cAMP-Regulated Cell Proliferation in Brown Preadipocytes

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Do the difficult things while they are easy and do the great things while they are small. A journey of a thousand miles must begin with a single step.

Lao Tzu
Abstract

As a prototypical second messenger, cAMP is involved in the regulation of multiple cell functions. cAMP has a well established inhibitory effect on cell proliferation in smooth muscle and epithelial cell types. However, there is accumulating evidence also for stimulatory effect on proliferation, mainly in endocrine cell types.

Mechanisms mediating the cAMP stimulatory effect are not well studied. cAMP, produced via β1-adrenoceptor activation, promotes cell proliferation in brown preadipocytes. Due to the importance of brown adipose tissue in energy metabolism and its implication in the treatment of obesity and type II diabetes, understanding the mechanisms of tissue recruitment has clinical implication for the treatment of these metabolic syndromes.

We found that the Erk1/2 family of MAPK, often involved in regulation of cell proliferation, can be activated in response to the stimulation of G protein-coupled receptors, including adrenergic receptors (α1-, α2-, β1- and β3-Adrenoceptors) and mitogenic lysophosphatidic acid (LPA) in primary cultured brown adipocytes. In contrast to the case e.g. in many immortalized cell lines and various primary cultured cells, EGF receptor transactivation is not employed in Erk1/2 activation by any G protein-coupled receptor tested in brown adipocytes. This suggests that EGF receptor transactivation is not an universal mediation process for GPCR activation of MAPK.

cAMP-activated cell proliferation in brown preadipocytes is mediated through PKA rather than Epac under serum-free conditions. This effect is independent of PI3K/Akt, mTOR or Erk1/2 MAPK pathways. Differential responses to two different MEK inhibitors PD98059 and U0126 suggested the involvement of a pathway sensitive to PD98059, but independent of the Erk1/2 family of MAPK. At the transcriptional level, by combining microarray and RT-qPCR, we have identified eight genes, under the regulation of cAMP, that may be involved in the further mediation of the cAMP effect on cell proliferation.

An understanding of cAMP-induced cell proliferation can be of importance both in metabolic and cancer research.
The thesis is based on the following papers and manuscripts, referred to by their Roman numbers:

I. Non-transactivational, dual pathways for LPA-induced Erk1/2 activation in primary cultures of brown pre-adipocytes
   Therese E. Holmström, Charlotte L. Mattsson, Yanling Wang, Irina Iakovleva, Natasa Petrovic, Jan Nedergaard

II. In brown adipocytes, adrenergically induced β1-β3-(Gs)-, α2-(Gi) and α1-(Gq)-mediated Erk1/2 activation is not mediated via EGF receptor transactivation
    Yanling Wang, Johanna M. Fälting, Charlotte L. Mattsson, Therese E. Holmström and Jan Nedergaard
    Submitted

III. cAMP-stimulated cell proliferation in brown preadipocytes is mediated by protein kinase A, but is independent of ERK1/2, PI3K and mTOR
    Yanling Wang, Masaaki Sato, Tore Bengtsson and Jan Nedergaard
    Manuscript

IV. Identifying novel genes involved in cAMP induced cell proliferation of brown preadipocytes
    Yanling Wang and Jan Nedergaard
    Manuscript
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Introduction

As a prototypical intracellular second messenger, the effect of cAMP on cell proliferation is perplexing and often under debate. The cell growth inhibitory effect of cAMP in many cell types has been intensely studied, including both the functional implications and cell signalling pathways, even with a therapeutic potential proposed for cancer therapy. However, a cAMP hyperplastic effect has been seen in an increasing number of cell types under physiological conditions, and a tumourigenesis effect under certain pathological conditions, for instance Carney complex. This has called for serious attention and further research endeavours regarding cAMP and cell proliferation. Only limited information is available regarding signal transduction pathways responsible for the cAMP proliferative effect, compared to its cell growth inhibitory effect.

Brown preadipocytes serve a good cell model for studying cAMP-induced cell proliferation in that the cells go through a proliferative stage before differentiation into mature adipocytes. cAMP stimulates the cell proliferation in the premature stage (referred to as brown preadipocytes in the thesis) rather than in the mature stage. Moreover, the study of brown preadipocyte proliferation would also help us understand the mechanism of brown adipose tissue recruitment, where proliferation mainly takes place in cells of the stromal vascular fraction, rather than in mature brown adipocytes.

In my studies, I have used brown preadipocytes and investigated the involvement or interaction of signalling pathways between cAMP and MAPK, PI3K and mTOR mitogenic signalling pathways. The transcriptional profile stimulated by cAMP in the proliferative stage of brown adipocytes was examined with microarray, and confirmed with RT-qPCR. mTOR complexes and PI3K are important in maintaining basal cell proliferation/cell survival, but are not crucial for cAMP-stimulated cell proliferation. Eight genes have been confirmed to be upregulated dramatically following cAMP stimulation of brown preadipocytes, and these are candidate genes for cAMP-stimulated cell proliferation.
1. cAMP signalling pathway

As the prototypical and most well studied intracellular second messenger, cAMP regulates multiple cellular functions. In mammalian cells, cAMP production is catalysed by adenylyl cyclase in response to G protein-coupled receptor activation by extracellular signalling molecules. Being viewed as linear and simple, the cAMP signalling pathway transduces a diverse range of biological effects ranging from memory, metabolism, cell proliferation etc., due to the existence of a diversified combination of multiple forms of signal transducers at all levels. Apart from that, the spatiotemporal regulation of cAMP signalling adds to the complexity at another level.

1.1 G protein-coupled receptors

G-protein coupled receptors, which share a common seven transmembrane helix structure, are involved in sensing and responding to almost every physiological stimulus transmitted by hormones, ions, nucleotides, lipids, peptides, light, taste and pheromones etc. [1]. Conformational changes happen in response to ligand binding, facilitating the interaction between the cytosolic C-terminal of the GPCR and G proteins, which are located in the vicinity to the plasma membrane. Whorton et al., with single molecule spectrometry and FRET, have shown that monomeric forms of rhodopsin and β2-adrenergic receptors are able to bind to G-proteins and catalyse the exchange of GDP for GTP in reconstituted high-density lipoprotein (rHDL) phospholipid bilayer particles [2]. However, homo- or hetero- dimers of signalling regulation are also reported for a series of GPCRs, e.g., β1-AR, muscarinic acetylcholine M1 receptor (M1R) and dopamine D2 receptor (D2R) [3].

Adrenergic receptors are a large family of G protein-coupled receptors [4, 5]. In brown adipocytes, several types of the α and β families of adrenergic receptors exist: α1, α2, β-adrenoceptors are expressed at different stages of cell maturation [6, 7]. Norepinephrine released from the sympathetic terminals plays crucial roles in multiple cell functions through its action of various adrenergic receptors [8]. We have focused in this study on the cAMP-generating β-family of adrenergic receptors and cell proliferation. Both β1- and β3-adrenoceptors are expressed in brown adipocytes; however different functions are carried out through the production of cAMP in the process of the development of this cell type [9, 10].
1.2 G proteins

Heterotrimeric G-proteins consist of a catalytic $\alpha$-subunit, and a regulatory $\beta\gamma$-subunit complex. Activation of a GPCR causes a conformational change in $G\alpha$, allowing for GTP binding and dissociation from the $\beta\gamma$-subunit complex, and advancing the GTP/GDP exchange rate for the G protein. The $G\alpha$ and $\beta\gamma$-subunit complex then act separately on their down-stream effectors. Based on the heterogeneity of the $\alpha$-subunit, G proteins are divided into 4 categories: $G_s$, $G_i$, $G_{q/11}$, and $G_{12/13}$, activating different downstream signalling pathways [1]. The alpha subunit of Gs and Gi, regulate adenylyl cyclase, and cAMP production, with stimulatory and inhibitory effects respectively. Activation of the alpha subunit of $G_{q/11}$ ($G_{\alpha q/11}$) activates phospholipase C, increases IP$_3$ levels, leading to Ca$^{2+}$ release from an endogenous calcium pool, initiating calcium signalling; at the same time, diacylglycerol (DG) can activate some protein kinase C isoforms, regulating both Ca$^{2+}$ and PKC signalling [11].

The rate of GTP hydrolysis is another aspect that affects the signal amplitude of G proteins. Similarly to small G proteins, the GTPase activity of the $G\alpha$-subunit is efficiently enhanced with the help of GTPase activating proteins (GAPs), which accelerate the GTPase activity by 30-2000 times. Downstream effectors including phospholipase C, Rho GEF and Phosphodiesterase act as GTPase activating proteins for heterotrimeric G proteins. Regulators of G protein signalling (RGS) are another family of proteins possessing GAP function. RGS advance the hydrolysis of GTP bound to the G-protein $\alpha$-subunit by causing conformational change in GTP*G$\alpha$ rather than by direct hydrolase activity [12].

1.3 Adenylyl cyclases

The adenylyl cyclases (AC) are a family of enzymes that catalyse the production of cAMP from ATP when activated by $G\alpha$. In eukaryotes, the AC family consists of 10 members. AC1-IX are hormone-sensitive, plasma membrane-binding isoforms, while ACX is a soluble isoform, not spanning the membrane. Membrane-spanning adenylyl cyclases have a pseudo-symmetry topology, consisting of a short cytosolic N-terminus, two repeats of membrane-spanning domains, each linked with a cytoplasmic domain. The two cytoplasmic domains (C1 and C2) contain a region of approximately 230 amino acid residues with roughly 40% identity but nearly identical tertiary structures. The heterodimerization between these two cytoplasmic domains, which form the catalytic core, has been the most prevalent model, constructed from a heterodimer composed of the C1 domain from purified ACV and the C2 domain from ACII [13]. The interface formed by these two domains is suggested to be the catalytic core with both the substrate-binding
site and the forskolin-binding site [14, 15]. The C1/C2 complex has only one forskolin-, one ATP- and one Ga-binding site; however, both C1 and C2 form homodimers, and the C1 homodimers were shown to have high affinity for ATP but low catalytic activity [16].

The GTP-bound α subunit of Gs binds mainly to the C2 domain in the cleft and activates all isoforms of transmembrane ACs [17]. Signals from the GPCRs are conducted into the cytosol by activating cAMP production in this process. α subunits of Gi, Gz and Go inhibit some isoforms of AC, while, depending on the isoform, Gβγ subunits can activate or inhibit AC. Other signalling molecules reported to regulate adenylate cyclase activity include Ca²⁺, Mn²⁺, Calmodulin, PKA and PKC [18].

Forskolin, a diterpene, is the most commonly used adenylyl cyclase activator. It is isolated from the Indian plant Coleus forskolii. Most membrane-spanning adenylyl cyclases are sensitive to forskolin, except for type IX and the soluble form ACX [19]. Forskolin exerts its action by interacting with the two cytoplasmic domains which form the catalytic core of AC. Ser 942 of the C2 (C-terminal) domain in AC was reported to be essential for interaction with the 7-acetyl group of forskolin, and a point mutation of this residue in AC IX restored its sensitivity to forskolin. In the presence of Gsα or forskolin, AC affinity for ATP is increased, which facilitates ATP conversion to cAMP [20]. One molecule of ATP is used for the generation of one molecule of cAMP and one molecule of pyrophosphate.

Adenylyl cyclase is an important signalling molecule for brown adipose tissue, and forskolin can imitate most of the effects of NE, although it is not clear about the subtype specificity for various adrenergic receptor effects. mRNAs encoding III, IV, V, VI, and IX types of AC were found to be expressed in brown adipose tissue [21]. Among them, Adenylyl cyclase III has been shown to be upregulated by neural stimulation and correlates strongly with adenylyl cyclase activity in brown adipose tissue. However, neuronal denervation did not change its mRNA expression level. Adenylyl cyclase III expression at the mRNA level was increased under hypothyroid conditions, in concert with an increase in AC activity [22]. Moreover, cAMP levels in response to NE differ during the process of cell maturation, implying the possible switch of AC isoform activation in the process of brown adipocyte differentiation [23].

1.4 Phosphodiesterase

Intracellular cAMP concentrations are controlled by the balance between its synthesis catalyzed by adenylyl cyclases and degradation by phos-
phodiesterases (PDE). PDE catalyses the hydrolysis of the 3’-5’-phosphodiester bond of cyclic nucleotides, and therefore degrades cyclic nucleotides, including cAMP and cGMP, which plays an important role in regulating intracellular second messenger levels for both cAMP and cGMP. Mammalian PDEs are a superfamily of enzymes encoded by 21 genes, which can be divided into 11 families (1-11) according to their structural similarity, kinetic properties and sensitivity to endogenous regulators and inhibitors. Structurally, they share a conserved C-terminal catalytic domain, while the regulatory N-terminal varies. Among these subfamilies, PDE4, 7 and 8 are cAMP-specific, while PDE5, 6 and 9 are cGMP specific [24, 25]; the others function for both cAMP and cGMP.

