Involvement of PI3Kγ in the Regulation of Erythroid Differentiation of K562 Cells

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1. INTRODUCTION

1.1 Background

Differentiation is a basic developmental process by which a cell or a number of cells that lack specific functions proliferate for a defined period and change their pattern of gene expression to produce specified cell types that perform highly specialised biochemical functions. Embryogenesis is the most striking form of differentiation, during which all specialised functions of the adult arise from a single fertilised egg.

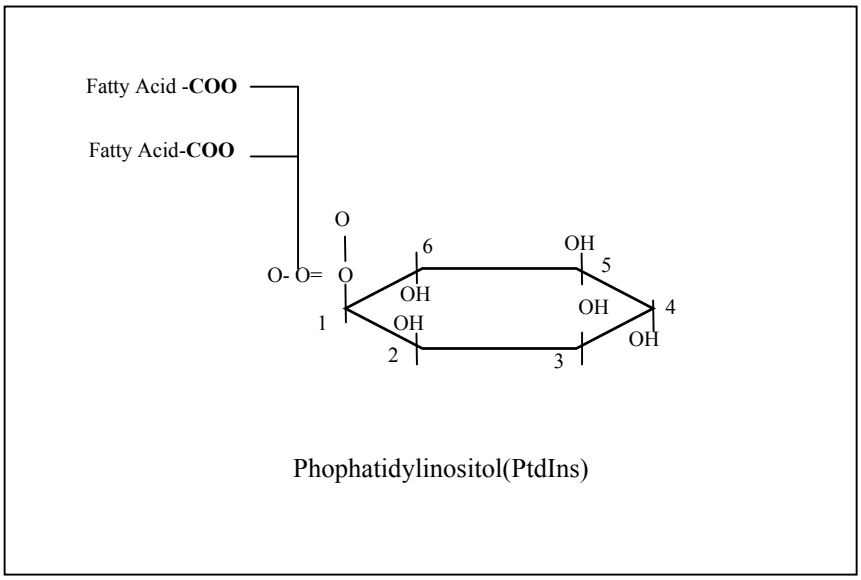
During normal differentiation, each step in the "proliferation-execute genetic differentiation program-stop proliferation" cascade is under stringent control from both extracellular and intracellular stimuli. For example, in haematopoietic tissue, stromal cells produce numerous cytokines, growth factors, and cell adhesion molecules, supporting the proliferation of hematopoietic progenitors and specifying their direction of differentiation. In the nucleus of differentiating hematopoietic progenitors, both lineage-restricted and ubiquitous transcription factors play critical roles in governing a highly organised gene expression program. Currently, one of the major tasks in cell differentiation study is to define the signalling events that link the extracellular stimuli to specific gene(s) expression.

It is known that the transfer of extracellular stimuli to their nuclear targets is mediated by various intracellular messengers or signalling pathways. Phosphoinositide 3-kinases (PI3Ks), are a family of ubiquitous enzymes that have been shown to be important mediators of intracellular signalling in mammalian cells. Previous work has demonstrated PI3Ks are important regulators of mitogenesis, cell survival, metabolic control, and cytoskeletal rearrangements. More recently, the role of PI3Ks in differentiation has begun to be revealed.

1.2 Phosphoinositide 3-kinases

Phosphoinositide 3-kinases (PI3Ks) are a large family of ubiquitous lipid kinases. To date, eight members of this family have been cloned and characterised in mammalian cells. A common feature of these enzymes is there ability to catalyse the phosphorylation on the 3-hydroxyl of the inositol ring of phosphoinositide (PI) and its derivatives. These enzymes differ in structure, and have distinct mode of regulation and *in vitro* lipid substrate

specificity. On the basis of these differences, they can be divided into three classes (Vanhaesebroeck, B et al., 1997).



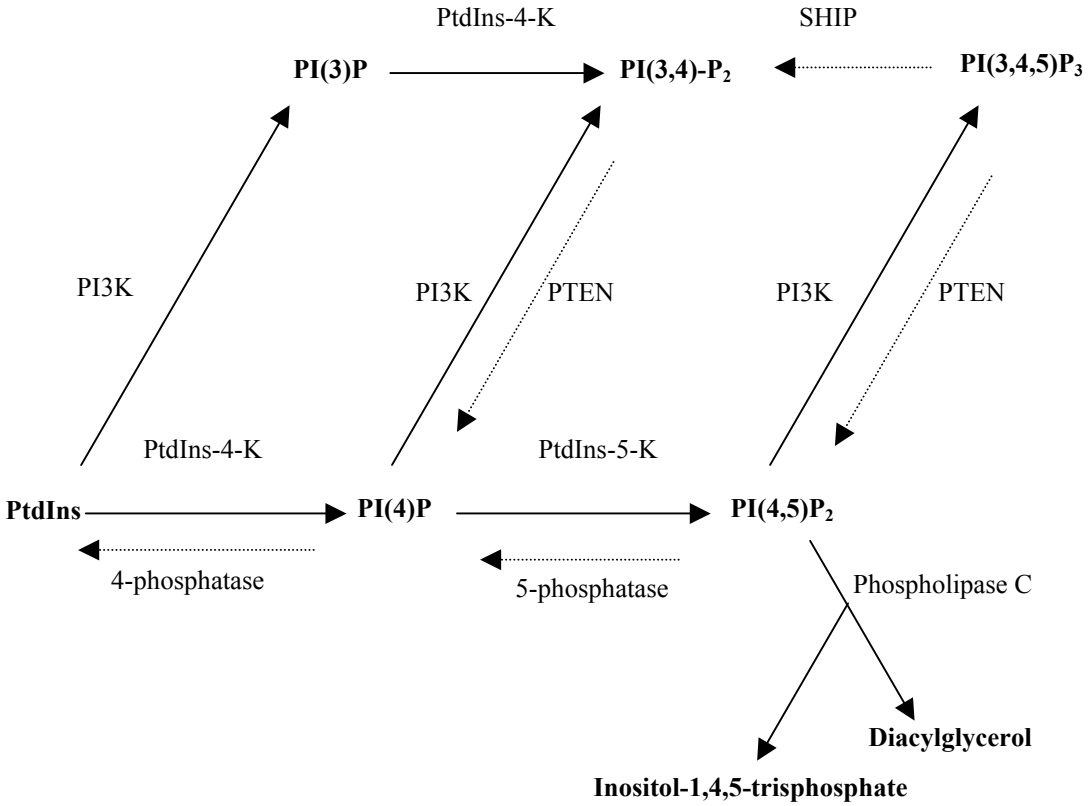


Fig.1 Reactions catalysed by PI3K and related enzymes for the synthesis and hydrolysis of phosphoinositides. The basic structure of PtdIns and its numbering system of the inositol ring are presented in the upper of this figure.

1.2.1 Class I PI3Ks

Among the PI3K family, the Class I PI3Ks are the most intensively investigated and best characterised. *In vitro* they utilise phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns(4)P), phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) as substrates to produce PtdIns(3)P, PtdIns(3, 4)P_2 and PtdIns(3,4,5)P_3 respectively. *In vivo,* their preferential substrate is PtdIns(4,5)P_2. Class I PI3Ks comprise four isoforms, namely, PI3Kα, β, γ, and δ. Based on the difference in mode of regulation and adaptor association, they are further subdivided into class IA and class IB PI3Ks (Vanhaesebroeck, B et al., 1997)

Class IA PI3Ks

The class IA PI3Ks are heterodimers consisting of a p110 catalytic subunit and an adaptor/regulatory subunit. In mammals, three isoforms of catalytic subunits have been identified, termed p110α (Hiles, ID et al., 1992), p110β (Hu, P et al., 1993), and p110 δ (Vanhaesebroeck, B et al., 1997). They are encoded by three separate genes, with molecular weights of about 110-120 kDa. p110α and p110β are widely expressed in mammalian tissues, in contrast p110δ shows a restricted distribution and is mainly found in leukocytes (Vanhaesebroeck, B et al., 1997). Despite the differences in tissue distribution, these catalytic subunits are quite similar in structure (Fig.2). A striking structural feature of these proteins is characterised by the presence of an N-terminal p85-binding domain. In the C-terminus, they also share a high degree of homology in the catalytic domain, which contains the binding sites for ATP and the PI3K inhibitor wortmannin. In addition, they also share a Ras–binding domain located in the N-terminal part, a C2 domain , and a phosphoinositide kinase domain (PIK). The C2 domain is conserved in all PI3Ks and is thought to mediate the interaction with the phospholipid membrane. The PIK domain is conserved in all lipid kinase enzymes, and is thought to mediate protein-protein interactions (Walker, EH et al., 1999).

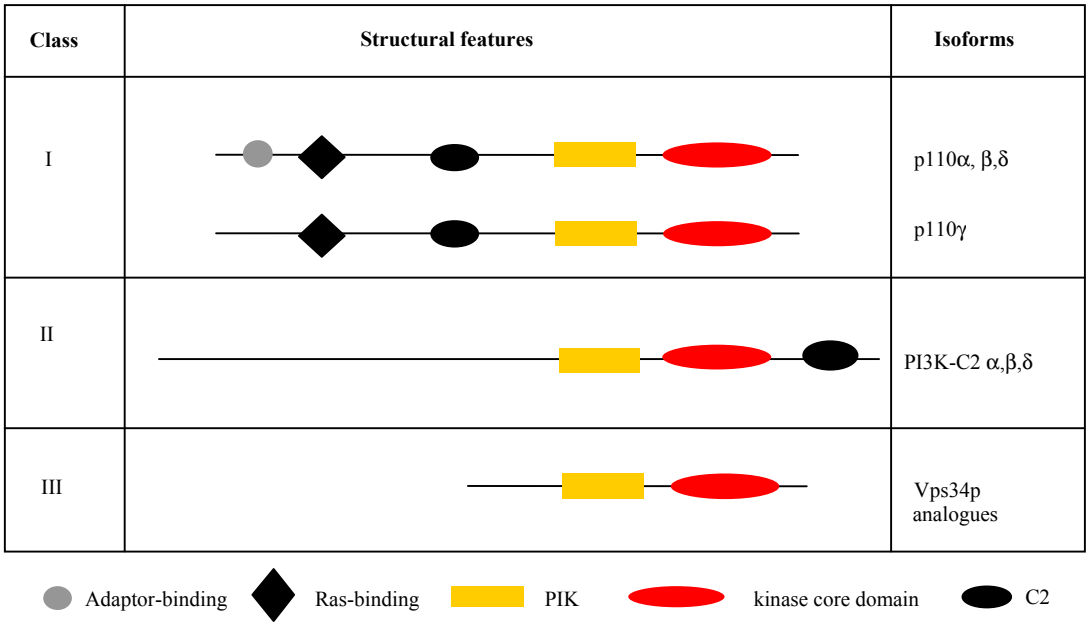


Fig.2 structural features of mammalian PI3Ks

The catalytic subunits are constitutively associated with an adaptor protein. There are three genes that encode at least five different adaptor proteins (p85α, p55α, p50α, p85β, and p55γ) (for an overview, see Okkenhaug, K, 2001). p85α and p85β, encoded by different genes, represent the full-length versions of the regulatory subunits. These two proteins are similar in structure. Each contains a SH3 domain, a bcr homology (BH) flanked by two proline-rich domains, two SH2 domains separated by an inter SH2 domain, which contains the p110 binding region. The two SH2 domains can bind specific phosphorylated tyrosine residues in activated receptor proteins or other tyrosine phosphorylated protein adaptors and thus to bring the p110 catalytic subunit to the membrane in proximity to its substrate. The SH3 domain mediates the interactions between p85 and proteins with proline-rich sequences, such as SOS (son of sevenless) and Cbl. These interactions create an alternative signal mechanism for receptors to recruit p110 to the membrane (Kivens, WJ et al., 1998). The BH domain of p85 is homologous to the Rho family GAPs. Some evidence has been presented that this domain can bind Cdc42, an interaction that may contribute to the activation of p110 (Jimenez, C et al., 2000, Vanhaesebroeck, B et al., 2001). Among the shorter versions of the regulatory subunits, p55 α and p50α are splicing variants derived from the same gene as p85α (Fruman, DA et al., 1996), whereas p55γ is encoded by a different gene. These short forms of regulatory subunits share the common SH2 domains

and inter SH2 domain structure with p85α and p85β, but lack the N-terminal half containing the SH3 domain, N-terminal proline-rich domain, and BH domain. Due to these structural differences, the longer p85 subunits have been shown to possess additional functions to that of the shorter forms. The main function of the regulatory subunits is to bring the p110 catalytic subunits to the membrane, where the catalytic subunit phosphorylates its lipid substrates. However, in the unstimulated state, a regulatory subunit plays a role in maintaining the thermal stability of the p110 catalytic subunit but also keeps it in low activity state (Yu, J et al., 1998). Intriguingly, there is increasing evidence to suggest that the regulatory subunits in fact can exert some effects independently of the catalytic subunit. For instance, p85α was shown to independently stimulate signalling pathways involved in actin cytoskeletal rearrangements (Jimenez, C et al., 2000, Okkenhaug, K et al., 2001).

In response to different stimuli, Class IA PI3Ks can be activated in various ways (for an overview, see {Wymann, MP et al., 1998}). One type of activation involves binding of p85 to the tyrosine phosphorylated sites of the activated receptor tyrosine kinases. For instance, PI3K binds to the PDGF receptor through the SH2 domain of p85 (Panayotou, G et al., 1992). Nonreceptor tyrosine kinases, such as src-family or JAK kinases, are also implicated in the activation of class I PI3Ks through the interactions with B- and T-cell antigen receptors, cytokine receptors, and several costimulatory molecules (such as CD28, CD2, CD19). In addition, class IA PI3Ks can also be activated by the small GTPase Ras (Downward, J., 1997; Rodriguez-Viciana, P., 1994, 1996). Under some circumstances, p110β can be activated by G-protein coupled receptors (Kurosu, H et al., 1997, Yart, A et al., 2002).

There is evidence that different activation mechanisms could recruit distinct PI3K complexes to distinct intracellular locations, which lead to specificity in signal transduction (Wang, Q et al., 1998). For instance, insulin–induced glucose transport in adipocytes is mediated by p110β but not p110α, despite the fact that both of them are present and can equally well associate with the adaptor subunits (Wang, Q et al., 1998). This concept is further reinforced by the observation that p110β can mediate insulin and lysophosphatidic acid (LPA) induced mitogenesis in fibroblasts, whereas the platelet derived growth factor (PDGF)-induced mitogenesis in these cells apparently does not require the activation of p110β (Roche, S et al., 1998).

In addition to their function as lipid kinases, PI3Ks have an intrinsic protein-serine kinase activity. Initial studies showed that p110α-mediated phosphorylation on p85α adaptor

reduces the lipid kinase activity of the heterodimer *in vitro* (Dhand, R et al., 1994). PI3Kδ has been shown to undergo autophosphorylation both *in vitro* and *in vivo* (Vanhaesebroeck, B et al., 1999), and the *in vivo* autophosphorylation induced by activated receptors (such as CD28) leads to a concomitant decrease in associated lipid kinase activity. Similar to p110α, p110β can also phosphorylate p85 adapator, albeit to a lower degree than does p110α (Beeton, CA et al., 2000). Despite the finding that class IA PI3Ks are able to phosphorylate insulin receptor substrate-1 (IRS-1) *in vitro* (Vanhaesebroeck, B et al., 1999), the physiological protein kinase targets have not been identified. Nevertheless, *in vitro* protein kinase activity is a distinguishing feature among the PI3K isoforms and may contribute to isoform-specific functions.

Class IB

Up to date, only one isoform of class IB PI3K has been identified. Initial studies in neutrophils (Stephens, L et al., 1994) and platelets (Thomason, PV et al., 1994) revealed that the accumulation of $PI(3,4,5)P_3$ occurs in response to activation of heterotrimeric G protein-linked receptors, implying the existence of a PI3K isoform different from classIA PI3Ks. Later on, by screening of the human bone marrow cDNA library, a novel PI3K isoform was cloned and characterised in our lab, namely, PI3Kγ (Stoyanov, B et al., 1995). In structure, PI3Kγ is similar to that of class IA p110 proteins, but its N-terminal part lacks the binding domain for the p85 adaptor family. Instead of binding p85 family protein, Stephens et al. found the porcine version of human PI3Kγ is complexed with a novel protein, termed p101 (Stephens, L et al., 1997). This protein shows no sequence homology to any known protein. There is some evidence showing that the lipid kinase activity of PI3Kγ is positively regulated by p101. Stephens et al, demonstrated PI3Kγ, when expressed in SF9 cells or COS-7 cells, exhibits a significant basal activity that is only slightly stimulated by Gβγ dimers, but when co-expressed with p101, the PI3Kγ activity is stimulated to a much greater extent by Gβγ dimers. In agreement with the above observation, Krugmann et al, generated a panel of catalytically active PI3Kγ fragments using a deletion approach, and demonstrated that those fragments incapable of binding p101 are insensitive to Gβγ-stimulation (Krugmann, S et al., 1999). Thus, according to these studies, the function of p101 seems to increase the sensitivity of PI3Kγ to Gβγ-stimulation. However, there are some other observations arguing against an indispensable role of p101 in Gβγ stimulation of PI3Kγ by showing that PI3Kγ, in the absence of p101,

can be substantially activated by G-proteins (Stoyanov, B et al., 1995; Leopoldt, D et al., 1998). In another study, it was demonstrated that purified PI3Kγ from SF9 cells has kinase activity towards several 3-phosphoinositides, addition of p101 increases its specificity for PI(4,5)P_2. Thus the authors proposed that p101 might be responsible for the lipid substrate selectivity of PI3Kγ in its Gβγ-stimulated state (Maier, U et al., 1999). Up to date , the precise molecular basis of p101 in the regulation of PI3Kγ activity is unclear.

In contrast to classIA PI3Ks which signal downstream of tyrosine kinases, PI3Kγ is mainly activated by agonists of G-protein coupled receptors (GPCR), including the bacterial chemotatic peptide formyl-Met-Leu-Phe (fMLP), complement factor 5, interleukin-8. GPCR agonists lead to the activation of heterotrimeric Gαβγ proteins by exchanging the GDP molecule bound to the Gα subunit for GTP, thus releasing the Gβγ heterodimer (Gilman, AG, 1987). The mechanism whereby PI3Kγ is activated by GPCR is not completely defined, but likely involves direct binding of Gβγ to the PI3Kγ/p101 heterodimers. Direct binding of Gβγ to PI3Kγ is evidenced by co-purification of Gβγ with a GST-PI3Kγ fusion protein (Leopoldt, D et al., 1998). Involvement of p101 in binding to Gβγ is supported by a deletion analysis of p101 which indicates the Gβγ–stimulated activity of PI3Kγ requires the N-terminus of p101 and correlates with the tightness of binding of p101 to PI3Kγ (Krugmann, S et al.,1999). In any case, Gβγ seems to play a important role in the activation of PI3Kγ (Stoyanov, B et al., 1995, Murga, C et al., 1998), and possibly by localising PI3Kγ to the membrane (Leopoldt, D et al., 1998; Murga, C et al., 1998) where its lipid substrates reside. In support of this hypothesis, a membrane-targeted PI3Kγ mutant was shown to constitutively produce PIP_3 (Bondeva, T et al., 1998). It should be noted that, only specific Gβγ dimers can activate PI3Kγ. $G\beta_5\gamma_2$, for instance, does not activate PI3Kγ (Maier, U et al., 2000)

To date, PI3Kγ has been implicated in several signalling pathways. In COS-7 cells, PI3Kγ was shown to mediate a Gβγ-dependent pathway leading to mitogen-activated protein kinase (MAPK) stimulation, and possibly through the activation of a tyrosine kinase-Shc-Grb2-Sos-Ras dependent pathway (Lopez-Ilasaca, M et al., 1997). Nevertheless, the putative tyrosine kinase activated downstream of PI3Kγ in this study remains to be identified. In contrast, it was reported that PI3Kγ can mediate a Ras-independent activation on MAPK in CHO cells treated with lysophosphatidic acid (LPA) , an agonist of GPCRs (Takeda, H et al., 1999). The authors showed that PI3Kγ activation leads to the stimulation of MEK through PKCζ, a downstream target of PI(3,4,5)P_3. In addition to

MAPK, PI3Kγ also mediates Gβγ-dependent activation of Jun kinase (JNK) in COS-7 cells (Lopez-Ilasaca, M et al. 1998). In endothelial cells, shear stress leads to both MAPK and JNK activation (Go, YM et al., 1998). However, in this study, the authors have demonstrated that PI3Kγ only mediates the JNK, but not the MAPK activation. Thus, it seems PI3Kγ can participate in different signalling pathways depending on the cell type and stimulus.

It should be noted, in addition to its lipid kinase activity, PI3Kγ was also demonstrated to possess a serine kinase activity. Similar to PI3Kδ (Vanhaesebroeck, B et al., 1999), PI3Kγ is able to autophosphorylate *in vitro* (Stoyanova, S et al., 1997). Furthermore, it was demonstrated that PI3Kγ can transphosphorylate the adaptor protein p101 and the mitogen-activated protein kinase kinase 1 (MEK-1) *in vitro* (Bondev, A et al., 1999). The lipid and protein kinase activities of PI3Kγ could be involved in distinct cellular processes. Using a panel of engineered PI3Kγ constructs, Bondeva et al. demonstrated that the lipid kinase activity is needed for the lipid production and the subsequent activation of PKB, whereas the protein kinase activity is responsible for MAPK activation (Bondeva, T et al., 1998). Furthermore, it was also found that the membrane-targeted form of PI3Kγ constitutively produces $PI(3,4,5)P_3$ and activates PKB but interferes with the MAPK activation, suggesting the lipid and protein kinase activities of PI3Kγ are related to its cellular localisation. Although PI3Kγ protein kinase activity was shown to be sufficient to activate MAPK in this transient transfection system, its direct target protein has not been firmly established. Importantly, further work is required to assess whether this protein kinase activity is also operative under more physiological conditions.

PI3Kγ appears to be present only in mammals where it shows a more restricted tissue distribution than class IA PI3Ks. It is predominantly expressed in cells of hematopoietic origin (Ho, LK et al., 1997). Accordingly, multiple roles have been revealed for PI3Kγ in the regulation of blood cell functions. Studies of mice lacking PI3Kγ showed decreased migration of neutrophils and macrophages in response to chemokines *in vitro* and *in vivo* (Hirsch, E et al., 2000; Sasaki, T et al., 2000). As a consequence, the mice had reduced inflammatory responses to bacteria. In neutrophils, the chemokine-induced oxidative bursts were impaired (Sasaki, T et al., 2000). In addition, the T cell mediated inflammatory and humoral immune responses were also compromised in PI3Kγ deficient mice. (Sasaki, T et al., 2000).

