Parathyroid hormone–related peptide is a naturally occurring, protein kinase A–dependent angiogenesis inhibitor

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Angiogenesis is a highly regulated process that results from the sequential actions of naturally occurring stimulators and inhibitors. Here, we show that parathyroid hormone–related peptide, a peptide hormone derived from normal and tumor cells that regulates bone metabolism and vascular tone, is a naturally occurring angiogenesis inhibitor. Parathyroid hormone–related peptide or a ten-amino-acid peptide from its N terminus inhibits endothelial cell migration *in vitro* and angiogenesis *in vivo* by activating endothelial cell protein kinase A. Activation of protein kinase A inhibits cell migration and angiogenesis by inhibiting the small GTPase Rac. In contrast, inhibition of protein kinase A reverses the anti-migratory and anti-angiogenic properties of parathyroid hormone–related peptide. These studies show that parathyroid hormone–related peptide is a naturally occurring angiogenesis inhibitor that functions by activation of protein kinase A.

Angiogenesis is an important physiological process that is exquisitely regulated by stimulatory and inhibitory factors[1–3]. Sustained pathological angiogenesis results from an overbalance of stimulatory factors and an insufficiency of inhibitory factors[1–3]. An understanding of the positive and negative factors that influence vascular proliferation may enable the development of new therapeutics to treat aberrant angiogenesis such as that occurring in cancer, arthritis, blindness and psoriasis[1–3].

Angiogenesis requires activation of quiescent endothelial cells by growth factors; degradation of basement membrane and dissociation of endothelial cells from the supporting vascular smooth muscle; proliferation, survival, migration and eventual differentiation of these cells into lumen-bearing structures that may also be lined with vascular smooth-muscle cells[1–3]. Effective inhibitors of angiogenesis may block one or more of these discrete processes. In fact, several naturally occurring inhibitors of angiogenesis have been developed as potential therapeutic agents for cancer[4–6].

Parathyroid hormone–related peptide (PTHrP) and the closely related parathyroid hormone (PTH) are peptide hormones that regulate serum calcium levels, vascular tone and bone formation[7–12]. Naturally occurring isoforms of PTHrP extend from amino acids 1–139, 1–141 and 1–173; the last is found only in humans[8]. Mice without PTHrP die shortly after birth and have skeletal abnormalities including shortened bones and increased bone vascularization[10–12].

Extracellular PTH/PTHrP activates a cell surface G protein–coupled receptor (GPCR) that then activates protein kinase A (PKA) and phospholipase C (refs. 13,14). This PTH1 receptor is expressed on many cells including chondrocytes, vascular smooth-muscle cells and endothelial cells[15–17]. Many of the physiological functions of PTHrP are mediated by activation of PKA (refs. 18–20).

PTHrP is also produced by many tumor cells; however, little is known about its effects on tumor vasculature. In contrast, PTHrP is known to cause increased serum calcium in cancer patients (humoral hypercalcemia of malignancy) and to promote local bone metastases[7,21,22]. Humoral hypercalcemia of malignancy results from PTHrP stimulation of osteoblasts to express RANKL, a factor that induces osteoclast differentiation from macrophages, subsequent degradation of mineralized bone and an eventual increase in serum calcium[23]. Local PTHrP-mediated bone degradation may contribute to tumor cell colonization of bone to form metastases[21,22]. Recent studies indicate that PTHrP also acts in an intracrine manner to stimulate tumor cell proliferation[24].

As studies indicate that PTHrP may promote tumor progression but also may negatively regulate bone vascularization, we sought to determine if PTHrP is involved in the physiological regulation of angiogenesis. Like other naturally occurring angiogenesis inhibitors such as thrombospondin-1 (ref. 1), endostatin[4], angiostatin[5] and tumstatin[6], PTHrP is produced in measurable quantities by tumor cells. We found that PTHrP inhibits growth factor– and tumor-induced angiogenesis. The angiostatic effects of PTHrP require activation of PKA by its G protein–coupled receptor. Once activated, PKA suppresses Rac activation, which in turn inhibits endothelial cell migration and angiogenesis. Our studies show a previously unknown signal transduction pathway that negatively regulates angiogenesis and indicate that localized administration of PTHrP or activation of PKA may be a useful strategy to inhibit diseases associated with angiogenesis.