PDEs are regulated both by protein-protein interactions, e.g. with Calmodulin and by phosphorylation by PKA, PKB, ERK and PKG [25]. PDEs are localized in both the cytosol and plasma membrane, endoplasmic reticulum, nuclear membrane. Compartmentalized PDEs play important roles in the spatial and temporal regulation of cyclic nucleotide signalling [26].
2. cAMP signalling effectors

2.1 Protein kinase A

Protein Kinase A (PKA) is the first discovered and most well-studied cAMP effector protein which could be directly activated by elevated cAMP levels. Protein Kinase A is a serine/threonine kinase which can phosphorylate a variety of signalling proteins when activated by cAMP, including transcription factors, small G proteins, protein kinases, etc., and thereby plays an important role in regulating multiple cellular functions ranging from cell proliferation, differentiation, cell migration, metabolism to gene regulation and so on.

PKA has a heterotetramer structure, consisting of a regulatory subunit dimer and two catalytic subunits. Two principle types of PKA exist in mammalian cells (PKAI and PKAII), which differ in their regulatory subunits (RI or RII). The regulatory subunits are encoded by 4 different genes, which share about 75% identity in their cAMP-binding pockets [27, 28], namely: PRKARIA, PRKARIB, PRKARIIA and PRKARIIB, differing in tissue distribution, subcellular localization and biological properties [29].

PKA subtype is decided by the regulatory subunit, whose expression is tissue specific, and different isoforms of PKA display different sensitivity to cAMP. PKA subtypes with α-regulatory subunits are more ubiquitously expressed compared to β-regulatory subunit-containing isoforms. Type I PKA is more sensitive to cAMP binding and activation than type II PKA [30]. Within each regulatory subunit, there are 2 cAMP binding sites. Thus, binding of 4 cAMP molecules to a PKA holoenzyme causes dissociation of the catalytic subunits from the regulatory subunits and thereby activation of the kinase activity. There is a docking/dimerization (D/D) domain at the N-terminal of the regulatory subunit, important for A-kinase anchoring protein (AKAP) binding and proper localization of the kinase activity [31]. All the regulatory subunits and catalytic subunits may exist at the same time in the same cell. They can form an intact holoenzyme in dynamic equilibrium that is regulated at several different levels. There are 3 types of catalytic subunits, each consisting of 1-3 isoforms [29].

PKA activity is regulated both temporally and spatially, which ensures its ability to carry out complicated cell functions in a regulated way. The role of A-kinase anchoring proteins (AKAP) has been increasingly appreciated in recent years for their function in anchoring PKA to different compartments.
of the cell. AKAPs are a family of functionally related but structurally diverse proteins defined by their ability to bind to PKA [32]. These anchoring proteins form regulation complexes with cAMP-signalling components as well as with molecules from other signalling pathways, facilitating cAMP signalling in a spatially regulated way, as well as interaction with other signalling pathways [33].

AKAPs are structurally diverse, but different isoforms share a common PKA-binding domain which interacts with the docking/dimerization domain located at the N-terminus of the regulatory subunit of protein kinase A [34], and possess unique targeting domains that direct the PKA-AKAP complex to a specific subcellular structure [35]. AKAPs function by anchoring PKA to specific subcellular compartments mainly through protein-lipid interaction, providing PKA proximity to its target substrates [35]. AKAPs also serve as scaffolding proteins for several other signalling enzymes, including PDE4 family, Epac, adenylyl cyclase, Rap1, ERK5, PKC, calcineurin and GPCRs, some of which are also substrates of PKA phosphorylation, providing complex but regulated interaction between these molecules [14, 36, 37].

Signalling complexes formed by scaffolding with AKAP allow cAMP signal both spatially and temporally. When the PKA-complex is directed to G-protein coupled receptors, that are substrates for PKA, for example AKAP79/150, which locates PKA to β-adrenergic receptors; this will lead to phosphorylation of this GPCR. This has been postulated to induce phosphorylation-dependent downregulation of the receptor activation, repressing cAMP synthesis and also changing other signalling profiles of the target GPCR [38, 39]. AKAP79/150 could also physically associate with AC, which is also a substrate of PKA, causing the termination of cAMP production, providing feedback inhibition of cAMP signal transduction [34, 40].

Protein kinase A in brown adipose tissue

RII β regulatory is predominantly expressed in the brain and adipose tissues, including both white adipose tissue and brown adipose tissue. An RII β KO mouse model has been generated, which showed a surprisingly lean phenotype despite normal food intake or feeding of a high fat diet [41].

Mice with an RII β KO compensated with increased RIIα, showed higher PKA activity at least in BAT, where RII β is normally the dominant isoform. These mice showed increased resting metabolism, remained lean, gaining about 50% weight compared to wild type mice on a high fat diet. An increased glucose disposal rate, accompanied by lower blood insulin levels, suggests that PKA plays an important physiological role in metabolism [41]. In brown adipose tissue, UCP1 expression was dramatically increased at the protein level, rather than at the mRNA level, potentially accounting for the
increased basal increase in oxygen consumption, although no change was seen at the mRNA level, indicating no increase in sympathetic nervous system activity [42]. In the double knock out of PKARII β -/- UCP1-/-, the increased resting oxygen consumption disappeared, demonstrating the regulation of PKA on UCP1 expression in regulation of brown adipose tissue. However, the major adiposity loss was not reversed in the double knockout mice, indicating that the increased UCP1 expression and basal respiratory rate are not the cause of leanness in the RII β -/- mice [43]. In a study in leptin-deficient ob/ob mice, with a deletion of RII β, the obese phenotype was partially rescued, the mice showed decreased adiposity, increased oxygen consumption, increased locomotor activity compared to the ob/ob mice, showing the importance of protein kinase A in the central nervous system as well as in adipose tissue for metabolism regulation [44]. Also the UCP1-/-, RII β/- mice showed hyperactivity at night [43]. The increased activity could therefore explain the leaner phenotype.

2.2 Epac

Epac, exchange protein directly activated by cAMP, also known as cAMP-GEF, is another important intracellular cAMP effector. It is a guanine nucleotide exchange factor for small G-proteins. The small G-protein Rap is most commonly regarded as the target of Epac. The Bos group [45] and the Kawasaki group [46] identified Epac separately with different methods. Bos and colleagues found this cAMP specific effector when they were searching the genomic database for additional genes with cAMP binding domains, while Kawasaki and colleagues identified this novel gene by differential display RT-PCR. They both identified this protein-encoding gene, which could bind cAMP with affinities similar to that of the regulatory subunit of type I PKA [45-47]. Since the discovery of Epac, it has been shown to mediate cAMP functions independent of protein kinase A.

Two isoforms of Epac have been identified, Epac1 and Epac2, which share similar structures. Epac proteins are composed of a regulatory domain at the N-terminus and a catalytic domain at the C-terminus. The regulatory domain consists of a CBD (cAMP binding domain) and a DEP (Disheveled, Eg110, Pleckstrin) domain. The catalytic domain, which possesses GEF activity, consists of a REM (Ras exchange motif), a Ras association domain (RA) and a CDC25 homology domain (CDC25HD). Structurally, Epac1 and Epac2 differ in that the regulatory-domain of Epac2 contains 2 CBD domains separated by a DEP domain and therefore 2 molecules of cAMP are need for its activation, whereas Epac1 has only1 CBD domain and 1 molecule of cAMP is thus needed for its activation. Similarly to PKA, the CBD domain of the regulatory subunit, by covering the Rap entry site in the
CDC25HD domain, keeps Epac in an auto-inhibited state, according to both biochemical and crystallographic studies [48, 49]. Deletion of the regulatory domains could convert Epac into a constitutively active state, which further confirmed its functional role [50]. cAMP binding to the CBD domain could repress the auto-inhibitory state and activate Epac. The DEP domain is involved in the localization of Epac, although the structure of cAMP-binding Epac has not been obtained yet [48, 51].

Figure 1. Domain structure of Epac protein. Epacs are multi-domain proteins, composed of a regulatory region at the N-terminal and catalytic region at the C-terminal. The regulatory region consists of DEP and CNB domains, while the catalytic region consists of REM, RA and CDC35HD domains. Epac2 has one more CNB domain than Epac1. (Modified from Gloerich M & Bos JL 2010 Annu. Rev. Pharmacol. Toxicol 50: 355-375).

Based on the fact that a highly conserved glutamate residue critical for cyclic nucleotide binding was absent in the Epac proteins, 8-pCPT-2O-Me-cAMP, a cell membrane-permeable cAMP analogue has been devised, which turned out to be a more potent activator than cAMP for Epac, with a 100 times lower activity for PKA, which ensured its specificity for Epac [52]. 8-pCPT-2O-Me-cAMP activated Rap by activating both Epac1 and Epac2 [48] and its ability to discriminate between PKA and Epac has been used to reveal some cAMP effects carried out specifically by Epac.

Activated Epac mainly functions to activate small G proteins by exchanging GDP for GTP, Rap has been generally regarded as the primary target of Epac. Activation of Rap by Epac is involved in cell proliferation mediated by cAMP in a certain cell types, regulating cell proliferation either positively or negatively depending on the cell type.
result of the activation and opening of CNG, which comprises part of the chemical-to-electrical signal transition.
3. Flawed cAMP signalling leads to endocrine tumourigenesis

Neuropeptides, including GHRH and CRH, act on the GPCRs of pituitary cells, activate stimulatory G-proteins and increase cAMP production. Activated cAMP signalling transmits proliferative signals in certain types of cells of the pituitary gland, somatotroph cells, for instance. Mutations in a series of cAMP-signalling pathway events have been implicated in multiple endocrine neoplasms, including McCune-Albright syndrome. In most cases, cAMP events lead to benign tumours, and cAMP only transmits mitogenic signals in some but not all cell types of the pituitary gland [56].

3.1 GPCR abnormality in endocrine tumourigenesis

Abnormal regulation of cell proliferation by GPCRs is an important mechanism for tumour formation and development. Some agonists for the GPCRs are mitogenic and stimulate cell proliferation by binding to their corresponding G-protein coupled receptors and inducing cell proliferation, such as lysophosphatidic acid (LPA), prostaglandins etc. In tumour progression, agonists released by the tumour cells themselves or from the circulation, that act on some GPCRs through autocrine or paracrine effects are common tactics for tumour cells to pursue continued abnormal cell proliferation [57]. In endocrine organs, abnormal GPCR stimulation, including both mutation and hyperactivation, which may lead to increased cAMP production, is detected in a variety of endocrine tumours acting with different mechanisms: One way is the “Illegitimate expression of membrane receptors” (IEMR), which are detected in adrenocortical tumours where cells express extra types of receptors than normal, leading to additional responses to agonists [58]. Another way is gain-of-function mutations of G protein-coupled receptors, caused by substitution of key amino acids, facilitating receptor transition from an inactive to an active conformation in the absence of ligands [59]. Activating mutations of GPCR have been detected in several types of endocrine tumours; activating mutations of thyroid-stimulating hormone receptors (TSHR) are found in about 80% of thyroid adenomas. In thyroid cells, TSHR activating mutation would lead to persistent activation of adenylyl cyclase and cAMP production [60]. Transfection of such mutant into FRTL-5 thyroid cell lines substituted for exposure to TSH, the mitogenic hormone for thyroid cells, and caused cell transformation and tumourigenesis in nude mice. Activating mutations in the adrenocorticotropic receptor (ACTHR) were also found in pituitary tumours and adrenal hyperplasia [61]. GPCR
mutations lead to MacCune Albright Syndrome and ACTH-independent macronodular adrenal hyperplasia [62].

3.2 G protein mutations and endocrine tumours

Both constitutive mutations and over-expression of Gsα have been detected in endocrine tumours. Pituitary tumours are rare monoclonal neoplasias derived from a certain cell type of the pituitary gland, and normally co-exist with a specific endocrine syndrome including acromegaly, Cushing disease or hyperthyroidism etc. Pituitary neoplasias result from replication of a single cell with a mutated gene (either activation of a proto-oncogene or inactivation of tumour suppressor genes) [63, 64]. Pituitary neoplasias are common in humans but with low penetrance and low clinical diagnosis.

A gain-of-function mutation in the Gsα subunit has been identified in 30-40% of human GH-secreting pituitary adenomas, which is also the most frequent mutation among all genetic abnormalities in pituitary tumours and autonomously functioning thyroid adenomas [65, 66]. It is the only mutation identified in a significant proportion of pituitary tumours, particularly in GH-secreting adenomas, and causes constitutive activation of the cAMP pathway [64], although it has not been proven to directly cause tumourigenesis of the pituitary gland. The Gsα subunit is encoded by the gene GNAS, where activating mutations at codons 201 and 227 have been identified, e.g. gsp has been linked to pituitary neoplasia. Point mutations at both codons lead to constitutively active adenylyl cyclase activity, increased cAMP levels due to decreased intrinsic GTPase activity of Gsα [59]. The gsp mutation was also revealed in a small proportion of non-functioning and ACTH-secreting pituitary adenomas, as well as in thyroid adenomas [67].