1.2.2 Class II PI3Ks

Class II PI3Ks are a recently identified subclass of PI3K family. They are large molecules about twice the size (170-220 kDa) of type I PI3Ks. So far, three isoforms of class II PI3Ks, PI3K-C2α (Domin, J et al.,1997), β (Arcaro, A et al., 1998), γ (Ono, F et al., 1998) have been identified in mammals. These enzymes share high similarity in structure. However they have different pattern in tissue distribution. The PI3K-C2α and PI3K-C2β are ubiquitously expressed, in contrast PI3K-C2γ is mainly found in the liver. The striking feature of these proteins in structure is characterised by the presence of C-terminal C2 domain which is lacking in class I PI3Ks. The C2 domain was originally defined as the second of four conserved regions within mammalian protein kinase C (PKC) which confers a Ca^{2+} sensitive phospholipid binding (Kaibuchi, K et al., 1989). Whereas the C2-like domain of class II PI3Ks has been shown to bind *in vitro* to phospholipids in a Ca^{2+}-independent manner (MacDougall, LK et al., 1995). The deletion of C2 domain increased the *in vitro* lipid kinase activity of PI3K-C2β (Arcaro, A et al., 1998) toward PI. It is possible that the C2 domain competes with the catalytic domain for binding to phosphotidylinositol substrate, thus behaving as a negative regulator of lipid activity of the enzyme. The catalytic domain is also distinct from that of the class I enzyme. A feature of this domain is its ability to utilise Ca^{2+} -ATP for the *in vitro* lipid kinase activity (Arcaro, A et al., 1998). The N-terminal regions of class II PI3Ks are the most divergent among these isoforms, and show no homology to those of class I PI3Ks. Although a Ras-binding fold exists in this regions, class II PI3Ks have not been found to associate with Ras (Arcaro, A et al., 1998). In contrast to class I PI3Ks which are mainly cytosolic, class II PI3Ks show a predominant membrane localisation. *In vitro*, they preferentially utilise PtdIns, PtdIns(4)P, but can, albeit poorly phosphorylate PtdIns(4,5)P_2. (Domin, J et al.,1997, Arcaro, A et al., 1998). However, it is still unclear which lipids they produce *in vivo*. Insulin (Brown, RA et al., 1999), EGF (Wheeler, M, et al., 2001), and integrin (Zhang, J et al., 1998) have been shown to be able to activate class II PI3Ks in *in vitro* lipid activity assay system. However, the signalling events linking the above stimuli to the activation of these PI3Ks are unclear. Besides, the physiological relevance of these enzymes still remains to be established. Only a recent study has shown that PI3K-C2β is involved in cell growth of a human small cell lung carcinoma (SCLC) cell line in response to SCF stimulation (Arcaro, A et al., 2002).

1.2.3 Class III PI3Ks

To date, a single class III PI3K catalytic subunit has been identified in mammals (Volinia, S et al., 1995) which is the homolog of the yeast vesicular-protein-sorting protein Vps34p (Schu, PV et al., 1993). The only substrate for this enzyme is PtdIns, both *in vivo* and *in vitro* (Volinia, S et al., 1995). This catalytic subunit exists in association with a Ser/Thr protein kinase (Vps15 in yeast, p150 in human). Vps15 has been shown to be essential for Vps34p localisation and activation (Stack. JH et al., 1995), and a similar role is defined for p150 in mammals (Panaretou, C et al., 1997). The major cellular function of class III PI3Ks has been implicated in vacuolar protein sorting in yeast and membrane trafficking processes in mammalian.

1.3 Signalling proteins downstream of PI3Ks

Upon activation in response to various of stimuli, PI3Ks phosphorylate inositol lipids at the D-3 position of the inositol ring to generate the 3-phosphoinositides PI(3)P, PI(3,4)P_2, PI(3,4,5)P_3 *in vitro*. These lipids in turn serve as second messengers and function in signal transduction by interacting with 3-phosphoinositide-binding modules present in a broad variety of proteins. Specifically, certain FYVE domains bind to PI(3)P, whereas certain pleckstrin homology domains bind PI(3,4)P_2 and PI(3,4,5)P_3.

FYVE domains were named after four of the proteins in which they are first identified: Fab1p ,YOTB, Vac1p and Early endosome antigen 1 (EEA1). They are cysteine-rich zinc-finger-like motifs that specifically bind PI(3)P but not PI(3,4)P_2 , PI(3,4,5)P_3 The FYVE domain-PI(3)P interactions are shown to be essential for vesicular trafficking (Corvera, S et al., 1998).

PH domains are structurally conserved modules of approximately 100 amino acids that was shown to be involved in protein-protein interaction and binding to PI(4,5)P2 (Lemmon, M. A et al., 1997). Now it is clear that PH-domains bind preferentially to PI(3,4)P_2 and PI(3,4,5)P_3 over other inositol lipids (Salim, K et al., 1996). To date, PH domain has been found to be present in numerous proteins. These proteins include serine/threonine kinase Akt and 3-phosphoinositide-dependent kinase-1(PDK1), certain guanine nucleotide exchange factors (GEFs), certain tyrosine kinases and phospholipase-C(PLC)γ .

The serine/threonine kinase Akt is one of the well-established targets of PI3K (Kandel, ES et al., Hay, 1999). Akt, also termed PKB, is the cellular homologue of the transforming viral oncogene v-Akt and bears significant homology to PKA and PKC (Bellacosa, A et

al., 1993). Akt contains an N-terminal PH domain, a central kinase domain with an activation-loop, a Thr308 phosphorylation site, and a conserved regulatory serine phosphorylation site Ser473 near the C terminus. The activation of Akt entails a complex series of events involving additional proteins. First, PI3Ks generated lipid production $PI(3,4)P_2$ and $PI(3,4,5)P_3$ recruit Akt to the plasma membrane through their affinity for the PH domain of Akt (Burgering, BM et al., 1995), where it is phosphorylated on Thr308 by another PH domain containing protein, PDK1 (Anderson, KE et al., 1998). Phosphorylation of Thr308 is a prerequisite for kinase activation, but phosphorylation of Ser 473 is required for full activation of Akt kinase. The mechanism by which the Ser 473 is activated is incompletely understood. Although the existence of a kinase that targets Ser 473, termed PDK-2, has been postulated (Anderson, KE et al., 1998), the identification for this kinase has not been successful.

The activation of Akt has been shown to mediate many cellular functions by subsequent phosphorylation of a host of other proteins. One of the major functions of Akt is to promote growth factor-mediated cell survival and to block programmed cell death or apoptosis. It was shown that Akt phosphorylates the proapoptotic factor, BAD, on a serine residue and prevents its binding to Bcl-2 family members Bcl-2 and Bcl-XL, thus releasing them for a survival response (Datta, SR et al., 1997; Brunnet, A et al., 2001). Subsequently, it was reported that Akt-induced phosphorylation of a death protease called caspase-9 prevents apoptosis by inhibiting the protease activity directly (Cardone, MH et al., 1998). In addition, Akt can interfere with cell death through transcription-based mechanisms. For instance, Akt induces the degradation of IκB by promoting IKKα activity and subsequently increase the activity of nuclear factor-κB (NF-κB) leading to increased synthesis of antiapoptotic proteins, such as c-myc and Bcl-X_L (Lee, HH et al., 1999; Romashkova, JA et al., 1999). It was also shown that Akt promotes cell survival by preventing the expression of proapoptotic factors such as Fas ligand through the phosphorylation and inactivation of the Forkhead transcription factor (Brunet, A et al., 1999).

Akt can also influence protein synthesis. Forkhead transcription factors, cAMP-response element binding protein (CREB), E2F, and NFκB have been shown to be targets for Akt (Brunet, A et al., 1999). Akt modulates the activity of multiple factors that are required for mRNA translation. Akt can induce initiation of translation through phosphorylation and inactivition the repressor of translation 4E-BP (eIF-4E binding protein), and subsequent activation of elongation/initiation factor eIF-4E (Gingras, AC et al., 1998; Miron, M et al.,

2001). The mammalian target of rapamycin (mTOR) has been shown to be directly phosphorylated by Akt, which in turn mediates activation of the elongation factor eEF2 (Wang, X et al., 2001). In addition to Akt, PDK1 may also contribute to protein synthesis. PDK1 is reported to activate p70S6 kinase (Pullen, N et al., 1998). The p70S6 kinase can regulate mRNA translation through the phosphorylation of 40S ribosomal protein S6 (Ballou, LM et al., 1991).

The cellular function of Akt is also involved in the regulation of cell–cycle entry. Cyclin D 1 is a substrate of glycogen synthase kinase 3 (GSK3), which when phosphorylated by GSK3, is targeted for degradation (Diehl, JA et al., 1998). However, phosphorylation of GSK3 by Akt results in the inactivation of GSK3, which allows cyclin D1 to accumulate. In addition, Akt can phosphorylate and inactivate Forkhead transcription factors. The inactivation of these transcription factors leads to the decreased transcription of cyclin-dependent kinase (CDK) inhibitor, $p27^{kip1}$ (Medema, RH et al., 2000, Schmidt, M et al., 2002). Thus, Akt might have several inputs in control of the cell cycle.

Some of the metabolic actions of insulin are also mediated by Akt. It enhances glucose uptake in insulin-response tissues by affecting the glucose transportors GLUT1, GLUT3 and GLUT4. Akt induces the expression of GLUT1, GLUT3 (Berthel, A et al., 1999; Hajduch, E et al. 1998) and the tanslocation of GLUT4 (Kohn, AD et al., 1996). It also activates glycogen synthesis through the inactivation of GSK3., which in turn leads to the activation of glycogen synthase (Cross, DA et al., 1997). In addition, Akt is shown to directly activate 6-phosphofucto-2-kinase (PFK2), which is an important regulator of glycolysis (Deprez, J et al., 1997).

The PH-domain of the Bruton's tyrosine kinase (Btk) is also a downstream target of class I PI3Ks. Btk is a member of Tec kinases, a family of nonrecptor tyrosine kinases. The PH domain of Btk has been found to bind selectively to $PI(3,4,5)P_3$ (Rameh, LE et al., 1997) and is thought to mediate the translocation of Btk to the plasma membranes where it is activated by Src family kinases or PDK1-related enzymes. Once activated, Btk-related kinases can phosphorylate and activate PLCγ. Btk activation is involved in B-cell development and mutations in its PH-domain have been found to be responsible for X-linked immunodeficiency in mice (Xid) and X-linked agamma-globulinaemia in humans (Fukuda, M et al., 1996).

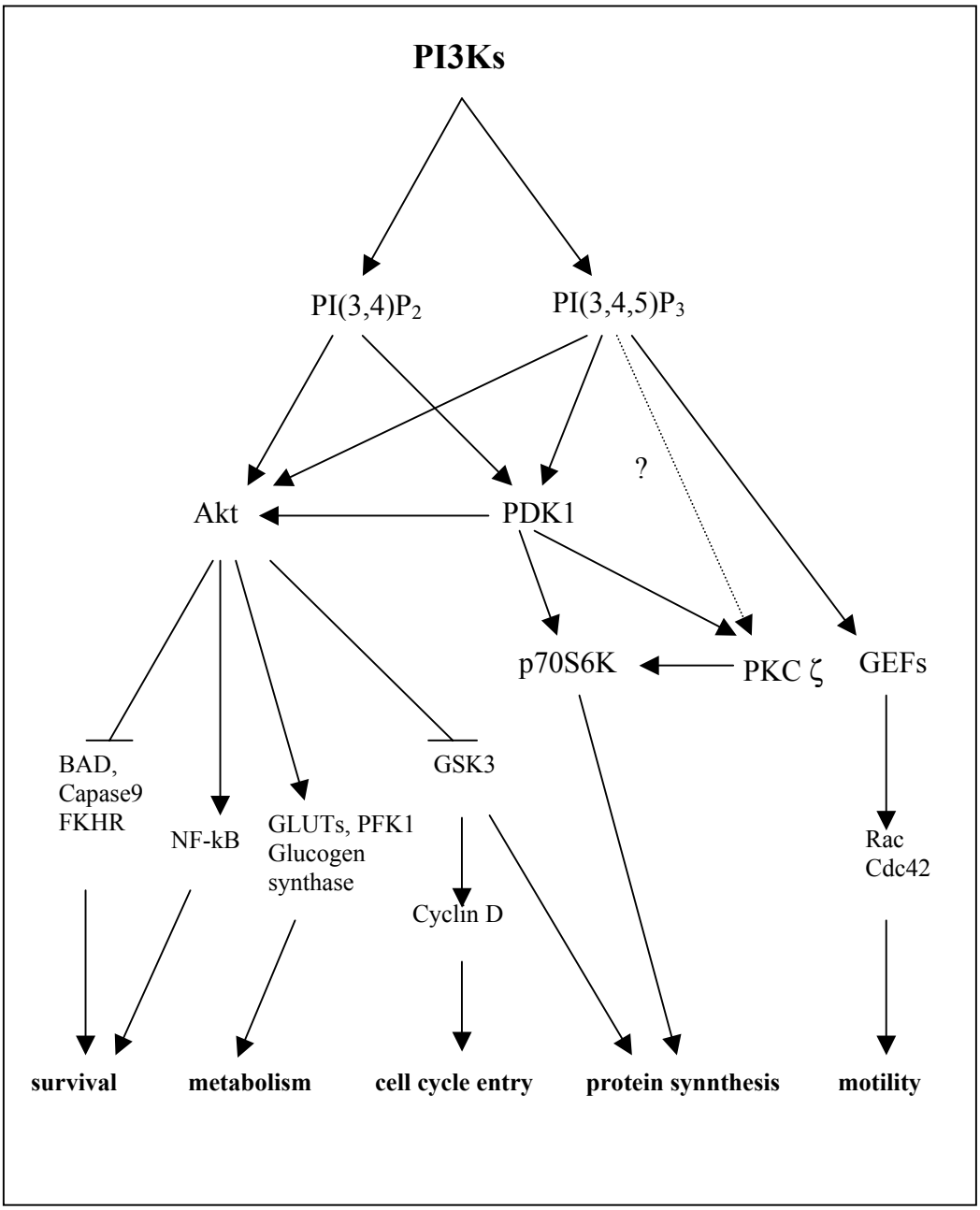


Fig.3 Main signalling pathways downstream of PI3Ks

PKC isoforms have also been shown to be the downstream targets of PI3Ks. However, the precise mechanism underlying the PKC activation by PI3Ks is unclear. Several studies have provided evidence that some of the atypical (ε,η) and novel PKC(ζ) isoforms, as well as the PKC related kinase (PRK) can bind to and are directly activated by $PI(3,4)P_2$ and/or $PI(3,4,5)P_3$ (Nakanishi, H et al., 1993; Palmer, R.H et al., 1995; Toker, A et al., 1994). Alternatively, it is proposed the activation of PKCs is controlled by PDK1, based on the

observation that PDK1 can phosphorylate critical residues in the activation loop of several PKC isoforms (Le Good , JA et al., 1998).

PH domains are also present in many guanine nucleotide exchanging factors (GEFs) specific for the Rho family of GTPases, which include Rho, Rac and Cdc42. $PI(3,4)P_2$ and $PI(3,4,5)P_3$ have been shown to bind to the PH-domain of the exchange factor VAV (Palmby, TR et al, 2002). The binding of $PI(3,4,5)P_3$ is thought to cause an allosteric activation of its exchange activity towards the small GTPases Rac and CDC42. Both GTPases are involved in regulation of cytoskeletal rearrangement and cell migration (Rameh, LE et al., 1999).

The PI3K signalling is negatively regulated by at least two types of phosphatases. The Src-homology 2(SH2)-containing phosphatases (SHIP1 and SHIP2) convert $PI(3,4,5)P_3$ to $PI(3,4)P_2$ by dephosphorylating the 5 position of the inositol ring. PTEN, another kind of phosphatase, dephosphorylates the 3 position of $PI(3,4,5)P_3$ and $PI(3,4)P_2$. Loss of PTEN or function has been found in a large fraction of human cancers, indicating that the uncontrolled PI3K signalling contributes to tumor formation (for an overview, see {Cantley, LC, 2002}).

1.4 PI3Ks and cell differentiation

1.4.1 Involvement of PI3Ks in hematopoiesis

Cell differentiation plays an important role in the developmental biology. An excellent model system in differentiation study is the hematopoietic system. The formation of different types of blood cells, hematopoiesis, is essential for the development and survival of an individual. During normal hematopoiesis, a small pool of pluripotent, hematopoietic stem cells gives rise to several blood cell types through a process of simultaneous lineage commitment, cell proliferation, and differentiation (Fig 4).

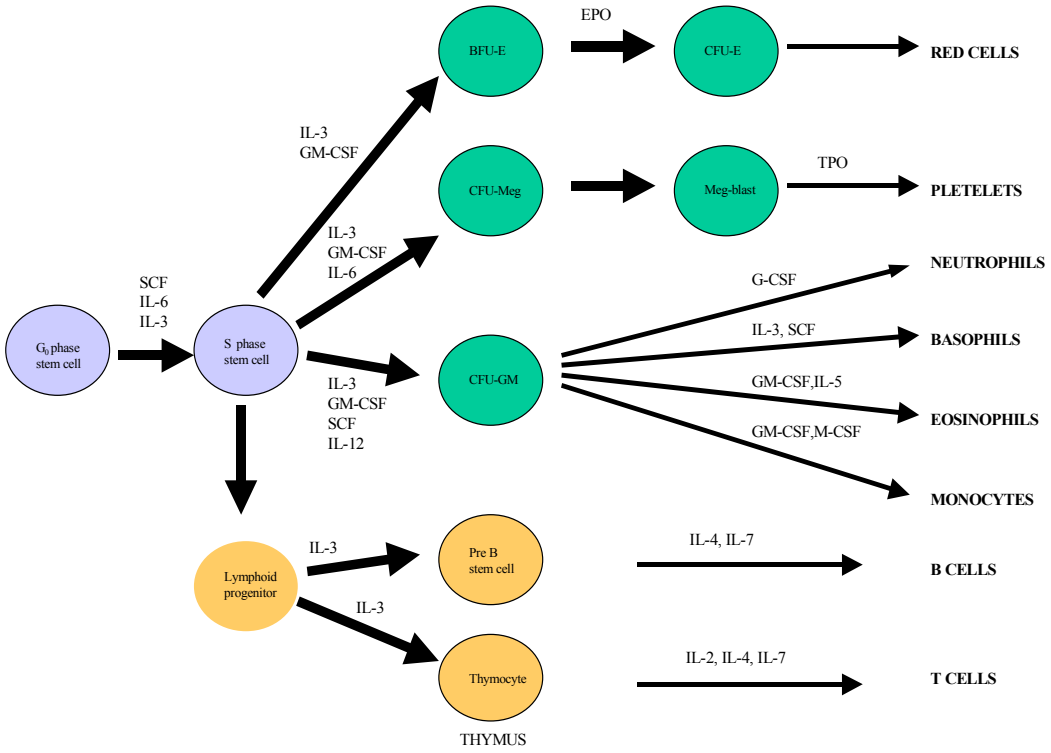


Fig.4 Scheme of hematopoiesis

This process is, at least in part, regulated by a complex network of hematopoietic growth factors (for an overview, see {Lotem, J et al., 2002}). Interleukin (IL)-3, also known as multi-colony-stimulating factor (multiple-CSF), stimulates the proliferation of multipotential progenitors as well as various lineage-committed progenitors, granulocyte colony-stimulating factor (G-CSF) also stimulates early progenitors in addition to granulocytes and macrophges. In contrast, erythropoietin (EPO), thrombopoietin (TPO), granulocyte colony-stimulating factor (G-CSF), and IL-5 predominantly stimulate erythrocytes, megakaryocytes, granulocytes and eosinophils, respectively (for an overview, see {Miyajima, A et al., 1999}). In addition, other cytokines such as interleukin (IL)-6 and stem cell factor are essential for the expansion of the primitive stem cell and the proliferation of various progenitors (Rappold, I et al., 1999). It should be noted multiple cytokines usually exhibits overlapping functions, because the receptors of these cytokines share some common structures. Currently, how these growth factors elicit varied developmental responses leading to different blood cell lineages has been only partially understood.

Once binding to their receptors, these cytokines are able to activate distinct intracellular signalling pathways. Among these well established signalling pathways involved in hematopoiesis are the Ras/Raf/MAPK pathway, and the Janus kinases (JAKs)/signal transducers and activators of transcription (STATs) pathway (for an overview, see {Miyajima, A et al., 1999}). Recently, several lines of evidence have shown that PI3Ks are also involved in the process of hematopoiesis.

The direct evidence implicating PI3K in hematopoiesis was derived from gene targeting studies. The class IA p85α regulatory subunit of PI3K has been knocked out by two independent groups (Fruman, DA et al., 1999; Suzuki, H et al., 1999). One strategy has led to the elimination of all the splice variants (Fruman, DA et al., 1999), whereas another eliminated the long (p85α) splice variant, but allowing for the expression of the shorter p50α and p55α isoforms (Suzuki, H et al., 1999). Both lines of p85 knockout mice have impaired pre-B cell development, reduced numbers of mature B cells in the spleen. These results suggest class IA PI3Ks play a role in the development of B cells. More recently, a defective B-cell development was also revealed in mice lacking the PI3Kδ (Jou, ST et al., 2002). It was shown that the percentages of pro-B cells ($CD43^+$ $B220^+$) were similar for wild type and PI3Kδ deficient bone marrow. However, the more mature B-cell subpopulation of $B220^+$ $CD43^-$ cells was significantly reduced in the bone marrow of PI3Kδ knockout mice. Taken together, these results point to an essential and nonredundant and stage-specific role for PI3Kδ in the development and differentiation of B cells.

In addition to B cells, recent studies have implicated PI3Ks in erythropoiesis, the process by which erythrocytes are formed. The process of erythropoiesis can be divided into a number of discrete parts: recruitment and commitment of pluripotent hematopoeitic stem cells to erythroid progenitor cells including erythroid burst-forming unit (BFU-E) and erythroid colony-forming unit (CFU-E), commitment of CFU-E to erythroblasts in the presence of EPO, and enucleation of the mature erythroblasts and their release into the bloodstream (Perry, C et al., 2002). Glycophorin A(GPA) is a marker for early erythroid differentiation, which has been most commonly used to define erythroid progenitors (Chasis, JA et al., 1992). Myklebust et al. have demonstrated that the formation of GPA-positive cells from $CD34^+$ stem cells were significantly inhibited by LY294002, the specific PI3K inhibitor (Myklebust, JH et al., 2002). In another study, it was shown that LY294002 not only inhibited the proliferation of erythroid progenitor cells (CFU-E), but also induced apoptosis of these cells as well (Haseyama Y et al., 1999).

Although the above data provide evidence that PI3Ks play an essential role in the earlier stages of erythroid differentiation, the isoform-specific role of PI3Ks in this process has not been well established. Recently, Kubota et al. have shown that the antisense p85α oligonucleotide inhibited the EPO-induced formation of CFU-E from normal human hematopoietic progenitor cells, implicating the class IA PI3Ks in erythropoiesis (Kubota, Y et al., 2001). It is proposed the binding of p85α through its SH2 domain to the tyrosine-phosphorylated EPO receptor is an essential step for the EPO induced erythroid differentiation signals. However, this *in vitro* result was not confirmed in p85α-deficient mice which show defects in B cell development but not in erythropoiesis (Fruman, DA et al., 1999; Suzuki, H et al., 1999). The authors suggested other molecules of PI3K family or other signalling molecules may compensate for the defect of p85α function in erythropoiesis during the early development of p85α-deficient mice. However, the precise reason for this discrepancy is still unclear.

