Deficiency in the secreted protein Semaphorin3d causes abnormal parathyroid development in mice

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Abstract

Primary hyperparathyroidism (PHPT) is a common endocrinopathy characterized by hypercalcemia and elevated levels of parathyroid hormone. The primary cause for PHPT is a benign overgrowth of parathyroid tissue causing excessive secretion of parathyroid hormone. However, the molecular etiology of PHPT is incompletely defined. Here, we demonstrate that semaphorin3d (Sema3d), a secreted glycoprotein is expressed in the developing parathyroid gland in mice. We also observed that genetic deletion of Sema3d leads to parathyroid hyperplasia, causing PHPT. In vivo and in vitro experiments using histology, immunohistochemistry, biochemical, RT-qPCR, and immunoblotting assays revealed that Sema3d inhibits parathyroid cell proliferation by decreasing the epidermal growth factor receptor (EGFR)/Erb-B2 receptor tyrosine kinase (ERBB) signaling pathway. We further demonstrate that EGFR signaling is elevated in Sema3d-/- parathyroid glands and that pharmacological inhibition of EGFR signaling can partially rescue the parathyroid hyperplasia phenotype. We propose that since Sema3d is a secreted protein, it may be possible to use recombinant Sema3d or derived peptides to inhibit parathyroid cell proliferation causing hyperplasia and hyperparathyroidism. Collectively, these findings identify Sema3d as a negative regulator of parathyroid growth.

Introduction

The parathyroid glands synthesize and secrete parathyroid hormone (PTH) to regulate serum calcium concentration in the body (1-4). In mice, there are two parathyroid glands located bilaterally in the neck and near the superior border of the thyroid gland. The parathyroid glands develop from the common parathyroid/thymus primordia, derived from the third pharyngeal pouch (5,6). As the development proceeds, parathyroid is separated from the thymus and becomes situated near or embedded within the thyroid gland (7,8). The early development and patterning of the parathyroid and thymus domains are controlled by a set of common regulatory genes (9-13). However, once the domains are specified within the same primordium, parathyroid cells express specific transcription factors such as glial cells missing 2 (Gcm2) (8). Gcm2 is essential for the differentiation and survival of parathyroid cells. Mice with Gcm2-deficiency develop parathyroid phenotype because their parathyroid precursor cells fail to proliferate and differentiate, and die via programmed cell death (8,14,15). Parathyroid hormone (PTH) gene expression is...
detected in parathyroid domain before its separation from the thymus, and is maintained throughout development (14).

Primary hyperparathyroidism (PHPT) is a common endocrine disorder caused by excessive secretion of PTH from the parathyroid glands (16,17). PHPT is the third most common endocrine disorder with a prevalence of 0.1–1.0%. It is more common in elderly females with a prevalence of up to 2.1% in postmenopausal women (16,18). In most cases of non-familial origin PHPT, hyperparathyroidism results from either benign single adenoma (80-85% cases) or multi-glandular parathyroid hyperplasia (15-20% cases) (16,17). In rare cases (<1%), parathyroid carcinoma has been associated with PHPT (19). The parathyroid gland maintains calcium homeostasis by sensing fluctuations in extracellular calcium levels through calcium-sensing receptor (CaSR) and responding to changes in PTH secretion (1-4). PHPT is characterized by increased parathyroid cell proliferation and calcium-insensitive hypersecretion of parathyroid hormone (20). Another form of hyperparathyroidism is secondary hyperparathyroidism (SHPT), in which parathyroid glands become enlarged and hyperactive in response to a condition outside of parathyroid causing hypocalcemia. SHPT is most frequently seen in patients with chronic kidney disease (21). In most primary or secondary hyperparathyroidism cases, increased serum PTH levels are associated with increased serum calcium levels, causing complications such as renal stones, neuropsychiatric disorders and bone abnormalities (16). Parathyroidectomy is the most common treatment for hyperparathyroidism. However, reoperative treatment for persistent or recurrent hyperparathyroidism remains technically challenging due to fibrotic scar and distorted anatomy that make it more difficult to identify the abnormal parathyroid glands (17,22).