Other gain-of-function mutations found in pituitary tumours, including common proto-oncogenes, include Cyclin E, Cyclin D1, Ras, PKC, PTTG, FGFR4 and so on, but with a much lower rate of occurrence than gsp. The mutant Gsα (Q227L) was found to advance cell growth in somatotrophic pituitary cell line GH3 cells [68].

The adrenal gland, under physiological conditions, is regulated by the pituitary gland through the release of adrenocorticotropic (ACTH), which regulates glucocorticoid secretion through cAMP. Increased steroid hormone release, which could lead to Cushing syndrome, is usually associated with adrenal cortical hyperplasia. ACTH-independent adrenal hyperplasia, macronodular adrenal hyperplasia (AIMAH), has been associated with gsp mutation in 40% of AIMAHa patients unrelated to McCune Albright Syndrome [69].
gsp oncogene mutations have also been found in some individuals with typical McCune Albright Syndrome (MAS) with several endocrine organ abnormalities, including precocious puberty, hyperthyroidism, hypercortisolism, growth hormone excess and hyperprolactinemia [70]. Patients with MAS have normal or even lower hormone levels but higher levels of response to the hormones, e.g. thyroid hyperplasia, indicating the role of abnormally active GPCRs or G-proteins.

In addition, overexpression of the stimulatory wild-type Gsα has been detected in neoplastic growth of the thyroid gland, and most human somatotrophic pituitary tumours are correlated with increased adenylyl cyclase activity or hyperactivated cyclic AMP response element binding protein (CREB) in those patients [71]. Overexpression of functional Gsα was found to be associated with increased hormone secretion in somatotrophinomas, causing elevated basal adenylyl cyclase activity but not increased cAMP production, possibly because of increased Gi protein expression and increased phosphodiesterase activity [72]. There are contradictory reports concerning the relationship between gsp oncogene mutations or overexpression of Gsα and pituitary cell proliferation. In the GH4 pituitary cell line, both conditional overexpression of Gsα and the gsp mutation, despite inducing increased intracellular cAMP, prolactin and GH levels, showed no effect on cell proliferation [73]. However, GH3 cells transfected with constitutively active Gsα showed enhanced cell proliferation and stimulated CREB activity via the protein kinase A pathway [74]. This shows the cell type-specific profile of the cAMP signalling, implicating changed activity of other cAMP regulating events, e.g. PDE, or possibly correction of the total outcome of the cAMP abnormality by other signalling pathways.

3.3 PDE mutations and endocrine tumours

**PDE and cell proliferation**

Under physiological conditions, phosphodiesterase activity increases with the elevation in cAMP concentration, which provides a negative feedback effect, aimed at bringing the cAMP concentration to a normal level. This feedback effect from PDE is an important part of cAMP signalling on cell differentiation, gene expression etc. [75]. PDE also plays important roles in the cAMP effect on cell proliferation. In FRTL-5 cells, PDE4 inhibition further promoted DNA synthesis increased by insulin, whereas DNA synthesis induced by both insulin and thyroid-stimulating hormone was inhibited with PDE4 inhibitors. PDE inhibitors switch from promoting to inhibiting cell cycle progression as [cAMP]i levels change from low to high, implying that phosphodiesterase is crucial in determining the cAMP effect on cell
proliferation through fine-tuning cAMP in a reasonable concentration range [76]. PDE mRNA is regulated by cAMP production, suggesting a cAMP self-regulation feedback by PDE action, and PDE is therefore an important mediator of the hormone desensitization process. This has been observed both in the physiological state and in pathological conditions. In endocrine disorders caused by Gs overexpression or gsp mutations, which lead to constitutive adenyl cyclase activation, both PDE expression and activity were dramatically elevated [77]. In McCune-Albright syndrome and pituitary and thyroid adenomas with increased intracellular cAMP concentrations, increased PDE activity may function to degrade cAMP to keep intracellular cAMP at a relatively stable level [26].

**Phosphodiesterase mutations and endocrine neoplasia:**

PDE mutations and the resulting change in [cAMP]i correlate with adrenal cortical hyperplasia. Phosphodiesterase-11A and -8B mutations were detected in patients with isolated adrenal hyperplasia, Cushing Syndrome and PPNAD (primary pigmented nodular adrenocortical disease). In a genome-wide association study, Stratakis’ laboratory discovered that inactivating mutations in the PDE11A isoform-4 gene were related to adrenocortical hyperplasia in individuals containing no other common mutations, such as PRKAR1A, the activating mutation in the PKA I regulatory subunit. In some cancer tissue samples, reduced PDE11A4 protein levels and enhanced CREB activation were also detected [78]. Horvath et al. reported mild micronodular adrenal hyperplasia and cyclical Cushing Syndrome with an R804H mutation in one patient. The phenotype was only seen in some of the family members carrying the same mutation, but with a significantly higher occurrence than in normal populations. This point mutation resides in a conserved region of the enzyme, which was supposed to be important for keeping the enzyme activity [79].

PDE8B is a cAMP-specific subtype, that has been found to be linked to isolated micronodular adrenocortical disease with bilateral adrenocortical hyperplasia in genome-wide association studies. A novel isoform of PDE8B, showing a lower activity in cAMP degradation, is found highly expressed in the adrenal gland. A point mutation P305H substitution in PDE8B was present in individuals with a mild to significant phenotype of Cushing Syndrome. Alternatively, in samples from isolated micronodular adrenocortical disease without any PDE mutations, the protein level rather than the mRNA level of PDE8B was shown to be consistently lowered [80].

In conclusion, PDE effects on cell proliferation are cell-type dependent. In many endocrine cell types, PDE inhibitors up-regulate cell proliferation via up-regulated cAMP concentrations, contrary to the expected negative effect on cell proliferation in other cell types. Phosphodiesterase mutations
are low penetrating mutations for adrenocortical hyperplasia, PDE11A and PDE8B seemed to predispose to a variety of lesions from isolated primary pigmented nodular adrenocortical disease with bilateral adrenocortical hyperplasia. Compromised cAMP degradation was detected in mutations of both isoforms, implicating the positive role of cAMP for cell proliferation.

3.4 PRKAR1A and endocrine tumours

Activating mutations in PKA have been identified in both endocrine hyperplasia and endocrine tumours. PRKAR1A encodes the protein kinase A regulatory subunit 1-α (RIα). Inactivating mutations of this gene lead to increased PKA activity. Several genetic defects of the PRKAR1A gene have been detected in patients with Carney complex (CNC). Carney complex is a familial multiple neoplasia syndrome characterized by the association of skin pigmentation, cardiac myxomas, and different endocrine tumours, particularly GH-secreting pituitary tumors. These patients develop a multiple neoplasia syndrome involving endocrine tumour formation, along with increased PKA sensitivity to cAMP stimulation in a large proportion of the patients with CNC (41%). A truncated RIα with a mutation in exon 6 of the gene was discovered, which showed increased PKA sensitivity to cAMP stimulation [81, 82]. These discoveries showed that PRKAR1A might function as a tumour suppressor gene for endocrine tissues.

In a transgenic mouse model, the PRKAR1A−/− was lethal in the embryonic stage, while PRKAR1A+/− heterozygous knockdown did not show any abnormality until older age, so a transgenic mouse model bearing a PRKAR1A antisense construct for exon 2 was developed. These mice developed thyroid lesions, mesenchymal and epithelial hyperplasia, as well as glandular ectasia, spindle cell schwannoma, squamous papilloma tumours, thus more resembling the symptoms of CNC patients than the heterozygous knockdown mice [83]. A tissue-specific knock-out of PRKAR1A in neural crest and pituitary gland caused increased cell growth and neoplasia. This further confirmed that a mutation in this gene is the causal factor for Carney complex and that PRKAR1A is a tissue-specific tumour suppressor gene.

Alternatively, an absence or low expression of the R1A subunit at the protein level, rather than altered gene expression, is also associated with GH-MAS, when no mutations in PRKAR1A at the mRNA level were found. The decrease of RIA is at least partly due to protein degradation by the proteasome, and a changed RI/RII ratio, which is suggested to be responsible for the difference in PKA effects on cell proliferation, since it is almost impossible to get elevated RIA levels in normal individuals due to a high degradation rate under physiological conditions. The decreased RIα was com-
pensated by increased RIIβ expression, leading to an increased PKAII/PKA1 ratio. The PKA activity in response to cAMP analogues was also increased. And therefore, a low ratio of RI/RII in the absence of a PRKAR1A mutation is also regarded as a mitogenic signal for transformed somatotrophs [84]. Increased PKA activity led to a mitogenic effect in somatotroph cells but the opposite effect was observed in non-functioning pituitary adenomas, indicating the cell-type dependent effect of PKA [85].

The molecular mechanism whereby PRKAR1A mutations regulate cell proliferation has been investigated in lymphocytes carrying a PRKAR1A mutation that induced higher PKA activity than in wild type cells. Activation of the cAMP-signalling pathway with forskolin or isoproterenol enhanced ERK phosphorylation in LPA-stimulated mutant cells, while ERK phosphorylation was inhibited in normal cells. The cell number also changed between mutant PKAR1α-expressing and wild type cells, indicating that constitutively active PRKAR1A increases cell proliferation through an ERK-dependent pathway [86]. However, an in-vitro model of PRKAR1α −/− MEF cells showed increased cyclin D protein levels independently of Ras/Raf/ERK and of the cyclin D mRNA level [87]. This showed regulation of cell proliferation by PKA, independently of the MAPK signalling pathway, although the underlying mechanism is not well formulated yet.
4. cAMP regulation of cell proliferation

4.1 Methods to study cell proliferation

**Flow Cytometry**

Flow cytometry is commonly used in immunology studies, as well as biological samples prepared in fluidic forms, for instance cells isolated from solid tissue. For cell proliferation, flow cytometry is a powerful tool for cell cycle studies. A wide range of DNA intercalating fluorophores have been applied to DNA ploidy analysis, including propidium iodide, Hoechst, DAPI, etc. In these protocols, the total DNA amount or DNA ploidy serves the primary criterion of cell cycle stage determination. Cells in G₀/G₁ phase have single ploidy of DNA, whereas cells of the G₂/M phases have double ploidy of DNA and therefore fluorescence doubles compared to cells in G₀/G₁, and cells that showed fluorescence between single ploidy DNA and double ploidy DNA are in S phase of the cell cycle [88]. DNA staining can also be used for apoptosis analysis, with minor changes in the process of data acquisition and analysis [89].

Since DNA content is the main defining factor for cell phase discrimination in this process, data analysis can easily be misleading due to the existence of cell aggregates. Therefore, it is routine in cell cycle analysis that other parameters, mainly the signal width, which reflects the size of each particle, is used to exclude cell aggregates from the final data analysis [90]. However, limitations still exist in that apoptotic (or necrotic) cells bias data analysis, since cells entering apoptosis from S phase or G2 phase can be categorised into a different cell phase, depending on the amount of total DNA left in the nucleus. Therefore, for apoptosis analysis, co-staining with some apoptosis markers is required.

For bivariate BrdU/DNA analysis; BrdU/EdU staining together with PI staining are used in cell proliferation experiments. BrdU or EdU are incorporated into newly synthesized DNA, providing parameters that reflect cytodynamic profiles of cell proliferation [90].

**Radiolabelled thymidine and thymidine analogues**

DNA synthesis is strictly regulated in actively replicating cells. During S phase of the cell cycle, four types of deoxyribonucleic acid moieties are incorporated with high fidelity into the newly synthesized DNA under the supervision of a proof-reading system. DNA replicates in a semi-conservative manner. After replication, each DNA chromosome is composed of an old strand and a newly synthesized strand [91]. Radiolabelled thymi-
dine and analogues of thymidine are readily incorporated into newly synthesized DNA and can be used for DNA synthesis analysis in cell proliferation assays [92].

**Tritium-labelled thymidine**: 3H thymidine can be detected with either radiography or scintillation and has been widely used in the measurement of cell proliferation. Thymidine incorporated into the newly synthesized DNA strand comes from both de novo synthesis and uptake mediated by the concentrative and equilibrative nucleoside transporters (ENT). In actively proliferating cells, inhibition of equilibrative nucleoside transporters (ENT) abolished 3H thymidine uptake, and the transporter level increased on the plasma membrane in actively proliferating cells of the human lung carcinoma cell line A549 implying that thymidine transport is rate-limiting factor for 3H thymidine incorporation analysis [93]. This could diminish the effect of stimulatory agents in experiments that used the scintillation method. Alternatively, the salvage pathways for TTP synthesis, which can be stimulated with β-adrenoceptor activation would also lead to a diminished effect of 3H thymidine incorporation [9].