The importance of PI3Ks in the regulation of erythropoiesis is also highlighted by the study on the cell transformation processes leading to erythroleukemia. PU.1 is a hematopoietic-specific Ets transcription factor, promoting differentiation of lymphoid and myeloid lineages. PU.1 dysregulated activation in erythroid progenitors can induce erythroleukemia in mice (Moreau-Gachelin, F et al., 1996). Barnache et al. showed that the overexpression of PU.1 in murine proerythroblasts induces their differentiation arrest and hyperproliferation, and the transformation ability of PU.1 is, at least in part, due to the subsequent activation of PI3Ks (Barnache, S et al., 2001). Thus the dysregulated activation of PI3Ks seems to interfere with the terminal erythroid differentiation. Paradoxically, Bavelloni et al. have demonstrated the dimethyl sulfoxide (DMSO) induced terminal differentiation of murine erythroleukemia cells was inhibited by LY294002 (Bavellooni, A et al., 2000). Thus the exact role of PI3Ks in the terminal differentiation of erythrocyte is unknown.

In addition to the above data, PI3Ks have been shown to be involved in other lineage differentiation processes. In this regard, the neutrophilic differentiation of HL60 cells induced by G-CSF was enhanced by wortmannin in a dose-dependent manner (Kanayasu-Toyoda, T et al., 2002). In another study, Baier et al. have demonstrated that PI3Kγ plays an essential and nonredundant role in the differentiation of myelomonocytic U973 cells, despite in PI3Kγ deficient mice the myeloid differentiation seems to be normal (Bayer, R et al., 1999; Hirsch, E et al., 2000; Sasaki T et al., 2000). It should be noted that a targeted gene disruption *in vivo* does not simply indicate a functional defect of the targeted

molecule, but provide a sum of alternative mechanisms by which other factors adapt to the unusual circumstances. The action of compensatory or redundant pathways may obscure the authentic *in vivo* role of the targeted gene.

1.4.2 Involvement of PI3Ks in differentiation of non-hematopoietic cells

The involvement of PI3Ks in differentiation is not only restricted to cells of hematopoietic origin, several lines of evidence have implicated PI3Ks in the differentiation processes in other cell types.

Rat pheochromocytoma PC12 cells are a model system for the differentiation of neuronal cells. After stimulation with nerve growth factor (NGF), neurite-specific genes are induced and neurite outgrowth occurs (Halegoua, S et al., 1991). It was shown that PI3K is activated in PC12 cells after NGF treatment, and inhibition of PI3K by the PI3K specific inhibitor wortmannin abrogated the NGF induced neurite outgrowth of these cells (Kimura, K et al., 1994). In addition, a dominant-negative mutant of p85 was also shown to have a similar inhibitory effect on neurite outgrowth as with wortmannin (Jackson, TR et al., 1996). Conversely, in the absence of NGF, neurite outgrowth could be partially induced by the microinjection of a constitutively active mutant of PI3K, and this effect of PI3K seemed to be due to the subsequent activation of the Rac-JNK pathway (Kita, Y et al., 1998).

A similar role for PI3K was also revealed in the myogenic differentiation. Chicken embryo myoblasts (CEM) are the precursors of chicken skeletal-muscle cells. CEM undergo myogenic differentiation *in vitro*. The oncogene p3k, coding for a homolog of the catalytic subunit p110α of PI3K with constitutive activity, was shown to strongly enhance the myogenic differentiation in cultures of CEM. This effect was abrogated by the use of PI3K inhibitor LY294002 (Jiang, BH et al., 1998). Another study on the mechanism by which insulin-like growth factors induce myogenesis in cultures of rat skeletal-muscle myoblast cell line L6E9 also identified PI3K as an essential component in this process (Kaliman, P et al., 1998).

While the above findings point to a positive role of PI3K in the regulation of cellular differentiation, in some other studies PI3K was shown to exert an opposite effect. A recent study by Ptasznik et al. has demonstrated that blockade of the basal level of PI3K activity in primary cultured human fetal pancreatic cells resulted in a robust activation of spontaneous endocrine differentiation. Thus, the primary role of PI3K in these cells seems to keep them in an undifferentiated state (Ptasznik, A et al., 1997). Similarly, a negative

role of PI3K in differentiation was also demonstrated in human colon cancer cells, HT29 and Caco-2 (Wang, D et al., 2001). In this study the authors showed the PI3K inhibitor wortmannin significantly enhanced the sodium butyrate induced differentiation of these cells in a time- and dose-dependent fashion, and the effect of wortmannin was mimicked by overexpressing the dominant negative mutant of p85. Interestingly, overexpression of PTEN also augmented the inductive effect of sodium butyrate on the differentiation of these cells, which suggests a contributory role of the lipid products of PI3K in intestinal cell differentiation.

In summary, available data suggest PI3Ks are important regulators for cellular differentiation in mammalian cells. A major change in the future work is to define the nonredundant role for each of the PI3K isoforms in a specific differentiation process. Furthermore, studies directed at downstream signalling elements coupled to PI3Ks should be of great value, as will the investigation of the transcription factors by which PI3Ks are linked to tissue-specific gene regulation.

1.5 Aim of the work

Phosphoinositide 3-kinase (PI3K) is a ubiquitous enzyme that has been shown to be an important mediator of various intracellular signalling pathways. In mammals, complexity arises by the expression of multiple PI3K isoforms which differ in structure, substrate specificity, binding partner and mode of regulation. Despite of this, much progress has been made in defining the role of PI3Ks in many aspects of cellular processes , such as cell proliferation, cell migration, cell cycle entry and survival. However, our knowlege about the role of PI3Ks in cell differentiation is relatively limited. Several lines of evidence have indicated PI3Ks can be positive or negative regulators of cell differentiation in different cell types. PI3Kγ, the only isoform of class IB PI3K is predominantly expressed in cells of hematopoietic origin. It was previously implicated in the retinoic acid induced differentiation of U937 cell line. This prompted us to make further investigations using human peripheral blood stem cells. These cells were induced to differentiate into several lineages by using different combinations of growth factors. The preliminary results showed a downregulation of PI3Kγ expression is specifically associated with erythropoietin-induced erythroid differentiation, indicating a possible involvement of PI3Kγ in this process. The aim of this work was to explore the role of PI3Kγ in erythroid differentiation in more details using K562 cell line as a model system. As PI3Kγ possesses both lipid kinase and protein kinase activities, we intend to make use of a panel of engineered PI3Kγ mutants to address which kinase activity is involved in this process. Furthermore, immunoprecipitation will be used to study the potential protein-protein interactions in attempts to define the downstream targets of PI3Kγ.

2. MATERTIALS AND METHODS

2.1 Materials

2.1.1 Cells

- $CD34^+$ stem cells

Isolated from peripheral blood of healthy adult donors.

- K562 Cell line

Human chronic myeloid leukemia in blast crisis, established from the pleural effusion of a 53-year-old woman with CML in blast crisis in 1970 (Lozzio and Lozzio 1975); The cells produce haemoglobin. They carry the Philadelphia chromosome with b3-a2 fusion gene. This cell line was purchased from DSM, Braunschweig, Germany.

2.1.2 Bacterial strains (E. coli)

Escherischia coli XL1-Blue (Stratagene, La Jolla, CA, USA) , used for plasmid amplification

2.1.3 Plasmids

- pcDNA3 empty vector (Invitrogen, GmbH, Karlsruhe,Germany)
- pcDNA3-PI3Kγ -wild type
  Encoding the full length PI3Kγ
- pcDNA3-PI3Kγ -K832R
  Mutant of PI3Kγ with mutation in wortmannin binding site, catalytically inactive
- pcDNA3-PI3Kγ -FRAP
  Swap mutant between PI3Kγ and mammalian TOR (FRAP)
- pcDNA3-PI3Kγ CAAX
  Membrane associated form of PI3Kγ by adding into C-terminus of PI3Kγ sequence of Raf/K-Ras-CAAX for membrane localisation
- pcDNA3-PI3Kγ -antisense
  Generated by inverting the orientation of the wild-type PI3Kγ full-length cDNA into pcDNA3.

- pcDNA3-GFP-PI3Kγ
  A cDNA of PI3Kγ subcloned in pEGFP-N1 Plasmid (Clontech, Heidelberg, Germany)

2.1.4 Primers for RT-PCR

- Primers used for an amplification of human p101:
  forward primer: 5' gacatgcacggaggaccgcatccag 3'
  reverse primer: 5' acagcaaggaactcggcctg3'

- Primers used for an amplification of human β-actin:
  forward primer: 5' ccgtaccactggcatcgtgat 3'
  reverse primer: 5' catactcctgcttgctgatc 3'

2.1.5 Antibodies

Antibodies for Western blot and Immunoprecipitation

- Anti-PI3Kγ monoclonal antibody, directed against the N-terminal fragment of PI3Kγ, used for Western blot (FSU facilities)
- Anti–PI3Kγ monoclonal antibody, clone 641, used for Immunoprecipitation (Eurogentech facilities, Berlin, Germany)
- Anti–PI3Kγ polyclonal antibody, directed against the peptide SOLPESFRVPYDPGN located in the C-terminal fragment of PI3Kγ, used for immunoprecipitatation (FSU facilities)
- Anti–PI3Kβ polyclonal antibody (s-19) (Santa Cruz Biotechnology, Inc., Heidelberg, Germany)
- Anti-p85 polyclonal antibody (Upstate biotechnology, Hamburg, Germany)
- Anti–HA monoclonal antibody (BabCO, Richmond, California, USA)
- Anti-phospho-Akt (S473) polyclonal antibody (New England Biolabs, Frankfurt, Germany)
- Anti-Akt1 polyclonal antibody (Pharmingen, Heidelberg, Germany)
- Anti-phospho-p42/44 MAPK monoclonal antibody (New England Biolabs, Frankfurt, Germany)

- Anti-pan-ERK monoclonal antibody (BD Transduction Laboratories, Heidelberg, Germany)
- Anti-vinculin mouse monoclonal antibody (Serotec LTD,UK)
- Horseradish-peroxidase-conjugated goat-anti-mouse IgG (Santa Cruz Biotechnology, Inc, Heidelberg, Germany)
- Horseradish-peroxidase-conjugated goat-anti-rabbit IgG (Santa Cruz Biotechnology, Inc, Heidelberg, Germany)

Antibodies for immunocytochemistry and FACS analysis

- Anti-human hemoglobinA polyclonal antibody (DAKO, Diagnostika GmbH, Hamburg, Germany)
- Anti-CD34-PC5 (phycoerythrin)monoclonal antibody (BD Transduction Laboratories, Heidelberg, Germany)
- Isotype control for IgG1-PC5 (Beckman /Coulter, Germany)
- Anti-CD61-FITC (fluoresceinisothiocyanate) monoclonal antibody (DAKO, Diagnostika GmbH, Hamburg, Germany)
- Anti-Glycophorin A-PE monoclonal antibody (DAKO, Diagnostika GmbH, Hamburg , Germany)
- Isotype control antibodies IgG1-FITC, PE (DAKO,Diagnostika GmbH, Hamburg, Germany)
- Streptavindin-Biotin-AP-conjugated anti-rabbit secondary antibody (DAKO Alkaline-phosphatase-kit 5005)

2.1.6 Medium and substances for cell culture

- RPMI1640 (BIOCHEM AG, Berlin, Germany)
- Iscoves modified Dulbecco medium (IMDM) (BIOCHROM KG , Berlin, Germany)
- Fetal calf serum (Life Technologies GmbH, Eggenstein, Germany)
- Recombinant human erythropoietin (EPO) (Gilag GmbH, Fresenius AG, Germany)
- Recombinant human thrombopoietin (TPO) (TEBU GmbH, Frankfurt, Germany)
- Recombinant human IL6 (Roche Diagnostics GmbH, Mannheim, Germany)
- Recombinant human granulocyte colony-stimulating factor (C-GSF) (AMGEN GmbH, Munich, Germany)
- Recombinant human stem cell factor (SCF) (AMGEN, Thousand Oaks, CA, USA)

- N-Acetyl-L-alanyl-L-glutamin (Biochrom KG, Berlin, Germany)
- Iron saturated transferrin (Sigma, Munich, Germany)
- Antibiotics: 100 U/ml penicillin/ 0.1 mg/ml streptomycin (Sigma, Munich, Germany)
- Cytosine arabinoside (Amersham Pharmacia Biotech, Freiburg, Germany)

2.1.7 Materials for silver staining

- Acetic acid glacial (Carl Roth GmbH & Co KG, Karlsruhe, Germany)
- Formaldehyde (37 %, w/v) (Carl Roth GmbH & Co KG, Karlsruhe, Germany)
- Methanol (Carl Roth GmbH & Co KG, Karlsruhe, Germany)
- Silver nitrate (Carl Roth GmbH & Co KG, Karlsruhe, Germany)
- Sodium carbonate (anhydrous) (Carl Roth GmbH & Co KG, Karlsruhe, Germany)
- Sodium thiosulfate (Carl Roth GmbH & Co KG, Karlsruhe, Germany)

2.1.8 Other reagents and materials

- Acrylamide, Rotiphorese Gel 30 (30 % Acrylamide / 0,8 % Bisacrylamide) (Carl Roth GmbH & Co KG, Karlsruhe, Germany)
- Actigel ALD Superflow kit (Sterogene, supplied in 20% Ethenal, 0.02%Sodium azide).
- [γ^{32}P]ATP, 3000 Ci/mmol (NEN Life Science, Brussels, Belgium)
- Benzidine dihydrochloride (Sigma, Munich, Germany)
- BioMax MR-1 Film (Amersham, Freiburg, Germany)
- Bradford-reagent (Sigma, Munich, Germany)
- CliniMACS separation system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)
- Dimethyl pimelimidate (Sigama, USA)
- Ethanolamine (Merck, frankfurt, germany)
- ECL-Kit (NEN Life Science, Brussels,Belgium)
- Gel drying film (Promega, Heidelberg, Germany)
- Geneticin (G418) (Invitrogen, Karlsruhe, Germany)
- Glycerol (Carl Roth GmbH & Co KG, Karlsruhe, Germany)
- LipofectAMINE PLUS^{TM} Reagent (Invitrogen, Karlsruhe, Germany)
- LY 294002 (Calbiochem Novabiochem, Bad Soden, Germany)
- NP-40 (Serva, Heidelberg, Germany)
- PD 98059 (Calbiochem Novabiochem, Bad Soden, Germany)
- Peptone (Becton Dickinson, Heidelberg,Germany)

- Protease inhibitors (Sigma, Munich, Germany)
- Protein A-Sepharose (Amersham Pharmacia Biotech, Freiburg, Germany)
- Protein G-Sepharose (Amersham Pharmacia Biotech, Freiburg, Germany)
- Polyvinylidene fluoride (PVDF)-membrane (Millipore, Eschborn,Germany)
- Qiagen plasmid-purification kit (QIAGEN GmbH, Hilden, Germany)
- Qiagen RNeasy Mini Kit (QIAGEN GmbH, Hilden, Gernany)
- Qiagen One-step RT-PCR Kit (QIAGEN GmbH, Hilden, Gernany)
- Sodium borate (Carl Roth GmbH & Co KG, Karlsruhe, Germany)
- Sodiumdodecylsulfate (SDS) (Carl Roth GmbH & Co KG, Karlsruhe, Germany)
- Sterile filters, 0.22 μm (Millipore Corporation, Eschborn,Germany)
- Triton X-100 (Carl Roth GmbH & Co KG, Karlsruhe, Germany)
- Tween 20 (Serva, Heidelberg, Germany)
- Yeast extract (Serva, Heidelberg, Germany)

2.1.9 Buffers and Solutions

All buffers and solutions were prepared with deionized water. Specifications in percentage are in weight per volume for dry substances and volume per volume for liquids.

Phosphate-buffered saline (PBS)

$Na_2HPO_4 \cdot 7\ H_2O$	4.3 mM
KH_2PO_4	1.4 mM
NaCl	137 mM
KCl	2.7 mM

TAE(1x)

0.04M	Tris-acetate
0.01 M	EDTA

Buffers for bacteria work

5 x KCM-buffer

KCl	500 mM
$CaCl_2$	150 mM
$MgCl_2$	250 mM

1×LB-medium

Bacto-Trypton (Peptone)	10 g
yeast extract	5 g
NaCl	10 g

fill up to 1L with H_2O

for LB-agar , add 15g of agar

TSB

2×LB	75 ml
DMSO	7.5 ml
1M $MgCl_2$	1.5 ml
1M $MgSO_4$	1.5 ml
PEG4000	15 g

Add to 150ml with H_2O and Steriled with 0.22 μm filter

Buffers for Western blot

Lysis buffer

20 mM HEPES (pH 7.5),
150 mM NaCl
1 0mM EGTA
1 % NP40
10 μg/ml leupeptin*
1 μg/ml pepstatin A*
1 μg/ml apportion*
1 mM benzamidine*
10 mM Pefabloc*
0.1 mM PMSF*
1 mM sodium-orthovanadate*
10 mM β-glycerolphosphate*
10 mM NaF*
1 mM DTT*
(* added freshly just before use)

5 x Loading buffer

50 % glycerol
500 mM Tris(pH: 6.8)
5 mM EDTA
10 % SDS
5% β-mercaptoethanol
0.1 % bromophenol blue

SDS-PAGE electrophoresis buffer

25 mM Tris
250 mM Glycine
0.1 % SDS

Semi-dry transfer buffer

TrisBase	48 mM
Glycine	39 mM
SDS	0.037 %
Methanol	20 %

TBS-Tween washing buffer(pH,7.5)

TrisHCl pH 7.5	20 mM
NaCl	137 mM
0.1 %	Tween-20

Membrane Strip-Buffer

Tris-HCl pH6.8	62 mM
β-Mercaptoethanol	100 mM
SDS	2 %

Coomassie-stain solution

Coomassie G250 (for membranes)
Coomassie R250 (for gels) 2 g/L

Acetic acid	10 %
Methanol	50 %

Destaining solution for Coomassie-stained gel

acetic acid	10 %
Methanol	25 %

PI3Kγ protein kinase reaction buffer

12.5 mM MOPS pH 7.5
7.5 mM $MgCl_2$
12.5 mM β-glycerolphosphate
10 mM NaF*
1 mM Sodium-orthovanadate*
(* added freshly just before use)

Silver staining solutions

- **Fixation solution**
  Methanol:Acetic acid: Water 50:5:45(v/v/v)
- **Sensitizing solution**
  Sodium Thiosulfate 0.02 %
- **Staining Solution**
  Silver nitrate 0.1 %
- **Developing solution**
  Formaldehyde 0.04 in 2 % sodium carbonate
- **Stop solution**
  Acetic acid 1 %

2.2 Methods

2.2.1 Plasmid preparation

Competent bacteria preparation

2ml LB-medium (without ampicillin) were inoculated with *E.coli* XL1-Blue and grown over night at 37°C. 1 ml of this culture was transferred to 150 ml fresh LB-medium without ampicillin. The culture was incubated on a shaker for 2-4 hours at 37°C until OD_{600} reaches 0.6. Then the cells were pelleted by centrifugation of the culture for 10 min at 5000rpm, and resuspended in 15 ml TSB. This suspension was then incubated on ice for 10 minutes, aliquoted and stored at -80°C.

Transformation of Competent bacteria and plasmid preparation

100 µl competent *E.coli* XL-1 blue cells were thawed on ice and mixed with a suspension containing 80 µl H_2O, 20 µl 5 x KCM-buffer and 0.1-10 µg plasmid DNA. The cells were then incubated on ice for 20-40 min and afterwards subjected to a heat shock at 42°C for 45 seconds. The bacteria were cultured in 1ml LB medium without ampicillin on a shaker for 1 hour at 37°C. Then the cells were pelleted by centrifugation for 10 min at 5000 rpm, and plated on a LB-agar plate containing ampicillin (100 µg/ml) and grown at 37°C over

night. From the plates, single colonies were picked and used to inoculate 2-5 ml LB-medium as a starter culture for plasmid preparation. Plasmid DNA was purified from bacterial cultures using Qiagen plasmid-purification kits following the manufacturer's instructions.

2.2.2 Isolation of $CD34^+$ progenitors from peripheral blood

The peripheral blood stem cells (PBSC) were collected on day 4, 5 and 6 after G-CSF (10 µg/kg body weight) treatment from healthy donors after informed consent by means of a Cobe spectra cell separator. The $CD34^+$ positive cells were isolated by positive selection using anti-CD34 antibody (GBEND/10) and magnetic cell sorting system for clinical use according to the manufacturer's instructions.

2.2.3 Culture of $CD34^+$ cells and induction of differentiation

CD34+ cells were cultured in Iscoves modified Dulbecco medium (IMDM), supplemented with 30% pre-tested fetal calf serum (FCS), 12 µl/ml N-Acetyl-L-alanyl-L-glutamine, 8 µl/ml iron saturated transferrin (27 mg/ml in IMDM), 10^{-6} M Mercaptoethanol, 100 U/ml Penicillin/100 µg/ml streptomycin.10 ng/ml SCF, 100 ng/ml IL6 at 37°C in a humidified atmosphere containing 5% CO_2. After expansion for 5 days, cell differentiation was induced by addition of 50ng/mL TPO or 4 U/ml EPO to the above culture medium in the presence of SCF/IL6 for the indicated time periods .

2.2.4 Flow cytometry analysis

Cells were washed once in PBS containing 2 % human serum albumin (PBS-A), and incubated with antibodies directed against the following antigens CD34, Glycophorin A, or CD61 for 30 minutes applied at the recommended concentrations. Control stainings were performed with isotype-matched control antibodies under the same conditions. After the staining, cells were washed once and resuspended in PBS-A. Fluorescence was analysed on a coulter EPICS profile II flow cytometer. Data acquisition and analysis were performed by "Flow mate" list mode software (DAKO).

2.2.5 K562 cell culture and induction of differentiation

K562 Cells were cultured in RPMI 1640 medium containing 10% fetal calf serum. Cultures were maintained at 37°C with 5% of CO_2. The cultured cells were passed every three days, seeding at a density of about 2×10^5 cells/ml. Cell counts were determined using the trypan-blue dye exclusion test.

For the induction of erythroid differentiation, exponentially growing K562 cells were seeded at a density of 2×10^5 /ml. A single-dose of freshly-prepared Ara-C was added at the seeding time. Cells were cultured for indicated time without changing the medium.

2.2.6 Assessment of erythroid differentiation of K562 cells

Benzidine staining

Benzidine staining was performed as described previously (Belhacene, et al. 1998). Briefly, 50 microliters of cell suspension ($0.5\text{-}1x10^6$ cells/ml) in PBS were mixed with 10µl of freshly- prepared benzidine reagent (0.6 % H_2O_2, 0.5 % acetic acid, and 0.2 % benzidine dihydrochloride) and incubated at 37°C for 5 min. The percentage of benzidine-positive cells (blue) was determined by light microscopic examination of at least 200 cells per sample. Each experiment was performed in triplicate, and results were averaged.

Immunocytochemistry for the detection of Haemoglobin A

Cells were washed twice in PBS and fixed with 4 % paraformaldehyde at 4°C for 30min. After 1 wash with PBS, cells were distributed on to slides by cytospin, and post-fixed with pre-cold (-30°C) acetone at 4°C for 2 minutes. Following 3 washes with PBS, cells were incubated with rabbit-anti-haemoglobin A antibody (1:2000, in PBS containing 2 % human serum albumin diluted) overnight at 4°C in a humid chamber. After 3 washes with PBS, cells were subsequently incubated with Streptavindin-Biotin-AP-conjugated anti-rabbit secondary antibody (DAKO Alkaline-phosphatase-kit 5005) for 30minutes. After 3 washes in PBS, the positive staining was visualised by incubated with the chromogen-substrate for Alkaline-phosphatase (accompanied with the kit) until a sufficient degree of staining is obtained. Finally, the slides were counterstained with hematoxilin for 3 minutes, and covered with glycerolgelatin.

