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REVIEW

Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets

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The cloning of the so-called 'parathyroid hormone-related protein' (PTHrP) in 1987 was the result of a long quest for the factor which, by mimicking the actions of PTH in bone and kidney, is responsible for the hypercalcemic paraneoplastic syndrome, humoral calcemia of malignancy. PTHrP is distinct from PTH in a number of ways. First, PTHrP is the product of a separate gene. Second, with the exception of a short N-terminal region, the structure of PTHrP is not closely related to that of PTH. Third, in contrast to PTH, PTHrP is a paracrine factor expressed throughout the body. Finally, most of the functions of PTHrP have nothing in common with those of PTH. PTHrP is a poly-hormone which comprises a family of distinct peptide hormones arising from post-translational endoproteolytic cleavage of the initial PTHrP translation products. Mature N-terminal, mid-region and C-terminal secretory forms of PTHrP are thus generated, each of them having their own physiologic functions and probably their own receptors. The type 1 PTHrP receptor, binding both PTH(1-34) and PTHrP(1-36), is the only cloned receptor so far. PTHrP is a PTH-like calciotropic hormone, a myorelaxant, a growth factor and a developmental regulatory molecule. The present review reports recent aspects of PTHrP pharmacology and physiology, including: (a) the identification of new peptides and receptors of the PTH/PTHrP system; (b) the recently discovered nuclear functions of PTHrP and the role of PTHrP as an intracrine regulator of cell growth and cell death; (c) the physiological and developmental actions of PTHrP in the cardiovascular and the renal glomerulo-vascular systems; (d) the role of PTHrP as a regulator of pancreatic beta cell growth and functions, and, (e) the interactions of PTHrP and calcium-sensing receptors for the control of the growth of placental trophoblasts. These new advances have contributed to a better understanding of the pathophysiological role of PTHrP, and will help to identify its therapeutic potential in a number of diseases.
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Keywords: PTHrP; nuclear localization; cardiovascular system; kidney; beta cell; calcium-sensing receptor; cell proliferation; development

Abbreviations: ANF, atrial natriuretic factor; BLC, blomstrand lethal chondrodysplasia; cyclic-AMP, cyclic adenosine monophosphate; CaSR, calcium-sensing receptor; CDK, cyclin-dependent kinase; FGF, fibroblast growth factor; HGF, hepatic growth factor; ICM, inner cell mass; N-, C-terminal, amino-, carboxy-terminal; NLS, nuclear localization sequence; PDGF, platelet derived growth factor; PHPIb, pseudohypoparathyroidism type Ib; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PTH, parathyroid hormone; PTH1R, type 1 parathyroid hormone receptor; PTH2R, type 2 parathyroid hormone receptor; PTHrP, parathyroid hormone-related protein; RIP, rat insulin promoter; SHR, spontaneously hypertensive rat; TIP39, tuberoinfundibular peptide of 39 aminoacids; VSM, vascular smooth muscle

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Introduction

The parathyroid hormone-related protein (PTHrP) study began in 1987 as the culmination of a 40 years search for the humoral factor responsible for the most common paraneoplastic syndrome, humoral hypercalcaemia of malignancy. Indeed, when abundantly produced by tumours and released into circulating blood, PTHrP stimulates, *via* an endocrine-like pathway, bone resorption and renal calcium reabsorption, by interacting with a common PTH/PTHrP receptor, now called type 1 PTH receptor, PTH1R. Limited sequence and conformational homologies between the 1-34 N-termini of PTHrP and PTH explained their ability to bind with equal affinity to a single receptor. Shortly after its discovery in 1987, it became obvious that this so-called PTH-related protein is actually markedly different from PTH in a number of ways. Many reviews of various aspects of the PTHrP field, have been published over the recent years (Grill *et al*., 1998; Jüppner, 1999; Karaplis & Kronenberg, 1996; Lam *et al*., 2000; Lanske & Kronenberg, 1998; Martin *et al*., 1997; Nguyen & Karaplis, 1998; Philbrick *et al*., 1996; Rabbani, 2000; Strewler, 2000; Wysolmerski & Stewart, 1998).

Despite their N-terminal homology and their calciotropic properties, PTH and PTHrP are the product of separate genes located on distinct chromosomes. In all species tested so far, the gene encoding PTHrP is more complex than the PTH gene (Figure 1). For instance, the human PTHrP gene (Figure 1) consists of 9 exons which give rise through alternative splicing to up to 12 transcripts and three different sized initial translation products (Figure 2), potentially in a tissue-specific manner, and under the control of three different promoters. Its expression is regulated by many hormones and growth factors. The instability motifs in the organization of the 3′-untranslated regions are reminiscent of those found in immediate–early genes, which encode cytokines and growth factors. PTHrP is undetectable in the circulating blood of normal subjects, but is produced in a paracrine/autocrine fashion during foetal and adult life by a number of normal cells and tissues, including epithelia, mesenchymal tissues, endocrine glands and the central nervous system, in which it is believed to play an expanding number of physiological roles through these autocrine/paracrine pathways. Lessons from PTHrP or PTH1R gene knockout mice, which die at birth or in utero, emphasize the critical role of PTHrP for normal life (Karaplis *et al*., 1994; Lanske *et al*., 1996).

In addition to its nearly ubiquitous expression, two other important features of PTHrP are further expanding the list of

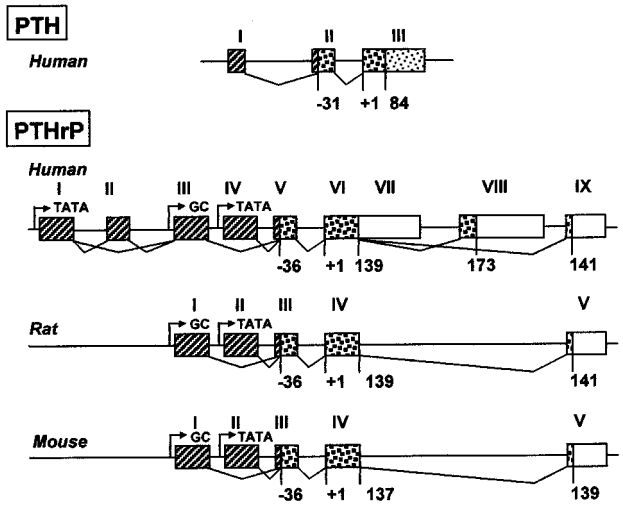

Figure 2 The three initial PTHrP isoforms arising from alternative splicing as shown in Figure 1, the posttranslational processing at multibasic endoproteolytic sites (multi-K/R), and the amino-terminal, mid-region and carboxy-terminal mature secretory forms of PTHrP, as they are understood at present. PTHrP(1-36) is the mature PTHrP species exhibiting PTH-like properties, not only in bone and kidney, but also in a number of other systems, including the cardiovascular system. This region contains the 1–13 region in which 8 aminoacid are homologous to the analogous region in PTH. Note also the bipartite nuclear/nucleolar targeted sequence (NTS, also referred to as "NLS") in the 88–106 region (see text).

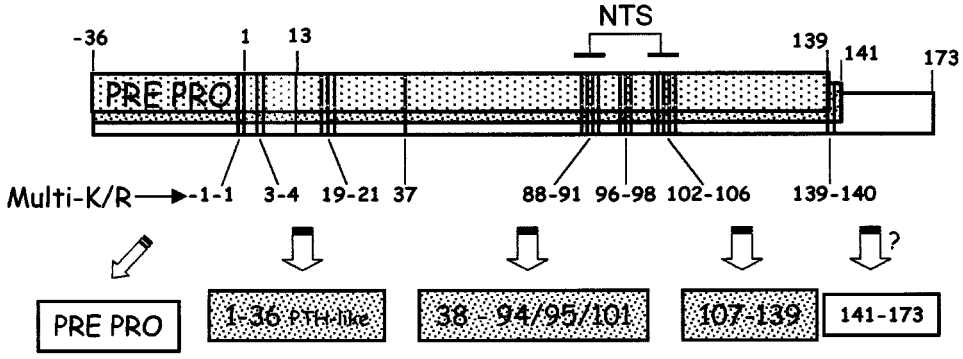

Figure 1 Structure of parathyroid hormone-related protein (PTHrP) gene in human, rat and mouse, as compared to the less complex human PTH gene. Key features of PTHrP genes are (a) the use of two (rat and mouse) or three different promoters (in human), indicated by arrows; the use of alternative splicing (splice options shown by bent lines connecting exons) which in human gives rise to three different coding-region protein isoforms of 139, 141 and 173 aminoacids in human or to a single protein of 141 aminoacids in rat and 139 aminoacids in mouse. Exons are shown as hatched boxes (5′-non coding exons), dotted boxes (mature coding region) or open boxes (3′-untranslated region). Like in growth factor or cytokine genes, the 3′-untranslated regions of the various PTHrP mRNAS contain multiple copies of an AUUUA motif, responsible for rapid degradation and short half-life. Moreover, the presence of TATA boxes and GC sequences in the promoting region suggests complex regulation of expression. In human, the PTHrP gene (15 kb) is located on the short arm of chromosome 12, while PTH gene is located on an analogous region on the short arm of chromosome 11; both chromosomes most likely arising from a common ancestral gene.

possible physiological roles. First, nascent PTHrP isoforms (Figure 2) are processed by members of the prohormone convertase family to at least three fragments: N-terminal PTHrP(1-36), structurally related to PTH; a mid-region PTHrP(38-94); and, a C-terminal PTHrP(107-139). Each has its own biological properties and probably acts through distinct receptors. Of these, only the PTH1R, which binds both PTHrP(1-36) and PTH(1-34), has been characterized in mammals. Second, findings accumulated over the past five years have demonstrated that PTHrP is able to act, not only *via* the classical autocrine/paracrine pathways, but also through a so called intracrine pathway, which involves the translocation of the nascent protein into the nucleus. Thus, PTHrP contains a basic bipartite nuclear/nucleolar localization sequence (NLS) in its 88–107 region similar to the NLS in viral and mammalian transcription factors. In a more recent study, a second tetrabasic KKKK(147–150) motif has been proven to determine intracrine regulatory effects of PTHrP(1–173) in human chondrocytes (Goomer *et al*., 2000). Nuclear localization of PTHrP was shown to regulate apoptotic cell death and cell proliferation. It should also be emphasized that PTH1R itself has been localized to the cell nucleus, suggesting that there may be a role for PTH and/or PTHrP in the regulation of nuclear events, either on the physical environment (nucleoskeleton) or directly on gene expression (Watson *et al*., 2000).

Overall, these studies indicate that PTHrP has multiple activities including control of foetal development, transepithelial calcium transfer, lactation, smooth muscle relaxation and cell growth. Thus, although PTHrP was discovered as a tumour-derived hypercalcemic factor, its primary physiological role is as a local regulator of many physiological processes. For the convenience of the reader, the main established and putative physiological roles of PTHrP classified by organ system, as well as the corresponding relevant bibliography, have been summarized in Table 1.

Receptors and peptides of the PTH/PTHrP system

The type I, PTH/PTHrP receptor (PTH1R)

Cloned in human, rat, and mouse, the PTH1R belongs to a distinct class of heptahelical G protein-linked, to which the receptors for secretin, calcitonin, glucagons and several other peptide hormones also belong (Jüppner, 1999). The PTH1R recognizes equally PTH(1-34) and PTHrP(1-36) and is expressed at high levels in bone and kidney in which it mediates the classical effects of PTH and PTHrP on Ca^{2+} homeostasis. PTH1R is also expressed at lower levels in a number of tissues, in which it mediates a large array of non-traditional paracrine and autocrine functions in response to locally produced PTHrP. Most of these functions have been summarized in Table 1. Although new members of the PTHR family have been discovered over the last few years, the PTH1R remains a primary target for research into new therapeutic drugs in diseases, such as hyperparathyroidism, humoural hypercalcemia of malignancy and osteoporosis.