**BrdU labelling**: A non-radioactive method was developed with the use of 5-Bromodeoxyuridine (BrdU), an analogue of thymidine, which can be detected with antibodies. The use of a histochemical method for the detection of BrdU facilitated the concurrent detection of other cell markers, which allow for the detection of cell lineage analysis etc., together with cell proliferation. A limitation with this method is that in order to detect the BrdU signal with antibody, cells have to be permeabilized and denaturation of DNA is required, which will more or less destroy cell structure and add variability to experimental results [92].

**EdU and “Click Chemistry”**: 5-Ethynyl-2’-deoxyuridine is a thymidine analogue in which a terminal alkyne group replaces the methyl group in the 5’ position of the pyrimidine ring, which is readily incorporated into cellular DNA during DNA replication. The terminal alkyne group is then detected through its reaction with fluorescent azides, in a cycloaddition (“click” chemistry) reaction catalyzed by copper [Cu(I)] [94]. Compared to the BrdU incorporation assay, azide is a small molecule and therefore permeable in live cells, and this gives easy access to incorporated EdU without any denaturation process, although the toxicity of Cu and the click reaction process in itself limits any further use of the stained cells.

**Total DNA measurement with double-stranded DNA staining**

A wide range of methods are available for total DNA measurement, including A260 absorption measurement, DNA intercalating fluorophores, digital polymerase chain reaction etc. Double-stranded DNA has a UV absorbance peak at 260 nm, and, based on this, DNA concentration can be estimated by the Beer-Lambert’s law (A260 = εcl, ε=50 for double-stranded DNA; c, the concentration of DNA; l, the pathlength of the cuvette). Howev-
er, contaminations from protein, RNA and phenol during the process of isolation of DNA can all interfere with the measurement, due to spectrum overlap, and therefore DNA concentration can be overestimated with this method. Digital polymerase chain reaction can give an approximate copy number of DNA, but is cumbersome and time-consuming [95]. When no strict DNA copy number is requested, DNA intercalating fluorochrome can provide a sensitive and relatively simple method for total DNA estimation. We have used Hoechst 33258 for total DNA measurement in our samples. Hoechst belongs to the bis-benzimide family of compounds, which bind preferably to the AT-pair in the minor groove of double-stranded DNA. Upon DNA binding, the excitation and emission spectra shift dramatically which facilitates the measurement [96].

In our studies, we have used Hoechst 33258 for total DNA measurement, $^3$H Thymidine incorporation with scintillation quantification and flow cytometry with PI staining.

4.2 cAMP inhibits cell proliferation in fibroblasts and smooth muscle cells

cAMP has been shown to have anti-proliferative effects in a variety of cell types, including smooth muscle cells, human hepatocellular carcinoma cells, neuron gliomas and T lymphocytes [97]. Depending on the cellular context, cAMP either inhibits the mitogenic signalling events, initiates apoptosis or advances cell differentiation, through interaction of cAMP with several well established downstream effectors.

4.2.1 Both PKA and Epac are implicated in the cAMP inhibitory effect

In the physiological state, cAMP signalling plays crucial roles in maintaining a proliferatively quiescent state in multiple smooth muscle cells in the presence of mitogenic factors [98]. Initially, PKA was commonly accepted as the mediator of the cAMP inhibitory effect on cell proliferation before the discovery of Epac [99]. The involvement of Epac was recently revealed with the application of PKA- and Epac-selective cAMP analogues. In primary cultured vascular smooth muscle cells, with the usage of cAMP analogues selective for PKA and Epac, both downstream effectors were shown to be necessary for cAMP inhibition of cell proliferation. Rap, which is the commonly accepted target of Epac, is not involved in this process, despite the detected activation by Epac. Further study showed that MAPK, including both ERK and JNK, are inhibited by PKA and Epac, suggesting the synergistic inhibition of MAPK by both PKA and Epac [100]. Epac involvement was also shown in inhibition of human airway smooth muscle cell
proliferation induced by β2-adrenergic receptor stimulation, which is widely used for the treatment of asthma [101].

4.2.2 cAMP inhibition of the MAPK pathway

Extracellular signal activation of ERK1/2 plays crucial roles in cell proliferation. Apart from the canonical activation by growth factors such as EGF and PDGF, GPCRs can also regulate ERK1/2 phosphorylation. Intense studies have been carried out to study the role of ERK1/2 phosphorylation in GPCR- and cAMP-mediated regulation of cell proliferation.

MAPKs are activated through various extracellular signals through different mechanisms, including growth factors and cell adhesion receptors. Canonically, growth factors bind to receptor tyrosine kinases (RTK) on the cell membrane leading to RTK auto-phosphorylation and recruitment of the GTP exchange factor Sos to the plasma membrane to catalyse the switching of GDP to GTP for Ras. GTP loading of Ras leads to a conformational change, and allows for Raf kinase binding [102]. Raf kinase regulation in vivo is an intricate, multistep process. GTP binding of Ras in the active state is necessary but not sufficient for Raf activation; phosphorylation by other kinases also contributes to Raf kinase activity. Raf kinases have restricted downstream effectors: MEK1/2, which then phosphorylate and activate ERK1/2 [103, 104].

4.2.2.1 PKA inhibits MAPK pathway by direct phosphorylation of Raf-1

Raf phosphorylation by protein kinase A applies either inhibitory or stimulatory effects depending on the action site. PKA inhibition of Raf-1 is reported in a couple of cell lines, where cAMP inhibits growth factor-activated ERK phosphorylation. The Raf kinase family comprises 3 members: Raf-1 (C-Raf), B-Raf and A-Raf. Growth factors activate Raf-1 through the phosphorylation of serine 338, tyrosine 341 etc. Protein kinase A phosphorylates Raf-1 on 3 sites in vitro: Ser 42, Ser 259 and Ser 621, inducing an inhibitory effect on Raf kinase activity. Although all three sites are phosphorylated by cAMP stimulation, only mutation of S259 led to a higher kinase activity in response to EGF+TPA stimulation and to resistance to cAMP inhibition of ERK kinase activity, which highlighted the possibility that Ser 259 is the main effector site for PKA/cAMP inhibition of C-Raf and thus inhibition of the MAPK signalling pathway as well [105].
4.2.2.2 cAMP inhibits Raf kinase activity through activation of Rap1

Rap1 is a member of the Ras family of small G proteins. The Rap1 protein shares roughly 50% amino acid homology with Ras, including the Rap effector binding domain. Rap1 is able to activate Ras-GDS, PI3K and Raf-1 in vitro, all of which are also Ras immediate downstream effectors, although most of the effector proteins differ for Ras and Rap [106].

cAMP inhibition of MAPK through the small G protein Rap1 is suggested as an alternative mechanism for the PKA inhibitory effect. In NIH3T3 cells, endogenous Raf-1 binding to HisRas was interrupted by cAMP stimulation in a PKA-dependent manner, demonstrating that cAMP induced an inhibitory effect in the process of Ras and Raf binding. Upon overexpression of Rap-GAP (GTPase activating protein), which inhibited native Rap1, Raf-1 binding to HisRas was recovered, even in the presence of isoproterenol. Therefore, it is suggested that native Rap1 can mediate the cAMP-mediated inhibition of Ras and MAPK interaction, with the consequence of lack of activation of the downstream effectors of Ras [107]. In addition, constitutively active Rap blocked Ras-dependent activation of ERK2 in Rat-1 fibroblasts [108]. It has been proposed that Rap binding, without activation of Raf-1, leads to an antagonistic effect to Ras, and to ERK inhibition, while Rap1 binding to B-Raf can stimulate ERK activation to increase cell proliferation or differentiation [109]. However, a direct binding of Rap1 and Raf1 could not be obtained, bringing into question the mechanism of the antagonising effect of Rap1 against Ras through a direct binding effect on Raf-1 [106].

Although Rap1 interference with Ras-Raf-1 binding has been shown repeatedly, how cAMP activates Rap1 is not known. Different mechanisms have been proposed, including PKA phosphorylation in fibroblasts of C3G, another GEF for Rap1, and PKA phosphorylation of Src kinase in NIH3T3
cells [110], PKA direct phosphorylation of Rap1 [111] and Epac activation of Rap1.

Contradictory effects on cAMP activation of ERK1/2 and its involvement in the anti-mitogenic effect of cAMP has been reported in human hepatocellular carcinoma cells. ERK1/2 phosphorylation by fetal bovine serum (FBS) is not inhibited by forskolin or 8-Br-cAMP. However, forskolin on its own can even activate ERK1/2 phosphorylation, even though it shows an inhibitory effect on FBS-induced cell growth and cell cycle progression, indicating that ERK1/2 activation is not always an indicator of an inhibitory effect of cAMP on cell proliferation [112, 113]. In this case, an inhibitory effect of cAMP/PKA on Akt (Ser473) phosphorylation, which is activated by mitogenic factors such as FBS, insulin or EGF, was suggested as the mediator of the cAMP anti-mitogenic effect [113].

Furthermore, cAMP anti-proliferation activity is also manifested by delay of cell cycle progression, downregulation of cell cycle proteins, including cyclin D1, cyclin D3, cyclin A, dephosphorylation of Rb protein and upregulation of p27kip1 [114, 115], implying that different signalling pathways are used in different cell types.

4.2.3 cAMP inhibition of anchorage-dependent signal transduction

cAMP, more specifically through PKA, is suggested to mediate anchorage-dependent MAPK activation. Soluble growth factors stimulated MAPK activation through RTK/Ras/Raf/MEK pathway more significantly when the cells adhered to extracellular matrix. Under suspension conditions, ERK2 phosphorylation is blocked by serum or by PDGF stimulation, even when normal activation of Ras (GTP/GDP exchange) and Raf occur. This can be reversed by plating the cells back to fibronectin-covered plates, indicating a requirement of adhesion for activation of MAPK. Transfection of constitutively active MEK, rather than Ras or Raf, activated ERK2 phosphorylation in suspended cells, showing that MEK phosphorylation of ERK is the target blocked by cells being in suspension [116]. Since cAMP brings about a cell growth inhibitory effect, as well as ERK inhibition, in fibroblasts and smooth muscle cells, the involvement of PKA was checked in this anchorage-dependent activation of MAPK. Experiments showed that PKA activity increased in a time-dependent manner with cell detachment, and inhibition of PKA induced anchorage-independent activation of the MAPK cascade, supporting the theory that PKA activity is involved in regulating adhesion-related growth factor signalling [117]. This effect is sensitive to cytochalasin D, suggesting a dependence of the PKA inhibitory effect on cytoskeleton rearrangement [118]. Moreover, fibronectin binding to integrin has been shown to inhibit cAMP level as well as PKA activity, through increased RhoA activity, which also supported the possibility that cAMP or PKA inhibition of adhesion-independent cell growth occurred via modifying the cyto-
skeleton in the human gastric cancer cell line SGC-7901 [119], although it is not clear how fibronectin regulated cAMP through its integrin receptors.

4.3 cAMP promotes cell proliferation in endocrine cells

CAMP production following GPCR activation was shown to play important roles not only in the pituitary gland but also in secondary endocrine organs including the adrenal gland, thyroid gland, gonads and ovary (lactotrophs) etc. CAMP produced by a variety of glycoprotein hormones including ACTH, TSH, FSH and LH regulate both cell differentiation and cell proliferation in the target cells of these organs. Apart from endocrine cells, cAMP also brought about cell proliferative effects in macrophages, Schwann cells, Swiss 3T3 fibroblast cells, hepatocytes in vivo etc. The signalling pathways by which cAMP promotes cell proliferation appear to be varied, and no consistent signalling pathway for all has been discovered.

4.3.1 Activation of MAPK in cAMP mitogenic effect

GPCRs, through the Gs, Gi, Gq and G12 families, regulate various MAPK signalling pathways [120]. The effect of Gsα activation on MAPK activation was studied in COS-7 cells transiently transfected with Gsα, and p44 (ERK1) activity was dramatically increased. The same increase was seen with forskolin activation, indicating that Gsα activation can activate ERK1/2 through a cAMP-dependent pathway [121]. In isoproterenol-induced salivary gland enlargement, ERK1/2 and p38 MAPK were both activated in this process [122]. Ligand activation of G protein-coupled receptors leading to ERK1/2 activation is also seen in physiologically relevant systems or in naturally occurring adenomas in a cAMP-dependent or -independent manner [123].

The activation of MAPK by the βγ subunit of the Gi- and Gq-proteins is one of the earlier well established mechanisms. The released βγ complex activates SHC through the tyrosine kinase c-Src [124] or through PI3K [125], recruiting Grb2 and SOS, and leading to Ras activation and the subsequent activation of Raf/MEK/ERK signalling. The cAMP signalling pathway, one of the main downstream signalling events of Gsα activation, has also been shown to interact with MAPK in PC12 cells [126], melanoma cells, and almost every cell type where cAMP activates cell proliferation. In most cell types where cAMP induced a proliferative effect, MAPK has been shown to be activated and functionally important for the mitogenic effect, although the mechanism is not clear.