2.2.7 Stable transfection of K562 cells

Exponentially growing cells were transfected using LipofectAMINE PLUS^{TM} Reagent

1. Pre-complex the DNA with PLUS Reagent: dilute 2-4 µg plasmid DNA into 250 µl serum-free medium, then add 5µl PLUS Reagent, mix gently and incubate at room temperature for 15 minutes.
2. Dilute 10µl LipefectAMINE Reagent into 250 µl serum-free medium in a second tube, and mix gently .
3. Combine the pre-complexed DNA and diluted 10µl LipefectAMINE Reagent; mix and incubate for 15 minutes at room temperature.
4. Add the above complex to a 60-mm tissue culture plate containing about 0. 5-1×10^6 cells in 1.5 ml serum-free medium.
5. After 3 hours incubation, the DNA containing medium was replaced by normal medium containing 10% FCS.

As negative controls, cells were mock-transfected without the addition of plasmid DNA.
48 hours after transfection, the cells were seeded in RPMI1640 medium supplemented with 1 mg/ml G418 for selection. Two to 3 weeks later, when mock-transfected cells died, cells were pooled and maintained in medium with 0. 4 mg/ml G418.

2.2.8 Western Blot

Preparation of protein extracts

Cells were pelleted by centrifugation at 1000 rpm for 4 minutes and washed twice with PBS, then lysed in an appropriate ice-cold lysis buffer depending on the subsequent sample preparation. The lysates were vortexed and kept on ice for 15 minutes. Cellular debris was removed by centrifugation at 14,000 rpm for 20 minutes at 4°C. The supernatant was then used for the assay.

Western blotting

Proteins were resolved by electrophoresis using appropriate percent sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel according to the molecular weight of the proteins of interest. After that proteins were transferred onto PVDF membrane using semi-dry transfer conditions. In semi-dry blotting, the gel and the membrane were sandwiched between

buffer-soaked 3MM Whatmann papers. The PVDF-membrane was pre-wetted in methanol for 10 seconds before it was submerged in transfer-buffer for 15 minutes to allow for equilibration. Proteins were transferred at a constant current of 2.5 mA/cm^2 for one hour. Thereafter, the membrane was blocked with TBS-Tween buffer containing 5 % dry milk or 1 % BSA for 30 minutes at room temperature. Following blocking, the membrane was incubated with primary antibody diluted in TBS-Tween buffer containing 1 % dry milk or 1%BSA for 1 hour at room temperature. Phosphospecific antibodies were incubated with the membrane over night (16 hours) at 4°C. The antibodies were diluted at concentrations as recommended by the manufacturers. The membrane was then washed three times with TBS-Tween, and, subsequently incubated for 1 hour at room temperature with appropriate HRP-conjugated secondary antibody diluted in TBS-Tween containing 1% dry milk. After three washes in TBS-Tween, the membrane was submerged for 1 minute in ECL solution, wrapped in Saran foil and exposed to Kodak Biomax films. Blots can be stripped by incubating the membrane in stripping buffer for 30 minutes at 50°C to remove the bound antibodies, and re-probed with other antibodies.

2.2.9 Purification of polyclonal anti-PI3Kγ antibody

Polyclonal antibody against PI3Kγ used for immunoprecipitation was purified from the immunized rabbit serum using Actigel ALD Superflow kit (supplied in 20% Ethanol, 0.02 % sodium azide).

- Preparation for the following buffers

1. 0.1 M Phosphate buffer , pH 7.4
   77.4 ml buffer A (0.1 M Na_2HPO_4)
   + 22.6 ml buffer B (0.1 M NaH_2PO_4)
2. 0.015 M Phosphate buffer, pH 7.4
3. 0.5 M NaCl, pH 7.2
4. 20 mM Glycine, pH 2.5
5. 1 M Tris, pH 8.0

- Peptide- coupling to Actigel ALD Superflow resin

1. Take 1 ml of suction-tried resin, wash the resin with 5 ml of 0.1 M Phosphate buffer for three times on a Buchner funnel, and pack the resin in a column.

2. Mix 100 µl of peptide (about 1mg) which was used for raising the PI3Kγ antibody with 100µl of ALD-coupling solution (1 M $NaCNBH_3$) and 800 µl of 0.1 M Phosphate buffer.
3. Apply the mixture to the column and let it stand overnight at 4°C.

- Purification of the antibody

1. Wash the column with 10 ml of 0.1 M Phosphate buffer for equilibration.
   Pass 10-15 ml of rabbit serum containing the anti-PI3Kγ antibody through the column.
2. wash the column with 20 ml of 0.015 M Phosphate buffer (pH 7.2), followed by 20 ml of 0.5 M NaCl (pH 7.2).
3. Elute the column with 20 mM Glycine (pH 2.5). Collect the eluate in 1.5 ml conical tubes containing 50 µl of 1 M Tris (pH 8.0). Mix each tube gently to bring the pH to neutral.
4. Determine the antibody concentration in each fractions by Bradford method and store the antibody in 50 % glycerol with 0.02 % sodium azide.

2.2.10 Coupling anti-PI3kγmonoclonal antibody to protein G- Sepharose

1. Take 2 ml of tissue-culture supernatant containing about 10-20 µg of monoclonal antibody, mix with 20 µl of protein G beads (prior to use, washed three times with 10 mM Tris (pH 8.0) to remove the PBS/ethanol solution the resin is stored in) at room temperature for 1hour with gentle shaking.
2. Wash the beads twice with 10 volumes of 0.2 M sodium borate (pH 9.0) by centrifugation at 5000 rpm for 2 minutes.
3. Resuspend the beads in 10 volumes of 0.2 M sodium borate (pH 9.0), add dimethyl pimelimidate to bring the final concentation to 20 mM.
4. Incubate for 30 minutes at room temperature with gentle mixing.
5. Stop the reaction by washing the beads once in 0.2 M ethanolamine (pH 8.0) and then incubate at room temperature for half a hour in 0.2 M ethanolamine (pH 8.0) with gentle mixing.
6. After the final wash, resuspend the beads in PBS with 0.01 Merthiolate.

2.2.11 Immunoprecipitation

For immunoprecipitation, 1x10 ^{8} of cells were pelleted and washed twice with PBS, then lysed in 3 ml of ice-cold lysis buffer. The soluble material was incubated with about 10 µg of antibodies for 2 hours at 4°C. Immune complex was then incubated with 30 µl of protein G or protein A-Sepharose for 1 hour at 4°C (prior to use, the Sepharose was washed three times with lysis buffer to remove the PBS/ethanol solution the resin is stored in). Alternatively, for special purpose, cell lysate was incubated directly with monoclonal antibody pre-coupled protein G beads for 1 hour at 4°C. After that the antigen-antibody-protein A/G complex was collected by centrifugation at 3000 rpm for 2 minutes. Immunoprecipitate was washed three times with lysis buffer. Pellet was then processed for *in vitro* protein kinase assay or resuspended in electrophoresis sample buffer for subsequent immunoblotting analysis.

2.2.12 PI3Kγ Protein Kinase activity assay

Cells were pelleted, lysed and subjected to anti-PI3Kγ immunoprecipitation. immunoprecipitates were washed three times with lysis buffer and once with protein kinase buffer. Pellets were then resuspented in 50 µl of protein kinase buffer, incubated in the presence or absence of 10 µM LY 294002 for 30 minutes at 30°C. The reactions were initiated by adding 20 µm ATP and 10 µCi [γ^{32}P]ATP to the protein kinase buffer. After 30-minute incubation at 37°C on a thermomixer, reactions were stopped by adding 12.5 µl of 5 × sample buffer. Samples were denatured at 95°C for 10 minutes and resolved on 8% SDS-PAGE gel. After silver or Coomassie staining, the gel was dried on a vacuum gel-dryer. The phosphorylated proteins were revealed by exposure of the gels to BioMax MR-1 Films.

2.2.13 Silver staining

Silver staining was performed as described by Shevchenk (Shevchenko, et al. 1996) with some modifications

1. After electrophoresis, fix the gel in fixation solution for 20 minutes.
2. Wash the gel with 50% methanol for 10 minutes, and with water for 30 minutes on a shaking platform.

3. Incubate the gel in sensitizing sulotion for 1 minute. Discard solution and quickly rinse the gel with two changes of water (1 minute each).
4. Incubate the gel in chilled 0.1% silver nitrate for 20 minutes at 4°C.
5. Discard the silver nitrate solution and quickly rinse the gel twice with water (1 minute each).
6. Incubate the gel in developing solution for 1-10 minutes until a sufficient degree of staining is obtained.
7. Stop the staining by discarding the developing solution and replace with 1% acetic acid , and dry the gel with Gel-drying film.

2.2.14 RNA isolation and RT-PCR

RNA isolation

Total RNA was isolated using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions. Briefly, cells were lysed in lysis buffer containing the highly denaturing agent guanidine isothiocyanate, which immediately inactivates RNAses to ensure the isolation of intact RNA. To shear genomic DNA and to reduce the viscosity, the lysate was passed through a 20 -G needle (Ø 0.9 mm) 5 to 10 times for homogenization. Add 1 volume of 70 % ethanol to the homogenized lysate to adjust conditions for the binding of RNA to the RNeasy column. Apply the lysate on the RNeasy column and centrifuge for 15 seconds at 12000 rpm to allow for the binding of RNA to the column membrane. After removing contaminants with washing buffer, the RNA bound on the column was eluted with 30 µl of RNase free water. Measure the RNA concentration and store it at -20°C.

RT-PCR

RT-PCRs was performed using the One-step RT-PCR Kit from Qiagen. The following conditions was used for each reaction:

- Reaction mixture for one reaction

RNA template	1 µg
5x reaction buffer	10 µl

Q-solution	10 µl
forward primer	50 pmol
reverse primer	50 pmol
dNTP	2 µl
RT-PCR enzyme	2 µl
ddH_2O	to 50 µl final volume

Thermal-cycler conditions

Reverse transcription:	30 minutes	50°C
Initial PCR activation:	15 minutes	95°C

3-Step cycling

Denaturation:	45 seconds	94°C
Annealing:	1 minute	56°C
Elongation:	1 minute	72°C
Number of cycles:	30 cycles	
Final elongation	5 minutes	72°C

The products of the amplified PCR fragments were electrophoreticaly separated on 1.5 % agarose gel in 1x TAE containing 0.5 µg/ml of ethidium bromide at 90 V. Gels were illuminated with UV light for visualisation.

3. RESULTS

3.1 Involvement of PI3Kγ in the erythroid differentiation of hematopoietic stem cells

Previous work from our lab has indicated that PI3Kγ plays a role in ATRA (all-trans retinoic acid)-induced U937 cell differentiation (Baier, R et al., 1999). However, it is unclear whether PI3Kγ is also involved in other lineage–specific differentiation processes. To address this issue, we first analysed the profile of PI3Kγ protein expression during the differentiation processes of human $CD34^+$ progenitor cells. $CD34^+$ cells are able to differentiate *in vitro* into different hematopoietic lineage's depending on the cytokine combination given. In this study, the $CD34^+$ cells were isolated from human peripheral blood. Before induced to differentiate, the cells were pre-cultured in the presence of SCF/IL6 for 5 days for expansion. Thereafter the day 5 cells were induced to differentiate by using different combination of growth factors.

For the induction of erythroid differentiation, day 5 cells were grown in medium containing SCF/IL6, and cultured in the presence of 4 U/ml of EPO for the indicated periods. At each time points, expression of glycophorin A, which is one specific marker for erythroid differentiation, was analysed by flow cytometry using direct immunofluorescence. As shown in Fig.5, glycophorin A-positive cells increased from 0.5 % at day 0 to 15 %, 38 % or 69 % after 4, 6, or 8 days in the presence of EPO.

For the induction of megakaryocytic differentiation, day 5 cells were grown in medium containing SCF/IL6, and cultured in the presence of 50 ng/ml of TPO for the indicated periods. At each time points, cells were collected and subjected to flow cytometric analysis of the cell surface expression of CD61, a marker for megakaryocytic differentiation. As shown in Fig. 6, $CD61^+$ cells increased from 1.4 % at day 0 to 9 %, 12 % or 30 % after 4, 6, or 8 days.

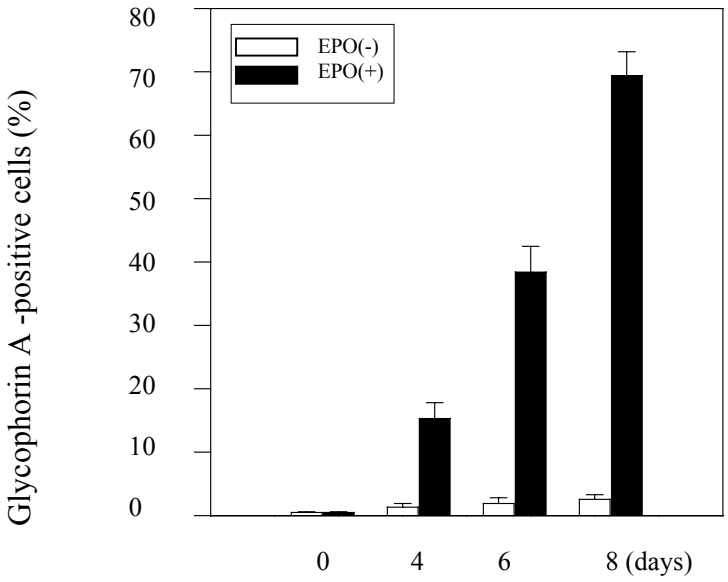


Fig.5 **Erythroid differentiation of CD34$^+$ stem cells induced by EPO**. CD 34$^+$ stem cells were pre-cultured in the presense of SCF/IL6 for 5 days for expansion. Day 5 cells were cultured in the presense of SCF/IL6, or in combination with EPO for the indicated time periods. The erythroid differentiation was determined by flow cytometric analysis for the cell suface expression of glycophorin A. Results represent the Mean±SEM of triplicate experiments.

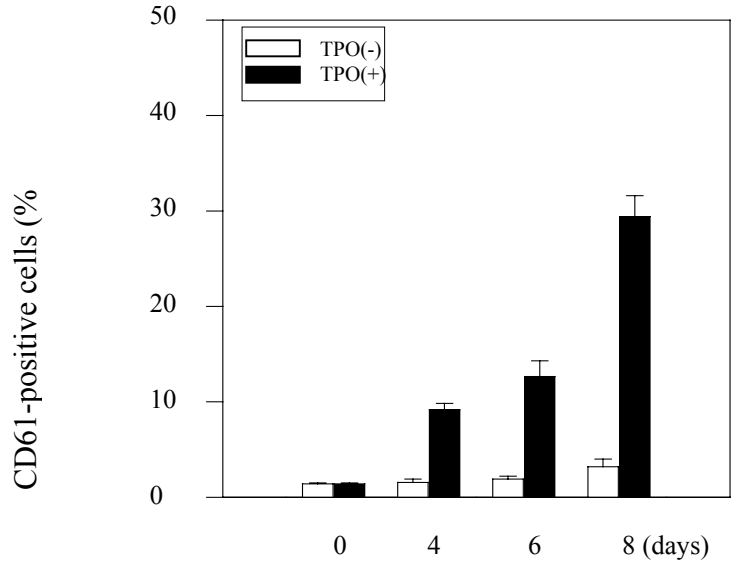


Fig.6 **Megakaryocytic differentiation of CD34$^+$ stem cells induced by TPO**. CD34$^+$ stem cells were pre-cultured in the presense of SCF/IL6 for 5 days for expansion. Day 5 cells were cultured in the presense of SCF/IL6, or in combination of TPO for the indicated time periods. The megakaryocytic differentiation was determined by flow cytometric analysis for the CD61 expression. Results represent the Mean±SEM of triplicate experiments.

In parallel with the flow cytometric analysis, cell samples were taken at each time points of the induction period, and frozen at –80°C. Finally, cells were lysed, and the expression of PI3Kγ was evaluated by Western blot. As shown in Fig.7, the PI3Kγ expression was significantly downregulated after EPO treatment. In contrast, TPO treatment did not significantly change the PI3Kγ expression. These results indicate an inverse correlation exists between the PI3Kγ protein level and the erythroid differentiation.

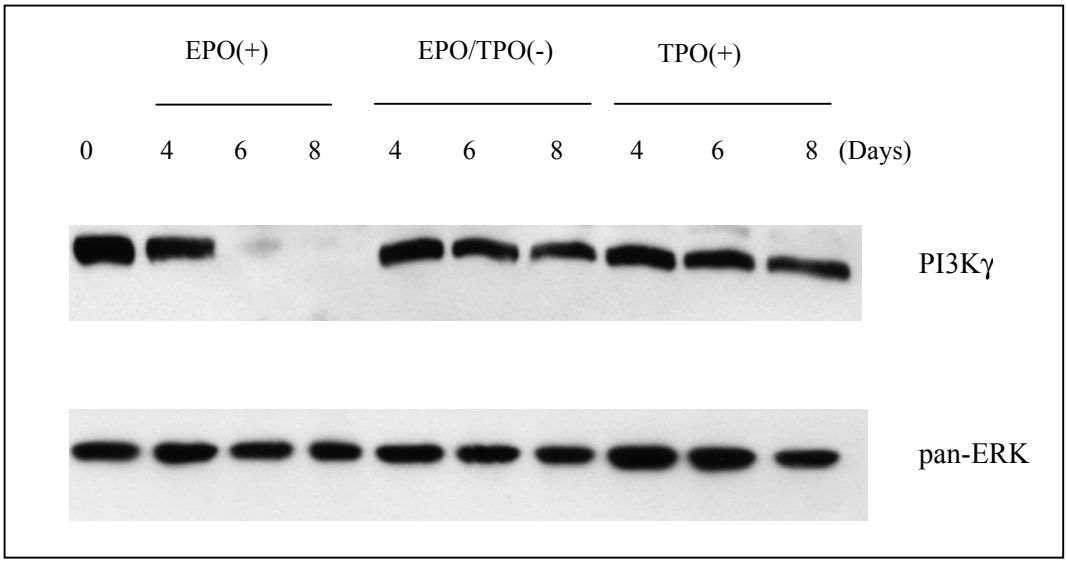


Fig.7 **EPO downregulates PI3Kγ expression in CD34$^+$ stem cells**. CD34$^+$ stem cells were pre-cultured in the presence of SCF/IL6 for 5 days for expansion. Thereafter these cells were cultured in the presence of SCF/IL6, or in combination of EPO or TPO. Whole cell extracts from the indicated time points were subjected to immunoblot analysis with the anti-PI3Kγ antibody. Equal loading was monitored by immunoblotting with an antibody against pan-ERK.

3.2 Involvement of PI3Kγ in the erythroid differentiation of K562 cells

Because of the difficulties in the isolation and maintenance of adequate numbers of CD34$^+$ stem cells, we have used the K562 cell line as a model system to explore the possible functional significance of PI3Kγ in erythroid differentiation. The K562 is a erythroleukemia cell line. These cells are pluripotent in that they are able to differentiate along both erythroid and megakaryocytic lineages in response to a variety of differentiation inducers (Leary, JF et al., 1987).

In order to clarify if PI3Kγ is down-regulated in K562 cells upon induced erythroid differentiation, we therefore analysed the PI3Kγ expression before and after Ara-c treatment, because Ara-C is known to be a potent inducer of erythroid differentiation in

K562 cells (Rowley, PT et al., 1981; Yamada, H et al., 1998). For the induction of erythroid differentiation, exponentially growing K562 cells were cultured in the presence of increasing concentrations of Ara-C from 0.1μg/ml to 50 μg/ml for 4 days. After that, the erythroid differentiation was determined by the benzidine staining for haemoglobin. As shown in Fig.8, at doses less than 10 μg/ml, Ara-C induced the erythroid differentiation in a dose-dependent manner. At the concentration of 5μg/ml, Ara-C almost achieved the maximum inductive effect, resulting in about 33 % of benzidine positive cells, this concentration was therefore used in subsequent experiments. In the absence of Ara-C, about 3% cells were benzidine positive, indicative of a minor spontaneous differentiation of K562 cells under normal culture conditions.

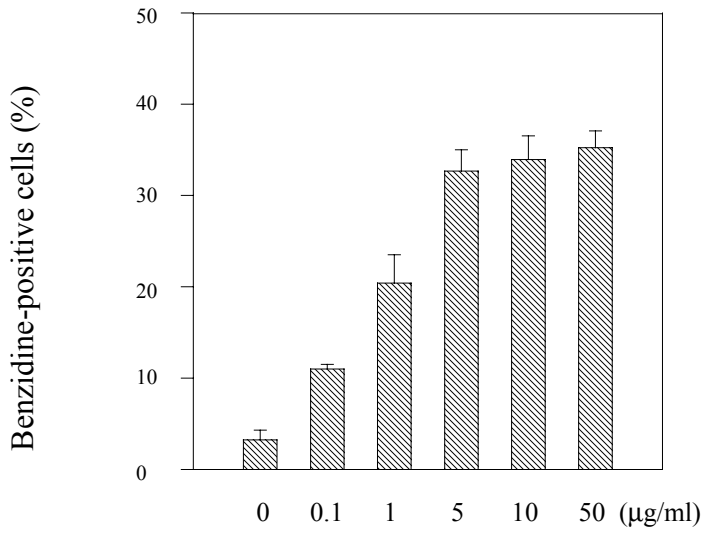


Fig.8 **Dose-dependent erythroid differentiation in K562 cells induced by Ara-C**. Exponentially growing K562 cells were seeded at a density of 2×10^5 cells /ml, and cultured in the absence or in the presence of increasing concentrations of Ara-C as indicated. After 4 days, erythroid differentiation was determined by benzidine staining. Results represent the Mean±SEM of triplicate experiments.

After Ara-C treatment, cells were lysed and analysed by Western blot to detect the expression of PI3Kγ. As shown in Fig.9A, Ara-C treatment significantly reduces the PI3Kγ expression in a dose-dependent manner, further confirming that the PI3Kγ expression is inversely correlated with the erythroid differentiation.

In order to know whether PI3Kγ is a specific isoform of PI3Ks which is associated with erythroid differentiation, the expression of PI3Kβ, and its adaptor protein p85 was also

analysed by Western blot upon Ara-C treatment. In contrast to PI3Kγ, the expression of PI3Kβ and p85 was not obviously affected by Ara-C (Fig. 9B).