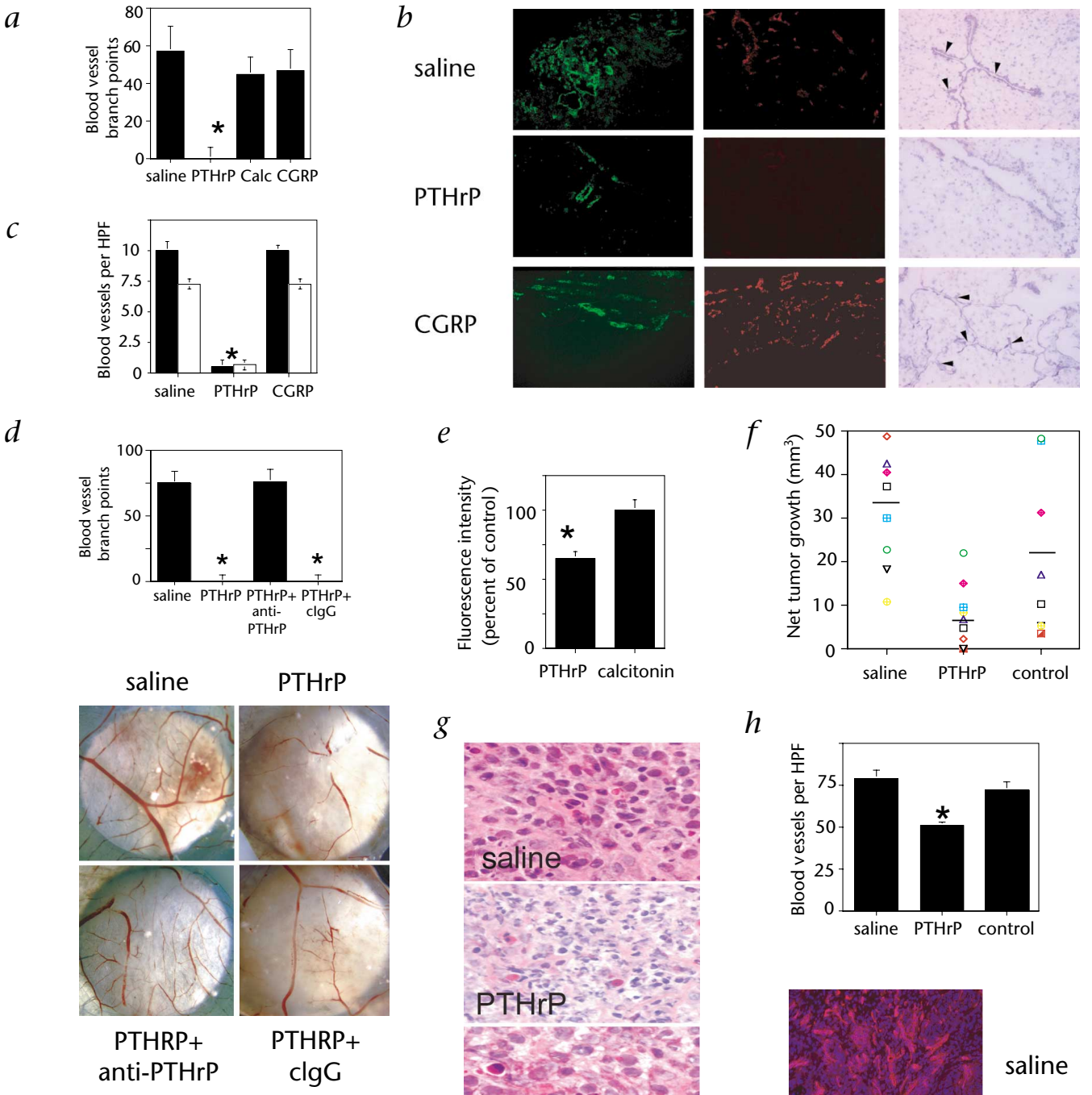
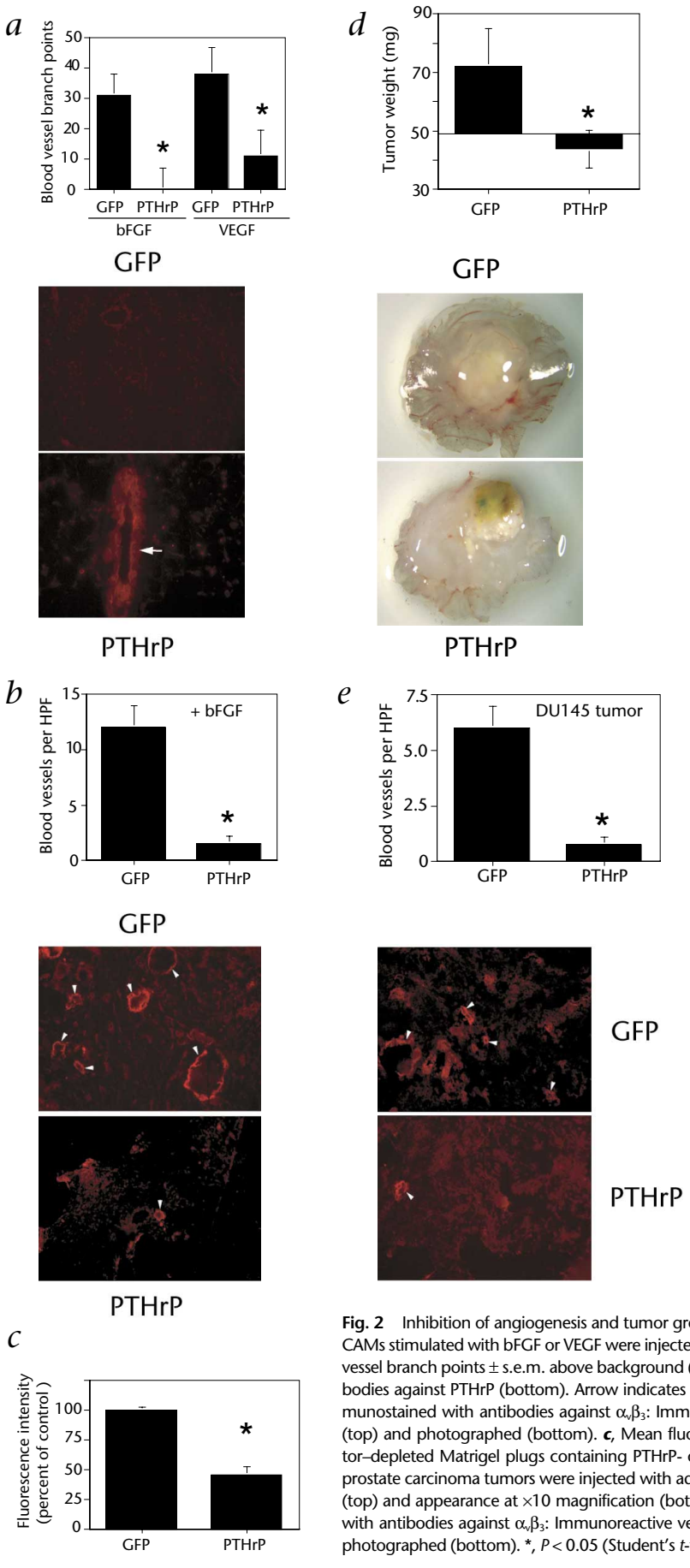


Fig. 1 PTHrP inhibits angiogenesis *in vivo*. ***a–c***, CAMs stimulated with bFGF or saline were treated with $PTHrP_{1–173}$, calcitonin (Calc) or calcitonin gene–related peptide (CGRP). *a*, Blood vessel branch points ± s.e.m. above background. *b-c*, Cryosections of CAMs immunostained with antibody against $\alpha_v\beta_3$ (left and ■) or against VWF (middle □) and then stained with hematoxylin and eosin (H&E; right).*c*, quantification of immunoreactive vessels per high-power field (HPF; magnification, ×200). ***d***, bFGF-stimulated CAMs treated with saline, $PTHrP_{1–173}$ or PTHrP with PTHrP-function-blocking (anti-PTHrP) or control antibodies (cIgG; bottom). Top, quantification of blood vessel branch points ± s.e.m. above background. ***e***, Mean fluorescence intensity in lysates of growth factor–depleted Matrigel plugs containing bFGF and PTHrP or bFGF and calcitonin. ***f–h***, Mice bearing PTHrP-negative DU145 tumors were treated with saline, PTHrP or a scrambled control peptide (control). *f*, Net tumor volume after treatment. Solid line, average tumor volume. *g*, Cryosections of tumors stained with H&E. *h*, Cryosections immunostained for expression of vascular antigen CD31 (red) and DNA (blue). Top, quantification of immunoreactive vessels. Bottom, ×200 magnification of cryosections. *, $P < 0.05$ (Student's *t*-test).