Expression of the PTH1R cDNA, either rat or opossum, in cultured cells, promotes a potent PTH(1-34)- or PTHrP(1-36)-induced stimulation of adenylyl cyclase, and a weaker stimulation of phospholipase C. The pharmacological evaluation of PTH(1-34) and PTHrP(1-36) reveals nearly identical potency in most *in vitro* assay systems. For instance, in a human, comparable increases in circulating PTH or PTHrP produce comparable effects on mineral ion homeostasis (Everhart-Caye *et al*., 1996). The significant sequence homology within the first 13 amino acid residues of PTH and PTHrP reflects the functional importance of the N-terminal residues in PTH1R signalling. This sequence homology decreases dramatically in the 14–34 region, and beyond residue 34 there is no further identifiable similarity.

It has been clearly demonstrated that, despite the absence of homology, the 15–34 regions of both PTH and PTHrP function as the principal ligand binding domain, interacting with the extracellular N-terminal domain of the PTH1R, while the early N-terminal portion of each ligand is required for bioactivity through the cyclic AMP/PKA pathway, owing to contacts with the extracellular loops of transmembrane helixes 5 and 6 of the PTH1R. In support of this concept, PTH or PTHrP species missing the 2 or 6 N-terminal residues, together with substitution of some other residues, are authentic antagonists of PTH(1-34)- or PTHrP(1-36)-induced cyclic AMP stimulations in most *in vitro* systems. For instance, [Asn^{10},Leu^{11},D-Trp^{12}]PTHrP(7-34) has been recognized as the most selective and potent antagonist of PTH1R in most *in vitro* assays. N-terminally-truncated PTH and PTHrP analogues may function as inverse agonists, in that they blunt cyclic AMP production in a dose-dependent fashion in cells expressing a constitutively active mutant of PTH1R associated with Jansen's metaphyseal chondrodysplasia. The precise ligand regions involved in the stimulation of PLC and PKC have not yet been clearly identified. A detailed exploration of the structure–activity relationship in PTH and PTHrP, as well as the PTH1R–ligand interactions have been extensively reviewed in recent years (Jüppner, 1999; Mannstadt *et al*., 1999; Orloff *et al*., 1994).

Other receptors for PTHrP(1-36) or PTH(1-34)

N-terminal PTHrP fragments also activate receptors in nonclassical (i.e. non-renal, non-skeletal) PTH target tissues, in which the signalling mechanisms may differ qualitatively from those of the cloned PTH1R. Thus, some evidence has been provided for the existence of a novel receptor for N-terminal PTHrP in keratinocytes, insulinoma cells, lymphocytes and squamous carcinoma cell lines (Gaich *et al*., 1993; Orloff *et al*., 1995). Although PTH(1-34) or PTHrP(1-36) activate both adenylyl cyclase and phospholipase C through the classical PTH1R in renal tubules and bone, both peptides have been proven to produce a large increase in intracellular free calcium, but not cyclic AMP in keratinocytes and squamous carcinoma cell lines, which do not express the classical PTH1R (Orloff *et al*., 1995), suggesting the existence of an alternate receptor for N-terminal PTH and PTHrPs. Such a receptor has, however, not yet been conclusively identified. By contrast, in PTH1R-expressing vascular smooth muscle (VSM) cells, PTHrP has been proven to stimulate cyclic AMP formation, but to be unable to induce intracellular calcium or inositol phosphate formation despite the presence of detectable Gαq. In these cells, transient overexpression of Gαq proteins conferred the ability of PTHrP to activate both the inositol phosphate/calcium and

Table 1 Established and emerging normal physiological functions of PTHrP classified by organ systems

Organ system	*Functions of PTHrP*	*References*
Cartilage[a]	Regulates endochondral bone development. Delays chondrocyte maturation and apoptosis, and accelerates growth of cartilage by promoting chondrocyte proliferation.	Chung *et al*., 1998 Amizuka *et al*., 1996
	A bipartite NLS in the 88–106 region targets PTHrP to the nucleus/nucleolus in chondrocytes where it regulates cell differentiaation and apoptose through an intracrine pathway.	Goomer *et al*., 2000 Henderson *et al*., 1995 Karaplis & Vautour, 1997
	A second NLS in the 147–150 region targets PTHrP to the nucleus/nucleolus in human chondrocytes where it determines intracrine regulatory effects of PTHrP (1–173) on chondrocyte PPi metabolism and matrix synthesis.	Lanske & Kronenberg, 1998 Vortkamp *et al*., 1996 Weir *et al*., 1996
Bone	Interacts with PTH1R to stimulate bone resorption.	
	PTHrP (107–139), a.k.a. osteostatin, inhibits osteoclastic bone resorption via a yet unidentified receptor.	Caverzasio & Bonjour, 1996 Cuthbertson *et al*., 1999
	Regulates Pi transport in osteogenic cells.	Ouyang *et al*., 2000
	Ensures tooth eruption, by resorption of the alveolar bone to allow passage of the newly developed tooth.	Philbrick *et al*., 1998
Skin Hair follicles	Interacts with PTH1R to delay terminal differentiation of epidermal keratinocytes, keratinisation and apoptosis.	Foley *et al*., 1998
	Promotes keratinocyte proliferation.	Guo *et al*., 1997
	Decreases the number of hair follicles through epithelial-mesenchymal interactions.	Holick *et al*., 1994 Wysolmerski *et al*., 1994
Smooth muscle[a]	Is rapidly induced by vasoconstrictors and mechanical stretching.	
	PTHrP interacts with PTH1R to induce strong myorelaxant effects in a number of smooth muscle containing organs.	Noda *et al*., 1994 Pirola *et al*., 1993 Steers *et al*., 1998
	Has been proposed to modulate parietal compliance in hollow organs.	
Vessels[a]	PTHrP and PTH1R are present in VSMC and endothelial cells.	
	Decreases vascular tone and blood pressure and is believed to regulate regional and systemic hemodynamics.	Maeda *et al*., 1999 Massfelder *et al*., 1997
	PTHrP (1–36) binds to membrane PTH1R to inhibit VSMC proliferation.	Massfelder *et al*., 1998 Qian *et al*., 1999
	A bipartite NLS in the 88–106 region targets PTHrP into the nucleus/nucleolus where it determines cell proliferation and delays apoptotic death through an intracrine pathway.	Roca-Cusachs *et al*., 1991 Wolzt *et al*., 1997
Heart[a]	Regulates heart development.	
	Exerts positive chronotropic effects.	Bui *et al*., 1993
	Induces inotropic effects which result from coronary dilation.	Burton *et al*., 1994 Deftos *et al*., 1993
	Is released by ventricular and especially by atrial myocytes.	Ogino *et al*., 1995
	Appears as a coronary endothelium-derived modulator of ventricular function.	Hara *et al*., 1997 Schlüter *et al*., 2000
Uterus	May regulate decidualization of endometrial stromal cell.	
	Is upregulated by mechanical stretch and oestrogen.	Ferguson *et al*., 1998
	Relaxes uterus and vascular smooth muscle, an effect potentiated by oestrogen.	Mitchell *et al*., 1996 Nowak *et al*., 1999
	May modulate implantation of the fertilized ovum and retention of the embryo.	Paspaliaris *et al*., 1995 Pitera *et al*., 1998
	Inhibits oxytocin-stimulated activity during pregnancy and prevents preterm labor.	Tucci & Beck, 1998 Williams *et al*., 1998
Placenta[a]	Mid-region of PTHrP interact with a yet unidentified receptor to maintain matemo-foetal gradients of calcium and magnesium	Curtis *et al*., 2000
	Interacts with Ca-binding proteins and Ca-sensing receptors to modulate trophoblastic growth and differentiation.	Kovacs *et al*., 1996 Tucci *et al*., 1996
Mammary gland, lactation	Necessary in epithelial-mesenchymal interactions during embryonic mammary development leading to nipple formation and branching morphogenesis.	Davicco *et al*., 1993
	Might participate in adolescent ductal morphogenesis.	Dunbar & Wysolmerski, 1999

Continued

Table 1 (*Continued*)

Organ system	*Functions of PTHrP*	*References*
	During lactation, is induced by prolactin, is released in mother's bloodstream, promotes calcium transport from blood to milk, increases mammary blood flow, and regulates maternal and neonate Ca-Pi metabolism.	Lippuner *et al*., 1996 Wysolmerski *et al*., 1995
Kidney[(a)]	May have a role in kidney maturation and glomerular development. Expressed with PTH1R in renal tubules, glomeruli, intrarenal arteries and arterioles. By interacting with PTH1R, mimics all PTH-like effects on tubules, including calcemic and phosphaturic effects. Decreases renal vascular resistance, renal blood flow and glomerular filtration rate. Exhibits proliferative effect on tubular cells and mesangial cells, where it may play a role in the normal glomerulus and in injured kidney. Displays paradoxical effects on renal VSMC proliferation (see 'smooth muscle'). Interacts directly with juxtaglomerular cells to stimulate renin release.	 Aya *et al*., 1999 Bosch *et al*., 1999 Henry *et al*., 1997 Massfelder *et al*., 1993 Massfelder *et al*., 1996a,b Saussine *et al*., 1993 Wolzt *et al*., 1997 Yang *et al*., 1997
Lung	A mediator of lung development through epithelial-mesenchymal paracrine loop. Is an autocrine inhibitor of growth and differentiation of alveolar type II cells *in vivo* and *in vitro*. Might also play a role in the production of surfactant by alveolar type II cells.	 Hastings *et al*., 2000 Ramirez *et al*., 2000 Rubin *et al*., 1994 Speziale *et al*., 1998 Torday *et al*., 1998
Gastrointestinal	Is expressed together with PTH1R in almost all structuro-functional regions of that system. Interacts with vasoactive intestinal factor receptors to relax the smooth muscle layers. Its expression is increased in smooth muscle layers in response to distension. Induces transcaltachia. It might regulate proliferation-differentiation equilibrium and contractility.	 Botella *et al*., 1994 Ito *et al*., 1994 Li *et al*., 1995 Yu *et al*., 1992 Zhou *et al*., 1992
Liver	May act as a hepatic developmental factor regulating hepatogenesis that is extinguished postnatally. Reexpressed during liver regeneration, endotoxic shock and hepatocellular carcinoma.	 Funk *et al*., 1997 Roskams *et al*., 1993 Roskams *et al*., 1995
Pancreas[(a)]	Is produced with PTH1R in insulin producing beta cell in which it mediates intracellular calcium. It delays beta cell apoptotic death and increases beta cell mass and insulin secretion.	 Gaich *et al*., 1993 Porter *et al*., 1998 Vasavada *et al*., 2000
Parathyroid gland	May replace PTH in foetal parathyroid glands to regulate calcemia. Is produced and released by the oxyphil cell lineage in adult thyroid and parathyroid glands. Unlike PTH, its secretion is not influenced by the medium calcium. It enchances low-calcium stimulated PTH secretion *in vivo* and *in vitro*. May function as a growth suppressor in human parathyroid.	 Connor *et al*., 1993 Kitazawa *et al*., 1992 Lewin *et al*., 2000 Matsushita *et al*., 1999 Okada *et al*., 1995 Tucci *et al*., 1996
Central nervous system (CNS)	Is expressed with PTH1R in a number of areas. May be involved in astrocytic differentiation, as well as in arginine-vaspressin secretion. It protects neurons against glutamate-induced exitotoxicity. Is a neuropeptide whose regualtion depends upon L-type voltage sensitive calcium channel activity. The gene is expressed under conditions that promote the survival of neurons. Displays central pressor effect in conscious rats.	 Brines & Broadus, 1999 Chattopadhyay *et al*., 2000 Fukayama *et al*., 1995 Holt *et al*., 1996 Nagao *et al*., 1998 Ono *et al*., 1997 Struckhoff & Turzynski, 1995 Weaver *et al*., 1995 Yamamoto *et al*., 1998

As a rule, only the most recent bibliography is shown. These citations refer to older key publications of the field. The various functions of PTHrP produced under physiopathological conditions, such as cancers, have not been considered in this table. Abbreviation used: NTS: nuclear targeting sequence; PTH1R: PTH/PTHrP receptor; VSMC: vascular smooth muscle cell. [(a)] indicates that more details for this organ are given in the text.

the cyclic AMP pathways (Maeda *et al*., 1996). Thus, a single PTH/PTHrP receptor, though capable of coupling to different G proteins, signals exclusively through a cyclic AMP-dependent pathway in vascular smooth muscle. This latter finding also indicates that the relative expression levels and probably intracellular locations of various G proteins determine which second messenger system will be driven by agonists in low PTH1R-expressing cells.