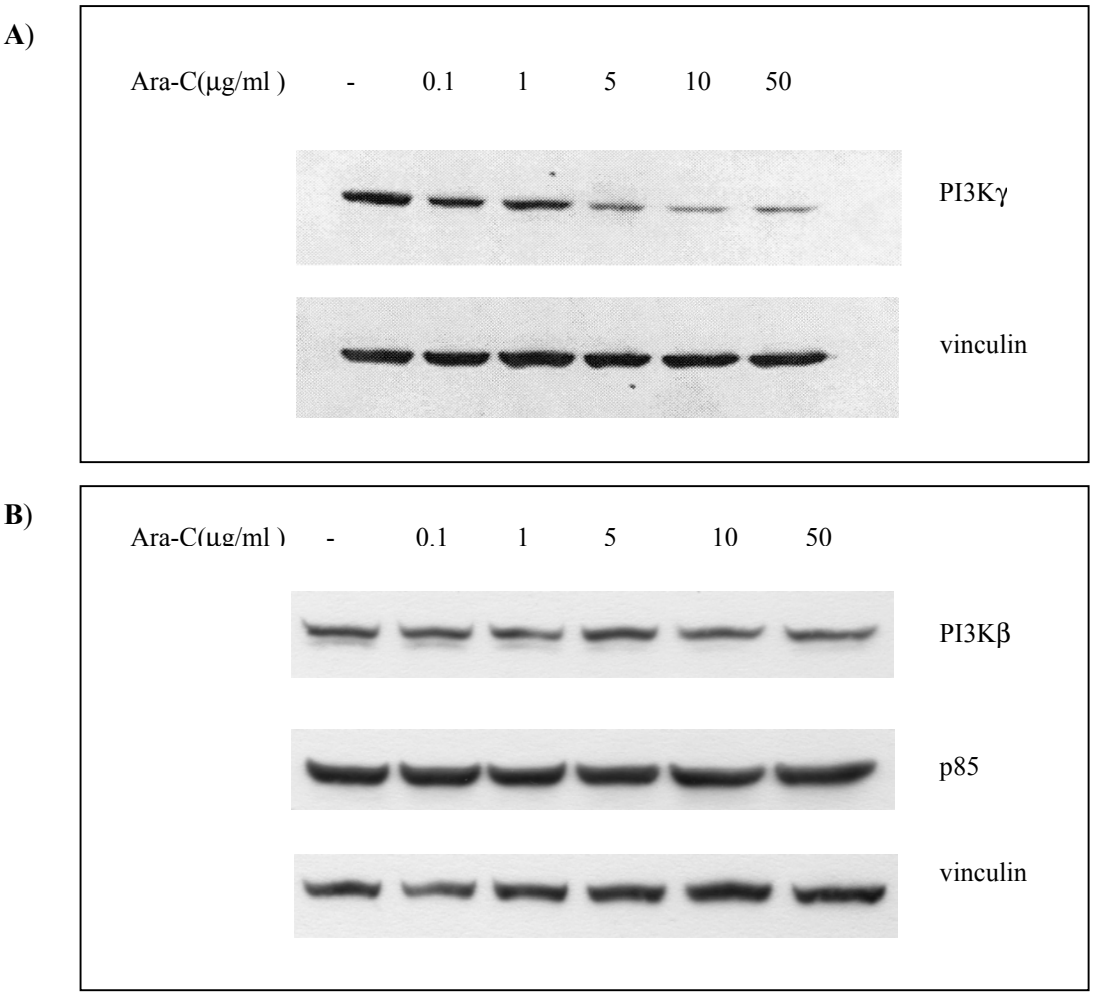


Fig.9 **Selective downregulation of PI3Kγ expression during erythroid differentiation of K562 cells**. Exponentially growing K562 cells were cultured in the absence or in the presence of 5μg/ml of Ara-C for 4 days. Immunoblots with antibodies against PI3Kγ, PI3Kβ, and p85 were performed. Equal loading of protein was monitored by immunoblottings with anti-vinculin antibody.

To further reveal the relationship of Ara-C induced decrease in PI3Kγ expression to the erythroid differentiation, a time-course study was performed. Exponentially growing K562 cells were left untreated or treated with 5 μg/ml of Ara-C from 24 to 72 hours. Erythroid differentiation was determined every 24 hours in triplicate using benzidine staining. At each time points, part of the cells was aliquoted, and frozen at –80°C. At the end of treatment, the PI3Kγ expression was determined by Western blot. As shown in Fig.10, the PI3Kγ expression was significantly decreased 72 hours post Ara-C treatment, concomitantly, an increase in benzidine-positive cells was also observed at this time point (Fig.11).

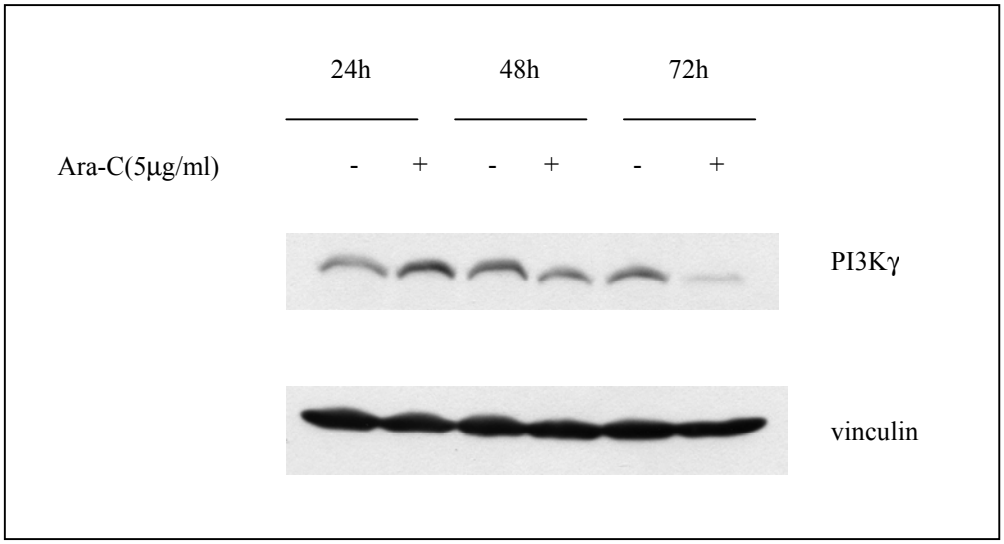


Fig.10 **Time-dependent downregulation of PI3Kγ expression during erythroid differentiation of K562 cells**. Exponentially growing K562 cells were cultured in the absence or in the presence of 5μg/ml of Ara-C for indicated time points. At each time point, cells were collected, and the cell lysates were subjected to Western blotting using anti-PI3Kγ antibody. Equal loading of protein was monitored by immunoblotting with anti-vinculin antibody.

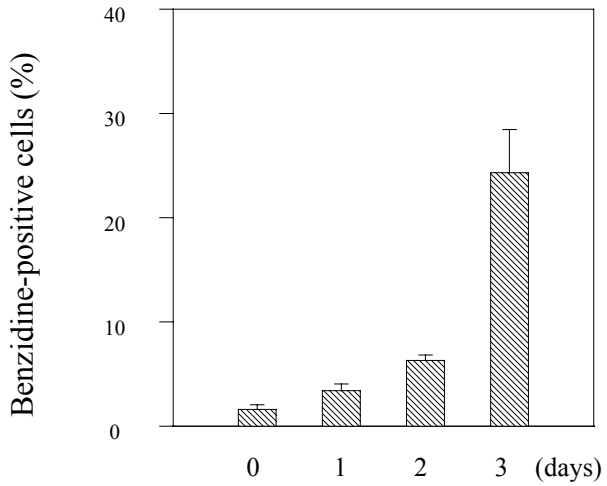


Fig.11 **Time-dependent erythroid differentiation in K562 cells induced by Ara-C**. Exponentially growing K562 cells were seeded at a density of 2×10^5 cells /ml, and cultured in the absence or in the presence of 5μg/ml Ara-C for the indicated time periods. Erythroid differentiation was determined by benzidine staining. Results represent the Mean±SEM of triplicate experiments.

3.3 Influence of suppressing endogenous PI3Kγ expression on the erythroid differentiation of K562 cells

Because the level of PI3Kγ decreases as the cells undergo differentiation, it is important to ascertain whether the differentiation event itself requires the downregulation of this protein. To address this issue, an antisense strategy was used in the present study. A plasmid encoding full-length PI3Kγ in antisense orientation was used. K562 cells was stably transfected with this plasmid. As negative controls, cells were stably transfected with an empty vector. The endogenous PI3Kγ expression was analysed after transfection by western blot. As shown in Fig.12A, cells stably expressing the antisense plasmid displayed a reduced PI3Kγ expression in comparison to the cells transfected with an empty vector, confirming that the expression of an antisense cDNA partially inhibits the endogenous PI3Kγ expression.

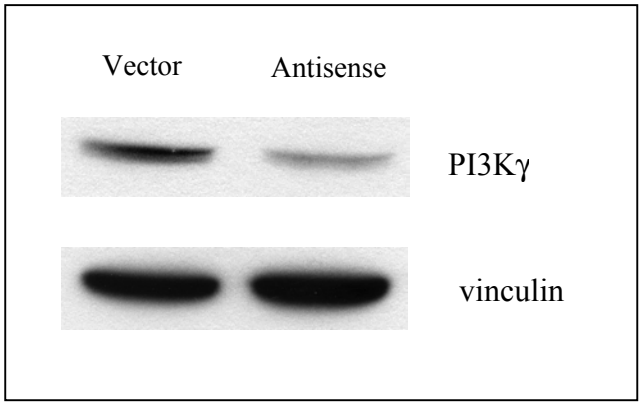


Fig.12A **Overexpression of PI3Kγ antisense cDNA reduces endogenous PI3Kγ expression**. K562 cells were stably tansfected with PI3Kγ antisense plasmid, or an empty vector (Vector) as described in "Materials and Methods". Protein levels of PI3Kγ expression were detected by Western blot using anti-PI3Kγ antibody. Equal loading of protein was monitored by immunoblotting with anti-vinculin antibody .

In order to confirm the inhibition of this construct on protein expression is specific for PI3Kγ, the protein level of PI3Kβ was also examined in parallel. As shown in Fig.12B, overexpression of this antisense construct did affect the endogenous PI3Kβ expression.

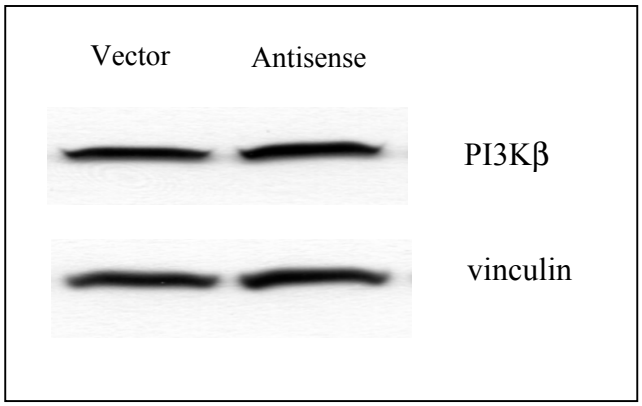
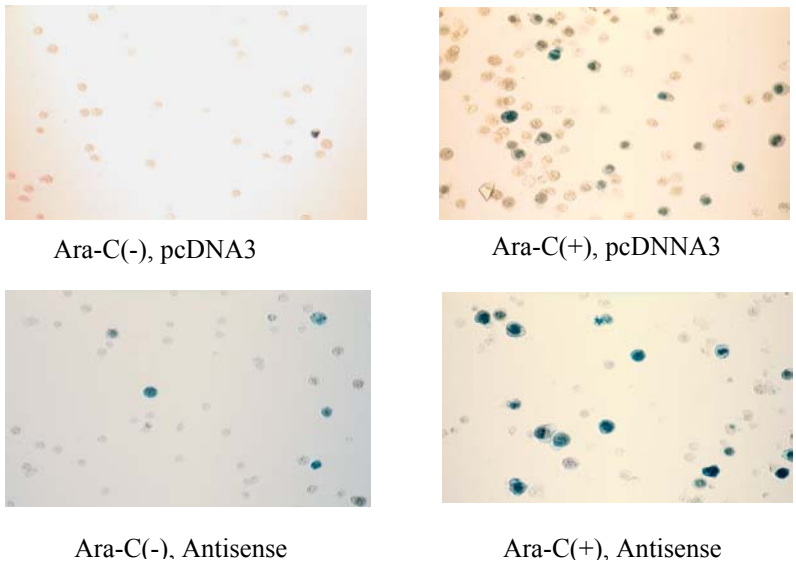


Fig.12B **Overexpression of PI3Kγ antisense cDNA does not affect endogenous PI3Kβ expression**. Lystes form cells stably tansfected with PI3Kγ antisense plasmid, or an empty vector were subjected to immunoblot analysis with the anti-PI3Kβ antibody. Equal loading was monitored by immunoblotting with an antibody against vinculin.

Subsequently, these stably transfected cells were analysed for their ability to produce haemoglobin, which was detected by benzidine staining and immunocytochemistry, respectively. Fig.13 shows the results of benzidine staining. Without Ara-C induction, about 3% of the cells transfected with an empty vector were benzidine-positive, which is similar to that of non-transfected parental cells (as shown in Fig.8). In contrast, the cells expressing the antisense of PI3Kγ displayed spontaneous differentiation of about 13%. Furthermore, after induction with 5 μg/ml of Ara-C for 4 days, the antisense transfected cells exhibited a significantly higher percentage of benzidine-positive cells than that of control cells tranfected with an empty vector (53% vs 27%).

A)



B)

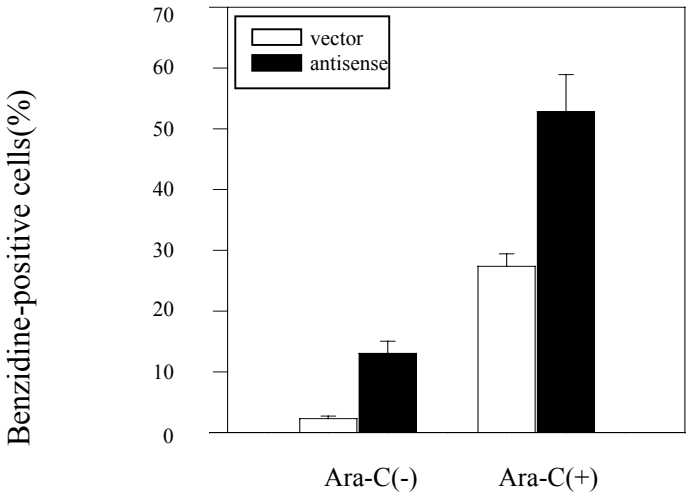
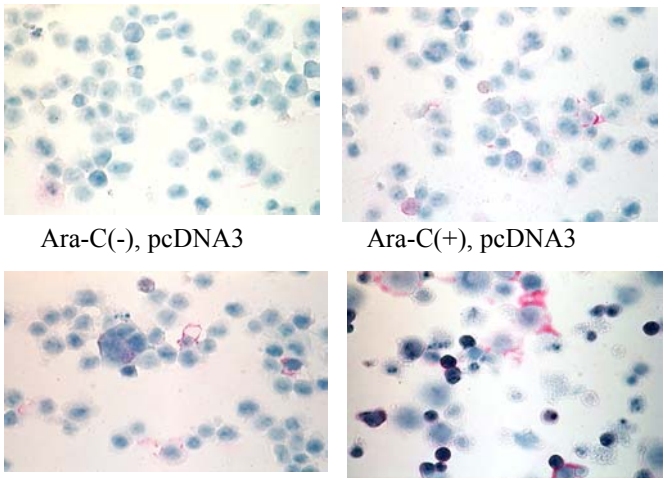


Fig.13 **Evaluation of erythroid differentiation by benzidine staining**. Exponentially growing K562 cells were cultured in the absence or in the presence of 5 μg/ml of Ara-C for 4 days. Erythroid differentiation was then detected by benzidine staining. A) representative pictures for benzidine staining (original magnification, 20 x). B) Quantitative presentation of benzidine-positive cells. Results represent the Mean±SEM of triplicate experiments.

The above results were further confirmed by immunocytochemistry using a specific antibody against haemoglobin A (Fig.14A). Compared with benzidine staining, this method seems to be less sensitive, as the percentages of positive cells were shown to be proportionally reduced (Fig.14B). Therefore, benzidine staining method was used in subsequent experiments. Taken together, these results indicate that suppressing the expression of PI3Kγ potentiates the spontaneous and promotes the Ara-C induced erythroid differentiation of K562 cells

A)



B)

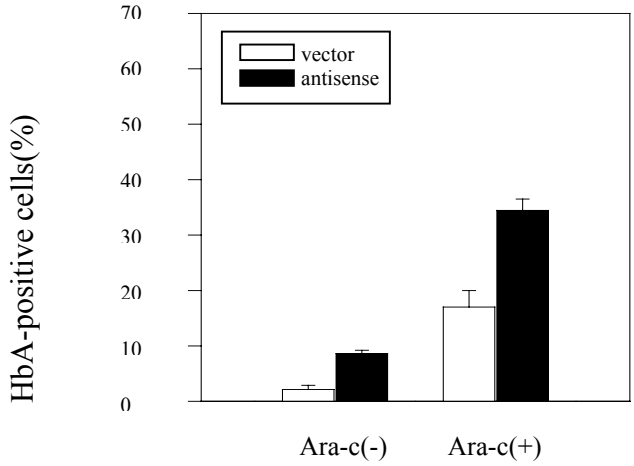


Fig.14 **Evaluation of erythroid differentiation by immunocytochemistry**. Exponentially growing K562 cells were cultured in the absence or in the presence of 5 µg/ml of Ara-C for 4 days. Erythroid differentiation was then detected by staining with an anti-hemoglobin A antibody A) Hemoglobin-containing cells appear red (original magnification, 20 x). B) Quantitative presentation of hemoglobin A-positive cells. Results represent the Mean±SEM of triplicate experiments.

3.4 Influence of overexpression of PI3Kγ on the erythroid differentiation of K562 cells

To gain further insight into the role of PI3Kγ in erythroid differentiation, K562 cells were stably transfected with wild type PI3Kγ or a dominant negative PI3Kγ-KR mutant. The expression of these proteins was detected by Western blot as shown in Fig.15.

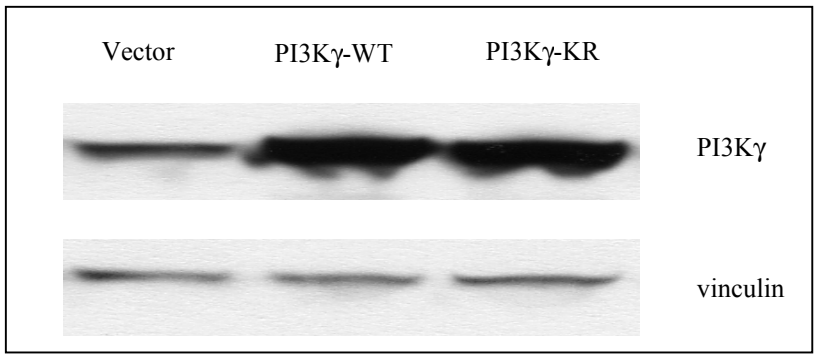


Fig.15 **Protein expression in K562 cells stably transfected with PI3Kγ constructs**. K562 cells were stably tansfected with wild type PI3Kγ (PI3Kγ-WT), PI3Kγ-KR mutant, or empty vector alone as described in "Materials and Methods". Protein levels of PI3Kγ expression were detected by Western blot using anti-PI3Kγ antibody. Equal loading of protein was monitored by immunoblotting with anti-vinculin antibody.

Subsequently, erythroid differentiation of these cells was evaluated. Exponentially growing cells were induced to differentiation by the addition of 5 μg/ml of Ara-C. 4 days post induction, erythroid differentiation was determined by benzidine staining. Fig.16 shows that the Ara-C induced erythroid differentiation was significantly inhibited in cells transfected with the wild type PI3Kγ in comparison to the vector controls. whereas Ara-C induced erythroid differentiation of cells transfected with PI3Kγ-KR mutant was significantly enhanced. Furthermore, cells transfected with PI3Kγ-KR mutant showed an obvious increase (about 10%) in spontaneous erythroid differentiation. These results indicate that PI3Kγ is a negative regulator for erythroid differentiation, and this function of PI3Kγ is related to its kinase activity .

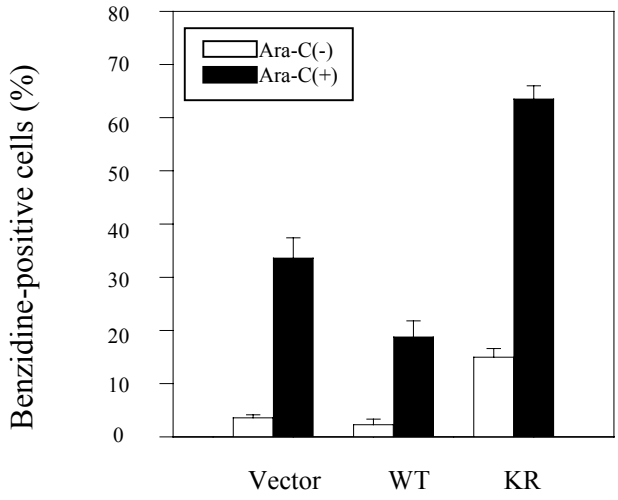


Fig.16 **Erythroid differentiation of stable transfectans overexpressing wild type PI3Kγ and PI3Kγ-KR mutant.** Exponentially growing K562 cells stably transfected wild type PI3Kγ and PI3Kγ-KR mutant were cultured in the absence or presence of Ara-C (5 μg/ml) for 4 days. Erythroid differentiation was then estimated by benzidine staining. Data are expressed as percentage of benzidine-positive cells. Results represent the Mean±SEM of triplicate experiments.

3.5 Relationship of PI3Kγ kinase activities to the erythroid differentiation of K562 cells

As PI3Kγ possesses both lipid an protein kinase activity, we therefore tried to figure out which activity is responsible for the observed inhibitory effect of PI3Kγ on the erythroid differentiation. For this purpose, we took the advantage of the PI3Kγ-FRAP mutant, which only retains the protein kinase activity, but lacks lipid kinase activity (Bondeva, T et al.,

1998). K562 cells were stably transfected with PI3Kγ-FRAP mutant. The expression of this protein was confirmed by Western blot as shown in Fig.17.

Erythroid differentiation ability of these cells was estimated subsequently. It was observed that erythroid differentiation of PI3Kγ-FRAP expressing cells in response to Ara-C induction was significantly reduced as compared to the control cells transfected with an empty vector (Fig.18). This result indicates the protein kinase activity of PI3Kγ is inversely correlated with the erythroid differentiation.

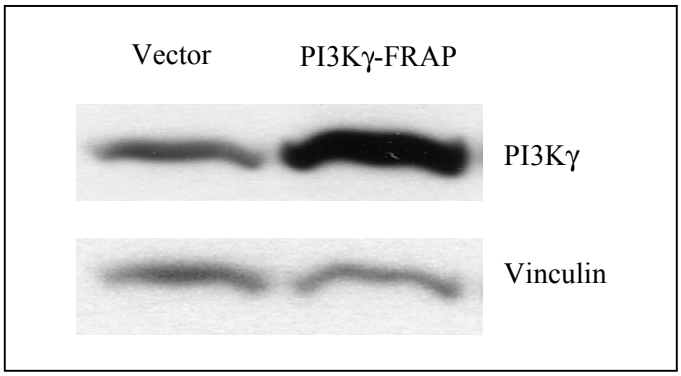


Fig.17 **Overexpression of PI3Kγ-FRAP mutant**. K562 cells were stably tansfected with wild type PI3Kγ-FRAP mutant, or empty vector alone as described in "Materials and Methods". Protein expression of PI3Kγ was detected by Western blot using anti-PI3Kγ antibody. Equal loading of protein was monitored by immunoblotting with anti-vinculin antibody.