Semaphorins are highly conserved secreted membrane-bound glycoproteins originally identified as axon guidance molecules in the developing nervous system (23). However, work in recent years has implicated them in a wide range of developmental, physiological, and pathological processes outside of the central nervous system including tumor progression (24-26). The primary receptors for semaphorins are plexins and neuropilins. Most membrane-bound semaphorins bind directly to plexins, whereas secreted class-3 semaphorins may require neuropilin as obligate plexin co-receptors (26,27). Increasing evidence has shown that semaphorins also can signal through a non-plexin receptor complex (28,29).

We have previously shown that Sema3d is expressed in the proepicardial organs and Sema3d-expressing proepicardial cells give rise to coronary vascular endothelium. While studying epicardial function of Sema3d, we noticed Sema3d was expressed in the third pharyngeal pouch, giving rise to parathyroid gland. Here, we report that Sema3d is expressed in the developing parathyroid gland and required for its proper development. Genetic deletion of Sema3d leads to PHPT due to the enlarged parathyroid gland. Molecular and biochemical analyses suggest that Sema3d inhibits parathyroid cell proliferation by decreasing EGFR/ErbB signaling pathway. We demonstrate that EGFR signaling is elevated in Sema3d−/− parathyroid glands and pharmacological inhibition of EGFR signaling can partially rescue the parathyroid hyperplasia phenotype. Since EGFR/ErbB signaling pathway is activated in the parathyroid glands in both PHPT and SHPT, Sema3d-EGFR/ErbB signaling axis may be targeted to treat parathyroid hyperplasia, especially in persistent or recurrent hyperparathyroidism after parathyroidectomy.

Results
Sema3d-deficient mice develop primary hyperparathyroidism
To determine the expression of Sema3d during parathyroid development, we performed expression and lineage tracing analyses in Sema3dGFPCre/− mice (30). Bright field and direct GFP fluorescence in E9.5 Sema3dGFPCre/− embryos demonstrated that Sema3d is expressed in the third pharyngeal pouch. Stronger GFP signals were observed in dorso-anterior domain of the third pouch that represents the parathyroid domain (Figure 1A). Similarly, when we crossed the Sema3dGFPCre/− line with the R26RloxP reporter line, strong β-galactosidase activity was observed in the third pharyngeal pouch (Figure 1B). Our results are consistent with a previous report suggesting that Sema3d is expressed in the parathyroid-specific domain of the common primordium that gives rise to the thymus and parathyroid (31). Thus far, Sema3d−/− mice survive and have abnormal pulmonary vein
observed in 40% (19/46) of
resembling parathyroid hyperplasia were
of
Sema3d
To determine whether elevated PTH levels in
but serum calcium levels remain normal (18,32).
Normocalcemic PHPT, PTH levels are elevated
calcium levels. However, in some cases of
hyperplasia, we isolated RNA from dissected
molecular changes associated with parathyroid
suggesting PHPT (Figure 2F). To determine the
compared with their littermate controls,
Gcm2 immunohistochemistry was performed to
mark the parathyroid glands in control and
Sema3d mice (Figure 2C). Significant increase
in the parathyroid area was observed in Sema3d−/−
mice (Figure 2D).