Evidence for an additional PTHR specific for PTHrP(1-36) has been observed in the rat supraaortic nucleus, in which it mediates a cyclic AMP-dependent stimulatory effect, on vasopressin release (Yamamoto *et al*., 1997). In these studies, PTH(1-34) was ineffective and unable to bind or to displace bound PTHrP. Interestingly, the 1-34 region of intraventricularly injected PTHrP has been shown to increase blood pressure in conscious rats (Nagao *et al*., 1998). However, the question as to whether this central pressor effect of PTHrP(1-141) is also driven by PTHrP(1-36) and PTH(1-34) through interaction with the PTH1R has not yet been elucidated. Finally, a receptor selectively binding PTHrP(1-36) and stimulating cyclic AMP has been identified recently in zebrafish (Rubin *et al*., 1999; Hoare *et al*., 2000). Whether a mammalian homologue of this receptor exists remains to be determined.

Receptor for mid-regional and C-terminal PTHrP species

The prohormone nature of PTHrP, with the documented existence of mid-region and carboxy-terminal secretory forms (see Figure 2), strongly suggests the existence of additional receptors recognizing these mid region and C-terminal PTHrP peptides, as these peptides do not interact with the PTH1R and have no PTH-like activities (see Table 1). These receptors have also not yet been identified.

The PTH2R

A second member of the PTHR family was recently identified and cloned from a rat cerebral cortex cDNA library by the group of Usdin in Bethesda, U.S.A., in 1995 (Usdin *et al*., 1995). A recent review from these authors focuses on the discovery of PTH2R and its endogenous ligand, and the current status of investigations aiming at the definition of their biological functions (Usdin *et al*., 2000). Briefly, the PTH2R displays 52% amino acid sequence homology with the PTH1R. Alignment of the PTH2R to sequences of other class II receptors reveals that this new receptor belongs to the same subfamily of the secretin and calcitonin receptors and suggests that its ligand may resemble PTH or PTHrP. Among various PTH and PTH-like peptides studied, PTH was the only peptide exhibiting significant activation of the cyclic AMP pathway in cells transfected with cDNA encoding the PTH2R. Binding and ligand specificity studies confirmed the high selectivity of PTH2R for PTH. It was therefore inferred that structural differences in the PTH and PTHrP determine selectivity for the PTH2R. Indeed, Phe^{23} in PTHrP (Trp in PTH) prevents binding to the PTH2R whilst the presence of His^{5} in PTHrP (Ile in PTH) blocked cyclic AMP signalling. Another important difference between PTH1R and PTH2R is the limited distribution of PTH2R as compared to the widespread distribution of PTH1R. Although less than PTH1R expression, immunohistochemical, as well as *in situ* hybridization studies reveal the brain as the primary PTH2R-expressing site. PTH2R has also been detected at discrete locations in pancreas, some peripheral endocrine cells, in cells close to the vascular pole of glomeruli in the kidney, and in vascular endothelium of most tissues.

The relatively high level of PTH2R expression in defined brain regions, together with older findings reporting immunoreactive PTH in the brain, suggested that locally produced PTH could act on PTH2R in this organ. However, in more recent studies, Usdin and colleagues were unable to detect PTH mRNA in brain (Usdin, 1997). This finding prompted the search for another endogenous PTH2R ligand in brain. Initial studies from Usdin's group using an extract prepared from bovine hypothalamus, provided experimental evidence for an endogenous PTH2R activating ligand distinct from PTH. After purification and sequencing, this novel species, called TIP39 (for tuberoinfundibular peptide of 39 residues), revealed a structure quite different from both PTH and PTHrP. TIP39 seems to selectively activate the PTH2R and the adenylyl cyclase pathway, with a potency greater than that of PTH. Compared to the activity found in the hypothalamic extract PTH is only a partial agonist for the PTH2R. This new ligand has little or no effect on the PTH1R. Thus, TIP39 appears to be the most likely endogenous PTH2R ligand.

As noted above, PTH1R antagonists have been developed by deletion of N-terminal residues of PTH and PTHrP. Recently, Hoare *et al*. (2000) prepared an N-terminal truncated peptide of TIP39: TIP(7-39), which was identified as a novel, selective and high-affinity PTH1R antagonist. This new peptide binds with higher affinity to the PTH1R than some of the previously described PTH1R antagonists (Hoare *et al*., 2000). It neither stimulates adenylyl cyclase nor increases intracellular Ca^{2+}. Such an antagonist will provide a new strategy for pharmacological studies.

PTH1 receptor and genetic disorders

Identification of spontaneous mutation represents another approach to understand PTH1R function. The present section aims in summarizing the main consequences of PTH1R mutations in human. PTH, by acting on the PTH1R localized in bone and kidney regulates calcium and phosphate metabolism (Kronenberg *et al*., 1998). Because of this, it was believed for many years that abnormality in PTH1R was the cause of pseudohypoparathyroidism type Ib (PHPIb) (Silve, 1995; Spiegel & Weinstein, 1995). Consistent with this possibility, these patients have renal resistance to PTH action, while the activity of the other Gsα-protein-coupled receptors appear normal. A number of groups have now presented convincing evidence against the existence of a defect in PTH1R gene in PHPIb. Thus, normal receptor function can be demonstrated in fibroblast cells from most patients (Suarez *et al*., 1995). Indeed, no mutations in the coding exons and intron/exon junctions of the receptor gene were found in PHPIb patients (Schipani *et al*., 1995b), nor mutations that would affect initiation, splicing or stability of transcripts expressed from P1 or P2 promoters (Bettoun *et al*., 1997; 1998). Furthermore, genes encoding type 1 and type 2 PTHR have been excluded by linkage analysis (Jan de Beur *et al*., 2000). Consequently, it has been postulated that a

regulatory defect in a gene controlling the expression of the PTH1R in renal proximal tubular cells may exist in PHPIb. Alternatively, tissue specific abnormality in Gsα expression or function could also produce this phenotype. Recent works support this latter possibility, and PHPIb has been associated with a GNAS1 imprinting defect (Liu *et al*., 2000; Jüppner *et al*., 1998).

Evaluation of PTHrP and PTH1R function using have demonstrated the roles of this receptor/ligand system in skeletal development (Strewler, 2000). Results from transgenic and knockout mouse models support the conclusion that PTHrP by acting on PTH1R plays a critical role in chondrocyte proliferation, differentiation and apoptosis, and thereby in endochondral bone formation. Recently two diseases, Jansen and Blomstrand chondrodysplasias have been associated with activating and inactivating mutations, respectively, in the PTH1R gene (Jüppner & Silve, 2000). Jansen chondrodysplasia is an autosomal dominant disorder characterized by short-limbed dwarfism associated with agonist-independent hypercalcemia and hypophosphatemia (Jansen, 1934). Three heterozygous nucleotide exchanges in the PTH1R were identified, which change a histidine at position 223 to arginine (exon M2) (H223R), a threonine at position 410 to proline (T410P) (exon M5), and an isoleucine at position 458 to arginine (I458R) (exon M7), respectively (Schipani *et al*., 1999). The three mutated residues are strictly conserved in all mammalian members of this receptor family, suggesting an important functional role for these three residues. Indeed, COS-7 cells transiently expressing PTH1R with either the H223R, T410P, or I458R mutation showed significantly higher basal accumulation of cyclic AMP than cells expressing the wild-type PTH1R (Schipani *et al*., 1999).

Blomstrand lethal chondrodysplasia (BLC) is an autosomal recessive disease that typically results in foetal death during the last trimester of pregnancy and is characterized by premature chondrocyte death (Blomstrand *et al*., 1985). The long bones are extremely short and poorly modelled, show markedly increased density and lack metaphyseal growth plates (Oostra *et al*., 2000). Compatible with the role of PTH1R/PTHrP signalling pathway recently described in mammary gland and tooth development of genetically manipulated mice (Philbrick *et al*., 1996; Wysolmerski & Stewart, 1998), defects in the development of these tissues have been recently demonstrated in two foetuses with BLC (Oostra *et al*., 2000). In these foetuses, nipples were absent, and no subcutaneous ductal tissue could be identified by histochemical analysis. Tooth buds were present, but developing teeth were severely impacted within the surrounding alveolar bone, leading to distortions in their architecture and orientation. Recently, four different defects in the PTH1R gene were described in genomic DNA from patients affected by BLC. One foetus, born from non-consanguineous parents, was shown to have two distinct abnormalities in the PTHR1 (Jobert *et al*., 1998). A nucleotide exchange in exon M5 of the maternal PTHR1 allele introduced a novel splice acceptor site which led to a mutant mRNA encoding an abnormal receptor that lacks a portion of the fifth membrane-spanning domain (amino acids 373 to 383; Δ373–383). Very low expression of the paternal PTHR1 allele from this patient was found. The defect responsible for this low paternal expression has not yet been identified. A nucleotide exchange that leads to a proline to leucine mutation at position 132 (P132L) in the N terminal portion of the receptor (exon E3) was identified in a second patient with BLC (Zhang *et al*., 1998; Karaplis *et al*., 1998). A homozygous deletion of G at position 1122 (exon EL2) was identified in a third case of BLC (Karperien *et al*., 1999). This mutation led to a shift in the open reading frame which resulted in a truncated protein that completely diverged from the wild-type receptor sequence after amino acid 364, and thus lacked transmembrane domains 5, 6 and 7, the connecting intra- and extracellular loops, and the cytoplasmic tail (Δ365–593). COS-7 cells transiently expressing PTHR1 with either the P132L, Δ373–383, or Δ365–593 mutations showed significantly lower accumulation of cyclic AMP in response to PTH, than cells expressing the wild-type PTHR1. Thus, BLC is associated with compound heterozygous or homozygous mutations that lead to mutant PTH1R with severely impaired functional properties.

To conclude, the most striking consequences of PTH1R mutations are abnormalities in endochondral bone formation that result in severe skeletal abnormalities in both Jansen and BLC. The evaluation of affected patients has furthermore provided new insights into the role of the PTH1R in other aspects of normal human development. Although, PTH1R has long been known to play an essential role in regulating calcium and phosphorus homeostasis by mediating the endocrine actions of PTH on bone and kidney, the importance of the PTH1R for normal bone development in humans has now been confirmed by the identification of mutations in the PTH1R gene in two rare chondrodysplasias.

Is there a nuclear function of PTHrP?