Thyrocytes are a cell model where cAMP stimulate cell proliferation. Studies have been carried out in different thyrocyte systems, including primary cultured canine thyrocytes and rat thyroid cell lines (FRTL-5, WRT
4.3.2 cAMP activation of ERK through B-Raf

In cells where cAMP promotes cell proliferation, PKA activates B-Raf, which can then phosphorylate and activate ERK. Contrary to the Rap1-mediated cAMP inhibition of ERK and cell proliferation through Raf-1 inhibition, Rap1 activation of B-Raf is proposed as an important mechanism mediating ERK activation and cell proliferation induced by cAMP [126]. Ras is implicated as the mediator of cAMP activation of ERK in melanocytes, in a cell type-specific manner [127].

As noted above, Raf proteins are a family of serine/threonine protein kinases, that includes three members: C-Raf, A-Raf and B-Raf (in the order of their discovery) [128]. Raf kinases are crucial for growth factor activation of MAPKs.

In the rat thyroid cell line FRTL-5, TSH, as well as the adenylyl cyclase activator forskolin, stimulated rapid and transient activation of ERK1/2, which is essential for ³H thymidine incorporation. TSH/forskolin significantly increased the GTP-bound form of Rap1, B-Raf translocation from the cytosol to the plasma membrane and colocalization with Rap1 within the same time frame (3 min) as ERK activation. This effect is PKA-independent, since PKA induced Rap1 phosphorylation at a much later time point (30 min). Knock down of both Rap1 and Ras decreased ERK activation, which had presumably occurred through cAMP activation of Epac. It was therefore proposed that cAMP stimulated MAPK through Rap or Ras activation of B-Raf in a PKA-independent manner in FRTL-5 cells [129].

The importance of Rap1 was further addressed with an in vivo mouse model with inducible expression of a stimulatory (G12V-Rap1b, GTPase deficient) form of Rap1 in the thyroid gland. These mice showed a cAMP-dependent oncogenic phenotype with progression from hyperplasia to nodular formation, demonstrating the importance of stimulation by cAMP for Rap1-mitogenic effects in specific cell types [130].

However, in Wistar rat thyroid cells, where ERK1/2 is also activated by TSH or forskolin activation through B-Raf activation, then ERK1/2 is activated by cAMP in a PKA-dependent manner. cAMP-activated ERK phosphorylation was abolished by Ras knockdown, rather than Rap1 knockdown, suggesting an alternative signalling pathway for ERK activation: cAMP/PKA/Ras/B-Raf [131].

Activation of ERK1/2 through Ras or Rap1 activation of B-Raf was also shown in cyst epithelial cells taken from patients with autosomal dominant
polycystic kidney disease (ADPKD). Compared to normal human kidney cortex (HKC), the cyst epithelial cells responded to cAMP with enhanced cell proliferation, which is not the case in cells from normal HKC. PKA-dependent activation of ERK was predicted to be involved in regulating cell proliferation and to contribute to cyst enlargement in ADPKD [132]. Further studies showed that Rap1 was expressed at a higher level in ADPKD cells than in normal HKC cells. Both Raf-1 and B-Raf were expressed, although only B-Raf activation by cAMP was inhibited by the PKA inhibitor H89. cAMP may thus act through the PKA-Rap1-B-Raf-ERK signalling pathway to promote cell proliferation [133, 134].

In human uveal melanoma cells, cAMP-elevating agents (prostaglandins) were shown to promote cell proliferation together with growth factors [135]. Constitutively active B-Raf is frequently detected in human uveal melanoma cells, and wild-type B-Raf was highly expressed in cell lines without B-Raf mutations. Gene silencing of B-raf rather than Raf-1 with siRNA significantly reduced ERK activation and cell proliferation, revealing the mitogenic effect of B-Raf. Protein kinase A was shown to be responsible for the activation of B-Raf. Cyclin D1 silencing inhibited cell proliferation in this cell type. Both PKA inhibition and B-Raf silencing greatly reduced cyclin D1 activity, suggesting the cAMP/PKA/B-Raf/cyclin D signalling pathway and cAMP activation of ERK to promote cell cycle progression [136].

However, in primary cultured dog thyrocytes, cAMP did not stimulate ERK1/2 phosphorylation. The cAMP mitogenic effect was demonstrated with a transgenic mouse model, Tg-A2aR (wild-type A2a-adenosine receptor under the control of the bovine thyroglobulin gene promoter), which developed a large goiter as well as strong stimulation of thyroid cell function, confirming the role of this receptor in vivo in regulating cell proliferation [137]. Insulin is required for the cAMP-mediated mitogenic effect in this cell type [138, 139], which has been shown to be a permissive effect for cAMP-stimulated cell proliferation. Moreover, insulin receptor expression, rather than IGF-1 receptor expression, is dependent on TSH or cAMP, which can compensate insulin dependence after long-term stimulation (16 h) [140]. In this more physiologically relevant system, TSH or forskolin per se do not activate ERK1/2 or any other member of the MAPK family, nor are there any additive effects on insulin-induced ERK activation. Nevertheless, inhibition of ERK1/2 with PD98059, a widely used MEK inhibitor, decreased TSH- or forskolin-induced ³H thymidine incorporation in a concentration-dependent manner, implying involvement of ERK1/2 in insulin/IGF cAMP stimulation of cell proliferation [141].
4.3.3 Epac activation of Rap1 as a mechanism for MAPK activation

In human umbilical vein endothelial cells, cAMP produced from the A2B adenosine receptor has been shown to be the signalling event responsible for ERK1/2 phosphorylation. ERK1/2 activation also occurs with an Epac-selective cAMP analogue, and both H89 and PKI failed to inhibit ERK1/2 phosphorylation, indicating that Epac, rather than PKA, mediated MAPK activation. At the same time, Rap1 and B-Raf are activated by cAMP stimulation. Therefore, it is proposed that cAMP activation of Epac led to ERK1/2 activation as a consequence of Rap1/B-Raf activation [142]. Rap1 activation by Epac1, which then activates B-Raf and MAPK signalling pathways, has also been proposed in several prostate cancer cell lines, where cAMP stimulates ERK1/2 activation and cell proliferation [143]. However, this mechanism may not be universal for several reasons. Firstly, more than one GEF, C3G (Crk SH3 domain Guanine nucleotide exchanger) has been found for Rap1, which can be activated indirectly by cAMP through PKA activation. The ability of Epac to activate ERK through Rap1 is dependent on the localization of Epac, i.e., localization to the plasma membrane promotes its ability for MAPK activation. Otherwise, Rap1 can be activated through C3G which is close to plasma membrane and promotes Rap1 activation of MAPK [144]. Secondly, Epac modulates multi-faceted effectors, including the atypical Ras, protein kinase B/Akt, phospholipase D, ion channels, regulators of the actin-microtubule networks etc., [145]. In B16 mouse melanoma cells, Ras activation, which was responsible for the cAMP activation of B-Raf/MEK/ERK1/2, was found to be independent of Sos, PKA and Epac, indicating the existence of a cell-type specific Ras activated by cAMP [127].

**Conclusion:** MAPK is an important interaction site for cAMP in its actions on cell proliferation, and it is usually activated by cAMP under circumstances where cAMP stimulates cell proliferation, and vice versa. Although there are no common signalling pathways for all cell types, there are still a few sites frequently involved in cAMP regulation of MAPK. Rap1 is activated by cAMP, but depending on the cell types, Rap1 acts on either Raf-1 to inhibit MAPK or on B-Raf to activate MAPK. Whether cAMP activation of Rap1 contribute to its opposing effect on MAPK in different cell types is still debatable.
4.3.4 cAMP and PI3K/Akt signalling interaction

Several glycoprotein hormones, ACTH, FSH, LH and TSH, which initiate cAMP signalling events through their GPCRs on the cell membrane, also activate the PI3K/PKB pathway.

**PI3K signalling pathways:**

PI3K/PKB (Akt) is a crucial pathway controlling both cell proliferation and cell apoptosis. PI3K, which is composed of a catalytic subunit p110 and a regulatory subunit p85, catalyses the phosphorylation of phospholipids at their 3'-position on the plasma membrane, specifically the conversion of PI(4,5)P2 (phosphatidylinositol 4,5-bisphosphate) into PI(3,4,5)P3 (phosphatidylinositol 3,4,5-trisphosphate). Upon receptor tyrosine kinases activation, activated PI3K catalyses the production of PIP3, which recruits PIP3-phospholipid binding proteins to the plasma membrane through binding to their PH domains [146]. Akt and PDK1 are two effector proteins recruited by PIP3 to the plasma membrane, where PDK1 phosphorylates Akt at Threonine308. It has been disputed if phosphorylation at Serine 473 of Akt is necessary for this activation, which is shown to be regulated by PDK1, ILK (integrin-linked kinase) and a series of other kinases. Recently mammalian target of rapamycin complex 2 (mTORC2) has been shown to activate Akt phosphorylation at Ser 473, although it is disputable whether it is essential for Akt kinase activity or function [147]. Activated Akt translocates to the nucleus, where it phosphorylates and activates a series of substrates, including forkhead transcription factors and components of cell apoptosis signalling such as Bad [148]. It may also influence GLUT1,3,4 glucose transporter localisation.
A genetically modified mouse model with a selective deletion of PTEN (phosphatase) in the thyroid follicular cells confirmed the mitogenic effect of constitutive activation of PI3K/Akt pathways [149]. PTEN is the negative regulator of the PI3K/Akt pathway. A PTEN heterozygous knockout caused Cowden disease, for which one of the symptoms is multi-nodular goitre, and increased risk of developing thyroid follicular cancer.

cAMP has been reported to regulate the PI3K/Akt signalling pathway with different read-outs depending on the downstream effectors abundance and distribution [150]. In Wistar rat thyrocytes, where cAMP stimulated membrane ruffling and PI3K activation independently of PKA-signalling pathways, Akt was activated and phosphorylated by both TSH and 8Br-cAMP stimulation. This was inhibited with LY294002 pre-treatment, suggesting that cAMP regulates PI3K/Akt in WRT cells, and this is also shown to be needed for cAMP-stimulated cell proliferation [151]. As a more recently characterised cAMP intracellular effector, Epac has been implicated in cAMP activation of Akt in several cases.

In primary cultures of thyrocytes, where PKA mediated the mitogenic effect of cell proliferation [152], Akt activity is required for cAMP-stimulated cell proliferation. TSH or forskolin (in the presence of 10 μg/ml insulin) stimulated cell proliferation, and this was inhibited by the Akt inhibitor wortmannin, implying PI3K/Akt activation by insulin as the complimentary signalling required for cAMP to stimulate DNA synthesis in canine thyrocytes [153], although TSH and forskolin themselves do not activate Akt.

In macrophages, cAMP signalling, one of the signalling pathways initiated by macroglobulin, has been shown to increase cell proliferation, in which both PKA and Epac are involved. Forskolin-stimulated cell proliferation was inhibited by a PKA inhibitor. However, Rap1, PI3K and Akt were activated by cAMP independently of PKA, co-immunoprecipitated with each other and with Epac, indicating the involvement of Epac1/Rap1 in Akt activation and function by locating it to the site of action on the cell membrane. Akt may function to inhibit cell apoptosis [154]. The association between Epac1 and Akt1, with Epac-selective cAMP analogue stimulation, was later confirmed by GST pull-down assays. CPT-2-O-Me-cAMP (specific for Epac) rather than 6′Bnz-cAMP (specific for PKA) activated Akt1 at Thr 308 and Ser 473 in association with Epac and activation in both plasma membrane and nuclear fractions, demonstrating that cAMP-binding to Epac initiated the Akt1 signalling pathway. Another candidate, TCL1 (T cell leukemia1) was also suggested for Akt activation. Application of Epac1 RNAi attenuated Akt activation and DNA synthesis, implicating Epac1/Rap1 activation in the Akt signalling pathway in cAMP regulation of macrophage proliferation [155].
PI3K/Akt signalling pathway has been demonstrated to be essential for cAMP-stimulated cell proliferation in a series of other cell types, including lactotrophs from Wistar rats [156], and for the glucagon-stimulated (mediated by cAMP) anti-apoptosis effect against bile acid in hepatocytes. In the latter, Epac-activated Rap1 mediated the cAMP pro-survival effect through activation of PI3K/Akt signalling events, although the mechanism involved is not known [157].