To determine whether parathyroid hyperplasia in
Sema3d−/− mice leads to hyperparathyroidism, we
measured serum PTH in 6 months old mice. We
observed elevated PTH levels in Sema3d−/− mice compared with their littermate controls (Figure 2E). In most cases of PHPT, increase serum PTH levels are associated with increased serum calcium levels. However, in some cases of normocalcemic PHPT, PTH levels are elevated but serum calcium levels remain normal (18,32). To determine whether elevated PTH levels in Sema3d−/− mice are associated with changes in calcium levels, we measured serum calcium levels in the same set of animals. We observed elevated calcium levels in Sema3d−/− mice compared with their littermate controls, suggesting PHPT (Figure 2F). To determine the molecular changes associated with parathyroid hyperplasia, we isolated RNA from dissected parathyroid gland from control and Sema3d−/− mice and performed qPCR analysis for parathyroid-specific genes such as parathyroid hormone (PTH), glial cells missing homolog 2 (Gcm2) and calcium-sensing receptor (CaSR). Expression levels of PTH and Gcm2 were significantly elevated in Sema3d−/− parathyroid compared with controls. However, the expression of CaSR was not altered in Sema3d−/− parathyroid glands (Figure 2G).

Sema3d inhibits parathyroid cell proliferation
To determine whether observed hyperplasia in
Sema3d−/− mice is due to abnormal parathyroid
cell proliferation, we performed Ki67 immunostaining on P0 and 6 months old parathyroid sections from control and Sema3d−/− mice. A dramatic increase in the number of Ki67-positive cells was noted in the Sema3d−/− parathyroid sections compared with controls at both time points. This suggests that abnormal cell proliferation is the primary cause of parathyroid hyperplasia (Figure 3A). To determine the molecular changes associated with increased parathyroid cell proliferation and hyperplasia in Sema3d−/− mice, we analyzed the expression of cell cycle regulators including cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitor (CDKIs) by qPCR on RNA isolated from microdissected parathyroid glands. We observed a 4-fold increase in the expression of Ccdn1 (cyclin D1), an oncogene known to be overexpressed in 20–40% of parathyroid adenomas (33-35). We also observed an increased in Cdkn2a (p21) and a modest decreased in Cdk4 expression in Sema3d−/− parathyroid (Figure 3B). Increased expression of Cdkn2a has been associated with tumor progression (36). Growing evidence suggests that depending on its environment, Cdkn2a can act as either tumor suppressor or an oncogene (37,38). No significant changes were observed in Ccdn2 (cyclin D2), Cdkn2c (p18), Cdkn1b (p27) and Cdkn1c (p57). To determine total RNA quality isolated from the microdissected parathyroid tissues, we performed qPCR analysis for thyroid stimulating hormone receptor (TSHR), troponin T1 (Tnnt1) and Sox9. This allowed us to detect possible contamination from thyroid, skeletal muscle and cartilage tissues, respectively. No significant changes were observed (Supplementary Figure 2). Together, these results suggest that Sema3d inhibits parathyroid cell proliferation in vivo by regulating the expression of cell cycle genes.

Since parathyroid tissues from mice are limited, we used a human parathyroid cell line shHPT-1 to dissect the molecular mechanism of Sema3d-mediated inhibition of parathyroid cell proliferation. shHPT-1 is established from a hyperplastic parathyroid gland surgically removed from secondary hyperparathyroidism (sHPT) patient (39). To determine whether shHPT-1 cells are sufficiently similar to primary parathyroid cells with regard to their semaphorin signaling pathways, we treated shHPT-1 cells with
recombinant Sema3d and analyzed proliferation by Ki67 staining. Consistent with the in vivo data, we see significant reduction in sHPT-1 cells proliferation in Sema3d-treated samples compared with controls. This suggests that Sema3d inhibits parathyroid cell proliferation both in vivo and in vitro conditions (Figure 3C).