PTHrP contains a nuclear localization signal (NLS)

As indicated previously (Introduction), PTHrP, unlike PTH, does not circulate in appreciable amounts in normal subjects, but rather exerts its biological actions locally. Signalling *via* the cell surface PTH1R is the mechanism by which PTHrP mediates most of its physiological effects, yet the possibility that some of these effects arise as a consequence of intracrine action of the peptide has also been considered (Henderson *et al*., 1995). The latter concept first arose from the observation that the PTHrP sequence contains a bipartite NLS, analogous to the prototypical nucleoplasmin NLS (Robbins *et al*., 1991). This sequence (amino acids 87–107 of the mature form, Figure 2) also bears homology to sequences in human retroviruses shown to target viral regulatory proteins to the nucleolus. When transiently expressed in COS-7 cells, PTHrP indeed localized, in part, to the nucleus. Nuclear translocation was entirely dependent on sequences encoding the putative NLS, demonstrating that it was functional and sufficient to target a protein to the nucleus. Studies *in vitro* as well as *in situ*, have subsequently revealed that endogenous PTHrP also localizes to the nucleolus (Henderson *et al*., 1995). Immunoreactivity for the protein was observed over the dense fibrillar component of nucleoli, a subnucleolar structure representing the major site of rRNA gene transcription. These initial observations were later confirmed in a human keratinocyte cell line (HaCaT) (Lam *et al*., 2000) and in VSM cells (Massfelder *et al*., 1997).

Mechanisms underlying translocation of PTHrP to the nucleus

To be targeted to the nucleus, a secretory protein may be internalized back to the cytoplasm and from there to the nucleus. The mechanism by which a secretory protein may gain access to the cytoplasm after secretion may involve endocytosis-dependent pathways. For instance, such a mechanism has recently been documented for angiogenin (Hatzi *et al*., 2000). Although endocytosis of PTH1R, with subsequent PTHrP degradation has been demonstrated (Huang *et al*., 1995; Lam *et al*., 2000), other studies do not support this concept. For instance, in CHO cells transfected with the PTH1R gene, Amizuka *et al*. (2000) have shown that there is no nuclear staining for PTHrP, even after addition of PTHrP to the culture medium, ruling out the possibility that secreted PTHrP is internalized *via* receptor-mediated endocytosis and subsequently directed to the nucleus. Since a novel splice variant of the PTH1R, that preferentially localizes to the cytoplasm, has been described by Joun *et al*. (1997), the possibility exists that cytoplasmic PTHrP can reach the nucleus by binding to an intracellular PTH1R variant. Such a pathway has been described for FGF2 (Maher, 1996). Alternatively, endocytosis of PTHrP might be mediated by a receptor different from PTH1R. The demonstration that full-length PTHrP(1-141) secreted from COS-1 cells transfected with PTHrP can be endocytosed and targeted to the nucleolus supports this concept. These results are particularly interesting since PTHrP endocytosis was NLS-dependent, but did not require the PTH1R (Aarts *et al*., 1999b). On the other hand, the observation that full-length PTHrP, the only PTHrP species that contains the entire NLS, is a minor secretory form of PTHrP (Soifer *et al*., 1992; Yang *et al*., 1994) does not support this concept. Thus, whether the NLS (or other motifs) is recognized at the cell surface by non-specific binding sites, allowing PTHrP to reach the cytoplasm and then the nucleus, remains an attractive but unanswered question.

While an autocrine pathway for nuclear localization of PTHrP has rarely been documented, there is more abundant evidence to suggest that PTHrP is not secreted prior to being translocated into the nucleus. A first possible mechanism for such a pathway is an alternative splicing leading to the generation of mRNA species that miss the exon encoding for the signal peptide. Since such alternative transcripts have never been documented, and because nuclear localization of PTHrP is observed in cells transfected with PTHrP cDNA that does not contain intron sequences (Henderson *et al*., 1995; Massfelder *et al*., 1997), such a hypothesis appears very unlikely.

A second possible mechanism for direct translocation of PTHrP to the nucleus involves alternative initiation of translation at non-AUG codons, particularly at CUG codons. Recently, Vagner *et al*. (1996) have demonstrated that alternative initiation of translation of FGF2 mRNA occurs at three downstream CUG codons in addition to the AUG start codon, lending strong support to that hypothesis. Thus, alternative initiation of translation resulted not only in the synthesis of the AUG-initiated isoform of FGF, but also of three CUG-initiated FGF variants that translocate to the nucleus, resulting in various biological effects. PTHrP mRNA contains four CUG codons within the secretory signal that have all the potential to serve as alternative post-translational start sites. This question has recently been tackled by Amizuka *et al*. (2000) who transfected CHO cells with a series of truncated forms of the rat PTHrP cDNA starting at the various CUG codons. They have thus shown that all of the CUG-initiated PTHrP variants were specifically localized into the nucleolus, whereas a construct containing only the AUG-initiated variant was present not only in the Golgi apparatus, but intriguingly also in the nucleolus. Therefore, it appears that the secretory signal of PTHrP is not sufficient to direct all the PTHrP molecules through the secretory pathway, resulting in some nuclear accumulation of the protein. These findings further raise the question of whether CUG-initiated PTHrP variants actually exist. Additionally, the fact that wild AUG-initiated variant was present in both the Golgi and the nucleolus strongly indicates that translocation of nascent PTHrP into the nucleus may take alternative pathways.

Finally, a third plausible mechanism for direct translocation of PTHrP to the nucleus is the retrograde translocation of the protein from the endoplasmic reticulum by specific proteins. The fact that PTHrP has been recognized as the only example of a secretory protein that undergoes proteolysis through the ubiquitin-proteosome machinery (Meerovitch *et al*., 1998) argues for such an hypothesis. It appears conceivable therefore that PTHrP, once in the endoplasmic reticulum, is translocated back to the cytoplasm by specific proteins acting as transport molecules and from there has access to the nucleus without being subjected to proteolysis. The observation that the expression of a PTHrP construct containing only the AUG initiating codon in CHO cells is targeted to both the secretory pathway and the nucleus (Amizuka *et al*., 2000) is in accordance with this possibility.

Timing and cellular functions of nuclear targeting of PTHrP

On the basis of the above findings it is apparent that PTHrP may employ several pathways, controlled at both spatial and temporal levels, to gain access to the nucleus. However, little is presently known about the events that determine the timing and degree of PTHrP nuclear translocation or the role it may serve in normal cellular function. In cultured HaCaT keratinocytes, PTHrP localized to the nucleus/nucleolus in G_1, but redistributed to the cytoplasm when cells were actively dividing. In contrast, untransfected and PTHrP-overexpressing A10 VSM cells, were reported to demonstrate PTHrP immunoreactivity in nuclei of cells which were dividing or were in the process of completing cell division (G_2 or M) (Massfelder *et al*., 1997, see also next section). Progression of eukaryotic cells through the cell cycle is often associated with a change in phosphorylation status of selected proteins and their subsequent redistribution between cytoplasmic and nuclear compartments. Accordingly, PTHrP was recently shown to be phosphorylated by cyclin-dependent kinase 2 (CDK2)/CDC2 but not by G_1-specific cyclin-CDK combinations, suggesting that PTHrP is phosphorylated in all stages of the cell cycle except G_0/G_1 (Lam *et al*., 2000). Moreover, phosphorylation negatively regulated its nuclear import which, in stark contrast to most other nuclear imported proteins, was mediated almost exclusively by the saturable transport receptor importin β and not importin α (Lam *et al*., 2000).

The nuclear target(s) of PTHrP have only recently begun to be explored. Since PTHrP localizes to the nucleolus where

ribonucleoprotein complexes form *in vivo*, Aarts *et al*. (1999a) asked whether endogenous, transfected, and *in vitro* translated PTHrP bind homopolymeric and total cellular RNAs. These authors thus demonstrated that PTHrP has the ability to bind to RNA *in vitro* through a core motif present in its NLS. These important observations document RNA binding by a secreted cellular protein and predict a role for PTHrP in regulating RNA metabolism that may be related to its localization in the nucleolus of some cells. Another interesting observation has been made by Watson *et al*. (2000). These authors demonstrated the presence of immunoreactive PTH1R within the nucleus of various tissues in a broad range of cell types, suggesting that PTHrP might act on nuclear function through binding to intranuclear PTH1R. Thus far, no studies have yet appeared describing the intranuclear/nucleolar target(s) of PTHrP.

Although the physiological implications for nuclear/nucleolar translocation of PTHrP remain to be fully defined, it would appear that it represents a mechanism by which this peptide growth factor modulates a number of cellular functions. Expression of PTHrP forms containing the NLS were reported to prolong survival of skeletal cells under conditions that promote programmed cell death (serum withdrawal) (Henderson *et al*., 1995) and stimulate proliferation of cultured VSM cells (Massfelder *et al*., 1997), as detailed in the next section. The most compelling evidence for a nuclear site of action for PTHrP, however, has come from gene targeting studies which indicate that PTHrP delays blood vessel and osteoblast invasion into hypertrophic cartilage, independent of PTH1R activation (Lanske *et al*., 1999).

From the foregoing discussion, it is becoming increasingly apparent that in order to prove a physiologic role for nuclear PTHrP signalling, it would be necessary to address the question in a relevant setting, namely the intact organism. This *in vivo* approach requires the development of an animal model which permits the selective study of a strategically placed mutation in the PTHrP gene that is not compatible with the generation of nuclear PTHrP forms while maintaining signalling through the cell surface PTH receptor. Such a genetically modified mouse strain with intact but mutated PTHrP alleles would be amenable for studies addressing the physiological consequences associated with impaired nuclear localization of PTHrP.

In summary, the effects of PTHrP were previously considered only in terms of its interaction with its N-terminal receptor. Studies now indicate that the capacity of PTHrP to influence normal cellular function must also be considered in terms of its effects at the level of the nucleus. It becomes essential, therefore, to understand the mechanism whereby PTHrP translocates to the nucleus and influences cellular function through its intracrine actions. Findings arising from these studies will have important implications for our understanding of PTHrP as a signalling molecule during embryonic and foetal development and as an oncoprotein during the progressive stages of neoplasia.

PTHrP in the cardiovascular system

Physiological and developmental actions of PTHrP

PTH has long been known to exert effects on the cardiovascular system. In the early 1900s, studies by Collip & Clark (1925) showed that systemic injection into dogs of extracts of parathyroid glands lowered systemic blood pressure. Since then numerous studies have unequivocally established the hypotensive/vasodilatory and cardiac effects of PTH (Mok *et al*., 1989). However, although the cardiovascular effects of PTH are unequivocal, their physiological significance has often been debated. This is in part because the concentrations of PTH required to produce vasodilation (1–100 nM), are substantially above those that normally circulate (low pM). It has been difficult therefore, to understand how physiological levels of this systemic hormone, which is synthesized exclusively in the parathyroid gland, could function in the local control of vascular tone. Also perplexing was the fact that patients with primary hyperparathyroidism and elevated circulating PTH levels often have high (not low) blood pressure that frequently returns to normal after parathyroidectomy. An apparent resolution for the seemingly contradictory biology of PTH on the cardiovascular system emerged with the discovery of PTHrP in 1987.

Studies over the last decade have begun to shed light on normal functions of PTHrP (Philbrick *et al*., 1996). In addition to its developmental roles, PTHrP is produced in abundance in smooth muscle where the protein functions to regulate contractility and proliferation. PTHrP is expressed in blood vessels in essentially all vascular beds from a wide range of species. The protein is produced predominantly in the smooth muscle layer of the vessel although its expression has also been reported in cultured endothelial cells (Rian *et al*., 1994; Ishikawa *et al*., 1994a). The sharp induction and rapid disappearance of PTHrP mRNA in association with serum stimulation of VSM cell proliferation is reminiscent of the behavior of cytokine and immediate early genes (Hongo *et al*., 1991). Among the most potent inducers of PTHrP are vasoconstrictors including angiotensin II, serotonin, endothelin, noradrenaline, bradykinin and thrombin; each of these agents induce PTHrP mRNA and protein levels over an identical time course as that observed for serum (Pirola *et al*., 1993). PTHrP is also induced in VSM in response to mechanical stimuli. PTHrP mRNA is transiently increased in rat aorta following distension with a balloon catheter (Daifotis *et al*., 1992). Flow motion-induced mechanical events induced by rocking or rotation of monolayer cultures of rat aortic VSM cells result in increased PTHrP mRNA expression (Pirola *et al*., 1994). As indicated above, PTHrP has been shown to replicate the vasorelaxant activity of PTH in many vascular beds, including heart (Nickols *et al*., 1989), kidney (Musso *et al*., 1989), placenta (Macgill *et al*., 1997; Mandsager *et al*., 1994) and mammary gland (Prosser *et al*., 1994). PTHrP appears to exert its vasodilatory actions by activating the PTH1R. This receptor is expressed in rat VSM beds (Nickols *et al*., 1990) and relaxation of aortic preparations is accompanied by an increased accumulation of cyclic AMP (Ishikawa *et al*., 1994b). Rat aortic VSM cells also express the PTH1R and respond to N-terminal PTHrP peptide fragments with an increase in cyclic AMP formation (Wu *et al*., 1993). Moreover, relaxation responses to PTH in aortic strip preparations are potentiated by phosphodiesterase inhibitors and forskolin (Nickols & Cline, 1987). Finally, stimulation of cyclic AMP-dependent protein kinase is associated with a reduction in cytoplasmic calcium and attenuated myosin light chain kinase activity (McDaniel *et al*., 1994).