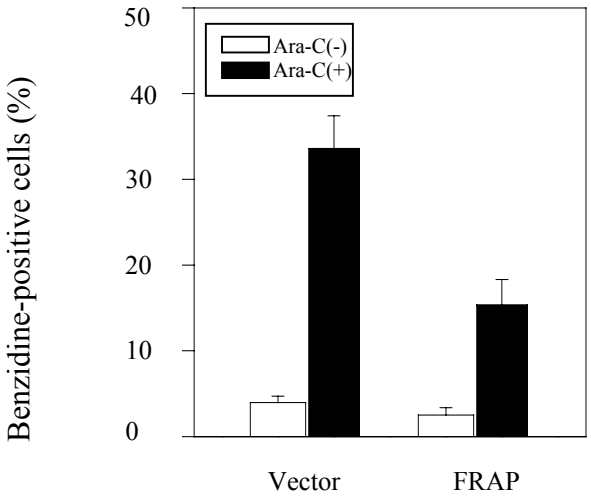


Fig.18 **Inhibitory effect of PI3Kγ-FRAP on erythroid differention**. Exponentially growing cells stably transfected with PI3Kγ-FRAP mutant, or empty vector alone were cultured in the absence or presence of Ara-C (5 μg/ml) for 4 days. Erythroid differentiation was then detected by benzidine staining. Data are expressed as percentages of benzidine-positive cells. Results represent the Mean±SEM of triplicate experiments.

In order to define the potential role of the lipid kinase activity of PI3Kγ in erythroid differentiation, the correlation between the lipid kinase activity of PI3Kγ and the erythroid differentiation in cells overexpressing PI3Kγ or PI3Kγ functional mutants was analysed. For convenience, activation of Akt, an effector enzyme downstream of PI3K (Franke , TF et al., 1995) was used as a readout for the evaluation of the lipid kinase activity. Exponentially growing cells under normal culture conditions were collected, and lysed directly in electrophoresis sample buffer. The Akt phosphorylation was detected by western blot using an antibody which specifically recognises the phosphorylated form of Akt (ser 473). As expected, overexpression of PI3Kγ-FRAP mutant did not affect the basal level of Akt phosphorylation (Fig.19). Interestingly, overexpression of the dominant negative PI3Kγ-KR mutant failed to inhibit the Akt phosphorylation, despite this mutant led to spontaneous erythroid differentiation. This suggests the effect of this mutant on erythroid differentiation is not caused by modulating the endogenous PI3Kγ lipid kinase activity. Intriguingly, overxepression of the wild type PI3Kγ was unable to activate Akt. As a positive control, overexpression of a membrane targeted PI3Kγ (PI3Kγ-CAAX) resulted in an elevated Akt phosphorylation. Taken together, these results indicate the lipid kinase activity of PI3Kγ is not correlated with the spontaneous erythroid differentiation.

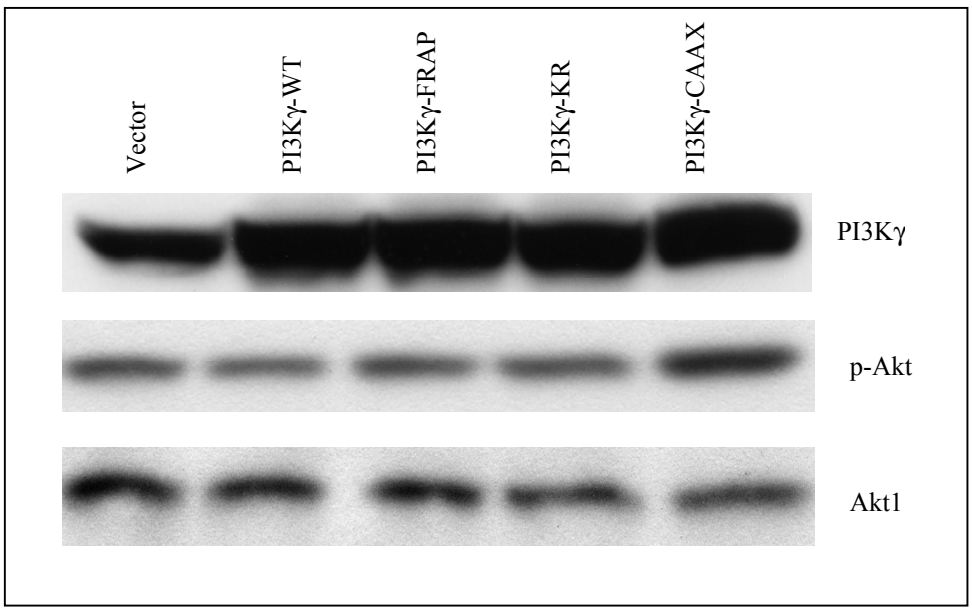


Fig.19 **Effects of overexpressing PI3Kγ and PI3Kγ mutants on basal Akt phospophorylation.** Exponentially growing cells stably tansfected with wild type PI3Kγ PI3Kγ-KR mutant, PI3Kγ-FRAP mutant, PI3Kγ-CAAX mutant, or empty vector were collected, lysed directly in electrophoresis sample buffer and analysed by anti-phospho-Akt (serine 473), and anti-PI3Kγ immunoblotting. Equal loading of protein was monitored by immunoblotting with anti-Akt1 antibody using the same-stripped membrane.

Furthermore, in order to know whether the lipid kinase activity of PI3Kγ is directly related to Ara-C induced erythroid differentiation, the effect of Ara-C on Akt phosphorylation was analysed. Exponentially growing cells cultured under normal conditions were subjected to Ara-C treatment from 30 minutes to 24 hours. As shown in Fig.20, Ara-C treatment did not significantly affect the Akt phosphorylation during the period observed .

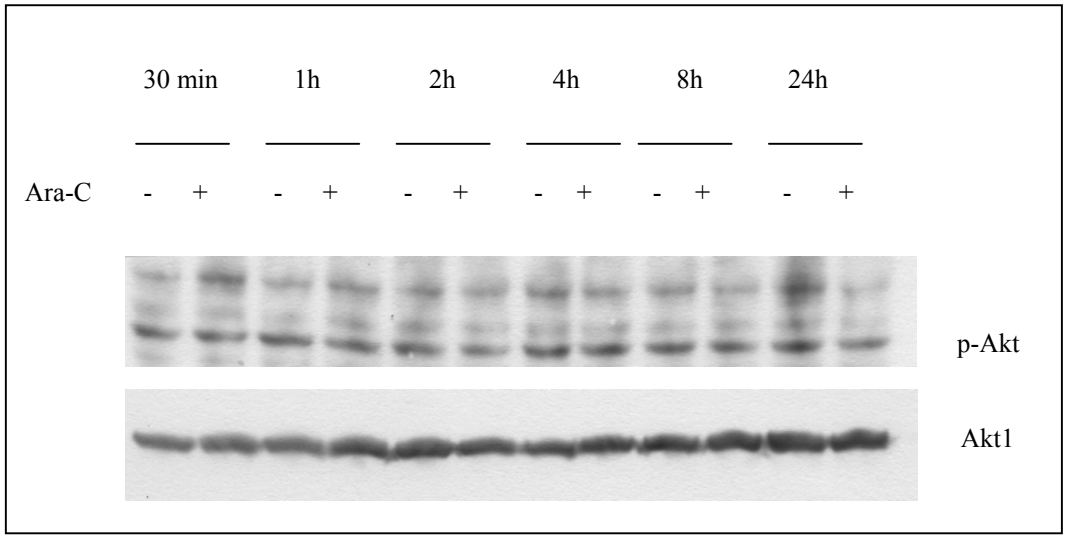


Fig.20 **Effect of Ara-C treatment on Akt phosphorylation**. Exponentially growing K562 cells were cultured in the absence or in the presence of 5 μg/ml of Ara-C for the indicated time periods. At each time point, cells were then collected, lysed directly in electrophoresis sample buffer, and analysed by anti-phospho-Akt immunoblotting. Equal loading of protein was monitored by immunoblotting with anti-Akt1 antibody using the same stripped membrane.

3.6 Expression of p101 in K562 cells

The activity of PI3Kγ has been shown to be regulated by its adaptor protein p101 (Stephens, et al., 1997). In this study, the expression p101 was also examined. Because of the lack of an antibody against p101, a Reverse Transciption Polymerase Chain Reaction (RT-PCR) was used to detect the messenger RNA of p101. Gene specific primers were designed against human p101 sequence. The size of the product expected was approximately 516 base pairs. β-actin primers were used to amplify a product of approximately 600 base pairs which was used as an internal control. Cells cultured under normal conditions were collected and total RNA was extracted using the Qiagen Mini Kit. RT-PCR was then performed using the one-step RT-PCR Kit from Qiagen as described under "Materials and Methods". To our surprise, no amplified p101 cDNA was detected in

K562 cells (Fig. 21). This result suggested that the function of PI3Kγ in K562 cells could be independent of p101.

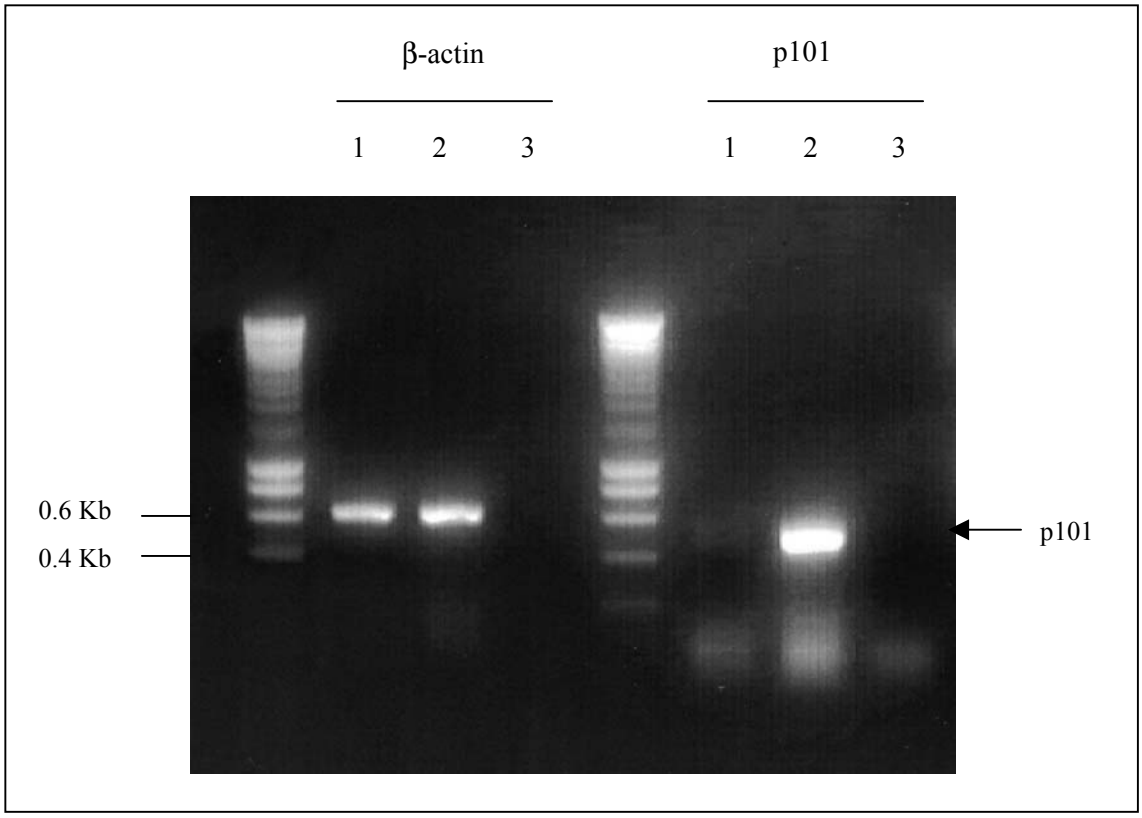


Fig.21 **Detection of p101 expression in K562 and U937 cells by RT-PCR**. p101 specific RT-PCR was performed using total RNA of K562 cells (lane 1). The total RNA of U937 cells was used as a positive control (lane 2). Reaction in the absence of the template RNA was used as a negative control (lane 3). The products of the amplified PCR fragments were electrophoreticaly separated on 1.5 % agarose gel containing 0.5 µg/ml of ethidium bromide, and visualised under UV light. The β-actin product was used as an internal control for the amount of total RNA used in the RT-PCR reactions.

3.7 Influence of PI3Kγ on MAPK activity in K562 cells

The PI3Kγ protein kinase activity has been shown to be sufficient to activate MAPK in COS-7 cells (Bondeva et al., 1998). Previous studies have also implicated MAPK pathway in erythroid differentiation of K562 cells (Racke, FK et al .,1997; Shelly, C et al., 1998; Witt, O et al., 2000). Therefore, we ask whether the observed effect of PI3Kγ on erythroid differentiation is associated with the MAPK signalling pathway. The activity of MAPK was evaluated by Western blot using an antibody that specifically recognises the activated, phosphorylated form of the ERK p42/44 MAP kinase.

First, we detected the basal level of MAPK activity and determined whether the basal level of MAPK activity could be affected by overexpressing PI3Kγ and its functional mutants. As shown in Fig.22, under normal culture conditions, K562 cell exhibits a detectable basal level of MAPK activation, which was inhibited by the MEK inhibitor PD98059. Consistent with previous study (Herrera, R et al., 1998; Kang. C., 1998), the inhibition of basal MAPK activity led to an increase in spontaneous erythroid differentiation (Fig.25).

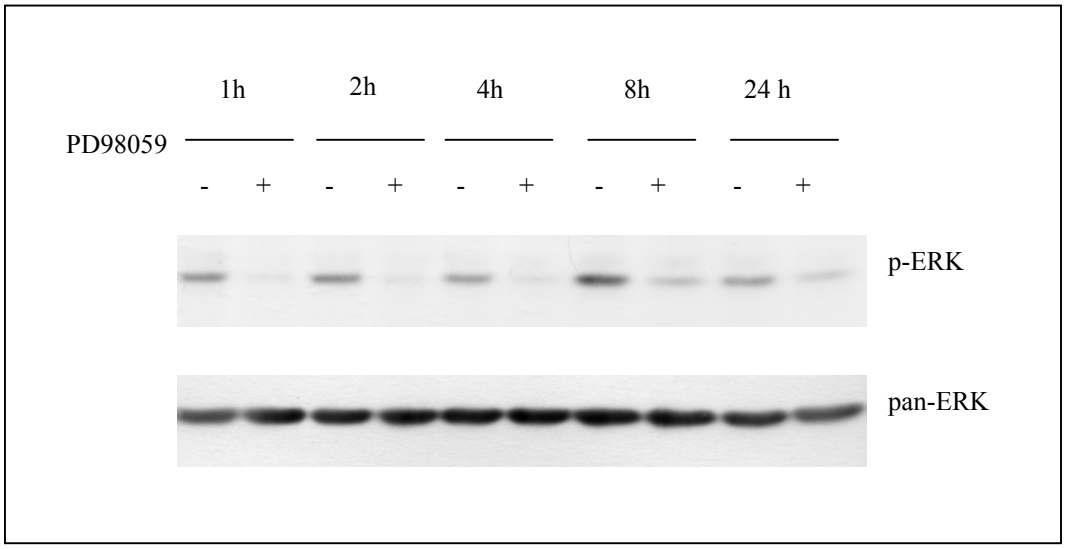


Fig.22 **Inhibition of basal MAPK activity by MEK inhibitor**. Exponentially growing K562 cells were cultured in the absence or in the presence of 20 μM PD98059 or an equivalent volume of DMSO for the indicated time periods. At each time point, cells were collected, lysed directly in electrophoresis sample buffer and analysed by anti-phospho-ERK immunoblotting. Equal loading of protein was monitored by immunoblotting with anti-pan-ERK antibody using the same stripped membrane.

However, we found that the basal level of MAPK activity was not affected by the overexepression of PI3Kγ and the functional mutants (Fig.23)

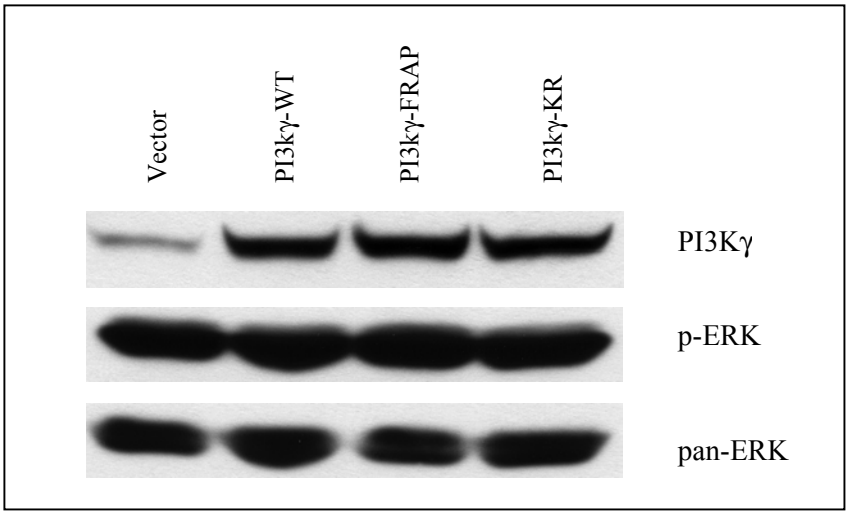


Fig.23 **Effect of overexpression of PI3Kγ or its functional mutants on the basal leve of MAPK activity**. Exponentially growing K562 cells stably transfected with wild type PI3Kγ (PI3Kγ-WT), PI3Kγ KR mutant (PI3Kγ-KR), PI3Kγ FRAP mutant (PI3Kγ-FRAP), or empty vector were lysed directly in electrophoresis sample buffer and analysed by anti-phospho-ERK immunoblotting. Equal loading of protein was monitored by immunoblotting with anti-pan-ERK antibody using the same stripped membrane.

Next, we have investigated the possible contribution of PI3Kγ to MAPK activity linked to Ara-c treatment. It was found that MAPK was activated 2 hours after Ara-C treatment, which lasted at least up to 24 hours. This stimulation was completely abrogated by PD98059 (Fig.24). Interestingly, inhibition of MAPK activity by PD98059 enhanced the Ara-C induced erythroid differentiation (Fig.25), pointing to a negative role for MAPK in Ara-C induced erythroid differentiation. We therefore compared the MAPK activities in cells stably transfected with PI3Kγ and its functional mutants upon Ara-C treatment. Cells were cultured in full medium in the presence of 5 μg/ml of Ara-C for 24 hours. After that, cells were lysed directly in electrophoresis sample buffer and analysed by anti-phospho-ERK immunoblotting. As shown in Fig.26, overexpression of wild type PI3Kγ or the PI3Kγ-FRAP mutant which are assumed to possess protein kinase activity did not increase the Ara-C induced ERK phosphorylation over that of the empty vector control. Furthermore the Ara-C induced ERK phosphorylation was not inhibited by overexpression of PI3Kγ-KR mutant. Thus, the MAPK pathway seems to be acting rather in parallel than downstream of PI3Kγ in K562 cells.

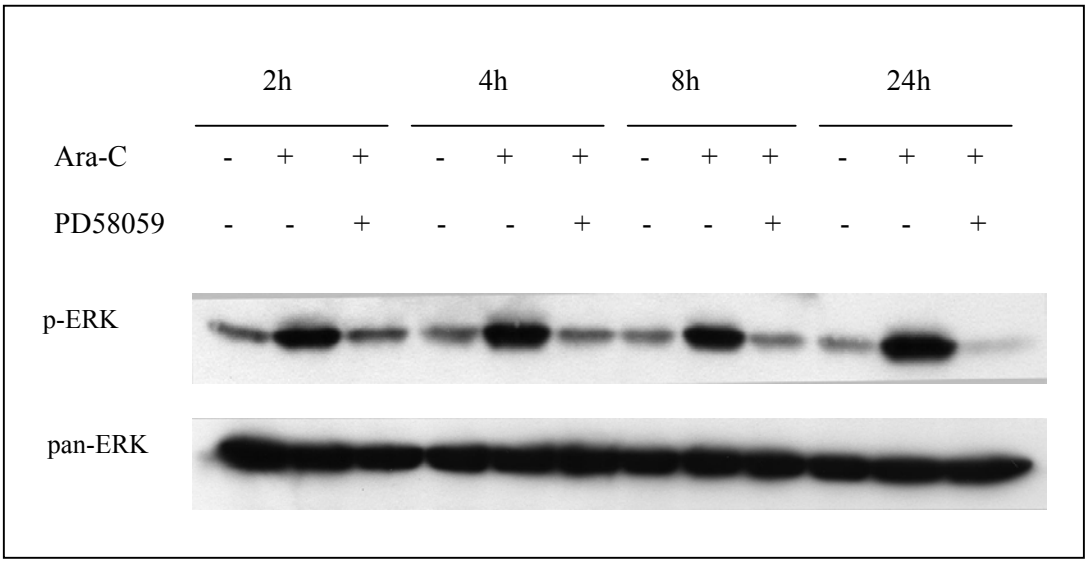


Fig.24 **Effect of Ara-C on MAPK activity**. Exponentially growing K562 cells were cultured in the absence or in the presence of 5µg/ml Ara-C with 20µM PD98059 or an equivalent volume of DMSO for the indicated time periods, PD98059 and DMSO were added 1hour before Ara-C treatment. Cells were then collected, lysed directly in electrophoresis sample buffer and analysed by anti-phospho-ERK immunoblotting. Equal loading of protein was monitored by immunoblotting with anti-pan-ERK antibody using the same stripped membrane.

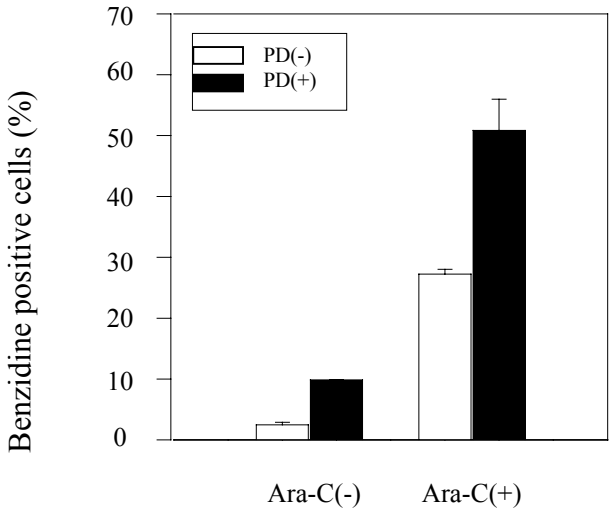


Fig. 25 **Inhibition of MAPK activity enhances erythroid differentiation of K562 cells.**Cells were incubated in the absence or in the presence of 5 µg/ml of Ara-C with a single dose of 10 µM PD98059 or an equivalent of DMSO for 4 days. PD98059 was added 1 hour before Ara-C treatment. The erythroid differentiation was determined by benzidine staining. Results are shown as the Mean± SEM of triplicate experiments.