**Sema3d inhibits EGFR signaling pathway to control parathyroid cell proliferation**

Semaphorins signal through their classical Plexin and neuropilin receptors, and other receptors such as receptor tyrosine kinases (28,40,41). Sema3d binds and signals through Neuropilin-1 (Nrp1) to regulate endothelial cells patterning during pulmonary vein development (30,42). However, Nrp1 is not expressed during parathyroid development, suggesting that Sema3d may signal through other receptors expressed by the parathyroid gland (31). In a candidate based screening, we found that Sema3d inhibits epidermal growth factor receptor (EGFR) signaling pathway. EGFR is a member of the ErbB family of receptor tyrosine kinases that includes ErbB2, ErbB3, and ErbB4. Ligand binding induces homo or heterodimerization of EGFR with other family members including ErbB2 and activation of intracellular tyrosine kinase through formation of an asymmetric kinase dimer. Activation of ErbB receptors results in activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling, leading to enhanced proliferation, primarily due to aberrant changes in cell cycle gene expression including cyclin D1 (43,44). Enhanced EGFR signaling has been associated with parathyroid adenomas and hyperplasia in both PHPT and SHPT (45-49).

To determine whether Sema3d inhibits parathyroid cell proliferation through the EGFR signaling pathway, we treated sHPT-1 cells with control and alkaline phosphatase (AP)-tagged Sema3d (Sema3d-AP) condition media and analyzed the expression of EGFR signaling components such as pEGFR (Y1045), pEGFR (Y992), EGFR, pErbB2 (Y1248), ErbB2, pAkt, Akt, pErk1/2, Erk1/2 and cyclin D1 by western blots. Sema3d-AP condition media treatment reduced the amount of total EGFR, as well as pEGFR (Y1045) and pEGFR (Y992), suggesting that Sema3d may inhibit EGFR signaling by inducing receptor endocytosis/internalization and degradation. In parallel with EGFR downregulation, ErbB2 and pErbB2 (Y1248) levels were also decreased after nanomolar quantities of Sema3d treatment. To determine whether the downstream components of EGFR pathway were also affected after Sema3d treatment, we analyzed expression and activation of Akt and Erk1/2. The phosphorylation level of Akt and Erk1/2 was reduced. However, the total amount of Akt and Erk1/2 was not changed. Cyclin D1, one of the known targets of ErbB signaling in cancer cells, was significantly reduced in Sema3d-AP treated cells (Figure 4A).

Next, we treated sHPT-1 cells with recombinant Sema3d at different time points and analyzed EGFR signaling components. Consistent with earlier observation, all signaling components were significantly down-regulated after Sema3d treatment (Figure 4B). Treatment of sHPT-1 cells with increasing recombinant Sema3d concentrations affected EGFR signaling pathway in a similar manner described above (Figure 4C-D).

**Inhibitory effects of Sema3d on ErbB2 is EGFR dependent**

Although Sema3d does not bind to ErbB2 alone, it can bind as part of a receptor complex. This suggests that changes in pErbB2 levels in sHPT cells after Sema3d treatment were likely from the impact of EGFR signaling pathway (Figure 4 and Supplementary Figure 3) (29). To determine whether the inhibitory effect of Sema3d on ErbB2 activation was EGFR dependent, we co-transfected HEK293T cells with wild-type ErbB2 and a plasmid expressing either wild-type EGFR or kinase-inactive EGFR. The transfected cells were then treated with recombinant Epidermal growth factor (EGF), in the presence/absence of Sema3d. Consistent with previous observations, when wild-type EGFR and wild-type ErbB2 were co-expressed, both receptors were phosphorylated in response to EGF (Figure 4E). Sema3d exposures inhibited EGF-induced phosphorylation of both EGFR and ErbB2. However, when kinase-inactive EGFR and wild-type ErbB2 were co-expressed, no EGFR phosphorylation and low level of ErbB2 phosphorylation was observed after EGF stimulation (Figure 4E). Addition of Sema3d did not change ErbB2 phosphorylation, suggesting that the inhibitory effect of Sema3d on ErbB2 activation is EGFR dependent (Figure 4E).