In addition to its effects on vascular tone, PTHrP also modulates VSM cell proliferation. The peptide attenuates

serum and platelet-derived growth factor-activated DNA synthesis in primary arterial vascular smooth muscle cells (Hongo *et al*., 1991; Jiang *et al*., 1995) and in A10 VSM cells stably expressing the PTH1R (Stuart *et al*., 2000). In both of these cell types, the antimitogenic effects require the PTH-like N-terminal portion of the molecule and are mimicked by dibutyryl cyclic AMP or forskolin. The mechanism for the antiproliferative effect of PTHrP involves the induction of the cyclin dependent kinase inhibitor $p27^{kip1}$, and impairment of the retinoblastoma gene product (Rb) which results in cell cycle arrest in mid G1 phase. Cellular levels of PTHrP fluctuate during the cell cycle and reach their highest levels in G2/M) (Okano *et al*., 1995). PTHrP also inhibits platelet-derived growth factor (PDGF)-directed migration of VSM cells *in vitro* (Ishikawa *et al*., 1998). The effects on VSM cell growth and migration *in vitro* are likely to be relevant to conditions under which VSM cell growth and migratory behaviour is altered *in vivo*.

The ability of PTHrP to modulate VSM cell growth suggested that the protein might function during the development of the cardiovascular system. Although the cardiovascular system appears to develop normally in the PTHrP knockout mouse, homologous deletion of the PTH1R results in a higher incidence in early foetal death at midgestation, coincident with the development of the heart and major blood vessels (Lanske *et al*., 1996). Moreover, transgenic mice expressing high levels of PTHrP and PTH1R in VSM, created by intercrossing the ligand and receptor overexpressing mice, die at day E9.5 with severe thinning of the ventricle and disruption of ventricular trabeculae (Qian *et al*., 1999). Mice with targeted overexpression of either PTHrP or PTH1R in smooth muscle have reduced sytemic blood pressure consistent with the prediction that PTHrP acts as a local vasodilator (Qian *et al*., 1999). In aortic ring preparations from the PTHrP overexpressing mice the relaxant effects of both PTHrP and acetylcholine were markedly attenuated. This suggests that local overexpression of PTHrP not only desensitizes the aviculture to PTHrP but also dampens relaxation to acetylcholine and perhaps other vasorelaxants. Thus, it appears that prolonged stimulation of the PTH1R and the consequent increase in cyclic AMP converge on signalling circuitry used by acetylcholine. As mentioned above, additional evidence for a role of PTHrP in heart and vascular development came from genetic disorders of PTH1R.

From the studies outlined herein, it is possible to construct a simple model for the mode of PTHrP action in VSM. In response to mitogenic, vasoconstrictor or mechanical signals, PTHrP is released and acts locally *via* a short feed back loop to activate the PTH1R and stimulate cyclic AMP in adjacent cells. Signalling pathways downstream of cyclic AMP impact on specific sets of genes which function to oppose the pressor (excitation/contraction coupling) and mitogenic (cell cycle) events. As mentioned above, induction of $p27^{kip1}$ with consequent inhibition of Rb phosphorylation would represent one such target for cyclic AMP induced cell cycle arrest.

Nuclear targeting of PTHrP and vascular smooth muscle cell proliferation

Studies of Massfelder *et al*. (1997) have proven that the ability of PTHrP to influence VSM cell proliferation may depend on where PTHrP is trafficked by the cell. In these studies, stable transfection of A10 cells with full-length PTHrP caused a marked increase of cell proliferation and nuclear localization of PTHrP, especially in cells that were undergoing cell division. In accordance with previous studies in chondrocytes (Henderson *et al*., 1995), a nuclear targeting motif, in the 88–106 region, was shown to be required for nuclear localization. Furthermore, nuclear localization was required for the acceleration of both cell cycle and cell proliferation. Among various N-terminal, mid-region or C-terminal fragments, only the N-terminal fragments added to wild-type A10 VSM cells exhibited antimitogenic effects which decreased with PTHrP length. Also, exposure of wild-type A10 cells to the secretory products of PTHrP-overexpressing A10 cells, failed to affect VSM cell proliferation. Overall, these results indicate that in A10 VSM cells overexpressing PTHrP, nascent PTHrP did stimulate proliferation through an intracrine pathway involving translocation of the peptide to the nucleus, to the exclusion of the classical paracrine pathway.

Such paradoxical effects of PTHrP have been observed in non-clonal cells as well. Indeed, in rat VSM cells cultured from intrarenal small arteries, PTHrP inhibits proliferation through an PTH1R-dependent mechanism and stimulates proliferation through an intracrine pathway which presumably involves nucleolar translocation of the peptide (Massfelder *et al*., 2001). Importantly, in these studies the paradoxical effects of PTHrP on cell proliferation were reversed in renal VSM cells cultured from isolated intrarenal arteries of spontaneously hypertensive rats (SHR) with established hypertension, i.e. PTH1R-dependent stimulation and intracrine inhibition of cell proliferation. The reversal of the paracrine proliferative effect was shown to be due to preferential coupling of the PTH1R to the Gi regulatory protein in SHR-derived renal VSM cells. The mechanism by which the growth stimulatory action of endogenous PTHrP was converted into an inhibitory effect in SHR-derived renal VSM cells remains unclear but presumably reflects a strain difference in the trophic effects induced by the translocation of the peptide into the nucleolus. It is therefore tempting to speculate that PTHrP may be involved in some of these events affecting different pathways between normotensive and genetically hypertensive rats. The response to this crucial question will undoubtedly be obtained from studies aiming at determining directly the molecular target(s) of PTHrP within the nucleolus of the VSM cell. Collectively, the findings of Massfelder *et al*. (2001) indicate that PTHrP might play, *via* an intracrine process, a beneficial role as a negative feedback regulator of renal vascular wall hyperplasia which contributes to the progression of the hypertensive state in the SHR model of genetic hypertension. Additionally, a new concept emerges from these results, according to which a single molecule may have opposite effects on VSM proliferation under physiological and pathophysiological conditions. This concept predicts a role for PTHrP in regulating vascular wall remodelling that may be related to its localization in the nucleus/nucleolus *in vivo*.

In other respects, VSM cell proliferation and migration into the lumen to form a neointima are responsible for the late complication following angioplasty of coronary and other arteries in subjects with atherosclerosis. PTHrP is upregulated in the media and neointima of angioplastied arteries (Ozeki *et al*., 1996), as well as in atherosclerotic,

partially occluded human arteries removed at the time of coronary artery bypass grafting (Nakayama *et al*., 1994). These events suggest that PTHrP may play a role, either stimulatory or contributory, in the pathogenesis of neointimal proliferation, migration, and restenosis following angioplasy. The observation that vascular proliferation rates are diminished in aortic smooth muscle of the PTHrP knockout mouse suggest that PTHrP is important in foetal smooth muscle proliferation as well (Massfelder *et al*., 1997).

These observations raise a number of questions. For instance, what are the regions of PTHrP, in addition to the NLS, which are required for nuclear targeting in VSM cells? While the NLS is required for nuclear entry, what regions of PTHrP are required for activation of the cell cycle once nuclear entry is achieved? That is, is the NLS alone capable of activating the cell cycle in vascular smooth muscle, or are there other regions of the molecule which are required? What are the nuclear partners for PTHrP once it enters the nucleus? Is it a transcription factor interacting with DNA, an RNA regulatory protein or does it interact with other nuclear proteins? Finally, is there a therapeutic opportunity for PTHrP in downregulating VSM proliferation, neointimal formation and restenosis following angioplasty or by preventing vascular hyperplasia? Recent findings obtained from the use of a variety of deletion mutants of PTHrP, lacking the signal peptide, the N-terminal, mid-region, NLS or C-terminal region, confirm that the NLS is necessary and sufficient for nuclear localization and that the C-terminal region of the molecule is required for activation of proliferation (deMiguel *et al*., 2000). These findings suggest that a more complete mapping will define nuclear actions, localization, and help to determine the nuclear partners of PTHrP, whether they be protein, DNA or RNA. In addition, the development of PTHrP constructs which can inhibit proliferation, and the development of such inhibitory molecules in viral gene delivery systems, may have therapeutic potential in limiting the rate of vascular restenosis following angioplasty (deMiguel *et al*., 2000).

PTHrP and the heart

PTHrP has been found in the myocardium (Deftos *et al*., 1993). The highest levels of expression were found in atria and vessels (Burton *et al*., 1994). Cardiomyocytes from the atrium have been shown to express and secrete PTHrP (Burton *et al*., 1994). In ventricular myocardium, however, the average level of its expression is lower. Moreover, ventricular cardiomyocytes do not express PTHrP (Schlüter *et al*., 2000). This expression pattern resembles that of atrial natriuretic peptide (ANF) which suggests a similarity in transcriptional regulation between ANF and PTHrP. PTHrP is abundantly expressed in coronary endothelial cells, another constituent cell type of myocardial tissue, neighbouring cardiomyocytes. Expression of PTHrP was found also in other types of endothelial cells, including those isolated from human umbilical veins (Rian *et al*., 1994), in simian virus 40-T-antigen-transformed rat lung vascular endothelial cells (Jiang *et al*., 1998) and the endothelium of both renal (Massfelder *et al*., 1996b) and myometrial-associated (Ferguson *et al*., 1998) vasculatures. Since endothelial cells can express the PTH1R (Nickols *et al*., 1990), they may represent targets for PTHrP. Thus, PTHrP seems to represent a possible autocrine/paracrine factor in the ventricle. The regulation of ventricular PTHrP expression remains to be elucidated. Angiotensin II was shown to induce the expression of PTHrP in smooth muscle cells (Pirola *et al*., 1993). Since angiotensin II seems to be a major player in the progression of myocardial hypertrophy one may speculate that angiotensin II also induces the ventricular expression of PTHrP, which may then act as a pro-hypertrophic cofactor. Evidence for a pivotal role for PTHrP in the progression of myocardial hypertrophy comes from experiments on isolated cardiomyocytes. In these cells, PTHrP exerts a hypertrophic effect which includes an activation of protein kinase C and the early response kinase pathway, leading to an acceleration of protein synthesis and re-expression of foetal type proteins (Schlüter *et al*., 1997). These effects of PTHrP are mediated through a protein kinase C-activating domain distinct from that used by PTH, i.e. the C-terminal located amino acids 107–111.

