schrum, 2009). Lung carcinoma is associated with the inflammation caused by cigarette smoking, asbestos or silica exposure (Kamp, 2009; Peebles et al., 2007). In addition, inflammatory bowel disease also correlates to colorectal cancer (Feagins et al., 2009).

The link between inflammation and cancer was revealed through genetic and biochemical studies. In Moore's study, they showed higher skin tumor incidence in TNFα sufficient mice than TNFα deficient mice after exposure to the 7,12-di-methylbenz[a]anthracene (DMBA) carcinogen (Moore et al., 1999). In addition, Gado et al's study showed that IL-6 deficient mice were resistant to plasmacytoma which was induced by the pristane oil (2,6,10,12-tetramethylpentadecane) (Gado et al., 2001). Thus, activation of P1A may enhance secretion of IL-6 and TNFα, and thereby increase the risk of tumor development. In order to test this hypothesis in vivo, genetically mutation mice can be used. One way is to cross breed $P1A^{+}Rag2^{-}$ mice with TNFα-deficient mice or IL6-deficient mice, and then the tumor incident rate will be compared between $P1A^{+}Rag2^{-}TNF\alpha^{-}$ mice (or $P1A^{+}Rag2^{-}IL6^{-}$) and $P1A^{+}Rag2^{-}$ mice. If cytokines indeed are involved in the tumorigenesis, I would expect to observe a lower tumor incidence in $P1A^{+}Rag2^{-}TNF\alpha^{-}$ mice (or $P1A^{+}Rag2^{-}IL6^{-}$) compared to $P1A^{+}Rag2^{-}$ mice. Another method is to use antibodies to neutralize cytokines in vivo. The tumor incident rate in

P1A+Rag2- mice should decrease after constitutive injections of anti-TNFα and anti-IL-6 antibodies.

Another possible mechanism of P1A in tumorigenesis is through the RalGEF/Ral pathway. RalA is a principal effector of RalGEFs involved in Ras-mediated transformation and tumorigenesis (Lim et al., 2005; Wolthuis and Bos, 1999). Although constitutively activated RalA does not induce oncogenic transformation on its own in rodent fibroblast cells, its expression enhances the transformation activities of both RasH and Raf in NIH3T3 cells through RalGEF/Ral pathway (Urano et al., 1996). Also, dominant-negative RalA mutants suppress Ras-induced transformation of NIH3T3 cells (Urano et al., 1996). The distribution of P1A was fully colocalized with RalA in a vesicular-like network in cytoplasm. However, after knock-down of P1A in P815 cells, RalA was dispersed away from the vesicle and then the vesicular localization of RalA was disappeared. It has been shown that the proper subcellular localization of RalA to vesicular bodies in the perinulcear region is required for cell transformation after ectopic Ras^{G12V} expression (Lim et al., 2005); therefore, RalA might control the complexity of cell signaling through its various subcellular localizations. Because P1A is responsible for the proper localization of RalA but not for regulation of RalA expression or activity, it is suggest that P1A might act as a protein scaffold which provides a spatially defined interaction matrix for other proteins.

I have demonstrated the interaction between P1A and the exocytic-related proteins in P815 cells and P1A transgenic thymocytes. The question that needs to be resolved is whether the association of P1A and these vesicle proteins is required for tumorigenesis in P1A Tg mice. To address this question, RalA-deficient or IQGAP1-

deficient mice can be used. If enhancement of exocytosis or interaction between P1A and RalA or P1A and IQGAP1 is essential in oncogenesis in P1A Tg mice, tumor incidence should decrease in $P1A^{+}Rag2^{-}RalA^{-}$ mice or $P1A^{+}Rag2^{-}IQGAP1^{-}$ mice compared to $P1A^{+}Rag2^{-}$ mice. Another alternative way is to produce a P1A mutation transgenic mouse train, specially a mutation in the N terminus of P1A, since I have demonstrated that the major binding site of P1A to IQGAP1 was in N terminus. The mutation site should alter the function of P1A, and therefore eliminating P1A's ability to bind its partners. The frequency of tumor formation in P1A mutation transgenic mice and P1A transgenic mice can be compared to elucidate whether the interaction of P1A and the exocytic-related proteins is necessary for tumorigenesis in vivo.