PTHrP suppresses angiogenesis *in vivo*

To evaluate the function of PTHrP in blood vessel development, we tested the effects of PTHrP on angiogenesis in the chick chorioallantoic membrane (CAM). We stimulated CAMs from 10-day-old chick embryos with basic fibroblast growth factor (bFGF) in the presence or absence of PTHrP amino acids 1–173 ($PTHrP_{1–173}$) or two other peptide hormones, calcitonin (a calcium-regulating peptide hormone[25]) and calcitonin gene–related peptide (CGRP, a vasodilatory peptide hormone[25]). Only PTHrP inhibited angiogenesis, whether analyzed by counting blood vessel branch points (Fig. 1*a*; $P < 0.01$) or the density of vessels positive for integrin αvβ3 (ref. 26) (Fig. 1*b-c*; $P < 0.0001$) or von Willebrand factor (VWF) (Fig. 1*b-c*; $P < 0.001$). Analysis of blood vessel morphology also indicated that PTHrP inhibited bFGF-induced vascular branching (Fig. 1*b*). PTHrP had no effect on the number, size or composition of pre-existing vessels, as determined by macroscopic evaluation of vessels in saline-stimulated CAMs or by examination of the number and nature of αvβ3- and VWF-positive vessels in saline-stimulated CAMs (data not shown). These studies indicate that PTHrP selectively prevents the formation of new microvessels. The half-maximal inhibitory dose of PTHrP was 0.01 μM (data not shown). This inhibition could be blocked by the addition of an antibody blocking PTHrP function (8B12) directed against amino acids 1–34 (ref. 27) (Fig. 1*d*). PTHrP also blocked bFGF-stimulated angiogenesis in the mouse (Fig. 1*e*; $P < 0.001$). These studies indicate that PTHrP functions as an inhibitor of angiogenesis.

PTHrP also inhibits tumor angiogenesis and growth. We treated nude mice bearing DU145 prostate carcinoma tumors (that did not express PTHrP) with daily intravenous injections of PTHrP (final serum concentration, 1 μM). PTHrP suppressed tumor growth (Fig. 1*f*; $P < 0.005$), induced tumor necrosis (Fig. 1*g*) and inhibited tumor angiogenesis (Fig. 1*h*; $P < 0.0001$). Whereas saline-treated tumors doubled in size during the 10-day study, PTHrP-treated tumors increased in size by only 30%. These studies indicate that PTHrP inhibits tumor angiogenesis as well as growth factor–induced angiogenesis.

Inhibition of angiogenesis by PTHrP gene delivery

To determine if gene delivery of PTHrP might be a useful strategy for the therapeutic inhibition of

Fig. 2 Inhibition of angiogenesis and tumor growth by PTHrP gene delivery. ***a*** and ***b***, Chicken embryos bearing CAMs stimulated with bFGF or VEGF were injected with adenoviruses expressing GFP or full-length PTHrP. *a*, Blood vessel branch points ± s.e.m. above background (top) and cryosections of treated CAMs immunostained with antibodies against PTHrP (bottom). Arrow indicates PTHrP-positive blood vessel. *b*, Cryosections of CAMs from *a* immunostained with antibodies against $\alpha_v\beta_3$: Immunoreactive vessels per microscopic field (×200) were quantified (top) and photographed (bottom). ***c***, Mean fluorescence intensity in lysates of bFGF-supplemented growth factor–depleted Matrigel plugs containing PTHrP- or GFP-expressing adenoviruses. ***d*** and ***e***, CAMs bearing DU145 prostate carcinoma tumors were injected with adenoviruses expressing GFP or full-length PTHrP. *d*, Tumor weight (top) and appearance at ×10 magnification (bottom) after 7 d. *e*, Cryosections of tumors from *d* immunostained with antibodies against $\alpha_v\beta_3$: Immunoreactive vessels were quantified per ×200 microscopic field (top) and were photographed (bottom). *, $P < 0.05$ (Student's *t*-test).

angiogenesis *in vivo*, we stimulated angiogenesis in chick CAMs with bFGF or vascular endothelial growth factor (VEGF). We then transduced CAMs by injecting adenoviruses expressing green fluorescent protein (GFP) or full-length $PTHrP_{1–173}$ (ref. 27) into the embryonic circulation. Virally expressed PTHrP, but not GFP, inhibited angiogenesis stimulated by bFGF ($P < 0.001$) or VEGF ($P = 0.01$), as shown by quantification of blood vessel branch points (Fig. 2*a*, top) or integrin $\alpha_v\beta_3$-immunoreactive vessels (Fig. 2*b*; $P < 0.0001$). PTHrP was detected in blood vessels (arrows) in CAMs transduced with PTHrP using an antibody directed against PTHrP C-terminal amino acids 109–141 (ref. 28) (Fig. 2*a*, bottom). Virally expressed PTHrP also inhibited angiogenesis in the adult mouse. Viral delivery of PTHrP inhibited bFGF-stimulated mouse angiogenesis (Fig. 2*c*; $P < 0.05$). Virally induced expression of PTHrP also inhibited tumor growth on the CAM (Fig. 2*d*; $P < 0.03$), indicating that locally delivered PTHrP can suppress tumor angiogenesis. PTHrP-treated tumors were smaller, more obviously necrotic and associated with fewer integrin $\alpha_v\beta_3$-positive blood vessels than GFP-treated tumors (Fig. 2*e*, $P < 0.0001$). Thus, virally expressed PTHrP can inhibit angiogenesis and may serve as a useful therapeutic angiogenesis inhibitor.

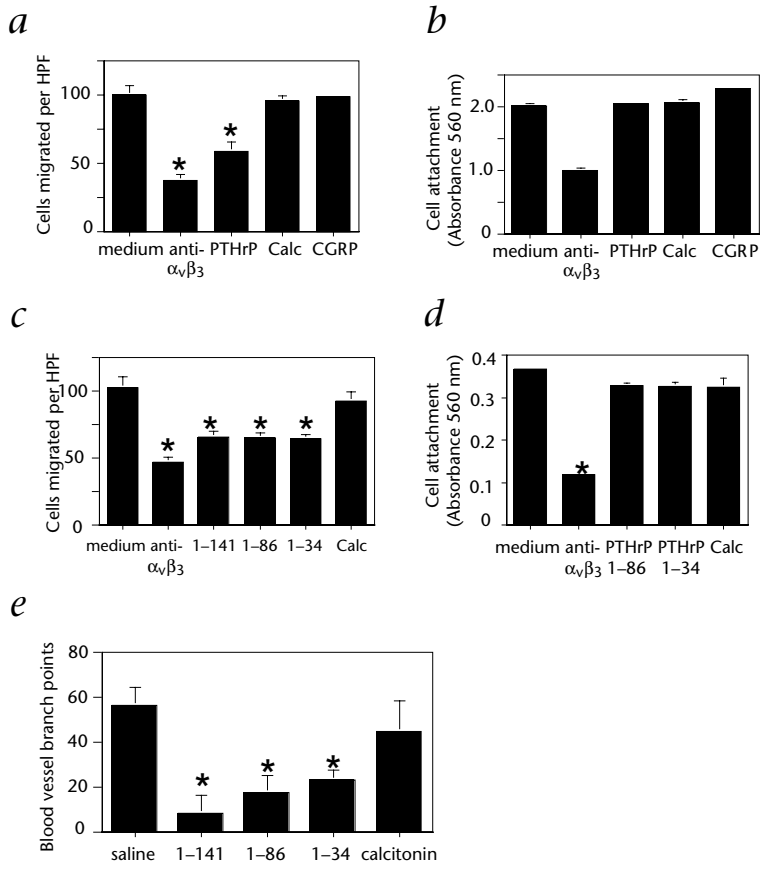


Fig. 3 The N-terminal 34 amino acids of PTHrP inhibit endothelial cell migration *in vitro* and angiogenesis *in vivo*. ***a*** and ***b***, Endothelial cell migration (*a*) and attachment (*b*) to vitronectin in the presence of medium, antibody against $a_v\beta_3$ (anti-$a_v\beta_3$), PTHrP, calcitonin (Calc) or CGRP. ***c*** and ***d***, Endothelial cell migration (*c*) and attachment (*d*) to vitronectin in the presence of medium, antibody against $a_v\beta_3$ (anti-$a_v\beta_3$), $PTHrP_{1–141}$, $PTHrP_{1–86}$, $PTHrP_{1–34}$ or calcitonin (Calc). ***e***, Blood vessel branch points in bFGF-stimulated CAMs treated with saline, $PTHrP_{1–141}$, $PTHrP_{1–86}$, $PTHrP_{1–34}$, and calcitonin. *, $P < 0.05$ (Student's *t*-test). HPF, high-power field.