4.3.5 cAMP activation of mTOR complexes and cell proliferation

Mammalian target of rapamycin is a conserved Ser/Thr kinase, belonging to the phosphatidylinositol kinase-related kinase (PIKK) family. Together with a series of other proteins, two complexes are formed with mTOR, namely mTOR complex1 and mTOR complex2, to regulate a diverse range of cellular processes, including protein synthesis, ribosome biogenesis, cell cycle, cell growth, gene transcription, autophagy and metabolism. mTOR complex I and II differ in their composition and sensitivity to rapamycin [158]. mTOR complex1 includes scaffolding protein RAPTOR (regulatory-associated protein of mTOR), mLST8 (mammalian lethal with Sec 13 protein 8) and Deptor (DEP-domain-containing mTOR-interacting protein), and recently PRAS40 has been identified as a novel member of this complex [159]. Multiple extracellular signals are integrated at mTORC1 to regulate cell behaviour, including nutrient changes, especially essential amino acids leucine and arginine, hormones and growth factors, for instance insulin, and energy status mediated by AMPK, etc.

![Diagram of mTOR complexes](image)

**Figure 5.** Composition and regulation of mTORC1 and mTORC2. From Wang & Proud, Journal of Molecular Cell Biology (2011), 3, 206–220. [159]

Despite extensive research, the regulatory mechanism governing the mTOR complexes are still not clear. A small GTPase Rheb (Ras homolog
enriched in brain), has been regarded as a main signalling regulator upstream of mTORC1 in a GTP-independent manner. Tuberous sclerosis complex ½ (TSC1/2) acts as a negative regulator for Rheb to inhibit mTORC1 activity, which is involved in hormone and growth factor activation of mTORC1 [160]. P70S6K1 and 4EBP1 are two well known effectors of mTORC1. mTORC1 phosphorylates p70S6K1 at Thr389, which regulates cell function at both the transcriptional and translational levels through multiple effector proteins: ribosomal protein S6, transcription factors, RNA helicas and translation initiation and elongation factors. Another downstream effector of mTORC1, 4EBP1, binds in a hypophosphorylated state to the eIF-4E mRNA cap-binding complex which prevents its assembly with the eIF-4F cap-binding complex and interferes with the translation initiation of target proteins. Through phosphorylation by mTORC1, 4EBP1 gets inactivated, releases eIF-4E mRNA binding protein which can then assemble with eIF-4F mRNA cap-binding protein and initiate cap-dependent mRNA translation. This mechanism only leads to the active translation of a selective group of proteins, which are reported to be under mTORC1 control through 4EBP1: mRNAs with cap-5′ untranslated regions (UTRs) that require unwinding by the eIF4F complex and/or lack an internal ribosome entry site [161]; 5′ TOP mRNAs, which have relatively short 5′ UTRs that contain stretches of pyrimidines, and encode for translational machinery proteins, are another class of such mRNAs [162].

The defining factor for mTOR complex 2 is Rictor which is mutually exclusive to Raptor in mTOR binding ability, the other components of the complex being mLST8 and Deptor, which are shared with mTORC1, mammalian stress-activated protein kinase interacting protein (mSIN1) and protein observed with Rictor-1 (Protor-1, also known as PRR5), the unique components of mTORC2. Compared to mTORC1, little is known about mTORC2, about its upstream regulators and the downstream effectors, except for Akt phosphorylation at Ser 473 and GSK regulation by mTORC2 [161].

Growth factors in the circulation reflect the metabolic status of an organism, which can be sensed by the mTOR complexes, and they regulate cell behaviour through mTOR. Different growth factors regulate mTORC1 activation through different mechanisms. Insulin or IGF1 play important roles in mTORC1 regulation. Upon ligand binding to the cognate receptor, PIP3 increases and this recruits PDK1 and Akt to the plasma membrane, followed by Akt phosphorylation by both PDK1 and mTORC2. Activated Akt translocates to the cytosol and phosphorylates TSC2 [163]. Phosphorylation of TSC2 in an unknown mechanism leads to its inactivation and loss of the GTPase activating protein activity towards Rheb. This will lead to enrichment of GTP-bound Rheb, resulting in an increased mTORC1 activity [161]. However, apart from Akt, TSC2 can be phosphorylated at multiple sites by
other kinases, for instance ERK1/2 phosphorylation and inactivation of mTORC2. The phosphorylation of TSC2 by multiple protein kinases provides a signalling crosstalk possibility from multiple signalling pathways [164].

**cAMP and mTOR complex activation:**

The mTORC1 signalling pathway has been shown to be regulated by the intracellular second messenger cAMP in a cell-type dependent manner. mTORC1 is activated in cells where cAMP stimulates cell proliferation or DNA synthesis, and is inhibited by cAMP where cAMP inhibits cell proliferation. cAMP stimulates S6 protein phosphorylation, which can be inhibited with rapamycin in Swiss 3T3 cells, Schwann cells, WRT cells, whereas no S6 protein phosphorylation is seen in NIH3T3 cells, showing a correlation between the cAMP effect on cell proliferation and mTORC1 activation [165].

In thyroid cell models, the mechanisms of cAMP activation of cell proliferation differ. In the primary cultured dog thyrocyte, which is most relevant to the physiological system, neither MAPK nor PI3K/Akt were activated [141, 153], whereas in FRTL-5 and WRT cells, MAPK was activated in a Rap1-dependent manner which was crucial for cAMP stimulated cell proliferation [129, 131]. The importance of Akt activation for thyrocyte proliferation has been demonstrated with PI3K inhibitors and in PTEN knockout mice, which develop thyroid neoplasm [166]. The only commonly activated signalling molecule is p70S6K1, implicating mTORC1 as the converging point for these different signalling pathways. An in vivo study showed that the mTOR inhibitor RAD1 inhibited the hyperplastic effect, rather than the hypertrophic effect of TSH in goitrogen-treated mice, indicating the crucial role of mTOR in cAMP stimulation of cell proliferation [167]. In PCCL3 cells, forskolin and TSH were shown to induce phosphorylation of p70S6K1 and 4EBP1 at several sites which can be prevented by rapamycin pretreatment, suggesting the activation of mTORC1. In this specific thyrocyte cell line, mTOR inhibition with rapamycin inhibited DNA synthesis, together with cyclinD3-CDK4 association, implying mediation of the cell cycle promoting effect of cAMP by mTORC1. For mTOR activation by cAMP, a PKA direct phosphorylation of PRAS40 rather than TSC2 phosphorylation was suggested as the upstream mediator of mTORC1 activation [168].

In primary cultured theca-interstitial cells, gonadotropin, which stimulated cell proliferation and cell cycle progression, also stimulated mTOR complexes, and gave protein phosphorylation on p70S6K1 at Thr389, Akt phosphorylation at both Ser473 and Thr308 and also ERK1/2 phosphorylation, together with upregulation of D type cyclins, which are required for G1-S phase progression. Both TSC2 and downstream effectors of mTOR complexes were inhibited by Akt inhibitors, and attenuated by adenylyl cyclase.
inhibition, whereas they were insensitive to inhibition with a MEK1/2 inhibitor, demonstrating a signalling pathway activating mTOR through PI3K/Akt activation, which is activated by cAMP [169].

mTOR activation during cAMP-stimulated cell proliferation is also reported in colon cancer cell lines [143], Ins-1 rat insulinoma line [170], and Sertoli cells [171] etc. In brown adipocyte, mTOR complexes have been shown to be regulated in an AMPK-dependent manner, and to be essential for cell differentiation at an early stage, which is implicated in brown adipogenesis within white adipose tissue [172]. In our study with primary cultured brown pre-adipocytes, we have shown that both p70S6K1 (Thr389) and Akt (Ser473) are phosphorylated by cAMP stimulation, indicating cAMP activation of mTORC1 and mTORC2 by cAMP.

4.3.6 In cell lines, GPCRs activate MAPKs through EGFR transactivation

Signal cross-talk is a common phenomenon for cells to carry out complicated cell functions. Recent years have seen a new mechanism for signal crosstalk between GPCRs and Receptor Tyrosine Kinases, especially through the EGF receptor, and in some cases the PDGF receptor. Receptor transactivation refers to the process of MAPK phosphorylation following stimulation by a GPCR agonist through the activation of EGFR (or PDGFR). Transactivation always takes place in a time frame of a few minutes. In this process, EGFR is activated by the release of an active RTK agonist after ectodomain shedding, which is a requirement for the switching of the agonist from a transmembrane precursor to an active agonist. In a variety of cell lines, GPCR ligands ANGII, endothelin-1, thrombin, ATP and LPA have been reported to transactivate EGFR [173].

In 1996, Daub et al., found that in Rat-1 cells, after stimulation with endothelin-1, or lysophosphatidic acid (LPA) or thrombin treatment, cell lysates immunoprecipitated with tyrosine-phosphorylated αEGFR, which could be blocked by a dominant negative form of the EGFR mutant (HERCD533) and by EGFR inhibitor AG1478, a selective tyrphostin. Further, GST-Grb-2 fusion pull-down assay showed that the GPCR ligands catalysed the association of Grb-2 protein with several tyrosine phosphorylated proteins, with molecular weights corresponding to EGFR and SHC. Complex formation was absent in HERCD533 mutated cells, as well as with AG1478 treatment, suggesting the activation of GPCRs phosphorylates EGFR at tyrosine, which then recruited Grb-2 and SHC to the plasma membrane [174].

Although the cell signalling pathway in GPCR activation leading to receptor tyrosine kinase signalling pathways is poorly defined, a group of met-
alloproteinases have been reported as crucial mediators during this transactivation process. It is proposed that all the EGFR ligands are expressed and displayed on the cell surface in the form of inactive transmembrane precursors, that require protein shedding to become functioning ligands, which can bind to certain EGFRs on the plasma membrane, activating the receptor tyrosine kinase activity and initiating intracellular signal transduction events [175]. These metalloproteinases were suggested as candidates responsible for the cleavage of the ectodomain of the precursors, including the matrix metalloproteinase (MMP) family [176, 177]. With the metalloproteinase inhibitor BB94, and siRNA of different ADAMs (a disintegrin and metalloproteinase), it was shown in ACHN cells (a kidney cancer cell line) and TccSup cells (a bladder cancer cell line), that ADAM 15 and ADAM 17 were activated and mediate the transactivation of EGFR tyrosine phosphorylation, following activation of different GPCR agonists, suggesting the possible involvement of metalloproteinases in EGFR transactivation [178].

**MAPK activation in an RTK independent manner by GPCR in primary cultured brown adipocytes**

In primary cultured brown adipocytes, we have shown that different adrenergic receptor ligands, as well as LPA, can activate MAPK in a short time-frame of 5 min (paper I and II). However, when the cells were preincubated with 1 µM of the EGF receptor inhibitor AG1478 for 1 h, ERK1/2 activation brought about by LPA, cirazoline, the α1-adrenoceptor agonist, clonidine, the α2-adrenoceptor agonist, CL316243, the β1-adrenoceptor agonist, was not inhibited. This especially holds true for norepinephrine, the physiological stimulant of brown adipose tissue, which stimulated ERK activation in both mature brown adipocytes and brown pre-adipocytes, suggesting that at least in this particular cell type, receptor transactivation is not employed as the main mechanism for MAPK activation, which is crucial for the functionality of the cell and tissue. Adrenergically activated ERK1/2 activation is also insensitive to Pertussis toxin, and therefore excluding the involvement of the βγ subunit from inhibitory G protein (Gi). Instead, a pharmacological study showed that β3-AR activated MAPK in a cAMP-, PKA- and Src-dependent manner [179].

4.4 cAMP regulates cell proliferation at the transcriptional level

One major cellular effect of cAMP is regulation of the transcription of a group of genes through the ubiquitously expressed transcription factor CREB (cyclic AMP response element binding protein). CREB is an important downstream effector of PKA. Upon ligand binding to G-Protein Coupled Receptors, Gsα is activated, leading to increased intracellular cAMP concentrations. cAMP binding to the regulatory subunit of protein kinase A, releases the activated catalytic subunit (C), some of which translo-
cates from the cytosol to the nucleus to activate CREB through phosphorylation at serine 133.

CREB is a ubiquitously expressed 43 kDa transcription factor containing a basic leucine zipper region, which is responsible for binding to double-stranded DNA. cAMP response element modulator (CREM), activating transcription factor 1 (ATF-1), and inducible cAMP early repressor belong to the same transcription factor family [180]. These transcription factors are encoded separately by 3 separate genes, although they share similar conservative functional domains: the glutamate-rich domain (Q-domain), the kinase-induced domain (KID), responsible for activation by kinases, and the functioning basic leucine zipper domain (bZIP). Each gene has several splice variants. CREB binds constitutively to the conserved cAMP response element, a palindromic sequence (TGACGTCA) in the promoter region of its target genes, and function as a transcription factor only after activation by upstream kinases. Once phosphorylated by protein kinase A, activated CREB can bind with the other members of this family (ATF and CREM) as a homo- or heterodimer to the cAMP response element of the target gene and modulate its gene expression. Phosphorylation of CREB at serine 133 by upstream kinases also permits the recruitment of coactivators, e.g. CREB-binding protein (CBP), and facilitates binding of other components of the transcription machinery [181]. CREB is phosphorylated at serine 133 by a series of other kinases, including Akt, ERK1/2, p38 MAPK, PKC, p90RSK and calcium/calmodulin-dependent kinases [182].

cAMP stimulation leads to phosphorylation of the majority of CREB attached to CREs near the promoter of the target genes, although transcriptional activation requires other regulatory partners. It is normally believed that CREB phosphorylation is not enough for transcriptional activation, rather it may function to recruit coactivators, for instance CEBP-binding protein (CBP), and facilitates binding of other components of the transcription machinery [181]. CREB is phosphorylated at serine 133 by a series of other kinases, including Akt, ERK1/2, p38 MAPK, PKC, p90RSK and calcium/calmodulin-dependent kinases [182].