Next, we tested whether Sema3d can inhibit EGFR-activated pathway, a condition associated with parathyroid hyperplasia in both PHPT and
SHPT (45-49). We treated sHPT-1 cells with different doses of NSC 228155, an EGFR activator (50) and performed western blot analyses on pEGFR (Y1045) and pEGFR (Y992) to determine the optimal dose required to activate the pathway. We observed that 100 µM of NSC 228155 significantly increased the level of pEGFRs (Figure 5A). Recombinant Sema3d inhibited NSC 228155-induced EGFR activation (Figure 5B). Cyclin D1 expression was also increased with NSC 228155 treatment, but it was decreased following Sema3d treatment (Figure 5B). To determine whether increased cyclin D1 expression correlated with increased cell proliferation, we treated sHPT-1 cells with NSC 228155 in the presence/absence of recombinant Sema3d, and analyzed their proliferation (Figure 5C). Activation of EGFR pathway by NSC 228155 resulted in significantly increased proportion (>80%, p<0.001) of Ki67 positive cells. However, addition of Sema3d significantly decreased (~30%, p<0.001) the number of Ki67 positive cells induced by NSC 228155 treatment. This suggests that Sema3d regulate cell cycle progression by signaling through EGFR receptor (Figure 5C). Together, both in vivo and in vitro results suggest that Sema3d inhibits EGFR signaling pathway to restrict parathyroid cell proliferation. A recent study by Aghajanian et al demonstrated that Sema3d activates ErbB2 signaling pathway in endothelial cells during coronary vasculature patterning (29). This indicates that depending on its biological context, Sema3d can act as either agonists or antagonists for ErbB signaling pathway (29).

Blocking EGFR signaling can partially rescue the parathyroid hyperplasia phenotype in Sema3d−/− mice

To determine whether Sema3d inhibits EGFR pathway in vivo, pEGFR immunohistochemistry was performed on parathyroid sections from control and Sema3d−/− mice. We observed elevated levels of pEGFR in Sema3d−/− parathyroid sections compared with controls (Figure 6A). Next, we tried to determine whether hyperplasia seen in Sema3d−/− mice was due to an abnormal increase in the EGFR signaling and whether blocking EGFR signaling can rescue the hyperplasia phenotype. To examine if elevated EGFR signaling resulted in parathyroid hyperplasia, we treated Sema3d−/− mice with either vehicle or Erlotinib, an EGFR signaling pathway inhibitor, and analyzed their parathyroid glands. We observed a significant size reduction of the parathyroid glands in Sema3d−/− mice treated with Erlotinib compared with vehicle-treated controls (Figure 6B). Consistent with reduced parathyroid size, we also observed that Ki67 positive cells were reduced in Erlotinib treated samples when compared with controls (Figure 6C). To determine whether the reduced parathyroid glands impact PTH levels, we measured serum PTH levels in Sema3d−/− mice treated either with vehicle or Erlotinib. PTH levels were significantly reduced after Erlotinib treatment (Figure 6D). Together, these results indicate that blocking EGFR signaling could partially rescue the hyperplasia and hyperparathyroidism phenotype seen in Sema3d−/− mice.

Discussion

The pathogenesis of parathyroid gland hyperplasia in hyperparathyroidism is poorly understood. Thus, a better molecular understanding is essential for prevention and therapeutic intervention. In this study, we demonstrate that Sema3d−/− mice develop PHPT from parathyroid hyperplasia. However, only ~40% of Sema3d−/− mice develop hyperplasia. We suspect that this incomplete phenotype is contributed by the genetic background, as phenotypes in other class 3 semaphorin knockouts are also strain dependent (51). Expression of parathyroid specific developmental genes such as PTH and Gcm2 was increased in Sema3d−/− mice. However, CaSR expression was not altered. Expression of CaSR enables the parathyroid cell to respond to changes in extracellular calcium concentration. CaSR activation by high extracellular calcium results in reduced PTH secretion. On the other hand, deactivation of CaSR by low extracellular calcium induces PTH secretion. Normal expression of CaSR in Sema3d−/− mice suggests that elevated PTH levels are primarily caused by increased gland size and not from individual parathyroid cells secreting more PTH.