PTHrP inhibits endothelial cell migration

To evaluate the function of PTHrP in endothelial functions *in vitro*, we tested the effects of $PTHrP_{1–173}$ and other peptide hormones on endothelial cell migration on extracellular matrix substrates such as vitronectin, collagen and fibronectin. PTHrP significantly inhibited cell migration on vitronectin (Fig. 3*a*), collagen and fibronectin (data not shown), yet had no effect on attachment to these proteins (Fig. 3*b*). Cell migration was inhibited 50% by PTHrP in the range of 1–10 μM. These studies demonstrate that PTHrP inhibits endothelial cell migration, but is not an inhibitor of integrin ligation.

Mapping of anti-angiogenic active sites of PTHrP

PTHrP is composed of several domains with distinct physiological properties[29]. To identify the domain(s) of PTHrP responsible for the anti-migratory and anti-angiogenic effects, we evaluated the effects of various fragments of the peptide hormone in migration and angiogenesis assays. Like $PTHrP_{1–173}$, fragments containing the N terminus (1–141, 1–86 and 1–34) inhibited migration (Fig. 3*c*), but not attachment (Fig. 3*d*), on vitronectin. Fragments lacking the N terminus, such as $PTHrP_{107–138}$, were not able to inhibit cell migration (data not shown). *In vivo*, $PTHrP_{1–141}$, $PTHrP_{1–86}$ and $PTHrP_{1–34}$ potently inhibited angiogenesis (Fig. 3*e*). As the first 34 amino acids contain the angiogenesis-inhibiting properties, we undertook further studies to identify the essential residues responsible for angiogenesis inhibition.

Studies have shown that the first 6 amino acids of PTH are required for activation of PTH/PTHrP receptor signaling, whereas the last 15 are required for high-affinity binding to the receptor[29]. Five of the first ten amino acids in PTH and PTHrP are identical, and structure predictions indicate these regions have similar conformations[30]. Therefore, to further delineate the active angiogenesis- and cell migration-inhibition sites on PTHrP, we compared the activities of fragments of PTHrP extending from amino acids 1–10, 1–34 and 15–34 to that of a peptide consisting of a scrambled version of 1–10. Whereas amino acids 1–34 and 1–10 were similarly effective in inhibiting cell migration on vitronectin, amino acids 15–34 and the scrambled version of 1–10 did not inhibit cell migration (Fig. 4*a*). The N-terminal PTHrP fragment 1–10 inhibited cell migration in a dose-dependent manner, with a maximum of 50% inhibition at a concentration of 10 μM. $PTHrP_{1–10}$ and $PTHrP_{1–34}$ also inhibited angiogenesis in the CAM assay, whereas the scrambled 1–10 peptide did not (Fig. 4*b*). $PTHrP_{1–10}$ was slightly less effective at inhibiting angiogenesis than $PTHrP_{1–34}$, however. $PTHrP_{1–10}$ also inhibited angiogenesis in a mouse model of angiogenesis, whereas the scrambled 1–10 peptide did not (Fig. 4*c*; $P < 0.01$). These studies indicate that the first ten PTHrP residues are essential for activation of the endothelial cell PTHrP receptor *in vitro* and *in vivo*.

Angiogenesis can be induced by many growth factors. To determine if PTHrP is a general inhibitor of angiogenesis, we evaluated the effect of

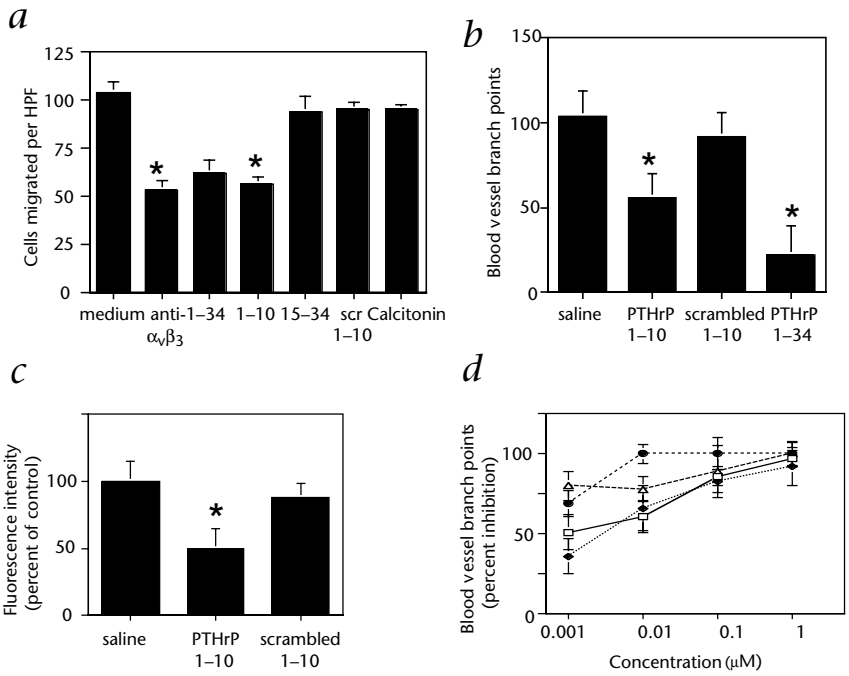


Fig. 4 PTHrP amino acids 1–10 are sufficient to inhibit endothelial cell migration *in vitro* and angiogenesis *in vivo*. ***a***, Endothelial cell migration on vitronectin in the presence of medium, antibody against $\alpha_v\beta_3$ (anti-$\alpha_v\beta_3$), $PTHrP_{1–34}$, $PTHrP_{1–10}$, $PTHrP_{15–34}$, a scrambled version of $PTHrP_{1–10}$ (scr. 1–10) or calcitonin. ***b***, Blood vessel branch points in bFGF-stimulated CAMs treated with saline, $PTHrP_{1–10}$, a scrambled version of $PTHrP_{1–10}$ or $PTHrP_{1–34}$. ***c***, Mean fluorescence intensity in lysates of growth factor–depleted Matrigel plugs containing purified bFGF and saline, $PTHrP_{1–10}$ or a scrambled version of $PTHrP_{1–10}$. ***d***, CAMs stimulated by bFGF (□), VEGF (△), IL-8 (◆) or TNF-α (●) treated with saline or $PTHrP_{1–34}$. Blood vessel branch points were counted and percent inhibition of saline control was determined. *, $P < 0.05$ (Student's *t*-test).