CREB has been found to be overexpressed or constitutively activated in different types of human cancers. Abnormal activation of CREB by cAMP/PKA is involved in different forms of tumourigenesis, especially in endocrine tissues [181]. CREB mRNA levels were elevated in GH-secreting adenomas [185], and Bertherat et al., found consistently elevated levels of Ser133-phosphorylated CREB in a series of GH-secreting adenomas [186]. There are a group of genes with CREs in their promotors which are upregulated in cells expressing gsp mutations, including the oncogene c-fos, Pit-1
and also CREB and ICER themselves, suggesting the possibility that gsp regulates cell proliferation through activation of some oncogene expression at the transcriptional level. Transfection studies showed that in PC12 cells, transfection with the gsp oncogene dramatically activated CRE-reporter genes and the immediate early gene c-fos, which has a CRE domain in its promoter region. This was inhibited by transfection of an inactivating PKA mutation, implying that one of the mitogenic effects of the gsp oncogenic effect was carried out by transcriptional upregulation of cell cycle protein expression [187].

In pituitary lactotrophs, where increased cAMP has been shown to stimulate cell proliferation in a PKA-dependent manner, forskolin was shown to activate cell proliferation and CREB activity, which was demonstrated with a CRE-controlled reporter gene. Both cell proliferation and CREB activity are suppressed by dopaminergic agonists. Furthermore, MCREB, a mutated CREB at Ser133 which functions as a CREB inhibitor, blocked forskolin-induced proliferation, suggesting that cAMP stimulation of CREB accounted for at least one aspect of its mitogenic effect, possibly by elevating cell proliferation-related genes [188]. cAMP was also shown to be essential for the LH-mediated anti-apoptotic effect seen in ovary surface epithelial cells [189]. The expression level of CREB increased dramatically in human ovarian adenocarcinoma, which is crucial for maintaining the cell proliferative state of SKOV-3 ovarian adenocarcinoma cell lines [190].

Different isoforms of CREM are related to cell growth with isoform- and cell type-dependent effects. The expression pattern of CREM is under the modulation of cAMP. The H295R cell line is derived from an invasive adrenal cortical carcinoma, in which chronic ACTH (adrenocorticotropic hormone) application activates the cAMP signalling pathway, leading to adrenal cortex hyperplasia. CREB expression differs between the normal adrenocortical cells and the H295 cell line. Western blot and RT-PCR studies demonstrate that CREB is not expressed in the H295R cancer cell line, whereas it is expressed in normal adrenal cells. Other members of this transcription factor family, Activating transcription factor-1 (ATF-1) and several forms of CRE modulator (CREM) are expressed, and cAMP transcriptional activity is preserved, implicating a compensatory effect for the loss of CREB on the transcriptional activity. An activator isoform, CREMτ was high in H295R cells compared to normal adrenal cortex. The loss of CREB and compensation by overexpressed CREM could be linked to cellular transformation [191].

Induced cAMP early repressor (ICER) is another member of this leucine zipper transcription family. ICER derives from an alternative internal promoter in the CREM gene, which is expressed as a direct effect of cAMP signalling. In cell types where cAMP inhibits cell proliferation, ICER func-
tions as a tumour-suppressor, through either CRE binding, competitively antagonizing CREB transcripational activity [192] or forming a complex with other transcripational modification effectors [193]. In either case, the transcripational activity decreased when ICER was present. ICER expression is induced with increased cAMP transcripational activity, which is assumed to play a negative feed-back role on the increased transcripational activity of CREB family transcripational factors, playing a crucial role in controlling the transient nature of cAMP transcripational activity [180]. Expression levels of ICER and CREB were compared at the mRNA level between gsp oncogene mutation-containing- (gsp+) and Gsα wildtype (gsp-) cells from growth hormone-secreting pituitary adenomas expression. The expression levels of both transcripational factors were significantly increased with gsp+, which would lead to constitutive cAMP signalling activation and could explain the phenotype [185].

ICER expression have been detected in brown adipose tissue from cold-exposed animals, as well as in primary cultured brown adipocytes. Several isoforms of ICER were induced by NE stimulation in primary culture of both preadipocytes and mature brown adipocytes in a time-dependent manner [194].

Cell proliferation realted genes upregulated through CREB in a cAMP-dependent manner

CREB, as a conserved and universal transcripational factor, is activated in multiple signalling pathways, integrating signals from a variety of stimuli. cAMP-activated PKA is a main activator of CREB phosphorylation; this signalling pathways was also revealed to activate cell proliferation through upregulation of oncogenic proteins. Indeed, a Genome-wide study showed that CREB has 4000 promoter binding sites in the human genome, with a similar occupation pattern in most tissues. Stimulation with cAMP increased the phosphorylation in most of the promoter-bound CREB, although only a small proportion were activated, partly dependent on whether CBP was recruited and on DNA methylation content [183].

Glucagon-like peptide has been found to increase cell proliferation or inhibit cell apoptosis in pancreatic ductal and β cells that express GLP1R. This has been tested with a series of cell lines (MIN6 cells, rat ARIP and human PANC-1 cells) [195] as well as primary cultured rat islet cells [196]. Knock-out of CREB in mice induced β cell apoptosis and diabetes, related to the lose of β cells. One gene that is down-regulated in CREB KO mice and in MIN6 cells is insulin receptor substrate 2. IRS2 was induced by the GLP1R agonist Exendin-4 and proposed to support β cell survival through increased PI3K/Akt signalling pathway [197]. Its down-regulation in the CREB knock-out mice indicates a role for cAMP signalling also in this pathway.
cAMP was shown to play an important role in regulating GLUT1 expression and glucose uptake in embryonic stem (ES) cells, which is crucial for ES cell proliferation and survival. cAMP regulates GLUT1 expression by increasing the complex formation of CREB/CBP/TORC2 and the transcriptional activity of this complex, which is necessary for the increased GLUT1 expression. Therefore, CREB regulation of GLUT1 has been proposed as an important mechanism for cAMP/PKA-induced stimulation of mouse embryonic stem cell proliferation [198].

In primary cultured brown adipocytes, most cellular effect induced by NE can be mimicked by forskolin, including transcriptional gene expression. CREB phosphorylation and activation can be upregulated with NE. Stimulation by both β-adrenoceptors, mediated by cAMP, as well as α1-adrenoceptors, mediated by TPA-sensitive protein kinase C, are involved in CREB phosphorylation by NE [199]. c-fos is an early oncogene activated in response to a wide range of mitogens. NE stimulated c-fos expression within 30 min. Forskolin had only a small inductive ability alone. However, both the Ca\(^{2+}\) ionophore A23187 and the α1-adrenoceptor agonist cirazoline potentiated the cAMP effect, implying that cAMP and Ca\(^{2+}\) signalling pathway synergistically activated c-fos transcription in brown adipocytes [200].

Another gene that is upregulated through cAMP activation is ribonucleoside reductase subunit R2 (RNR2). RNR catalyses the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which is the rate-limiting step in the synthesis of all four deoxyribonucleotides. RNR2 expression is upregulated by NE and forskolin in a time-dependent manner, which can be inhibited by H89, PP2 (a Src inhibitor) and PD98059 pre-treatment, indicating that PKA, activated by cAMP, mediated the transcriptional activity, which is further mediated through Src and ERK1/2 kinases [201]. VEGF is another cAMP/PKA-mediated gene that is proven to be upregulated in primary cultured brown adipocytes in response to NE stimulation, and in vivo experiments showed upregulation in response to cold acclimation, implying a role in the angiogenesis process during brown adipose tissue recruitment in response to cold [202, 203].
5. cAMP and brown adipose tissue

5.1 Brown adipose tissue recruitment

Under chronic cold acclimation conditions, the total DNA of brown adipose tissue increases 6-8 fold over a period of 3-4 weeks [204]. A thymidine autoradiograph study showed almost no DNA synthesis in mature brown adipocytes, whereas the so-called ‘reticuloendothelial cells’ of rat brown adipose tissue, lying within the expanding vascular spaces of the tissue, showed the most significant response during the cold acclimation response, starting from 16 h after cold exposure up to 16 days [205]. Later, a study on the effect of sympathetic innervation showed that in the surgically denervated lobe of brown adipose tissue, $^3$H-thymidine incorporation was dramatically diminished compared to the intact lobe, in response to cold exposure, which showed a 6 fold-induction in $^3$H-thymidine incorporation. Moreover, NE administration compensated for surgical denervation, indicating that the major hyperplastic effect is from the sympathetic nervous system, and probably adrenergic activation by NE served as the main mitogenic effector. Interstitial cells, brown preadipocytes and endothelial cells all contributed to the cold exposure- and NE administration-induced DNA synthesis, whereas nothing was from mature brown adipocytes [206, 207].

Brown preadipocyte proliferation is an important aspect of brown adipose tissue recruitment because it increases the number of cells potentially able to differentiate into functional brown adipocytes, thus enlarging the nonshivering thermogenesis capacity. During the recruitment process, not only the number of brown preadipocytes increases, but also the number of microvascular endothelial cells, which predispose for the increased vascularization. Also, a $^3$H-thymidine incorporation tracing study showed that the total $^3$H-thymidine labelling stabilized after 2 days of cold acclimation; however, the $^3$H-labelled cells shifted among the 4 types of cells present in brown adipose tissue. Radioactively labelled cell number decreases with time in interstitial cells and brown preadipocytes, whereas labelling in mature brown adipocytes increased progressively, implying that interstitial cells are the stem cells, which develop into brown preadipocyte, then into mature brown adipocytes [207]. A lineage (E-cadherin) tracing study with C57Bl6 mice suggested that the origin of brown adipocytes was from vascular endothelial cells, suggesting that endothelial cells not only proliferate to coordinate with the increased need for neovascularization, but also contribute to the formation of brown adipocyte precursors [208].

Norepinephrine released from the sympathetic nervous system is the dominating factor for the innervation of brown adipose tissue, regulating cell
differentiation, proliferation etc. NE functions through various adrenergic receptors on the plasma membrane. Brown adipocytes have α1-, α2-, β3- and β1- adrenoceptors expressed and these mediate the physiological function of norepinephrine. A study to characterise the adrenergic receptor subtypes involved in cell proliferation showed that injection of the β-adrenoceptor antagonist propranolol, rather than α-adrenoceptor antagonist phenolamine, abolished ³H-thymidine incorporation induced by norepinephrine treatment in brown adipose tissue, implying that NE-stimulated cell proliferation is dependent on β-adrenoceptors [209].

5.2 Adrenergic and non-adrenergic regulation of brown preadipocyte proliferation

An effect of NE on brown preadipocyte proliferation is also demonstrated in primary culture, although the reported downstream mediators differ. Bronnikov et al. used brown adipose tissue from young mice to delineate this problem. In accordance with the in vivo studies, NE induced a prominent increase in DNA in primary cultured brown preadipocytes but not in mature brown adipocytes. Administration of pafenolol, a selective pharmacological antagonist of β1-adrenoceptors, inhibited the NE effect, implying mediation via β1-adrenoceptors. Furthermore, several cAMP-elevating treatments, including forskolin, cholera toxin and stimulatory cAMP analogues imitated the NE-induced total DNA increase [9].

Moreover, cell apoptosis was dramatically diminished demonstrated as a decrease in DNA fragmentation in cold-exposed mice compared to mice at near thermoneutral temperatures, indicating that increased cell survival may also contribute to the enlargement and recruitment of brown adipose tissue. Chronic NE treatment of primary cultured brown preadipocytes (from day 4-6) dramatically increased cell survival, which was imitated by cirazoline (α1-adrenoceptor agonist), isoprenaline (β-adrenoceptor agonist) and forskolin (cAMP). ERK1/2 is activated by all of them, and ERK1/2 inhibition blocked the anti-apoptosis effect of NE, suggesting the involvement in brown adipocyte survival of MAPK activation by NE through G protein-coupled receptors [210].