Although P1A was originally express in ES cells, it seems that P1A is not critical in the early embryonic stem cell proliferation and differentiation. First, the expression of ES marker genes important for self-renewal and pluripotency were comparable between P1A-null and control ES. Second, there was no effect on ES cell proliferation in vitro and in vivo when P1A knocked out. Third, P1A-null cells were still able to differentiate into the 3 germ layers. Nevertheless, a role for P1A in embryogenesis cannot be ruled out. Our attempt to generate mice with targeted mutation of P1A gene has been unsuccessful and the ES cells with floxed P1A gene are incapable of germline transmission.

By global gene expression profile, I revealed that the genes with the most decrease in expression level in the P1A knocked out ES cells, RapGEF3, TGases, dynein and RhoBTB1, all have been shown to be related to membrane trafficking. This suggested that P1A might also function as a regulator of exocytosis or vesicle trafficking during ES cell development. Moreover, whether P1A influences the later stages of

embryogenesis and development is still unexplored and unknown. Therefore, further investigation is required to reveal the molecular function of P1A during embryonic or fetal development. To establish the P1A knock out mouse strain is probably the best solution to address this question.

In addition, I found that P1A not only interacted with exocytosis-related proteins but also with AP2 which is involved in endocytosis. Furthermore, another P1A partner, RalA has been suggested to travel between endocytic and exocytic membrane compartments and might be controlled by the specific location of target proteins. Therefore, it is also interesting to extend the study of P1A in endocytosis pathway. It is highly possible that the function of P1A may be related to overall vesicle trafficking system.

Finally, by definition, onco-fetal antigens are recognized by cancer-reactive lymphocytes because they are capable of inducing immune response. Despite widespread alterations in cellular components in tumor cells, only a few tumor antigens are known to be recognized by cytotoxic T lymphocytes. It is therefore plausible that studying the function of onco-fetal antigens may provide valuable insights for understanding immune response to cancer cells. Since it is generally agreed that recognition of tumor antigens requires cross-priming, which involves transfers of tumor cellular components into professional antigen-presenting cells such as dendritic cells (Fig 5B), it is of interest to speculate that those proteins whose up-regulation facilitates such transfer may be preferentially recognized by the immune system. In this context, the function of P1A in facilitating exocytosis, as described herein, makes it worthwhile to test its role in cross-priming of tumor-specific cytotoxic T cells. In order to test whether P1A is important for

T cell cross-priming, another tumor antigen in P815 cells, P815E, can be used (Bilsborough et al., 1999). First, P815 control cells or P1A knocked down P815 cells will be injected into DBA/2 mice. After 10 days, lymph node cells will be harvested and the specific P815E T cells will be stained with anti-CD8 antibody and P815E tetramer. The ability of T cell priming between the control and the P1A knocked down group will be compared according to the amount of specific P815E T cells. If P1A can enhance the T cell priming, the number of specific P815E T cells in the P1A knocked down group should be less compared to the control group.

In conclusion, I showed that the prototype onco-fetal protein, P1A, could promote tumor formation under condition of immunodeficiency. I also revealed that P1A interacted with several vesicle trafficking proteins, such as RalA, AP2, IQGAP1 and Rac1; thereby suggesting that P1A functions in regulation of vesicle trafficking in cells. Finally, one of possible mechanisms that accounts for the tumor-promoting effect of P1A is elevated secretion of cytokines, such as TNFα and IL-6, which has been found to correlate with cancer development. My results demonstrated a relationship between onco-fetal proteins and tumorigenesis which might be involved in the membrane trafficking pathway (Fig 5A). In addition, the fact that P1A promotes exocytosis may also offer insights why it is readily recognized by the immune system (Fig 5B).