$PTHrP_{1–34}$ on angiogenesis stimulated by bFGF, VEGF, interleukin (IL)-8 and tumor necrosis factor (TNF)-α (Fig. 4*d*). Angiogenesis induced by each growth factor was inhibited by $PTHrP_{1–34}$, with complete inhibition occurring at 1 μM. The half-maximal inhibitory concentration for each growth factor was 0.001 μM or lower. Thus, PTHrP is a potent general inhibitor of angiogenesis.

Mechanism of PTHrP anti-angiogenic effects

To establish the mechanism by which PTHrP inhibits cell migration *in vitro* and angiogenesis *in vivo*, we evaluated the effects of PTHrP on endothelial cell signal transduction. Like PTH, PTHrP interacts with the PTH1 receptor, a G protein–coupled receptor expressed on endothelial cells[17] that activates PKA (refs. 13,14). $PTHrP_{1–34}$, as well as a cell-permeable cAMP (dibutyryl cAMP), rapidly stimulated PKA activity in endothelial cells (Fig. 5*a*). PKA activation was detectable in as few as 2 minutes, with maximum activity 15 minutes after stimulation with both cAMP and PTHrP. To determine if the anti-migratory properties of PTHrP result from signals transduced through PKA, we evaluated the effects of $PTHrP_{1–34}$ on cell migration in the presence and absence of the PKA inhibitor N-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinoline sulfonamide (H89) (Fig. 5*b*). This PKA inhibitor blocked the anti-migratory properties of PTHrP ($P < 0.01$). Furthermore, expression of a mutationally inactive form of PKA (dnPKA), which blocks PKA activation[31], suppressed the PTHrP- and cAMP-mediated inhibition of endothelial cell migration (Fig. 5*c*; $P < 0.0001$). In fact, direct activation of PKA by cAMP ($P < 0.0001$) or by transient transfection with the PKA catalytic subunit ($P < 0.0001$) also inhibited endothelial cell migration (Fig. 5*d*). We were able to detect expression of both transgenes by western blot analysis of lysates of transfected cells (Fig. 5*e*). These results indicate that PTHrP inhibition of migration is PKA dependent and that activation of PKA blocks endothelial cell migration.

The small GTPase Rac is essential in regulating cell motility by influencing actin assembly and lamellipodia extension[32]. Cell adhesion as well as growth-factor stimulation upregulate Rac activity in endothelial cells[33]. Activation of Rac was blocked by PTHrP, by expression of activated PKA (Fig. 5*f*) and by cAMP (not shown). Overexpression of mutationally active Rac (V12 Rac) overcame the inhibition of cell motility mediated by PTHrP ($P < 0.007$), cAMP (Fig. 5*g*; $P < 0.003$) or the PKA catalytic subunit (Fig. 5*h*; $P < 0.006$) . These studies indicate that PTHrP activation of PKA inhibits cell migration by inhibiting Rac activation.

Signal transduction pathways that promote cell migration often also promote cell survival[34]. As PTHrP inhibits the migration of endothelial cells, it is possible that it inhibits their survival. In fact, PTHrP induced apoptosis of endothelial cells in a dose-dependent manner (Fig. 5*i*). As with the effects of PTHrP on cell migration, PTHrP-induced apoptosis was PKA dependent, as expression of dominant negative PKA suppressed this induction of cell death (Fig. 5*j*), and suggested that direct activation of PKA may induce apoptosis in endothelial cells. Endothelial cell expression of the catalytic subunit of PKA or exposure to dibutyryl cAMP indeed induced apoptosis in endothelial cells (Fig. 5*k*). These studies indicate that PTHrP not only inhibits endothelial cell migration, but also induces apoptosis of endothelial cells in a PKA-dependent manner.

PTHrP inhibition of angiogenesis is protein kinase A dependent

Our studies indicate that activation of PKA *in vivo* may inhibit angiogenesis. We therefore evaluated the involvement of PKA in the inhibition of angiogenesis by PTHrP. Either pharmacological or genetic inhibition of PKA reversed the PTHrP inhibition of angiogenesis. A selective PKA inhibitor (H89) blocked the suppression of angiogenesis induced by $PTHrP_{1–34}$ (Fig. 6*a*; $P < 0.0002$). Expression of mutationally inactive PKA (dnPKA) also reversed the inhibitory effects of PTHrP (Fig. 6*b*; $P < 0.0003$). Furthermore, activation of PKA by cAMP (Fig. 6*c*; $P < 0.0003$) or by expression of the catalytic subunit of PKA in the CAM (Fig. 6*d*; $P = 0.0005$) potently inhibited angiogenesis. These studies demonstrate that PTHrP inhibits angiogenesis by activating PKA. Recent studies have shown that Rac activity is required for angiogenesis[35]. Taken together, these studies indicate that activation of PKA in endothelial cells *in vivo* causes Rac inactivation and probably inhibition of cell migration *in vivo*. In addition, PTHrP or PKA may inhibit angiogenesis by inducing apoptosis of proliferating endothelial cells *in vivo*. In fact,