However, primary cultures made from 20-day old rat foetuses revealed a different mechanism of NE mitogenic action and phosphorylation of ERK1/2. ERK1/2 phosphorylation was activated by β-, α1- and α2- adrenoceptor activation but not by forskolin. In cell proliferation, ³H-thymidine incorporation was only activated by β-adrenoceptor activation, no effect was seen from cAMP activation, which was confirmed by expression of a cell proliferation marker PCNA [211], implying that NE brought about a mitogenic effect in foetal brown adipocytes in a β-adrenoceptor-dependent manner, but independently of cAMP. The differential cell signalling pathways probably reflect the different cell origin, both the specifies difference and the develop-
mental stage difference of the cultured cells could contribute to the difference; for instance, the composition of G protein-coupled receptors could differ in brown adipocyte precursors before and after birth.

Although its physiological function is under debate, non-adrenergic signalling also stimulates cell proliferation. A series of growth factors have been shown to stimulate cell proliferation in different types of brown adipocytes, including IGF-1 [212], EGF, PDGF, bFGF, aFGF and vasopressin [213]. Growth factors bFGF, EGF and vasopressin synergistically stimulated DNA synthesis together with norepinephrine, implying the need of both NE and growth factors for a full mitogenic effect on brown preadipocyte proliferation. Growth factors EGF and PDGF also significantly increased DNA synthesis of primary cultured brown preadipocytes of mouse origin, although it appears to be a relatively smaller effect, based on ³H-thymidine incorporation data [214]. It was also revealed that EGF and PDGF activated different signalling pathways, although they both ended up activating ERK1/2 phosphorylation as the converging signalling event, which was crucial for cell proliferation [214].

5.3 NE stimulates cell proliferation through autocrine and paracrine effects

As a secretory organ, brown adipose tissue produces a series of macromolecules, functioning in autocrine, paracrine or endocrine manners, which constitute an important part of brown adipose tissue function [8]. A series of fibroblast growth factor family members have been found to be expressed in brown adipose tissue and subject to adrenergic regulation, including Fgf 2 (or bFGF) [215], Fgf 16 [216], and Fgf 21 [217], all of which demonstrate metabolically beneficial effects in different ways. Fgf 2, whose expression is increased by NE stimulation and is increased in cold-acclimated blood plasma, is involved in NE-stimulated brown preadipocyte proliferation [215]. Fgf 16, which is mainly expressed in brown adipose tissue, also showed close relevance with embryonic brown adipose tissue enlargement [218]. In our microarray study, which was later confirmed by RT-qPCR analysis, Fgf 18, another member of this family, was upregulated at the mRNA level by NE and cAMP treatment, with the cell function yet to be explored.

Apart from the classical mitogenic growth factors, arachidonic acid, a polyunsaturated fatty acid, also showed mitogenic effects on primary cultured brown preadipocytes from rats. Arachidonic acid potentiated DNA synthesis on its own, as well as synergistically with norepinephrine. By the use of pharmacological inhibitors, protein kinase C, through ERK1/2 activation, was shown to be crucial for brown preadipocyte proliferation induced by arachidonic acid, and seemed to be involved in fibroblast growth factor-induced cell proliferation. It has been shown that NE, acting through the second messenger cAMP, activates perilipin and the hormone-sensitive li-
pase, which leads to increased arachidonic acid release [219], implying that arachidonic acid constitutes another aspect of the autocrine or paracrine effects of NE, through the release of mitogenic fatty acids [220].
Summary and Conclusion

Cells integrate extracellular signals in order to make a mitogenic decision. As a universal second messenger, cAMP demonstrates either positive or negative effects on cell proliferation, depending on the cell type. On one hand, cAMP functions to balance the mitogenic signal from growth factors, which contribute to fine-tune cell proliferation behaviour under physiological conditions. On the other hand, in endocrine cells, cAMP production from G protein-coupled receptor activation contributes to cell proliferation, typically showing a correlation between a neoplasia-related endocrine syndromes and activated cAMP signalling activity.

In this thesis, I have reviewed the cell signalling pathway mediating both the positive effect and negative effect of cAMP on cell proliferation in different types of cells. cAMP signalling interaction with several signalling pathways has been studied in cells where cAMP positively regulates cell proliferation, including MAPK, PI3K and mTOR complexes. Significant differences between cell lines and primary cultured cells have been presented in thyrocytes in respect to cell signalling. However, studies carried out with primary cultures, which represent more physiological situations, are relatively limited. The differences between primary cultures and cell lines in cell signalling pathways mediating cell proliferation exist beyond cAMP, for instance the involvement of EGFR transactivation in GPCR activation of MAPK. In the present study, we used primary cultured brown preadipocytes as a model to study the cell signalling in cell proliferation.

Brown preadipocytes survive and go through a proliferation stage, followed by an automatic differentiation process in culture medium without supplementation of so-called differentiation cocktails. In the precursor stage, NE and cAMP applied mild cell proliferation-stimulating and anti-apoptosis effects, which make this cell model appropriate for studying the cell signalling pathways of cAMP and adrenergic stimulation.

In **paper I**, we have studied the signalling pathway mediating LPA activation of ERK1/2 phosphorylation in brown adipocytes. LPA is a common mitogen, acting through a family of GPCRs (LPR1-6), inducing ERK1/2 phosphorylation. EGFR transactivation has been proposed as a paradigm for LPA-activation of ERK1/2 in various cell lines. In primary cultured brown adipocytes, we found that LPA indeed activated ERK1/2 in a short time frame, as it does in many cell lines; however, EGFR transactivation was not needed for this activation process. Instead, PI3K/Akt and Gi/PKC are involved in this process. This indicates that EGFR transactivation is not a universal mechanism for ERK1/2 activation by GPCRs, especially under more physiological conditions.
In paper II, we investigated the involvement of EGFR transactivation in adrenergic receptor signalling, in both mature brown adipocytes and preadipocytes. With the use of NE and selective agonists for α1-, α2-, β3-, and general β-adrenoceptors, we found that ERK1/2 can be phosphorylated following stimulation of all the adrenergic agonists mentioned above. Surprisingly, pretreatment with AG1478 did not lead to any significant inhibitory effect on ERK1/2 phosphorylation induced by NE and the α1-, α2-, β3-agonists, even though EGF stimulation was almost totally abolished by AG1478, which again proved that transactivation of EGFR is not a common mechanism for GPCR activation of MAPK. Moreover, we did not detect a crucial effect of ERK1/2 kinase activation for cAMP-stimulated cell proliferation in brown preadipocytes, where β1-adrenoceptor is the main mediator of the cAMP signalling pathway. Since transactivation mostly converges on ERK1/2 activation, this would then suggest that transactivation may not be involved in β1-adrenoceptor (cAMP)-stimulated cell proliferation in primary cultured brown preadipocytes.

In this study, we used AG1478, a commonly used tyrphostin for the inhibition of the EGF receptor, and a tendency to decreased phosphorylation of ERK1/2 was seen in some experiments. This could be either explained as an inhibition of the basal constitutive EGF receptor activity or as unspecific inhibition of ERK1/2 phosphorylation by AG1478. Therefore, a direct test of the occurrence of the EGF receptor phosphorylation by adrenergic agonists and inhibition by AG1478 would be more informative. However, we have tested commercial antibodies for phospho-EGF receptor at both tyr1173 and tyr1148 and neither of them gave any detectable signal, even with EGF stimulation of brown adipocytes (data not shown). Moreover, several mechanisms of EGF receptor transactivation have been suggested, including ecto-domain shedding of precursor EGF with ADAM 17 and 19, etc. [221]. To further elucidate the mechanism underlying of the absence of EGF receptor transactivation by adrenergic receptors, a study of the activity of these metalloproteases could provide further insight into the difference between transactivation of ERK1/2 in cell lines and the lack of transactivation in primary cultured cells.

In paper III, the cell signalling pathway mediating cAMP-stimulated cell proliferation in brown preadipocytes was studied.

Considering the strong mitogenic effect of serum, we have tested the effect of cAMP on cell proliferation under three different serum conditions: 10 % and 2 % new-born calf serum (NCS) and serum-free medium supplemented with fatty acid free bovine serum albumin (FFA-BSA). A tendency to increased cell proliferation in response to norepinephrine stimulation was seen with Hoechst binding assay when 10 % new-born calf serum was present in the medium. After lowering the serum concentration to 2 % in the culture medium, a statistically significant response to both norepinephrine
and forskolin stimulation was reached. Further replacing NCS with FFA-BSA stabilized the results showing a more readily reproducible response to forskolin stimulation. Therefore, in paper III and IV, we performed experiments under serum-free conditions and with 2 % NCS medium, respectively.

We investigated if insulin is required for cAMP-stimulated cell proliferation since this had been shown in thyrocytes [139]. We also distinguished effect of cAMP from the cAMP-mediated anti-apoptosis effect reported before [210]. The roles of ERK1/2 MAPK, PI3K/Akt, and mTOR complex activity were examined with pharmacological tools. Inhibition of PI3K/Akt and the mTOR complexes did not significantly inhibit cell proliferation. PD98059, which prevents ERK1/2 phosphorylation through the inhibition of MEK1/2 showed a significant inhibitory effect on proliferation but not the alternative ERK1/2 inhibitor U0126, which completely abolished ERK1/2 phosphorylation by forskolin but did not inhibit proliferation. One interpretation could be that MAPK is not crucial for the cAMP mitogenic effect, and that PD98059 inhibits cell proliferation through effects other than inhibition of ERK1/2 phosphorylation.

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However, the involvement of PI3K was only based on the lack of inhibitory effect of pharmacological inhibitors. This may simply reflect the lack of function of the inhibitors, since we did not check PI3K downstream effectors other than Akt phosphorylation at Thr308, which was not dramatically activated by forskolin stimulation. Besides, side effects of most PI3K inhibitors on other members of the PI3K related kinase family, e.g. wortmannin binding to mTOR has been reported [222], stressing the necessity of validating the PI3K effect with more precise methods. Gene knockdown with RNAi would be a more preferable method, although we have not succeeded in knocking down PI3K, because of technical problems in transfection of primary cultured brown adipocytes.
The ideal inhibitor should show a minimal inhibitory effect on the basal (in this case, vehicle-treated DNA amount), while showing significant inhibitory effect on a specific drug action (in this case, forskolin) on the biological process measured (in this case, total DNA amount). However, a dramatic decrease was seen on the basal level of total DNA with most inhibitors, for instance, mTOR inhibition with both rapamycin and KU0063794 induced a dramatic basal inhibition of total DNA amount. Applying gene knockdown with RNAi would also help to exclude the possible side-effects of these inhibitors on other proteins crucial for cell survival. This implies the necessity of screening for the optimal working concentration of the inhibitors in this specific cell type.

Another drawback with the use of protein kinase inhibitors is that the inhibition of the kinase activity was only tested 5-10 min after stimulation, whereas the assessment of cell proliferation was tested after 32 h with the measurement of total DNA. The efficiency of the inhibitors could have terminated much earlier than the forskolin stimulatory effect, leading to a compromised inhibitor effect.

**In paper IV**, we investigated how cAMP regulates brown preadipocyte proliferation at the transcriptional level. The impact of NE, which triggers cAMP production in brown preadipocytes, on the transcriptional profile was investigated in a microarray study of mRNA expression in both brown preadipocytes and mature brown adipocytes. Based on the differential response to NE between brown preadipocytes and mature cells, genes were selected for further validation with RT-qPCR, following both NE and forskolin stimulation. Eight protein-encoding genes that were markedly upregulated with both NE and forskolin stimulation were selected, which are possibly involved in cAMP-induced cell proliferation at the transcriptional level.

In the process of data analysis of microarray data, genes that had been upregulated in mature brown adipocytes by norepinephrine stimulation were excluded from further study. This may risk excluding target genes that have mitogenic effect on brown preadipocytes, but are generally upregulated through increased cAMP transcriptional activity, since cAMP is increased in both mature brown adipocytes and preadipocytes. Indeed, no immediate early genes that function generally as proto-oncogenes or cell cycle regulating proteins appeared in the preliminary candidate gene selection from the microarray data analysis, although it is of course possible that they are not involved in the cAMP mitogenic effect. Moreover, further functional studies are absolutely necessary to define the roles of the proteins encoded by these genes, in the process of cell proliferation.
Conclusion:
The ERK1/2 family of MAPK, which are generally important for cell proliferation, are activated by stimulation with both adrenergic receptors and LPA receptors. Although the mechanism of activation is still not clearly known, it is evident that EGFR transactivation does not contribute significantly to the process of ERK1/2 activation in primary cultured brown adipocytes. We therefore conclude that transactivation is not a universal mechanism in GPCR activation of MAPK. In addition to the anti-apoptosis effect on brown preadipocytes, cAMP also stimulated cell proliferation. ERK1/2 is indeed activated by forskolin stimulation, although it does not seem to play a crucial role in cAMP-induced cell proliferation. Other signalling pathways that we have tested, including PI3K/Akt, and mTOR complexes, do not play important roles either, and therefore, further studies are required to clarify the situation.
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