Clear evidence has been provided by several laboratories that many cardiac effects of PTHrP can not be mimicked by PTH, which normally shares the structural and functional aspects with PTHrP. For example, PTHrP is able to activate the adenylate cyclase on ventricular cardiomyocytes and subsequently exerts a positive inotropic effect (Nickols *et al*., 1989; Schlüter *et al*., 1997). PTH, however, neither increases the contraction amplitude of isolated cardiomyocytes, nor does it exert a positive inotropic effect on isolated perfused rat hearts or ventricular preparations (Nickols *et al*., 1989; Ogino *et al*., 1995). This suggests an unusual structure–function–relationship for PTHrP on ventricular cardiomyocytes. In line with this observation, PTH(1-34), but not PTHrP(1-34), activates protein kinase C in ventricular cardiomyocytes. The difference between both peptide hormones could be explained by a structural difference at position 29, where alanine replaces glutamine in the PTHrP molecule (Schlüter *et al*., 1996). These two findings suggest either the expression of a different type of PTHrP receptor or an unusual receptor–signalling–coupling in this cell type. Different responsiveness to PTHrP and PTH has also been found in experiments focusing on the PTHrP effect on coronary flow. Thus, Nickols *et al*. (1989) found that PTHrP was more potent than PTH to increase coronary flow on isolated perfused rat hearts. However, other cardiovascular effects of PTHrP, i.e. its positive chronotropic effect, can be mimicked by PTH.

With regard to the cardiovascular effects of PTHrP described above, there is still discussion in the literature concerning their significance in *in vivo* conditions. For example, a positive inotropic effect of PTHrP could not be demonstrated in working heart preparations under experimental conditions in which the coronary flow was held maximally dilated by NO-donors (Ogino *et al*., 1995). This observation strongly suggests that the positive inotropic effect of PTHrP is mainly due to coronary vasodilation or positive chronotropic effect of PTHrP or, alternatively, indicate a cross-talk between the cardiac effects evoked by NO and PTHrP. To answer these questions one needs additional *in vivo* experiments, probably on other species as well, in which coronary flow and beating frequency will be held constant during the application of PTHrP.

The expression of PTHrP by coronary endothelial and smooth muscle cells raises the question as to whether PTHrP

released by these cells affects cardiac functions. Under basal and static culture conditions coronary endothelial cells do not release PTHrP (Schlüter *et al*., 2000). They do so, however, under energy depleting conditions. Moreover, PTHrP is released from hypoxic perfused hearts. Both findings suggest that the release of PTHrP from hypoxic perfused hearts is due at least in part to a release from energy depleted endothelial cells. The mechanisms by which energy depletion provokes the release of PTHrP needs further analysis. A related question is which forms of PTHrP are secreted by coronary endothelial cells? The biochemical characterization of endothelial derived PTHrP revealed that PTHrP is glycosylated. Glycosylation has also been reported for PTHrP secreted from epidermal keratinocytes (Wu *et al*., 1991). Therefore further studies are required to characterize the cardiac activity of authentic glycosylated PTHrP in comparison to synthetic PTHrP without such modifications.

In conclusion, PTHrP seems to be an important regulator of cardiac performance. This conclusion is based on its cardiac effects, i.e. its positive chronotropic and inotropic effect and its effect on coronary flow. These effects are, at least in part, distinct from those achieved by PTH. Therefore, the physiological effects of PTHrP on the heart are specific for the PTHrP and distinct from other PTHrP/PTH targets.

Physiological and developmental actions of PTHrP in the kidney

Expression and physiological actions in the glomerulo-vascular system

The glomerulo-vascular system plays a key role in the maintenance of volume homeostasis by the kidney. The formation of the glomerular filtrate is controlled by resistance changes in the pre- and postglomerular vasculature. Results accumulated over the last 14 years, strongly suggest that PTHrP belongs to the vasoactive factors which regulate renal blood flow and glomerular filtration rate through changes in renal vascular tone and renin release.

First evidence for renal expression of PTHrP transcript came from the studies of Ikeda *et al*. (1988) and Soifer *et al*. (1993). PTHrP protein and mRNA have also been detected in the renal cortex (Largo *et al*., 1999), in glomeruli (Massfelder *et al*., 1996b; Yang *et al*., 1997), in renal VSM and endothelial cells and in podocytes, but not in glomerular endothelial and mesangial cells (Largo *et al*., 1999; Massfelder *et al*., 1996b; Soifer *et al*., 1993). Cultured renal VSM cells derived from rat preglomerular small arteries continue to express PTHrP mRNA and protein in culture (Endlich *et al*., 2001; Massfelder *et al*., 2001).

Early studies have demonstrated the presence of common binding sites for PTH(1-34) and PTHrP(1-34) in renal arterioles (Nickols *et al*., 1990). The expression of several splice variants of the PTH1R gene in the whole kidney has been demonstrated by Urena *et al*. (1993). The expression of PTH1R is driven by two promoters in the mouse (P1 and P2) and by three promoters in humans (P1 to P3), among which P1 is active in the kidney only (McCuaig *et al*., 1995). Moreover, splice variants of the PTH1R, lacking the signal peptide and the first exon, have been detected in rat and human kidney (Jobert *et al*., 1996; Joun *et al*., 1997). PTH1R mRNA has also been found in glomeruli (Yang *et al*., 1997). However, in agreement with the *in vivo* situation, PTH1R expression could not be detected in cultured mesangial cells (Bosch *et al*., 1999; Lee *et al*., 1996). In mouse renal vessels and podocytes, PTH1R gene is transcribed exclusively by use of the kidney-specific P1 receptor (Amizuka *et al*., 1997; Lee *et al*., 1996). Thus, PTH1R gene transcription in the glomerulo-vascular system could be adjusted independently from other organs. The PTH1R is also expressed in podocytes and endothelial cells of peritubular capillaries (Amizuka *et al*., 1997). Renal VSM cells and podocytes continue to express the PTH1R in culture (Endlich *et al*., 2001; Lee *et al*., 1996; Massfelder *et al*., 2001).

That PTHrP(1-36) is a vasodilatory factor was first demonstrated in the isolated renal artery (Winquist *et al*., 1987), in the isolated perfused kidney (Musso *et al*., 1989), and in isolated renal arterioles (Trizna & Edwards, 1991). In the split hydronephrotic rat kidney model, which allows the *in situ* visualization of intrarenal arteries, PTHrP(1-36) has been proven to dilate the preglomerular vessels and to augment glomerular blood flow (Endlich *et al*., 1995). In normal anesthetized rats, intrarenal infusion of PTHrP(1-36) increased renal blood flow, glomerular filtration rate and diuresis (Massfelder *et al*., 1996a). The vascular effects of PTHrP(1-34) have also been investigated in humans (Wolzt *et al*., 1997). At doses which did not affect systemic blood pressure, PTHrP(1-34) increased renal plasma flow, by about 40%. Thus, PTHrP(1-36) is among the most potent renal vasodilators in humans. PTHrP(1-36) and PTH(1-34) have been reported to induce renal vasodilation with similar potency (Endlich *et al*., 1995; Massfelder *et al*., 1996b; Musso *et al*., 1989; Winquist *et al*., 1987). In some of these studies, the addition of N-terminally truncated PTHrP and PTH fragments antagonized the renovascular effects of PTHrP(1-36). Thus, PTHrP(1-36) acts on the renal vasculature through the PTH1R. In terms of the signalling pathway of the PTH1R, it has been shown that PTHrP(1-36) stimulates cyclic AMP production in isolated renal microvessels (Musso *et al*., 1989). Furthermore, studies in the isolated perfused rabbit kidney have implicated both cyclic AMP and nitric oxide of non-endothelial origin in the vasodilating action of PTHrP(1-36) (Massfelder *et al*., 1996b).

Despite the potent vasodilating properties of PTHrP(1-36), its physiological function in the renal vasculature has remained unclear. PTH1R antagonists neither affected baseline renal blood flow in the anaesthetized rat nor in the isolated perfused kidney of normo- as well as hypertensive rats PTHrP, suggesting that PTHrP does not influence the tone of renal vessels under baseline conditions (Fiaschi-Taesch *et al*., 1998; Massfelder *et al*., 1996a). On the other hand, the observed reduction of systemic blood pressure in transgenic mice which overexpress PTHrP in smooth muscle, suggests that PTHrP is capable of reducing vascular resistance (Maeda *et al*., 1999). Consequently, one could image that under certain pathophysiological situations, intrarenal PTHrP concentration might be high enough to cause renal vasodilation. Several pathophysiological states are known to be accompanied by increased PTHrP expression. Renal ischemia, cyclosporine treatment, and a model of tubular interstitial damage have been found to increase tubular PTHrP (Garcia-Ocana *et al*., 1998; Largo *et al*., 1999; Soifer *et al*., 1993), which could act on neighbouring vessels.

Enhanced PTHrP expression and prolonged survival after PTHrP blockade has been reported in endotoxic shock (Funk *et al*., 1996). Furthermore, PTHrP might be involved in glomerular hyperfiltration in diabetes and in compensatory glomerular hyperfiltration due to nephron loss. Finally, PTHrP is known to be upregulated in the aorta by vasoconstrictors (Hongo *et al*., 1991; Pirola *et al*., 1993), in response to mechanical stress (Noda *et al*., 1994), in hypertensive animals (Noda *et al*., 1997), and after vessel injury (Ozeki *et al*., 1996; Pirola *et al*., 1994). It should be stressed however that the expression of the PTH1R have not been explored in these studies. Therefore, the possibility exists that downregulation of PTH1R is responsible for the absence of the vasodilator action of endogenous PTHrP. Alternatively, PTHrP might predominantly regulate VSM cell proliferation (see the above section) instead of VSM tone. Thus, the precise function of PTHrP in the renal vasculature remains elusive.

PTHrP(1-36) has been shown to induce renin secretion from isolated perfused rat kidney, directly from juxtaglomerular cells (Saussine *et al*., 1993). In these studies PTH displayed lower potency than PTHrP, raising the question as to whether PTHrP triggers the release of renin *via* binding to the PTH1R. The signalling pathway of PTHrP in juxtaglomerular cells has not fully been explored. The so-called 'calcium paradox' for renin release predicting that a decrease, and not an increase of cytosolic calcium triggers the release of renin from juxtaglomerular cells has been proven to be true for PTHrP-induced renin release as well (Saussine *et al*., 1993). Since juxtaglomerular cells are myoepithelial cells in nature, it can be hypothesized that the signalling pathways of PTHrP are similar in juxtaglomerular and VSM cells. Moreover, increased cyclic AMP production is well recognized as the common intracellular signal for renin secretion in juxtaglomerular cells. Thus, PTHrP could be a paracrine and/or autocrine modulator of renin release. Of note, the macula densa, which participates in the control of renin secretion, has been shown to express PTHrP (Massfelder *et al*., 1996b; Yang *et al*., 1997). Since vasoconstrictors and mechanical stress upregulate PTHrP expression (Hongo *et al*., 1991; Noda *et al*., 1994; Pirola *et al*., 1993), PTHrP could limit vasoconstrictor- and mechanical force-mediated renin suppression.

As mentioned above, under physiological condition mesangial cells neither express PTHrP nor the PTH1R and the only source of PTHrP in the glomerulus appears to be podocytes. However, PTHrP(1-34) has been reported to relax mesangial cells in culture, associated with a decrease in myosin light-chain phosphorylation (Bosch *et al*., 1999). Furthermore, mesangial cells proliferate in response to PTHrP(1-34) (Bosch *et al*., 1999; Soifer *et al*., 1993). The signalling pathway of PTHrP(1-34) in mesangial cells has not been examined. Since the PTH1R could not be detected in mesangial cells, it has been suggested that mesangial cells possess a novel PTHrP receptor (Bosch *et al*., 1999). Concerning the role of PTHrP in mesangial cells, Largo *et al*. (1999) have recently shown that the mesangium starts to express PTHrP at a rather high level in an experimental model of tubular interstitial damage. Therefore, PTHrP could be involved in mesangial proliferation in glomerulosclerosis.