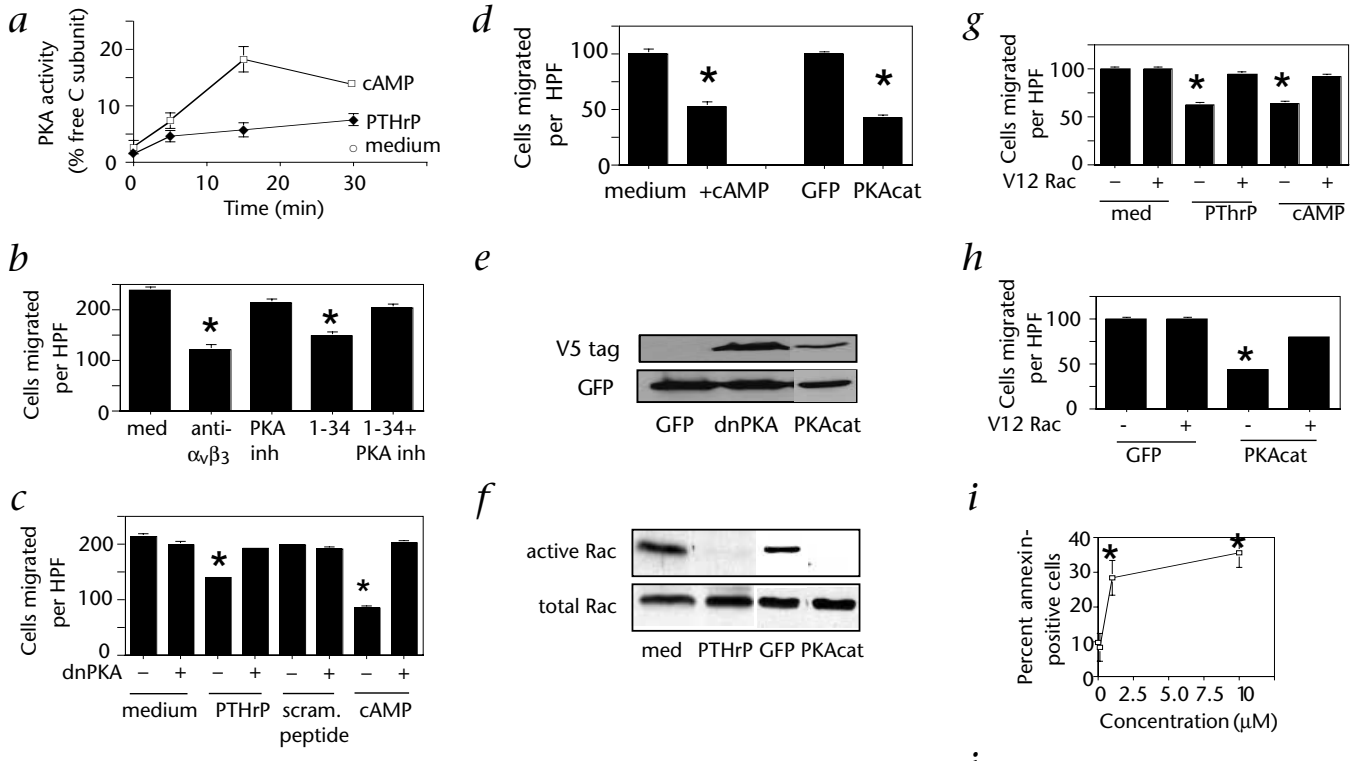


Fig. 5 PTHrP inhibits migration by blocking Rac activation in a PKA-dependent manner. ***a***, PKA activity in endothelial cells in the presence of culture medium (●), PTHrP (◆) or dibutyryl cAMP (□). Specific activity is expressed as percent of total PKA that can be directly activated by cAMP in cell lysates. ***b***, Endothelial cell migration on vitronectin in the presence of culture medium, antibody against αvβ3 (anti-$\alpha_v\beta_3$), $PTHrP_{1-34}$, the protein kinase A inhibitor H89 (PKA inh) or PTHrP combined with H89. ***c***, The effect of a mutationally inactive PKA (dnPKA) on cell migration in the presence of PTHrP, a scrambled control peptide (scram. peptide) or cAMP. ***d***, The effect of cAMP and transient transfection with the PKA catalytic subunit (PKAcat) on endothelial cell migration. ***e***, Western blots of lysates of cells transfected with GFP, dnPKA or PKAcat, incubated with antibodies against V5 tag and GFP. ***f***, Rac activity in endothelial cells treated with culture medium or PTHrP and in cells transfected with activated PKA or GFP. ***g***, Migration of endothelial cells expressing mutationally active Rac (V12 Rac; +) or GFP (–) in the presence of culture medium, PTHrP or cAMP. ***h***, Migration on vitronectin of endothelial cells transfected with PKAcat, V12 Rac plus PKAcat, GFP or V12 Rac plus GFP. ***i***, Quantification of annexin V–positive endothelial cells cultured on vitronectin substrates in the presence of PTHrP. ***j***, Quantification of annexin V–positive endothelial cells transfected with GFP (■) or dnPKA (□) and treated with culture medium, PTHrP or scrambled control peptide. ***k***, Quantification of annexin V–positive endothelial cells transfected with GFP or PKAcat and treated with culture medium (med) or cAMP. *, $P < 0.05$ (Student's *t*-test).

TUNEL staining of PTHrP-treated CAMs showed that this hormone induced apoptosis in bFGF- but not saline-stimulated endothelial cells *in vivo* (Fig. 6*e*). Expression of the PKA catalytic subunit in bFGF-stimulated CAMs or exposure of CAMs to cAMP also induced endothelial cell apoptosis *in vivo* (Fig. 6*f*). These studies indicate that PTHrP inhibits angiogenesis by directly and specifically inducing PKA-dependent endothelial cell apoptosis and, furthermore, that activation of PKA by hormonal, pharmacological or genetic means is a potent methods to inhibit angiogenesis.

Discussion

PTHrP is a well-characterized peptide hormone with physiological effects on vascular smooth muscle, bone and tumor cells[7–12]. We have shown here that PTHrP and an N-terminal ten-amino-acid fragment inhibit endothelial cell migration and angiogenesis, without affecting quiescent blood vessels. PTHrP also inhibits tumor growth by inhibiting angiogenesis. We have also seen similar effects with PTH (data not shown). PTHrP activates PKA, thereby suppressing activation of Rac and cell migration and inducing endothelial cell apoptosis. Furthermore, induction of PTHrP through gene therapy may be a useful approach to angiogenesis inhibition. These are the first studies to our knowledge to directly examine involvement of PTHrP in angiogenesis regulation.

PTHrP regulates vascular tone by relaxing vascular smooth muscle[7]. Our studies indicate that the vasodilatory effects of PTHrP are distinct from its effects on angiogenesis, as treatment of tissues undergoing angiogenesis with CGRP, another vasodilatory hormone[25], has no effect on the number or quality of vessels during angiogenesis. The effects of PTHrP on angiogenesis are specific, as neither CGRP nor calcitonin, two peptide hormones with related functions that are evolutionarily well conserved, inhibit angiogenesis. Although PTHrP has no effect

on the number or size of pre-existing smooth muscle or pericytes (smooth muscle–like cells associated with capillaries) *in vivo*, it does inhibit a bFGF-induced increase in desmin- and smooth muscle actin–positive vessels *in vivo* (data not shown). Like endothelial cells, desmin- and smooth muscle actin–positive pericytes or smooth-muscle cells proliferate in response to angiogenic growth factors[36]. Unlike endothelial cells, however, PTHrP does not induce apoptosis in smooth-muscle cells or pericytes (data not shown). These studies suggest that the main target of PTHrP during angiogenesis inhibition is the endothelial cell. The PTHrP-mediated inhibition of pericyte proliferation during angiogenesis may thus be secondary to its effect on endothelial cells. However, it is possible that PTHrP directly affects pericytes during angiogenesis by an alternative mechanism.

PTHrP may have important functions in the regulation of angiogenesis during embryonic development, particularly during bone formation. Mice without PTHrP die shortly after birth and have skeletal abnormalities that include shortened bones, accelerated osteogenesis and increased bone vascularization[10–12]. PTHrP promotes the proliferation of chondrocytes, suppressing chondrocyte hypertrophy and angiogenesis and thereby maintaining avascular regions of cartilage[10–12]. During normal endochondral bone formation, avascular cartilage is gradually replaced by highly vascularized and mineralized bone. Disruption of the carefully regulated patterns of vascularization can impair bone formation. For example, disruption of the pro-angiogenic activity of VEGF in bone by systemic administration of a soluble VEGF receptor suppresses vascular invasion of the hypertrophic cartilage and delays bone mineralization[37]. Additionally, mice deficient in MMP-9, a matrix metalloproteinase that is required for angiogenesis, show delayed osteogenesis, elongated bones and suppressed bone vascularization[38]. Together, these studies indicate that PTHrP, VEGF and MMP-9 function in opposing manners to regulate bone vascularization. Further detailed studies of developing tissues from PTHrP-null mice may identify additional tissues in which PTHrP regulates vascular development. Thus, PTHrP is probably essential in the negative regulation of angiogenesis during embryonic development.