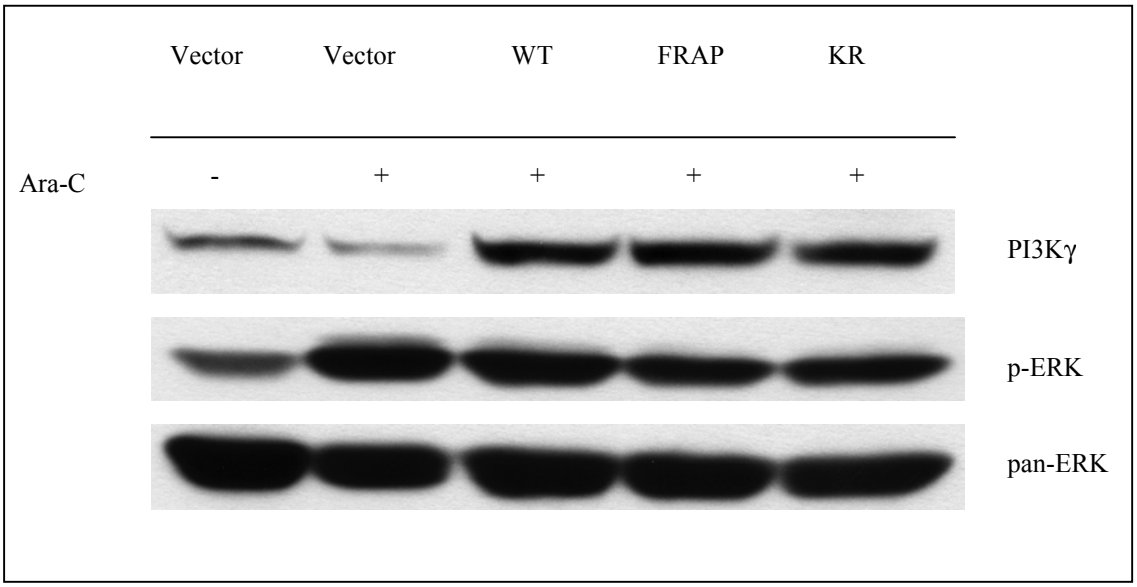


Fig. 26 **Effect of overexpression of PI3Kγ or its functional mutants on Ara-C induced MAPK activity**. Exponentially growing cells stably tansfected with wild type PI3Kγ (WT), PI3Kγ-KR mutant (KR), PI3Kγ-FRAP mutant (FRAP), or an empty vector (vector) were treated with 5 μg/ml of Ara-C for 24 hours. Immunoblotting was performed with anti-phospho-ERK, anti-pan-ERK and anti-PI3Kγ antibodies. Equal loading of protein was monitored by immunoblotting with anti-pan-ERK antibody using the same stripped membrane.

3.8 Downstream targets for PI3Kγ protein kinase activity

Our results demonstrated that both the PI3Kγ wild type and PI3Kγ-FRAP are capable of inhibiting the Ara-C induced erythroid differentiation of K562 cells. As the common feature of these proteins is their shared protein kinase activity, we therefore tried to address the question: what is the target(s) of this type of activity? At the first step, we performed anti-PI3Kγ immunoprecipitation experiments using two different types of antibodies to detect the binding partners for PI3Kγ. The polyclonal antibody is directed against the c-terminal peptide of the PI3Kγ, a monoclonal antibody is mapped to recognize the N-terminal part of the protein. To increase the amount of PI3Kγ, a GFP-fusion PI3Kγ was transiently expressed in K562 cells. Immunoprecipitations were performed using 10^6 to 10^8 of cells for each sample. After extensive washing, the immunocomplexes were separated on 8% SDS-PAGE gel and stained with silver for the detection of the associated proteins. As shown in Fig.27, both antibodies were able to precipitate GFP-PI3Kγ (approximately 158 kDa) and the endogenous PI3Kγ (approximately 120kDa). Together with PI3Kγ, several other bands were co-precipitated. These proteins are absent in the immunoprecipitate when the blocking peptide (blocking the PI3Kγ binding sites in the

polyclonal antibody) was used prior to immunoprecipitaion, which was used as a negative control.

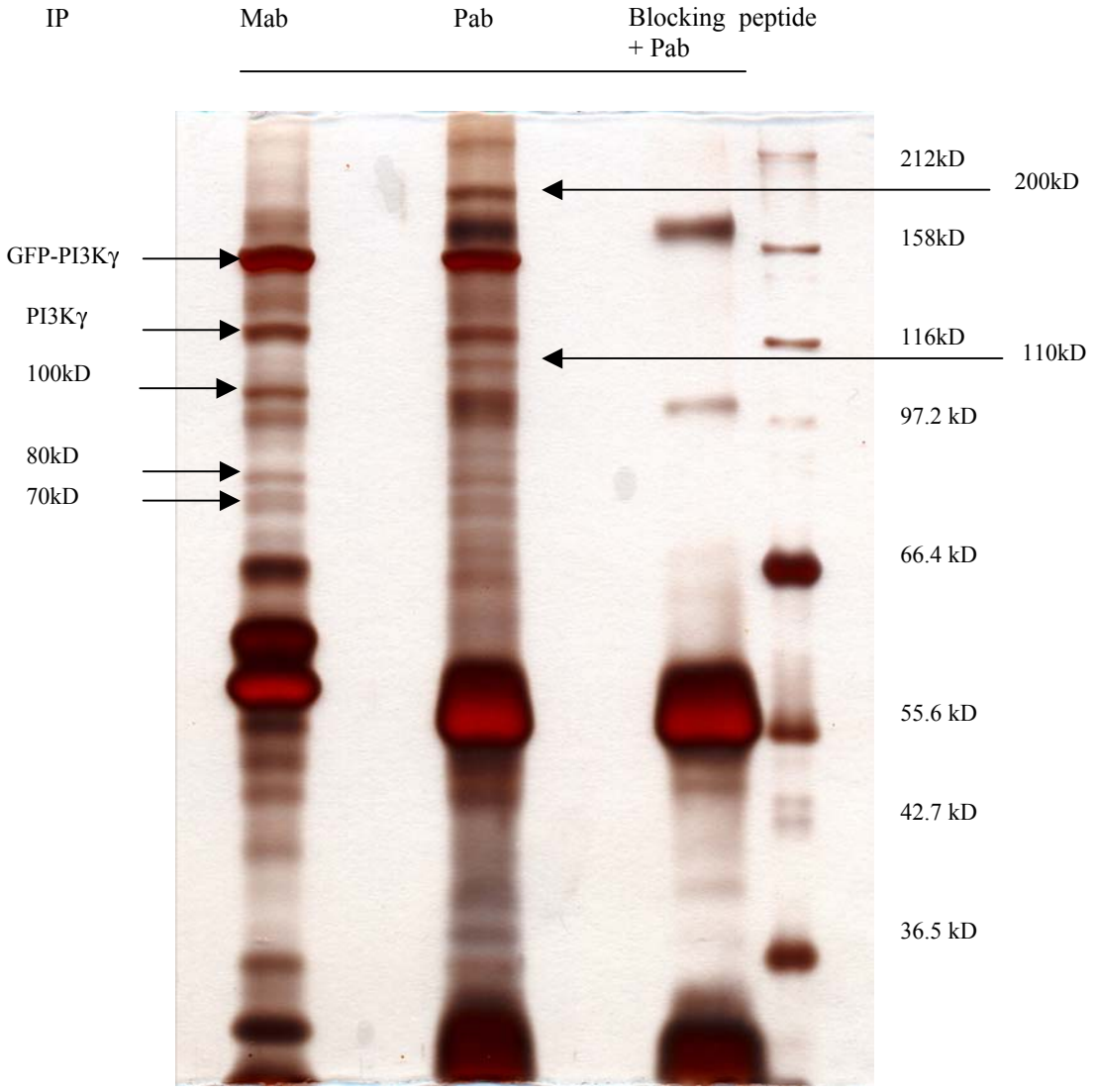


Fig.27 **Detection of PI3Kγ associated proteins**. 1x10^8 cells stably expressing GFP-PI3Kγ were lysed and the cleared lysates were used for PI3Kγ immunoprecipitation using either monoclonal or polyclonal antibody. As a negative control, anti-PI3Kγ polyclonal antibody was preincubated with the blocking peptide (20 µg/1µg antibody) before addition to the cell lysate. After extensive washing, precipitated proteins were separated on 8 % SDS-PAGE and revealed by silver staining.

It should be pointed out that different proteins were detected in complex with PI3Kγ depending on the antibody used for immunoprecipitation. The monoclonal antibody pulled down mainly three proteins with molecular weight of about 100 kDa, 80 kDa, and 70 kDa, respectively. In addition to these proteins, the polyclonal antibody precipitated two additional bands with molecular weight approximately 200 kDa and 110 kDa. We have

attempted to identify these binding partners of PI3Kγ, however we were unable to pull down sufficient amount of protein(s).

In subsequent experiments, we tried to clarify: are their targets for PI3Kγ protein kinase activity present in the co-precipitated proteins? For this purpose, immunoprecipitations were performed using the monoclonal antibody. The immunocomplexes were further subjected to *in vitro* kinase assay using [γ-^{32}P]ATP to check if any of the associated proteins can be phosphorylated by PI3Kγ *in vitro*. The reactions for *in vitro* protein kinase assay were carried out for 30 minutes in the absence or in the presence of the PI3Ks inhibitor LY294002 (10 μM). After that, proteins were separated on SDS-PAGE gel. The gel was then stained with Coomassie, dried and exposed to film. As shown in Fig.28, the precipitated proteins were visualised on the Coomassie stained gel (right panel), the left panel displayed the phosphorylation pattern of these proteins. PI3Kγ exhibits intrinsic protein kinase activity, as evidenced by its autophosphorylation. As expected, the autophosphorylation of PI3Kγ was inhibited by LY294002. In addition to PI3Kγ, the phosphorylation of two other proteins with molecular weight of approximately 70 kDa and 50 kDa was also sensitive to LY294002.

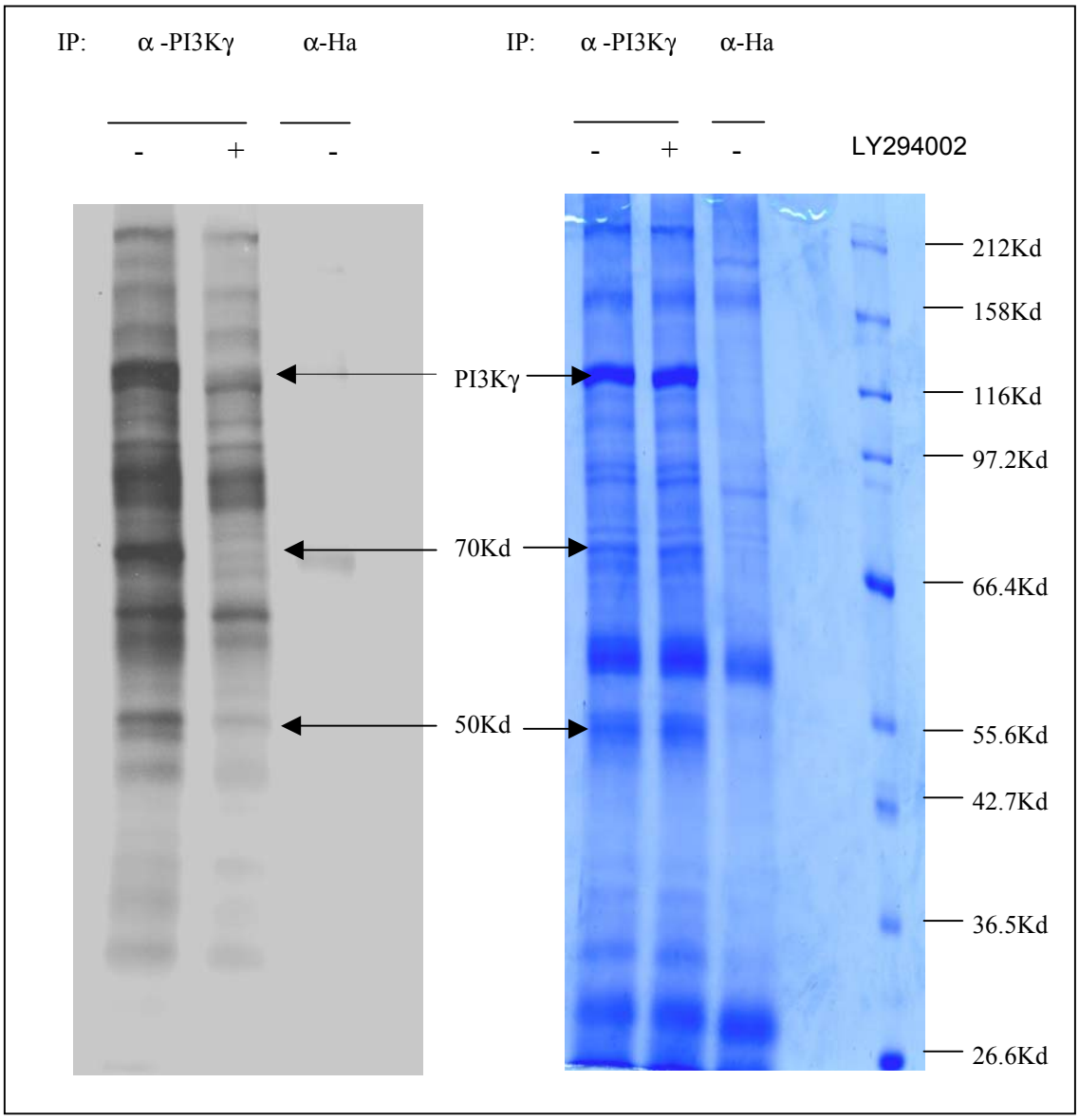


Fig.28 ***In vitro* PI3Kγ protein kinase assay**. 1x10^8 non-transfected K562 cells for each sample were lysed and the cleared lysates were subjected to immunoprecipitation using anti-PI3Kγ monoclonal antibody which was covalently-coupled to protein G beads. An isotype-matched monoclonal anti-HA antibody was used as a negative control. After the immunoprecipitation, the immunoprecipitates were subjected to *in vitro* protein kinase assay as described under 'Material and Methods'. After that , the proteins present in the immunoprecipitates were separated by 8 % SDS-PAGE followed by Coomassie staining (right panel). The phosphorylation of PI3Kγ and the associated proteins were visualised by exposure to the dried gel to film (left panel).

In this manner, we were able to demonstrate that PI3Kγ is involved in a complex of proteins in K562 cells, and some of the associated proteins show to be the direct targets for its intrinsic protein kinase activity under *in vitro* conditions. Further investigations are needed to identify these proteins and to clarify the physiological relevance of these interactions for the erythroid differentiation process.

4 DISCUSSION

Hematopoiesis, the differentiation of hematopoietic stem cells and progenitors into various lineages, is essential for the development and survival of a normal individual. New blood cells belonging to different cell lineages are formed from stem cells during embryogenesis and throughout the lifetime of the adult to replace cells that have completed their life span. Abnormalities in the normal developmental program for blood cell formation result in hematological diseases including leukemia. Understanding the cellular and molecular controls of normal blood development will make it possible to answer questions about the origin and treatment of these diseases.

Cell differentiation can be regulated at several levels. One level is the regulation of intracellular programs that are controlled by intracellular signal messengers. As important mediators of intracellular signalling pathways in mammalian cells, PI3Ks have been implicated in cell differentiation. Depending on cell type, both negative and positive roles for PI3Ks in differentiation have been revealed (Kimura, K et al., 1994; Ptasznik, A et al., 1997). However, little is known about the role of PI3Ks in hematopoietic stem cell differentiation.

In the present study, the role of PI3Ks in hematopoiesis was investigated using both normal hematopoietic stem cells and K562 cell line. By using various approaches, we revealed a nonredundant role for PI3Kγ as a negative regulator of the erythroid differentiation in K562 cells. Furthermore, we demonstrated that PI3Kγ performs this function presumably via its protein kinase activity.

4.1 PI3Kγ is a negative regulator of the erythroid differentiation of K562 cells

PI3Kγ is preferentially expressed in cells of hematopoietic origin. Previous work in our lab has shown that a specific up-regulation of PI3Kγ isoform precedes ATRA (all-trans retinoic acid)-induced cell differentiation in U937, a myeloid leukemia cell line (Baier, R et al., 1999). This provides the first evidence that PI3Kγ could be involved in hematopoietic cell differentiation, and raises the question whether PI3Kγ contributes to any other lineage specific differentiation process.

To answer this question, we screened the expression profile of PI3Kγ protein during the differentiation of $CD34^+$ progenitor cells derived from human peripheral blood. It was observed that PI3Kγ expression is significantly downregulated after induction of the CD34+ cells with EPO, which predominantly led to the development of erythroid

differentiation. In contrast, the PI3Kγ expression was not obviously affected when these cells were induced to megakaryocytic differentiation by TPO (Fig. 7), despite megakaryocytic and erythroid are closely linked lineage's *(*Debili, N et al., 1996*)*. These preliminary results provide the first clue that PI3Kγ is associated with erythroid differentiation.

Due to the difficulties in the isolation and maintenance of adequate numbers of hematopoietic stem cells, we therefore made further investigation using K562 cells. K562 is a human erythroleukemia cell line. Similar to hematopoietic stem cells, these cells are pluripotent in that they are able to differentiate along a megakaryocytic, erythroid, or, to a lesser extent, monocytic lineage (Lozzio, CB et al., 1975). Erythrroid differentiation of K562 cells can be induced by a number of chemical agents including cytosine arabinoside (Ara-C), hemin, and butyric acid (Leary, JF et al, 1987). Importantly, *in vitro* differentiation of these cells recapitulates many aspects of the normal erythroid terminal differentiation programs, including haemoglobin synthesis and proliferation arrest. Thus, this cell line is a useful model for in vitro differentiation studies.

To document that PI3Kγ is also downregulated in K562 cells upon induced erythroid differentiation as it is in induced erythroid differentiation of normal stem cells, K562 cells were induced to undergo erythroid differentiation by Ara-C. As shown in Fig.8, after treatment with Ara-C for 4 days, K562 cells were partially induced to erythroid differentiation, as evidenced by the haemoglobin synthesis. This process was accompanied by significant decrease in PI3Kγ expression. In contrast to PI3Kγ, the expression of PI3Kβ and its adaptor protein p85 was not significantly affected. Together with the results from $CD34^+$ cells, these findings indicate that PI3Kγ is a specific isoform of PI3Ks that is involved in erythroid differentiation.

To define the potential role of PI3Kγ in erythroid differentiation, it is important to ascertain whether this process can be affected by suppressing or increasing the PI3Kγ expression level. To achieve the specific suppression of PI3Kγ expression, an antisense strategy was used. The decrease in endogenous PI3Kγ, by stable expression of PI3Kγ antisense cDNA in K562 cells increased spontaneous and Ara-C induced erythroid differentiation. Conversely, overexpression of the wild type PI3Kγ blocked the effect of Ara-C on erythroid differentiation. These results indicate PI3Kγ is a negative regulator of erythroid differentiation in K562 cells. Furthermore, as overexpression of a dominant negative

PI3Kγ mutant displayed an effect opposite to that of the wild type PI3Kγ, we conclude that the observed inhibitory effect of PI3Kγ seems to be associated with its kinase activity.

In contrast to our results, previous studies have suggested the PI3Ks seems to play a positive role in erythropoiesis. In this regard, Myklebust, et al. have shown the PI3K inhibitor LY294002 suppresses the EPO-induced formation of glycophorin A positive erythroid cells from $CD34^+$ progenitors (Myklebust, JH et al., 2002). In addition, the terminal differentiation of murine erythroleukemia cells induced by dimethylsulfoxyde (DMSO) was also shown to be blocked by LY294002 (Bavelloni, A et al., 2000). A possible explanation for this apparent discrepancy could be due to the use of PI3K inhibitors in the above studies, which unselectively inhibit the activity of all PI3K isoforms. It is possible that other isoforms of PI3Ks may play a permissive role for the erythroid differentiation process during which the inhibition of PI3Kγ activity is needed. In consistent with this hypothesis, it is reported that inhibition of the overall PI3Ks activity by LY29002 induced erythroid precursor cells to undergo apoptosis (Haseyama,Y et al., 1999).

4.2 PI3Kγ negatively regulates the erythroid differentiation presumably via its protein kinase activity

PI3Ks are dual specificity enzymes. In addition to their function as lipid kinases, PI3Ks have an intrinsic protein kinase activity. The protein kinase activity of PI3Ks was initially discovered by the finding that p110α can phosphorylate the p85α regulatory subunit, which results in a reduced lipid kinase activity of the heterodimer *in vitro* (Dhand, R et al., 1994). Thereafter, protein kinase activity of other class IA PI3K isoforms has been revealed. PI3Kδ has been shown to undergo autophosphorylation both *in vitro* and *in vivo* (Vanhaesebroeck, B, 1999), and the *in vivo* autophosphorylation induced by activated receptors (such as CD28) leads to a concomitant decrease in associated lipid kinase activity.

In addition to class IA PI3Ks, PI3Kγ, the only class IB isoform of PI3K, also possesses an intrinsic protein kinase activity. Similar to PI3Kδ, PI3Kγ is able to autophosphorylate *in vitro* (Stoyanova, S et al., 1997). Furthermore, it has been demonstrated that PI3Kγ is able to transphosphorylate the adaptor protein p101 and the mitogen-activated protein kinase kinase 1 (MEK-1) *in vitro* (Bondev, A et al., 1999). To define the individual function

played by the lipid kinase and protein kinase of PI3Kγ, the use of PI3K inhibitor is not suitable, because they apparently interfere with both activities. To circumvent this situation, Bondeva et al. generated a PI3Kγ mutant construct, termed PI3Kγ-FRAP, by replacing a sequence within the conserved catalytic core of PI3Kγ with the corresponding sequence from FRAP (a member of target of rapamycin family without assigned lipid kinase activity) (Bondeva, T et al., 1998). The resultant PI3Kγ-FRAP protein does not have lipid kinase but still retain protein kinase activity. Taking advantage of this mutant, in the present study, we overexpressed PI3Kγ-FRAP in K562 cells, and its effect on erythroid differentiation was observed. Our results showed that the overexpression of PI3Kγ-FRAP inhibits the Ara-C induced erythroid differentiation of K562 cells as effectively as the wide type PI3Kγ (Fig.16, 18). Because the common feature of these two proteins is the shared protein kinase activity, thus the observed inhibitory effect of PI3Kγ on erythroid differentiation in K562 cells is presumably due to its protein kinase activity.

To address whether the lipid kinase activity of PI3Kγ is uncoupled from the erythroid differentiation process, we first compared the lipid kinase activities in cells stably transfected with PI3Kγ and its functional mutants. For convenience, the activation of Akt, an effector enzyme downstream of PI3K (Franke, TF et al., 1995) was used as a readout of lipid kinase activity. As expected, overxepression of PI3Kγ-FRAP mutant did not activate Akt because this mutant does not have a lipid kinase activity. Intriguingly, even overexpressing a wild type PI3Kγ was not sufficient to increase the Akt phosphorylation under conditions used in this work. Furthermore, the basal level of Akt phosphorylation was not inhibited by overexpressing the dominant negative PI3Kγ-KR mutant. Thus, despite cells stably transfected with PI3Kγ and its functional mutants exhibited different potentials for erythroid differentiation, these different potentials were not correlated with their lipid kinase activities. In addition, treatment of K562 cells with Ara-C from 30 minutes up to 24h did not obviously affect Akt phosphorylation, suggesting that Ara-C has no direct effect on the lipid kinase activity of PI3Ks. Taken together, these results indicate the lipid kinase activity does not account for the inhibitory effect of PI3Kγ on the erythroid differentiation in K562 cells.

In contrast to our results, ATRA increased the overall PI3K lipid products which was apparently correlated with the upregulation of P13Kγ expression in U937 cells (Baier, R et al., 1999). In this study, the authors suggested the elevated lipid kinase activity resulted in activation of MAPK, which is supposed to be responsible for ATRA-induced

differentiation in these cells. It is possible that PI3Kγ may function either as lipid kinase or protein kinase to regulate distinct cellular responses depending on the cell type and the cellular context.