Genetic inactivation of Sema3d leads to activated EGFR signaling pathway and cyclin D1, a cell cycle regulator and an oncogene. This results in increased parathyroid cell proliferation. Cyclin D1 binds and activates CDKs that phosphorylate several downstream proteins to ensure cell cycle progression through the G1/S checkpoint. The role of Cyclin D1 in parathyroid tumorigenesis is well established. Cyclin D1 is overexpressed in 20–40% of parathyroid adenomas (33-35).
However, these numbers are much higher (~80%) in sporadic parathyroid adenomas from Asian Indians. Cyclin D1 is also activated in a subset of parathyroid adenomas from chromosomal rearrangement, bringing the **Cyclin D1** gene under the influence of PTH promoter region (34). Studies in transgenic mice have demonstrated that targeted overexpression of **Cyclin D1** in parathyroid tissues can lead to parathyroid adenomas (33).

Semaphorins signal through their classical Plexin and neuropilin receptors. However, Semaphorins can also signal through other receptors such as receptor tyrosine kinases (28,40,41). Our data suggest that Sema3d can modulate cell behavior by regulating the receptor tyrosine kinases activities. Growth factor receptor tyrosine kinases, including EGFR and ErbB2, play an important role in mediating cancer growth and survival. EGFR is a member of the ErbB family of receptor tyrosine kinases that also includes ErbB2, ErbB3, and ErbB4. Upon ligand binding, ErbB family members can form homo or heterodimer and activate downstream MAPK and PI3K/Akt signaling pathway to regulate cell proliferation. Our data demonstrate that Sema3d can inhibit both EGFR and ErbB2 signaling pathways. Down-regulation of both total and phosphorylated form of EGFR suggests that Sema3d may inhibit EGFR signaling by inducing receptor endocytosis/internalization and degradation. Different posttranslational modifications such as tyrosine and serine/threonine phosphorylation, ubiquitylation and acetylation regulate EGF receptor endocytosis (52). Among the class 3 semaphorins, both Sema3a and Sema3e induce endocytosis of Plexin receptor/receptor complex during axon guidance (53,54). Recent work by Aghajanian et al. demonstrated that Sema3d does not bind to ErbB2 alone (29). However, it can bind as part of a receptor complex, suggesting that inhibitory effects of Sema3d on ErbB2 activation are likely from EGFR signaling pathway (29). Using plasmids expressing either wild type or kinase-inactive form of EGFR, we demonstrate that the inhibitory effects of Sema3d on ErbB2 are EGFR dependent. In contrast to the inhibitory effect of Sema3d on RTKs, another class 3 semaphorin, Sema3c, can activate RTKs during prostate cancer progression. This suggests that depending upon its biological context, class 3 semaphorins can act as either agonists or antagonists on the RTK pathway (40).

Our results demonstrate that pharmacological blocking of EGFR signaling can partially rescue the parathyroid hyperplasia phenotype seen in Sema3d knockout mice. It would be interesting to explore whether Sema3d-EGFR signaling can be therapeutically targeted to inhibit parathyroid hyperplasia in both PHPT and SHPT conditions. Parathyroidectomy is the most common treatment for hyperparathyroidism. Since the neck’s anatomy was distorted with fibrosis following the initial surgery, there were technical challenges in identifying and safely removing the abnormal parathyroid glands. Therefore, pharmacological treatment for persistent or recurrent hyperparathyroidism may be a good option to address this issue. Administration of EGFR inhibitors have shown beneficial effects. However, systemic inhibition could also suppress renal EGFR signaling and compromise kidney function (49). In contrast to its broad expression during embryonic development, Sema3d expression in adult is enriched in the spleen and thyroid gland (55). In this context, Sema3d may be a potential therapeutic target to treat parathyroid hyperplasia given its more limited tissue expression in adult mice. Nonetheless, the possible off-target effects of systemic or targeted delivery of Sema3d needs to be determined.