PTHrP may regulate the progression of solid tumors by inducing humoral hypercalcemia of malignancy and local bone metastases[7,21–22]. However, as with other naturally occurring tumor-derived angiogenesis inhibitors[39], release of PTHrP into the circulation by primary tumors may suppress the growth of distant metastases. In addition, PTHrP may directly influence tumor cell proliferation, as indicated by its expression in prostate cancer cells, where it seems to activate nuclear receptors to promote tumor cell proliferation[24]. Intracellular PTHrP may

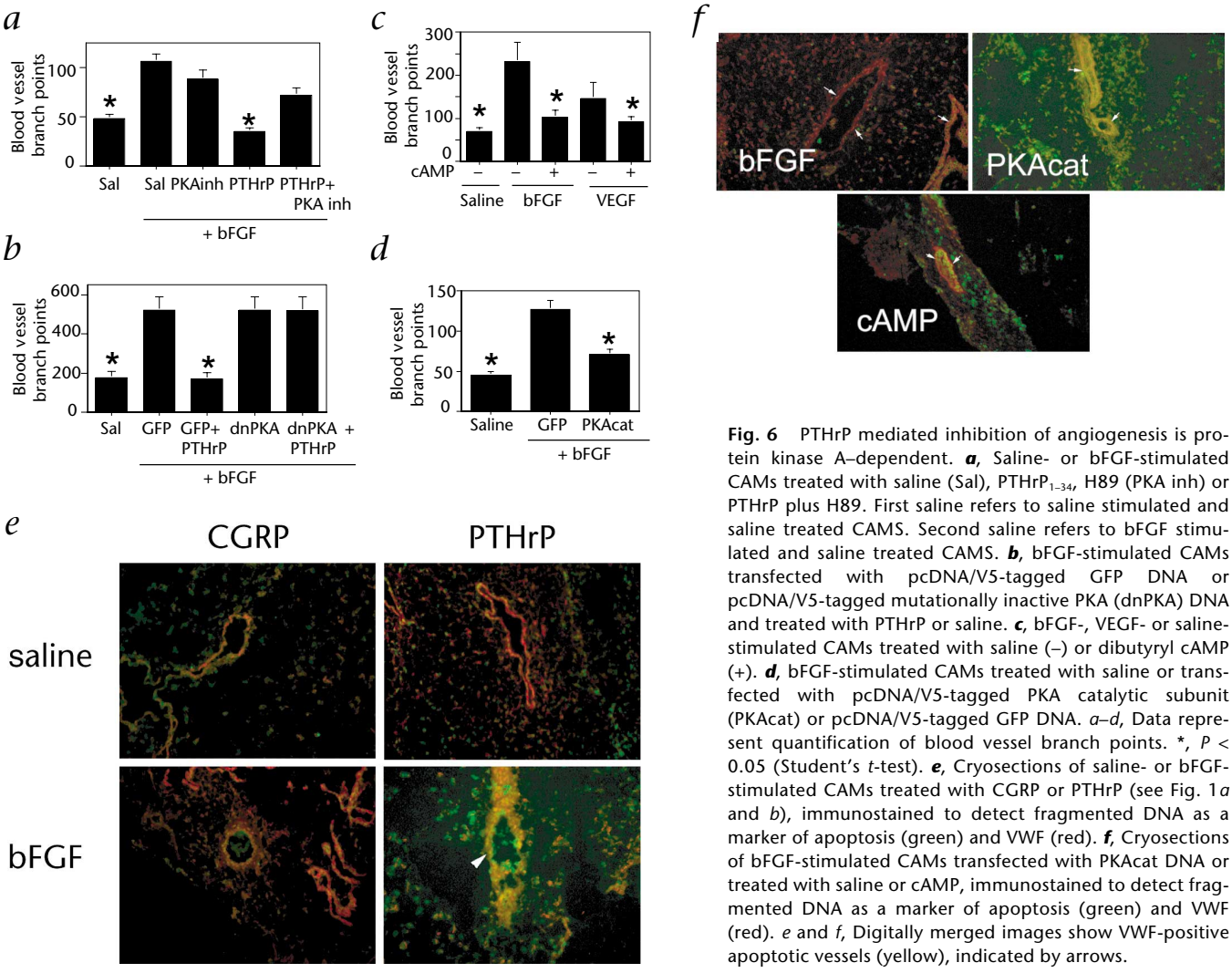


Fig. 6 PTHrP mediated inhibition of angiogenesis is protein kinase A–dependent. ***a***, Saline- or bFGF-stimulated CAMs treated with saline (Sal), $PTHrP_{1–34}$, H89 (PKA inh) or PTHrP plus H89. First saline refers to saline stimulated and saline treated CAMS. Second saline refers to bFGF stimulated and saline treated CAMS. ***b***, bFGF-stimulated CAMs transfected with pcDNA/V5-tagged GFP DNA or pcDNA/V5-tagged mutationally inactive PKA (dnPKA) DNA and treated with PTHrP or saline. ***c***, bFGF-, VEGF- or saline-stimulated CAMs treated with saline (–) or dibutyryl cAMP (+). ***d***, bFGF-stimulated CAMs treated with saline or transfected with pcDNA/V5-tagged PKA catalytic subunit (PKAcat) or pcDNA/V5-tagged GFP DNA. *a–d*, Data represent quantification of blood vessel branch points. *, $P < 0.05$ (Student's *t*-test). ***e***, Cryosections of saline- or bFGF-stimulated CAMs treated with CGRP or PTHrP (see Fig. 1*a* and *b*), immunostained to detect fragmented DNA as a marker of apoptosis (green) and VWF (red). ***f***, Cryosections of bFGF-stimulated CAMs transfected with PKAcat DNA or treated with saline or cAMP, immunostained to detect fragmented DNA as a marker of apoptosis (green) and VWF (red). *e* and *f*, Digitally merged images show VWF-positive apoptotic vessels (yellow), indicated by arrows.

promote tumor angiogenesis by inducing expression of IL-8 (ref. 24). In fact, a previous study indicating that PTHrP promotes angiogenesis showed only that conditioned medium from PTHrP-transfected tumor cells can induce angiogenesis[40]. Our studies with purified PTHrP and with PTHrP-transfected tumor cells indicate that PTHrP promotes angiogenesis only when it acts in an intracrine manner. As an anti-angiogenic agent, PTHrP may be useful therapeutically if delivered at high concentrations locally. Our studies using adenovirally expressed PTHrP indicate that local administration of PTHrP may be an effective strategy to inhibit angiogenesis and tumor growth.