4.3 The effect of PI3Kγ on the erythroid differentiation is independent of p101

Class I PI3Ks are all heterodimers containing a 110-kDa catalytic subunit and a regulatory subunit. The class IA members p110 α, β, and δ are associated with a p85 family regulatory subunit. The primary function of p85 is to recruit the catalytic subunits to the plasma membrane, where the catalytic subunit phosphorylates its lipid substrates. In addition, p85 also stabilises the catalytic subunits by protecting them from degradation (Yu, J et al, 1998). PI3Kγ does not interact with a p85 family adaptor. Instead, PI3Kγ has been shown to be associated with a p101 regulatory subunit which has no homology with any known protein (Stephens, L et al., 1997). Up to date, the functional significance of p101 remains elusive.

In present study, the expression of p101 was examined by a method of RT-PCR. Surprisingly, no p101 expression could be detected in K562 cells (Fig 21). The precise reason for the absence of p101 in K562 cells is unknown. Nevertheless, this suggests the observed effect of PI3Kγ on erythroid differentiation of K562 cells is independent of p101. Previously, Stephens et al. showed that, in the presence of p101, the Gβγ mediated lipid kinase activity of PI3Kγ was significantly enhanced (Stephens, L et al., 1997). Recently, it was demonstrated the membrane recruitment of PI3Kγ mediated by p101 is essential for the activation of PI3Kγ in response to Gβγ stimulation (Brock, C et al., 2002). Consistent with this notion, we found overexpression of a membrane-targeted form of PI3Kγ mutant (PI3Kγ-CAAX) resulted in an elevated Akt phosphorylation, whereas overexpression of the wild type PI3Kγ did not (Fig. 19). It is tempting to speculate, the absence of p101 in K562 cells may provide a cellular context which predisposes PI3Kγ to function as a protein kinase in the cytosol.

4.4 The effect of PI3Kγ on the erythroid differentiation is not mediated by MAPK

As our results support the inhibitory effect of PI3Kγ on erythroid differentiation could be related to its protein kinase activity, it raises the question: what is the signalling evens that act downstream of this protein kinase activity? A possible candidate is the ERK/MAPK

pathway. Previous work in our lab has demonstrated that the protein kinase activity of PI3Kγ is sufficient to activate MAPK in COS-7 cells (Bondeva, T et al., 1998). Furthermore, available data also implicate ERK/MAPK in erythroid differentiation. For example, it has been shown the TPA-induced sustained activation of MAPK not only commits these cells to megakaryocytic differentiation, but also suppresses the transcription of haemoglobin, a marker of erythroid differentiation (Lumelsky, NL et al., 1991).

In present study, we demonstrated that Ara-C treatment resulted in ERK phosphorylation (Fig. 24). The specific MEK inhibitor PD98059 completely abrogated Ara-C induced ERK phosphorylation, and consequently enhanced erythroid differentiation (Fig. 25). Furthermore, inhibition of the basal level of MAPK activity by PD98059 increased the spontaneous erythroid differentiation (Fig. 25). Consistent with the previous study (Shelly, C et al., 1998), our results confirm MAPK could function as a negative regulator of erythroid differentiation of K562 cells. Therefore it is tempting to speculate that the inhibitory effect of PI3Kγ on erythroid differentiation might be mediated by MAPK.

Next, we tested whether the protein kinase of PI3Kγ contributes to the baseline and Ara-C induced MAPK activation. The activity of MAPK was evaluated in Western blots using an antibody that specifically recognises the activated, phosphorylated form of the ERK p42/44 MAP kinase. As shown in Fig. 23 and Fig. 26, the baseline and Ara-C -induced ERK phosphorylation was neither increased by overexpressing PI3Kγ wide type or PI3Kγ-FRAP, nor inhibited by the overexpressing the dominant negative PI3Kγ-KR mutant. These results indicate that the protein kinase activity of PI3Kγ does not contribute to the MAPK activation in this system. Thus the ERK/MAPK pathway seems to act in parallel rather than downstream of PI3Kγ to convey an inhibitory effect on erythroid differentiation.

4.5 Attempts to identify the downstream targets for PI3Kγ protein kinase activity

As PI3Kγ fails to activate MAPK in K562 cells, it excludes MEK1 as the substrate for PI3Kγ protein kinase activity in this system, though MEK1 has been previously shown to be phosphorylated by PI3Kγ *in vitro* (Bondava, T et al., 1998*)*. This prompted us to search for the direct target (or targets) of PI3Kγ protein kinase activity. For this purpose, immunoprecipitation using a PI3Kγ specific antibody and subsequent *in vitro* protein kinase assay were performed. Using the monoclonal antibody, we have detected several

proteins that were co-precipitated with PI3Kγ (Fig. 28). These proteins are believed to be constitutively associated with PI3Kγ because they were not present in the immunoprecipitate using a control HA-antibody that failed to pull down PI3Kγ. When the anti-PI3Kγ immunoprecipitate was subjected to *in vitro* kinase assay, we found two proteins with molecular weights of about 70 kDa, and 50 kDa were phosphorylated. Importantly, the phosphorylation of these two proteins was abrogated by the PI3K inhibitor LY294002, strongly suggesting the phosphorylation of these proteins are catalysed by PI3Kγ. Thus these two proteins could be direct downstream substrates for PI3Kγ protein kinase activity. Due to the limited sensitivity of immunoprecipitation method, currently we can not exclude the existence of other substrates for PI3Kγ protein kinase activity in this system. Actually we have detected additional proteins present in anti-PI3Kγ Immunoprecipitate using polyclonal antibody (Fig. 27). Because the polyclonal antibody apparently inhibits the intrinsic activity of PI3Kγ, it is currently unknown whether these proteins can be phosphorylated by PI3Kγ or not. Nevertheless, these results confirm the existence of potential substrates downstream of PI3Kγ protein kinase activity in K562 cells

The molecular events that links the phosphorylation of the downstream proteins of PI3Kγ protein activity to erythroid differentiation is currently unknown. In fact, erythroid differentiation is a complex and finely-tuned multi-step process. It is now widely accepted that this process in controlled by two groups of functionally antagonistic transcription factors (Perry, C et al., 2002). The normal erythroid differentiation requires not only the activation of differentiation-promoting factors, but also the concomitant suppression of differentiation-inhibiting factors as well (Nerlov, C et al., 2000). In this regard, PU.1, a member of Ets family of transcription factors, has been shown to be an inhibiting factor for erythroid differentiation (Rekhtman, N et al, 1999). Repression of PU.1 function is a critical step for erythroid differentiation, because overexpression of PU.1 in erythroblast blocks the terminal erythroid cell differentiation (Deljado, MD et al., 1998). Interestingly, Rekhtman et al. have demonstrated that ectopic expression of PU.1 in erythroid cells blocks their maturation, while normal erythroid differentiation of these cells can be restored by overexpression of GATA-1, an erythroid differentiation-promoting transcription factor (Rekhtman, N et al., 1999). As the activities of transcription factors are to be regulated by phosphorylation, it is possible that the protein kinase activity of PI3Kγ may exert its effect through a mechanism of triggering a phosphorylation cascade which ultimately affects the activities of these transcription factors.

Based on our results, a model for PI3Kγ mediated signalling mechanism could be depicted as following: without induction, PI3Kγ may constitutively bind with and phosphorylate some downstream proteins via its protein kinase activity. After phosphorylated by PI3Kγ, these proteins may trigger a signalling pathway which conveys an inhibitory effect on erythroid differentiation and keep the cells in undifferentiated state. After induction, the expression of PI3Kγ is downregulated and its inhibitory effect is removed, thus allowing for the terminal differentiation of the cells.

4.6 Conclusion

In the present study, we established a specific role for PI3Kγ as a negative regulator of erythroid differentiation of K562 cells. Interestingly, it has been recently shown that the inhibition of PI3K activity in primary cultured fetal pancreatic cells results in a robust activation of endocrine differentiation (Ptasznik, A et al., 1997). The blockade of PI3K was also shown to enhance the neutrophilic differentiation in HL60 cells (Kanayasu-Toyoda, T et al, 2002). Despite the isoform-specific role of PI3Ks was not clarified in these studies, at least these data indicate the negative regulation of cellular differentiation by PI3Ks may be a general phenomenon. Nevertheless, other authors have previously shown a positive involvement of PI3Ks in PC12 pheochromocytoma (Kimura, K et al., 1994), SH-SY5Y human neuroblastoma (Lopez-Carballo, G et al., 2002) and U937 (Baier, R et al., 1999) cell lines differentiation. Together, all available data suggest that PI3Ks may play dual roles as both positive and negative regulators of cellular differentiation depending on cell type.

Up to date, almost all the established cellular functions of PI3Kγ are believed to be related to its lipid products. In the present study, we demonstrated PI3Kγ plays a negative role in erythroid differentiation of K562 cells, and the function of PI3Kγ in this process is mediated by its protein kinase activity. To our knowledge, this is the first time that a physiological relevance for PI3Kγ protein kinase activity has been established.

4.7 Outlook

In the present study, we clearly demonstrate that PI3Kγ plays a negative role in the regulation of erythroid differentiation in K562 cells. However, in mice lacking functional PI3Kγ (PI3Kγ $^{-/-}$ mice) (Hirsch, E et al., 2000; Sasaki, T et al., 2000), the erythropoiesis

seems not to be obviously affected, as evidenced by the normal count of erythrocytes in the peripheral blood. Presently, the precise reason for this is unknown. One possibility is that PI3Kγ may affect erythropoiesis in a differentiation-stage dependent manner. In the present study, PI3Kγ was shown to affect the haemoglobin synthesis, suggesting an involvement of PI3Kγ in the later phase of erythroid differentiation. It is possible PI3Kγ may only facilitate the terminal differentiation of the committed erythroid precursor cells, but has no effect on the generation of these erythroid precursor cells from the multipotent progenitors. To test this hypothesis, a close examination of the kinetics of erythroid differentiation of hematopoietic stem cells from PI3Kγ $^{-/-}$ mice will be helpful. On the other hand, K562 is a leukemia cell line, and it is well known that the arrest of differentiation is a common feature of tumor cells. Thus a more speculative explanation is that the observed inhibitory effect of PI3Kγ on erythroid differentiation in K562 cells may reflect an intrinsic property of PI3Kγ in leukemogenesis. To test this concept, future investigation is needed to determine if the PI3Kγ protein kinase activity can produce the same inhibitory effect on the erythroid differentiation of normal blood stem cells. Furthermore, in the present study, we detected two proteins that are constitutively associated with PI3Kγ and phosphorylated by PI3Kγ *in vitro*, implying they are direct targets for PI3Kγ protein kinase activity. Thus, these proteins could be the possible candidates that mediate the inhibitory effect of PI3Kγ on erythroid differentiation *in vivo*. Future work is needed to identify these proteins and elucidate how the phosphorylation of these proteins by PI3Kγ affects the molecular evens linked to erythroid lineage–specific gene(s) expression.

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SUMMARY

Cell differentiation is a complex, multi-step process that can be regulated at several levels. One level is the regulation of intracellular programs that are controlled by intracellular signal messengers. As important mediators of intracellular signaling, PI3Ks have been implicated in cell differentiation. Depending on cell types, both positive and negative roles for PI3Ks have been revealed. PI3Kγ is the only isoform of class IB PI3Ks, which is preferentially expressed in cells of hematopoietic origin.

In the present study, the role of PI3Kγ in erythroid differentiation was investigated. PI3Kγ expression was found to be rapidly downregulated in human hematopoietic stem cells when these cells were induced to undergo erythroid differentiation by EPO. This finding was further corroborated in K562 cells. K562 is an erythroleukemia cell line, which is able to differentiate along the erythroid lineage in response to a variety of chemical agents. It was observed that PI3Kγ expression was downregulated along erythroid differentiation of K562 cells induced by Ara-C, a potent differentiation inducer. In contrast, the expression of PI3Kβ and its adaptor protein p85 was not significantly affected by Ara-C treatment, suggesting that PI3Kγ is a specific PI3K isoform related to the erythroid differentiation process.

In subsequent experiments, a negative role of PI3Kγ in erythroid differentiation was established by using antisense and overexpression strategies. It was found that antisense inhibition of the endogenous PI3Kγ expression increased the spontaneous and Ara-C induced erythroid differentiation, whereas overexpression of wild type PI3Kγ attenuated the inductive effect of Ara-C.

As overexpression of the dominant negative PI3Kγ-KR mutant displayed an effect opposite to that of the wild type PI3Kγ, we conclude that observed effect of PI3Kγ on eythroid differentiation is linked its kinase activities. PI3Kγ possesses both lipid kinase activity and protein kinase activity. To distinguish which activity is responsible for the inhibiton of erythroid differentiation, the effect of the PI3Kγ-FRAP mutant, which possesses protein kinase activity, but lacks lipid kinase activity, was evaluated. It was observed that overexpression of this mutant inhibited the Ara-C induced erythroid differentiation. Thus, the inhibitory effect of PI3Kγ on erythroid differentiation is presumably due to its protein kinase activity. This concept was further substantiated by the following findings: (i). overexpression of the dominant negative PI3Kγ-KR did not affect the basal endogenous PI3Kγ lipid kinase activity, despite overexpression of this mutant increased the

spontaneous erythroid differentiation. (ii). Ara-C has no direct effect on lipid kinase activity of PI3Ks. Thus the lipid activity of PI3Kγ seems to be uncoupled from the erythroid differentiation process.

The activity of PI3Kγ has been shown to be subject to the regulation of its adaptor protein p101. In the present study, the expression of p101 was examined by the method of RT-PCR. No detectable p101 expression was found at mRNA level in K562 cells. Thus, the observed effect of PI3Kγ on erythroid differentiation is independent of p101.

As the PI3Kγ protein kinase activity has been previously shown to be sufficient to activate MAPK, a possible involvement of MAPK in mediating the effect of PI3Kγ in this system was investigated. Overexpressing PI3Kγ wild-type or PI3Kγ-FRAP did not increase the basal or Ara-C induced MAPK activity over the vector controls. In addition, the basal and Ara-C induced MAPK activity was not inhibited by the overexpression of the dominant negative PI3Kγ-KR mutant. These results indicate that the protein kinase activity of PI3Kγ does not contribute to the MAPK activation in this system.

To search for the direct target (or targets) for PI3Kγ protein kinase activity, anti-PI3Kγ immunoprecipitation and subsequent *in vitro* kinase assay was performed. Several proteins were coprecipitated with P13Kγ. *In vitro*, the phosphorylation of two of these proteins with molecular weight of approximately 70 kDa and 50 kDa was abrogated by the PI3Ks inhibitor LY294002, suggesting these two proteins are downstream substrates for the PI3Kγ protein kinase activity *in vitro*. These two proteins are the possible candidates that mediate the inhibitory effect of PI3Kγ on erythroid differentiation *in vivo*.

To conclude, in the present study, we revealed a nonredundant role of PI3Kγ as a negative regulator for erythroid differentiation in K562 cells. This is the first time that a role of the protein kinase activity of PI3Kγ in a physiological condition has been established. The identification of these substrates for PI3Kγ protein kinase activity and the elucidation of their downstream signalling events linked to the erythroid-specific gene(s) expression are the aim of our future investigations.

ZUSAMMENFASSUNG

Zelldifferenzierung ist ein komplexer, mehrstufiger Prozess, der auf verschiedenen Ebenen reguliert werden kann. Eine Ebene ist die Regulation von intrazellulären Programmen, die durch intrazelluläre Signal-Botenstoffe kontrolliert werden. Für PI3-Kinasen als wichtige Mediatoren der intrazellulärer Signaltransduktion wird eine Beteiligung an der Zelldifferenzierung angenommen. Abhängig vom Zelltyp konnte für PI3-Kinasen sowohl positiver als auch negativer Einfluß gezeigt werden. PI3Kγ ist die einzige Isoform der Klasse 1B PI3-Kinasen, welche bevorzugt in hämatopoietischen Zellen expremiert wird.

In der vorliegenden Arbeit wurde die Rolle der PI3Kγ bei der erythroiden Differenzierung untersucht. Eine schnelle Absenkung der PI3Kγ Expression nach Induktion der erythroiden Differenzierung durch EPO konnte gezeigt werden. Diese Beobachtung konnte in K562 Zellen weiter bestätigt werden. K562 ist eine erythroleukämische Zelllinie, welche in der Lage ist, in Reaktion auf eine Vielzahl an chemischen Agenzien die erythroide Differenzierung zu durchlaufen. Es wurde beobachtet, dass die PI3Kγ Expression während der Ara-C - induzierten erythroiden Differenzierung von K562 Zellen herab reguliert wird. Im Gegensatz dazu, wird die Expression von PI3Kβ und deren Adaptorprotein p85 durch Ara-C Behandlung nicht signifikant beeinflußt. Dies ist ein Hinweis darauf, dass die Funktion der PI3Kγ bei der erythroiden Differenzierung Isoform-spezifisch ist.

In folgenden Experimenten wurde durch Verwendung von Antisense - und Überexpressions - Strategien eine negative Rolle der PI3Kγ während der erythroiden Differenzierung etabliert. Es wurde gezeigt, dass die Unterdrückung der endogenen PI3Kγ Expression durch Antisense-RNA zu einer Erhöhungen sowohl der spontanen als auch der Ara-C induzierten erythroiden Differenzierung führt, während die Überexpression von Wildtyp-PI3Kγ den induzierenden Effekt von Ara-C abschwächt.

Da Überexpression der dominant negativen PI3Kγ-KR Mutante verglichen mit Wildtyp-PI3Kγ den gegenteiligen Effekt hat, schließen wir, dass für den PI3Kγ - Effekt deren Kinaseaktivitäten wesentlich sind. PI3Kγ besitzt sowohl Lipid - als auch Proteinkinaseaktivität. Um zu unterscheiden, welche von beiden für die Inhibierung der erythroiden Differenzierung verantwortlich ist, wurde der Effekt der PI3Kγ-FRAP Mutante, welche nur Proteinkinaseaktivität besitzt, untersucht. Es wurde beobachtet, dass Überexpression dieser Mutante die Ara-C induzierte erythroide Differenzierung inhibiert. Demnach ist für den inhibitorischen Effekt der PI3Kγ anscheinend deren Proteinkinaseaktivität nötig. Diese Annahme wurde durch die folgenden Beobachtungen

untermauert: 1. Überexpression der dominant negativen PI3Kγ-KR Mutante ist ohne Einfluß auf die basale endogene PI3Kγ Lipidkinaseaktivität, obwohl die Überexpression dieser Mutante die spontane erythroide Differenzierung erhöht, 2. Ara-C hat keinen direkten Effekt auf die Lipidkinaseaktivität von PI3-Kinasen. Die Lipidkinaseaktivität scheint also unabhängig von der erythroiden Differenzierung zu sein.

Für PI3Kγ wurde gezeigt, dass ihre Aktivität der Regulation durch das Adaptorprotein p101 unterliegt. In der vorliegenden Arbeit wurde die Expression von p101 mittels RT-PCR untersucht. Auf der Ebene der mRNA wurde in K562 Zellen keine detektierbare p101 Expression gefunden. Der beobachtete Effekt der PI3Kγ auf die erythroide Differenzierung ist demnach unabhängig von p101.

Da kürzlich beschrieben wurde, dass die Proteinkinaseaktivität der PI3Kγ für die Aktivierung der MAPK ausreichend ist, wurde die mögliche Beteiligung der MAPK an der Vermittlung der PI3Kγ Effekte in diesem System untersucht. Überexpression der PI3Kγ WT oder der PI3Kγ-FRAP erhöht weder die basale noch die Ara-C induzierte MAPK Aktivität über das Kontrollniveau. Die basale und die Ara-C induzierte MAPK Aktivierung wird auch nicht durch Überexpression der dominant negativen PI3Kγ-KR Mutante inhibiert. Diese Ergebnisse weisen darauf hin, dass die Proteinkinaseaktivität der PI3Kγ in diesem System nicht an der Aktivierung der MAPK beteiligt ist.

Um das direkte Substrat (oder Substrate) der PI3Kγ Proteinkinaseaktivität zu identifizieren, wurden anti-PI3Kγ-Immunpräzipitationen, gefolgt von einem *in vitro* Kinaseassay, durchgeführt. Mehrere Proteine werden mit PI3Kγ copräzipitiert. *In vitro* wird die Phosphorylierung von zwei dieser Proteine mit einem Molekulargewicht von etwa 50 und 70 kDa durch den PI3K-Inhibitor LY294002 verhindert. Dies weist darauf hin, dass diese beiden Proteine *in vitro* nachgeschaltete Substrate der Proteinkinaseaktivität der PI3Kγ sind. Diese beiden Proteine sind mögliche Kandidaten für die Vermittlung der inhibitorischen Effekte der PI3Kγ auf die erythroide Differenzierung *in vivo*.

In der vorliegenden Arbeit beschreiben wir die Rolle von PI3Kγ als negativer Regulator der erythroiden Differenzierung in K562 Zellen. Erstmalig wurden Hinweise für eine physiologische Funktion der PI3Kγ Proteinkinaseaktivität gefunden. Die Identifizierung der gefundenen Substrate der PI3Kγ Proteinkinaseaktivität und die Aufdeckung der nachgeschalteten Signalwege, die zur Expression von erythroid-spezifischen Genen führen, sind das Ziel weiterer Untersuchungen.

APPENDIX

Abbreviations

Ara-C	cytosine arabinoside
ATP	adenosine 5'triphosphate
ATRA	all-trans retinoic acid
BCR	breakpoint cluster region
Btk	Bruton's tyrosine kinase
C-GSF	granulocyte colony-stimulating factor
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine-tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene-bis (oxyethylenenitrilo) tetraacetic acid
EGFR	epidermal growth factor receptor
EPO	erythropoietin
ERK	extracellular signal regulated kinase
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FRAP	FKPB12 and rapamycin-associated protein
GAP	GTPase activating protein
GDP	guanosine di-phosphate
GEF	guanidine-nucleotide exchange factor
GPCR	G-protein coupled receptor
GST	glutathione-S-transferase
GTP	guanosine tri-phosphate
HEPES	(N- (2-hydroxyethyl) piperazine-N´-(2-ethanesulfonic acid))
IL6	interleukin 6
IRS	insulin receptor substrate
JNK	c-Jun N-terminal kinase
Jun	oncogene of the avian sarcoma virus 17 (ju-nana, japanese:17)
LPA	lysophosphatidic acid
MAPK	mitogen activated protein kinase
MEK	MAPK/ERK kinase

MOPS	3-(N-morpholino)-propanesulfonic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD98059	AMF, 2'-amino-3'-methoxy-flavone
PDK	phosphotidylinositol dependent kinase
PE	phycoerythrin
PH	pleckstrin homology
PLC	phospholipase C
PKB	protein kinase B
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PtdIns	phosphatidylinositol
PTEN	phosphatase and tensin homologue deleted on chromosome 10
SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulfate-polcralamine gel electrophoresis
SH2	src homology 2
TOR	target of rapamycin
TPA	tetradecanoylphorbol 13-acetate
TPO	thrombopoietin

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Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Jena, den 17.03.2003

Yuantao Liu

Erklärung zur Bewerbung

Hiermit erkläre ich, dass ich mich mit der vorliegenden Arbeit an keiner anderen Hochschule um den akademischen Grad Doctor rerum naturalium beworben habe und dass ich weder früher noch gegenwärtig die Eröffnung eines Verfahrens zum Erwerb des o.g. akademischen Grades an einer anderen Hochschule beantragt habe.

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