Podocytes express both the PTH1R and PTHrP. Thus, PTHrP appears as an autocrine hormone in podocytes. Earlier studies showed that PTHrP(1-34) stimulates cyclic AMP production in isolated glomeruli (Massfelder *et al*., 1993). These findings indicated that PTHrP might activate the cyclic AMP pathway in podocytes. Recent findings, obtained in a podocyte cell line, demonstrate clearly that PTHrP(1-36) *via* the PTH1R activates the cyclic AMP pathway, but does not increase intracellular Ca^{2+} concentration (Endlich *et al*., 2001). This podocyte cell line, which expresses both PTHrP and the PTH1R, might be a valuable model to progress towards understanding PTHrP function in podocytes. While several conditions, i.e. ischemia, cyclosporine treatment, and tubular interstitial damage, increase PTHrP expression in tubules, nothing is known about stimuli that alter PTHrP levels in podocytes.

Developmental actions of PTHrP in the kidney

In mammals, renal development proceeds in three stages. The first two stages lead to the formation of transient structures, the pronephros and the mesonephros, while the third stage gives rise to the metanephros, which is the permanent kidney. Although some experiments indicate that PTHrP works as a growth factor for primary renal cells *in vitro*, the role of the PTHrP system in the developing kidney *in vivo* has received little attention. During mesonephros development, PTHrP protein and mRNA have been shown to be expressed in the mesonephric duct and in developing mesonephric tubules in human (Burton *et al*., 1990; Moseley *et al*., 1991), as well as in rat (Campos *et al*., 1991). In humans, there is some discrepancy regarding the expression of immunoreactive PTHrP in developing mesonephric glomeruli. In the rat, however, *in situ* hybridization studies clearly demonstrate the absence of PTHrP mRNA in developing mesonephric glomeruli. On the other hand, abundant expression of PTHrP protein or mRNA in the collecting duct system and in the developing tubules has been proven in metanephros development. Strong expression of PTHrP mRNA in the developing rat glomeruli has also been reported. After birth, in rat, PTHrP mRNA is still expressed in the tubules, but at much lower levels, while no signal could be detected in both glomeruli and collecting duct system.

The expression of the PTH1R has also been studied in rat developing kidney by *in situ* hybridization (Campos *et al*., 1991). During mesonephros development PTH1R has been shown to be expressed in mesonephric blastema-derived from developing tubules, but not in the Wolffian duct. During the development of the metanephros, the mature tubules and glomeruli were strongly labelled. On the other hand, no signal could be detected in the nephrogenic area, as well as in the collecting duct system. The same pattern of expression persisted in the adult kidney, where PTH1R mRNA was expressed in both tubular and glomerular structures, especially in the macula densa, but not in the collecting duct system. In the glomeruli, the labeling was distributed in the region corresponding to the localization of podocytes and of parietal epithelial cells, while mesangial cells were not stained. The recent study performed by Aya *et al*. (1999) during mouse kidney maturation are in agreement with these findings. In these studies PTHrP mRNA was abundantly expressed in the collecting duct, urothelium of the pelvis, as well as in immature elements including the S-shaped body, the ureteric bud and glomerulus. By contrast, the expression

of PTH1R mRNA was markedly lower during maturation than after the completion of the maturation process. Thus, PTH1R mRNA was not detected in the collecting duct, urothelium of the pelvis, or nephrogenic area in embryonic day 16 or at birth.

It therefore appears that the expression of PTHrP localizes preferentially in the nephrogenic area and in differentiating mesenchyme, while PTH1R is mainly localized in mature structures with the exception of the collecting ducts. Collectively, these data suggest that during renal development, PTHrP could act through pathways which do not involve the PTH1R.

PTHrP in pancreatic islets: transgenic and viral approaches to increasing pancreatic beta cell mass in mammals

For years, many investigators believed that pancreatic beta cells were terminally differentiated and unable to replicate. It is now very clear that this is not accurate. Indeed, replication rates in pancreatic beta cells under normal circumstances are such that in any given 24 h period, approximately 2–3% of beta cells divide (Finegood *et al*., 1995; Scaglia *et al*., 1995). Moreover, rates of pancreatic beta cell division can be upregulated by physiological conditions which raise the blood glucose concentration and which therefore require additional insulin production. For example, during pregnancy, pancreatic beta cell mass increases by a factor of two to three in response to the insulin resistance induced by pregnancy. Similarly, increased caloric intake, weight gain, and subtotal pancreatectomy all induce a rapid upregulation in the normal beta cell replication rate (Bonner-Weir *et al*., 1989; Finegood *et al*., 1995; Scaglia *et al*., 1995). These events have developmental and physiological significance. Perhaps more interestingly, they have therapeutic implications as well, in the sense that if the replication rate of pancreatic beta cells could be accelerated, then pancreatic beta cell mass could in theory be increased, and this might prove to be useful in strategies designed to treat diabetes mellitus.

At present, pancreatic islet transplantation for diabetes is used infrequently, primarily because of a lack of a large supply of human pancreatic islets for transplantation, and also because when pancreatic islets are transplanted, their survival is relatively brief (Robertson *et al*., 1998; Slover & Eisenbarth, 1997). This is true despite sufficient immunosuppression to permit survival of an accompanying kidney transplant. These findings suggest that strategies designed to increase beta cell replication *in vitro* or *in vivo* may prolong the survival of pancreatic islet transplants, and may therefore be helpful in the long-term management of patients with diabetes.

Parathyroid hormone-related protein (PTHrP) is normally produced in the pancreatic beta cell, and also induces intracellular calcium responses in cultured beta cells, indicating that these cells may have some sort of paracrine/autocrine physiologic role involving PTHrP (Gaich *et al*., 1993). In order to determine what that role might be, we developed transgenic mice in which PTHrP was targeted to, and overexpressed in, the pancreatic beta cell under the control of the rat insulin promoter (RIP) (Vasavada *et al*., 1996; Porter *et al*., 1998). These RIP–PTHrP mice display a progressive, life-long increase in pancreatic islet mass, islet number and beta cell number, a combination of events which leads to insulin-induced hypoglycemia. This increase in islet mass is not due to an acceleration of replication (Porter *et al*., 1998), but instead appears to be due to a reduction in beta cell death rates (Vasavada *et al*., 2000).

The RIP–PTHrP mouse is the first example in which an islet-targeted growth factor has been demonstrated to augment islet mass and function. These studies prompted the search for other growth factors which might be able to augment islet mass and function. *In vitro* studies in other laboratories demonstrated that hepatocyte growth factor (HGF) may be important in islet proliferation during foetal development, and that it is a potent beta cell mitogen when added to cultures of neonatal rat islets (Otonkoski *et al*., 1996). With these thoughts in mind, we developed three lines of RIP–HGF mice using the same strategy described in the preceding paragraph. As predicted, like the RIP–PTHrP mice, the RIP–HGF mice displayed an increase in islet number and islet mass, which collectively result in insulin-mediated hypoglycemia (Garcia-Ocana *et al*., 2000). Interestingly, unlike the RIP–PTHrP mice in which 'beta cell hyperplasia' appears to result from reduction in beta cell death rates, islet hyperplasia in the RIP–HGF mice is associated with a 2–3-fold increase in beta cell proliferation rates, and thus serves as a beta cell mitogen *in vivo* (Garcia-Ocana *et al*., 2000).

A third potential islet growth factor is placental lactogen, which has been suggested to play a role in upregulating beta cell mass during pregnancy, and which has been shown to drive beta cell mitogenesis in short-term tissue culture (Sorenson & Brelje, 1997). Using a similar strategy to that described above, we developed RIP–mPL-1 mice which overexpress murine placental lactogen-1 (mPL-1) in the beta cell. As with the other two models described above, the RIP–mPL-1 mice also demonstrate an increase in islet mass and number, associated with accelerated proliferation (Vasavada *et al*., 2000). As with the other two models, increased insulin production results in hypoglycemia (Vasavada *et al*., 2000).

These three models represent the only three examples of transgenic delivery of islet targeted growth factors which are able to augment islet mass and result in hypoglycemia. Interestingly, while the end result is the same in each of the three models (increased numbers and size of islets, and resultant insulin-mediated hypoglycemia), the mechanisms through which they arrive at this phenotype differ: the RIP–PTHrP mouse appears to result from accumulation of beta cells with a prolonged life span, without identifiable increases in beta cell proliferation. In contrast, both the RIP–HGF and RIP–mPL-1 mice develop increased islet mass and number as a result of increased beta cell proliferation rates. Moreover, the cellular signalling pathways involved are likely to differ significantly: PTHrP typically acts *via* G-coupled protein receptors to activate cyclic AMP or PKC/PLC/intracellular calcium pathways and nuclear targeting pathways; HGF acts in most tissues *via* MAP kinase and JAK–STAT pathways; and, PL acts *via* cytokine family receptors coupled to JAK2/STAT5. These findings suggest that the effects of the three peptides may be additive or synergistic, a possibility which remains unexplored.

These findings in transgenic mice suggest that these three growth factors may be useful in augmenting human islet mass

and function, if suitable means of delivering them to the pancreatic islet could be defined. Preliminary studies suggest that the three growth factors can indeed be delivered to the pancreatic beta cell using viral gene delivery strategies (Takane *et al*., 2000). It remains to be defined as to whether these approaches can be implemented to enhance the mass and/or function of human islets either *in vitro* prior to transplant, or *in vivo* following transplant. These areas merit further study.

Physiological role of PTHrP in placenta and interaction with Ca^{2+}-sensing receptors in placental trophoblast

Parathyroid hormone-related protein (PTHrP) and the calcium-sensing receptor (CaSR) were sought and discovered primarily because of their imputed significance in calcium ion homeostasis. The molecular cloning and characterization of these proteins has opened new avenues to the understanding of their physiological functions as well as into pathological ramifications arising from gene mutations conferring inactivating or activating mutations on the respective protein. However, it was not anticipated that these two proteins would be so widely expressed in tissues that are not involved in mineral homeostasis and that they would come to be appreciated as having functions that are unrelated to extracellular ion constancy. Indeed, most functions of PTHrP have little to do with calcium homeostasis (Wysolmerski & Stewart, 1998), and the CaSR is now recognized to be involved in regulating a variety of cellular processes ranging from hormone secretion, modulation of ion and water channel activity, chemotaxis, and most relevant for the present review, cellular proliferation and differentiation (Squires, 2000).

Physiological roles of PTHrP in the placenta

As mentioned in the Introduction, post-translational processing of the initial translation products gives rise to a family of mature secretory forms of PTHrP, an N-terminal peptide, a mid-region molecule, and a C-terminal protein (Figure 2). Emerging evidence supports roles for N-terminal and mid-region peptide effects on the placenta. Putative functions of PTHrP during pregnancy include uterine smooth muscle relaxation and vasodilatation (Curtis *et al*., 2000; Macgill *et al*., 1997), regulation of placental calcium transfer (Kovacs & Kronenberg, 1997), and normal foetal development. N-terminal forms of PTHrP inhibit acetylcholine-induced uterine contractions in the rat (Barri *et al*., 1992). Levels of PTHrP decreased acutely in the amnion and myometrium at the time of onset of labour in humans (Ferguson *et al*., 1992) suggesting that N-terminal PTHrP may have a role in regulating the onset of labour. Contradictory evidence failed to disclose a change in the expression of PTHrP mRNA or protein with term labour or rupture of the foetal membranes in humans (Curtis *et al*., 2000). Rather, upregulation of mRNA and protein in foetal membranes at term suggests an important role in late pregnancy. This role may be related to the second and more well-established function of PTHrP to stimulate placental calcium transport.

Calcium concentrations are generally maintained at higher levels in foetal than in maternal circulation and both PTH and PTHrP play major roles in this action (Kovacs & Kronenberg, 1997). PTHrP exerts a dominant role in placental calcium transport. This has been demonstrated most convincingly in ewes and mice. Parathyroidectomy in foetal lambs eliminates this gradient. Infusion of PTH or PTHrP(1-34) into the foetal circulation does not restore the gradient or correct the foetal hypocalcemia (Care *et al*., 1990). In contrast, infusion of PTHrP that includes the mid-region residues 35–86, e.g., PTHrP(1–86), (1–108), or (1–141) restores the placental calcium gradient, suggesting that the mid-region of PTHrP, establishes and maintains the calcium gradient. The infusion of mid-regions peptides PTHrP(67–86) and, to a lesser extent, PTHrP(75–86) directly into the foetal circulation also stimulates placental calcium transport.