PTHrP activates G protein–coupled transmembrane receptors, thereby stimulating adenyl cyclase and activating protein kinase A (refs. 13,14). Many of the physiological functions of PTHrP, including vascular smooth-muscle relaxation, are PKA dependent[18–20]. Our current studies demonstrate a function for PKA in the inhibition of endothelial cell migration and angiogenesis by PTHrP. These studies indicate that local activation of PKA may also be a means to inhibit angiogenesis and diseases characterized by angiogenesis.

Previous studies have shown that activation of PKA by cAMP can inhibit cell spreading, cytoskeleton formation and intracellular signaling[41,42]. Our data indicate that activation of PKA leads to inhibition of the small GTPase Rac, which is required for cell migration *in vitro*[32,33] and for angiogenesis *in vivo*[35]. As Rac activity is required for angiogenesis *in vivo*, our studies indicate that PTHrP blocks angiogenesis by inhibiting Rac in a PKA-dependent manner.

Our data demonstrate that PTHrP is a natural angiogenesis inhibitor and that its local delivery may be a useful strategy to inhibit pathological angiogenesis. More importantly, our studies demonstrate that activation of PKA by hormonal, genetic or pharmacological means is an effective strategy for suppressing angiogenesis.

Methods

Reagents. N1–GFP (green fluorescent protein) reporter and GFP-expressing adenovirus vectors were obtained from D. Cheresh. Mouse PKA catalytic subunit and dnPKA (RImut) cDNA (from S. Taylor and S. McKnight) were subcloned into topoTA–pcDNA 3.1 V5/His by PCR-based TA cloning according to manufacturer's directions (Invitrogen, Carlsbad, California). Myc-tagged V12 Rac was obtained from M. Schwartz. The sequence and orientation of constructs were verified by DNA sequencing. Cells (5×10^6) were transfected by electroporation with a total of 30 μg DNA (20 μg expression vectors, 2 μg N1–GFP and 8 μg pBluescript as carrier DNA) in 300 μl endothelial basal medium at 300 V and 450 μF. Expression levels of the transfected PKA subunits or GFP were assessed by western blot analysis of cell lysates using antibodies against V5 or GFP. Cells were cultured for 48 h before being used in experiments.

Chorioallantoic membrane angiogenesis assays. CAMs of 10-day-old chicken embryos (McIntyre Poultry, Ramona, California) were stimulated with 30 ng bFGF, VEGF, IL-8 or TNF-α (Genzyme,Cambridge, Massachusetts) or saline as described[43,44]. CAMs were treated with 20 μl 0.001–10 μM PTHrP peptide fragments 1–173, 1–141, 1–86, 1–34, 15–34, 1–10 or scrambled 1–10 (HLQAHSVEDL), calcitonin, CGRP or saline 24 h after the stimulation. Alternatively, 1 μM PTHrP was applied together with 10 μg function-blocking antibody directed against PTHrP (8B12) or 480 nM H89, a selective PKA inhibitor. Embryos stimulated with bFGF or VEGF were also injected intravenously with 1×10^7 plaque-forming units of adenoviruses expressing GFP or $PTHrP_{1–173}$ (ref. 27). Additionally, bFGF-stimulated CAMs were transfected by application of 4 μg N1–GFP, dnPKA or PKA catalytic subunit plasmid DNA. In some experiments, 50-mg fragments of DU145 prostate carcinoma cell tumors were placed on CAMs. Embryos were injected intravenously with 1×10^7 plaque-forming units of adenoviruses expressing GFP or PTHrP, and tumors were excised after 7 d. Ten embryos were used per group. Representative CAMs were photographed at ×10 magnification. Blood vessel branch points were counted in each CAM at ×30 magnification. Statistical analyses used the paired Student's *t*-test. Unfixed CAMs were 'flash-frozen', cut into cryosections and immunostained with antibodies against PTHrP (9H7), αvβ3, smooth-muscle actin, desmin and VWF. CAMs were also stained by the terminal deoxinucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) method to detect fragmented DNA together with antibodies against VWF or desmin. All thin sections were photographed at ×200 magnification. Experiments were done five to ten times.

Mouse angiogenesis and tumor assays. Mouse angiogenesis assays were done as described[45] incorporating saline or 1 μg/ml bFGF in growth factor–depleted Matrigel. Peptides were incorporated in the Matrigel at a concentration of 10 μM and adenoviruses at a concentration of 1×10^7 plaque-forming units per 400 μl Matrigel. Then, 3 d after injection of Matrigel, mice were injected intravenously with FITC–*Griffonia* (*Bandeiraea) simplicifolia* lectin. Plugs were then removed and fluorescence intensity was measured. Ten mice were used per group. Statistical analyses used the paired *t*-test. Nude mice were also inoculated with 1×10^6 PTHrP-negative tumor cells. Then, 2 weeks later, mice were treated for 10 d with daily injections of saline, PTHrP or a scrambled control PTHrP peptide (1 μM final concentration). Tumor volume was measured at days 0 and 10. Tumors were excised, cryopreserved, sectioned, immunostained for expression of CD31 and photographed at ×200 magnification. Ten mice were used per group. Statistical analyses used the paired *t*-test. Institutional approval was obtained for all animal experimentation.

FITC–annexin V staining. Culture plates were coated with 10 μg/ml vitronectin at 4 °C for 16 h and blocked with denatured BSA. Human umbilical vein endothelial cells (HUVECs) were incubated on plates in the presence or absence of 10 μM PTHrP or scrambled peptide for 18 h, then incubated with FITC–annexin V for 15 min in the dark. Washed cells were fixed in 1% paraformaldehyde, then counterstained with DAPI (4,6-diamidino-2-phenylindole). The percentage of annexin-positive cells in five microscopic fields was determined at ×200 magnification by fluorescence microscopy.

Cell culture, migration and adhesion assays. HUVECs were grown in endothelial growth medium (containing 2% fetal bovine serum, bFGF and VEGF; Clonetics, San Diego, California). To render HUVECs quiescent for Rac assays, monolayers of endothelial cells were incubated for 18 h in endothelial basal medium supplemented with 0.1% serum (Clonetics, San Diego, California). Cell migration and adhesion assays on ECM substrates were done as described before[41,42].

PKA assays. PKA activity was measured in HUVECs treated with 10 μM PTHrP or 250 μM cAMP essentially as described[41]. Culture medium was removed from cell monolayers and was replaced with fresh culture medium containing 10 μM PTHrP or 250 μM cAMP. PKA activity was measured 2–30 min later.

Rac assays. Rac activity was measured by PAK-binding-domain, PBD-affinity precipitation as described[33] (Upstate Biotechnology, Syracuse, New York). Endothelial cells were maintained in suspension for 2 h before being plated on vitronectin-coated plates for 30 min in the presence or absence of 10 mM PTHrP or cAMP.

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Competing interests statement

The authors declare they have no competing financial interests.

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