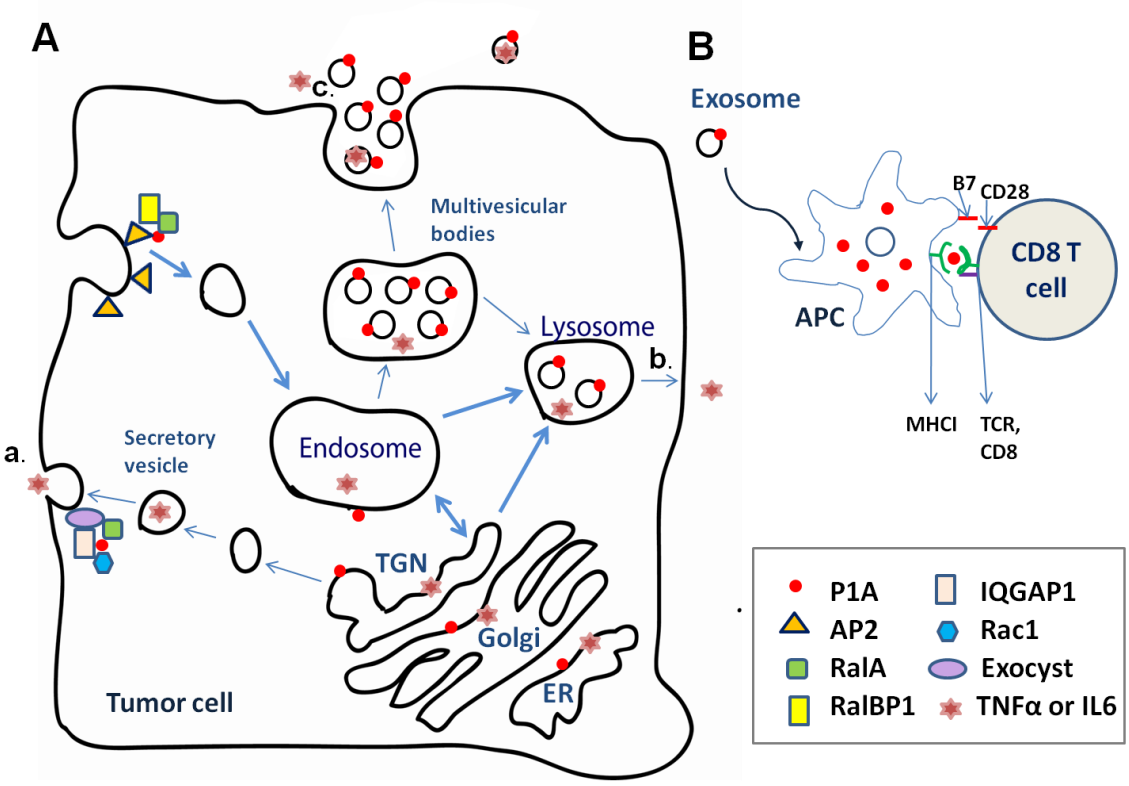

Figure 5-1. Illustration of P1A-related cellular process

A. P1A is involved in vesicle trafficking pathway. P1A has been shown to localize in membrane compartments, such as ER, Golgi and exosomes. I demonstrated that P1A interacts with IQGAP1, RalA, AP2 and Rac1. RalBP1 has been shown to interact with AP2 and RalA. a, b and c represent different secretion routes for cytokines. a. constitutively or regulated secretory vesicles, b. secretory lysosomes, c. exosomes (secretion of endocytic region, like endosomes). B. P1A is recognized by tumor specific CD8 T cells. Recognition of tumor antigens requires cross-priming, which involves transfers of tumor cellular components into an antigen-presenting cell (APC) such as a dendritic cell. P1A can be transferred from a tumor to an APC through exosomes.

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