The most trenchant observations establishing the role of PTHrP in regulating placenta calcium come from the work of Kovacs (Kovacs & Kronenberg, 1997), who reported that placental calcium transport is impaired and serum calcium levels are low in PTHrP–null mice as compared with their heterozygous or normal littermates. *In utero* intraperitoneal infusion of PTHrP(1–86) or PTHrP(67–86) stimulated placental calcium transport and restored the foetal–maternal calcium gradient by elevating foetal serum calcium. In contrast, injection of the foetal mice with various PTH peptides or with N-terminal PTHrP(1–34) had no effect on the calcium gradient. These findings confirm the importance of PTHrP in foetal calcium homeostasis and in placental calcium transport and unequivocally establish the importance of mid-region PTHrP in mediating this process. Further insight into the role of mid-region PTHrP in regulating placental calcium transport was obtained by taking the corollary approach, viz., studying mice lacking the PTH1R. As in the case of the PTHrP null animals, the receptor-null foetuses were hypocalcemic. These findings emphasize the importance of mid-region PTHrP in placental calcium transport and are compatible with the view that a distinct mid-region PTHrP receptor mediates these effects. Overall, these findings support the view that foetal serum calcium regulates the circulating level of foetal PTHrP (Abbas *et al*., 1994). As will be discussed below, preliminary evidence suggests that the CaSR may mediate the effects of ambient calcium concentration on PTHrP secretion by trophoblasts.

A third important role of PTHrP is on growth and differentiation. Although PTHrP has profound actions on the growth and differentiation of bone, mammary gland, and several other tissues (Strewler, 2000; Wysolmerski & Stewart, 1998), less is known of its effects on gestational tissues. PTHrP is expressed in many human and murine foetal and gestational tissues including amnion, placenta, and myometrium (Curtis *et al*., 2000; Ferguson *et al*., 1992). Within the placenta, PTHrP is expressed by placental syncytial trophoblasts (Curtis *et al*., 2000).

PTHrP and Ca^{2+}-sensing receptor: effects on trophoblast growth and differentiation

Trophoblasts are the parenchymal cells of the placenta. They form part of a multilineage that arises from progenitor stem cells. In mice, the trophoblast lineage appears at the

blastocyst stage as the trophectoderm, a sphere of 50–60 epithelial cells that surround the inner cell mass (ICM) and the blastocoel. After implantation, the ICM forms the embryo and some extraembryonic membranes, whereas the trophectoderm is restricted to the foetal portion of the placenta and the trophoblast giant cells. The trophectoderm forms two spatially distinct domains. The mural trophectoderm ceases to proliferate and becomes primary trophoblast giant cells. The polar trophectoderm directly contacts the ICM, remains proliferative, and forms the extraembryonic ectoderm, the ectoplacental cone, and the secondary giant cells of the early conceptus.

Four differentiated cell phenotypes are derived from trophoblast stem cells. These differentiated cells are spatially organized in the mature chorioallantoic placenta into the junctional zone that abuts the decidua on the maternal side, and the labyrinth zone that is located at the foetal interface (Soares *et al*., 1996). The placental locations and functions of differentiated trophoblasts are summarized in Table 2.

In mice, trophoblasts appear at the blastocyst stage as the trophectoderm. These specialized epithelial cells surround the inner cell mass and the blastocoel. After implantation, the inner cell mass forms the embryo, while the trophectoderm forms the foetal portion of the placenta and trophoblast giant cells. The trophectoderm is spatially polarized with the polar trophectoderm in direct contact with the inner cell mass and the mural trophectoderm migrating away from the inner cell mass. Polar trophectoderm cells in contact with the inner cell mass are highly proliferative, whereas those in the mural trophectoderm exhibit diminished growth and differentiate to form giant cells.

The precise orchestration and the mechanisms responsible for the cellular interactions that control trophoblast growth, differentiation, and invasion are uncertain. Previous studies reported that human placental cytotrophoblasts were sensitive to alterations of extracellular Ca^{2+} (Hellman *et al*., 1992; Juhlin *et al*., 1990; Lundgren *et al*., 1997). However, it is now clear that the glycoprotein megalin, which exhibits sensitivity to extracellular calcium, was responsible for the observed effects. In subsequent studies, however, Bradbury *et al*. (1998) demonstrate that the CaSR is expressed in human placental cells. Because the CaSR is now known to control growth and differentiation in a number of different cell types, studies were initiated to explore the interplay between extracellular Ca^{2+} and PTHrP on rat trophoblasts using Rcho-1 cells a rat trophoblast cell line derived from a transplantable choriocarcinoma, capable of differentiating from stem cells into trophoblast giant cells (Faria & Soares, 1991; Soares *et al*., 1996).

In these studies (Friedman & Magyar, unpublished data), Rcho-1 cells grown in RPMI supplemented with 20% FCS normally exhibited a doubling time of less than 10 h. Increasing extracellular Ca^{2+} from 0.3 to 5 mM suppressed cell proliferation in a concentration dependent fashion with an EC_{50} for inhibition of 1.8 mM Ca^{2+}, which corresponds to foetal serum ionized Ca^{2+} levels (Magyar *et al*., 2000). Addition of 10 μM NPS R-467, a type II calcimimetic, to standard, serum-supplemented RPMI for 48 h decreased proliferation by 50%. These findings suggested that the CaSR, a member of the class 3 superfamily of G protein-coupled membrane receptors, may participate in regulating growth of Rcho-1 cells. Conditioned culture medium was collected after the initial 24 h and PTHrP(1-36) was determined by IRMA. Raising extracellular Ca^{2+} decreased endogenous PTHrP(1-36) release in a step-wise fashion. To test if endogenous PTHrP release was responsible for cell proliferation, it was found that addition of 10 nM PTHrP(1-36) increased proliferation by 6 fold. Addition of 1 μM PTH(7-34) concurrently with PTHrP(1-36) blocked this increase consistent with an action on the PTH1R. The presence of the PTH1R was confirmed as was the expression of both CaSR and PTHrP.

Since the CaSR couples PLC, which hydrolyzes phosphatidylinositol and, in turn, causes the release of Ca^{2+} from intracellular stores, the effects of CaSR ligands on intracellular calcium ($[Ca^{2+}]_i$) in Rcho-1 cells was examined. Raising extracellular Ca^{2+} from 0.3 to 1.6 mM or addition of Gd^{3+} caused transient elevations of $[Ca^{2+}]_1$ measured ratiometrically with fura-2, consistent with the signal transduction mediated by the CaSR.

Trophoblasts also exhibit an invasive phenotype and the invasive behaviour of the differentiating Rcho-1 cells has been demonstrated (Peters *et al*., 1999). To begin to define the mechanisms participating in endometrial invasion and remodelling, it was theorized that the conspicuous acidification by Rcho-1 cells in culture is mediated by the vacuolar H^+-ATPase, as it is in bone (Gluck *et al*., 1998). Over a period of 72 h, extracellular pH of the medium bathing Rcho-1 cells decreases by 7.4 to 6.9. Addition of the macrolide antibiotic bafilomycin A1, a specific vacuolar H^+-ATPase inhibitor, suppressed the fall of pH to 0.1 unit. The presence of the 16 kD C-subunit of the vacuolar H^+-ATPase in Rcho-1 cells was verified. When this subunit is knocked out by gene targeting and the resulting heterozygous mice were intercrossed there were no homozygous offspring (Inoue *et al*., 1999). A few $-/-$ embryos were found at pre-implantation stages of embryonic development, up to day 3.5 post-coitus. Taken together, these results suggest that the H^+V-ATPase plays an essential role in implantation. We speculate that the low pH may activate an acid optima metalloproteinase such as MMP-3.

Thus, Ca^{2+} exerts an anti-mitogenic effect on Rcho-1 cells. Increasing extracellular Ca^{2+} diminishes PTHrP(1-36) release and subsequent Rcho-1 cell proliferation. In contrast, PTHrP is mitogenic. The presence of CaSR, PTHrP and PTH1R mRNA was demonstrated. The CaSR was identified as a candidate molecule in rat trophoblasts that mediates calcium

Table 2 Location and function of differentiated trophoblasts

Cell	*Location*	*Function*
Trophoblast giant cell	Junctional and labyrinth zones	Hormone synthesis, endometrial invasion
Syncytiotrophoblast	Labyrinth zone	Nutrient and waste transport
Spongiotrophoblasts	Junctional zone	Endocrine
Glycogen cell	Junctional zone	Energy store

signalling during differentiation. The balance of growth and differentiation in Rcho-1 cells may be achieved by opposing anti-mitogenic effects of Ca^{2+} mediated by the CaSR and the autocrine action of PTHrP on the PTH1R to promote growth and delay differentiation. Acidification of the culture medium is due to H^+ secretion mediated by the vacuolar H^+-ATPase. The interaction, if any, between CaSRs and H^+-V-ATPase-mediated acidification is not known.

Conclusions and future perspectives

The present review focused only on certain aspects of the biology of the PTHrP field. In the 13 years since its discovery however, the PTHrP system has been explored in essentially all of the main physiological systems throughout the developing and adult body. The accumulating findings highlight an impressive array of established or potential functions of PTHrP as a poly-hormone, as well as the breadth of the mechanisms involved in these functions, ranging from the classical endocrine or auto/paracrine processes up to sophisticated intracrine pathways that involve complex cellular trafficking. Thus, PTHrP appears as a multifunctional protein that can be simultaneously secreted and targeted to the nucleus. This latter aspect raises new concepts in cellular protein trafficking and a number of important questions which will have to be solved for a complete understanding of the roles of PTHrP in most cells. How, and under what (patho)physiological circumstances PTHrP leaves the classical endoplasmic secretory pathway and is translocated to the nucleus/nucleolus, what are the NLS-containing PTHrP fragment(s) translocated to the nucleus/nucleolus, and what are the intranuclear/intranucleolar targets of PTHrP, are undoubtedly among the most crucial questions of the PTHrP field. These questions are already the subject of intense investigations in a number of laboratories and promise to attract even more effort in the near future.

The dominant role of PTHrP as a developmental factor has been well established in bone, skin and mammary gland. Such a role appears relevant in most other organs as well, including the cardiovascular system and the kidney. In this latter system, consistent results point to a key homeostatic role that PTHrP may play in the heart as well as in systemic and regional hemodynamics. Overall, the widespread properties of the PTHrP system may prove to have a broad spectrum of possible therapeutical indications in a variety of diseases, including cancer, osteoporosis, renal insufficiencies, diabetes, interstitial fibrosis, arterial hypertension and vascular (re)stenosis-atherosclerosis.

Although null mutation in the germ lines of the PTHrP or PTH1R genes in mice emphasized the critical developmental role of PTHrP, the early lethality of the homozygous animals obscured the potential roles these genes may play in specific physiological systems in the adult animal. As a surrogate for studying the actions of locally produced PTHrP, transgenic mice that overexpress PTHrP in specific cells have been developed. These studies further strongly supported PTHrP as an authentic regulator of the vascular morphogenesis and tone of the pancreatic islet mass and insulin secretion. The up-to-date techniques of molecular biology will undoubtedly allow the generation of mouse models for conditional inactivation of the PTHrP gene using the *Cre* recombinase-*loxP* technology to examine the consequences of ablation of PTHrP and other genes of the PTHrP system in a variety of cell types in adult mice. This type of investigation is just beginning and will drastically increase the knowledge of the PTHrP system and should open new avenues for the design of new drugs.

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