Because class 3 semaphorins are secreted proteins, it is possible to use recombinant semaphorins or derived peptides to inhibit parathyroid cell proliferation causing hyperplasia. A recent study by Casazza et al demonstrated that systemic and targeted delivery of Sema3A inhibits tumor progression in multiple mouse models (56). However, the inhibitory effect of systemic or targeted Sema3d delivery on parathyroid tumor progression remains to be investigated. Our finding that Sema3d inhibits EGFR signaling to restrict aberrant parathyroid tumor cell proliferation may also be relevant to other tumor types. Aberrant activation or overexpression of EGFR receptor is reported in many tumors (57). For example, overexpression of EGFR receptor is observed in 60% of the high-grade gliomas (Glioblastoma) (58). SEMA3D has been shown to inhibit glioblastoma growth. The **SEMA3D** expression is reduced in glioblastoma as compared with low-grade gliomas, suggesting that its loss is involved in tumor progression (59). However, the signaling pathways mediating these effects of **SEMA3D** in gliomas have not been identified. Thus, future
work in this direction is required for the development of develop anti-tumor therapeutic strategies targeting the semaphorin signaling pathway.

Experimental procedures

Experimental animals
All mice were maintained on a mixed (C57BL/6 and Sv/129) genetic background. Both male and female mice were used for analysis. Sema3dGFPCre allele has been previously described (30,60). Heterozygous mice (Sema3dGFPCre/+ ) were crossed together to generate Sema3dGFPCreGFPCre (Sema3d−/−) mice. Sema3d+/+ or Sema3dGFPCre/+ mice were used as control. The SingHealth institutional animal care and use committee approved all animal protocols.

Histology and immunohistochemistry
After genotyping of the pups from Sema3d heterozygous intercross, we randomly selected 12 control and 12 Sema3d−/− mice from the cohort for histological analysis. Histology and immunohistochemistry were performed as described previously (61-65). Briefly, the thyroid, parathyroids, and trachea tissues were dissected en bloc in PBS and fixed in 4% paraformaldehyde overnight at 4°C. The tissues were washed with PBS, dehydrated in an ethanol series, and stored in 100% ethanol at 20°C. To examine the parathyroid glands, serial paraffin sections were cut to a thickness of 5-10 µm and processed for either morphological hematoxylin/eosin staining or immunohistochemistry. For immunohistochemistry, the sections were deparaffinized in xylene, and slides were deparaffinized in xylene, dehydrated in ethanol and rehydrated in water. Antigen retrieval using Bull’s eye decloaker (Biocare Medicals, Catalog no. BULL1000 MX) or Tris-based solution (Vector lab, Catalog no. H-3301) was performed according to the manufacturer’s instructions. Endogenous peroxidase activity was blocked with hydrogen peroxide (3%) treatment. Slides were washed in PBS and blocked in blocking buffer (5% serum) for 1-2 hours at room temperature. Slides were incubated overnight with primary antibody diluted in blocking buffer. Next day, slides were washed and a secondary antibody was applied for 2 hours at room temperature. Primary antibodies used were anti-GCM2 goat polyclonal (Santa Cruz Biotechnology, catalog no. sc-79495), anti-Ki67 rabbit monoclonal (Abcam, catalog no. ab16667) and anti-pEGFR-Y1045 rabbit polyclonal (Cell signalling, Catalog no. 2237). Secondary antibodies used were ImmPRESS® HRP Anti-Goat (Vector lab, Catalog no. MP-7405-50) or Anti-Rabbit (Vector lab, Catalog no. MP-7401-50). The sections were washed and staining was visualized using DAB substrate kit (Vector lab, Catalog no. SK-4100).

Biochemical analyses
At the indicated age, control and Sema3d−/− mice were anesthetized and blood was collected by cardiac puncture for plasma separation. Plasma PTH levels were measured using the mouse Intact PTH ELISA kits (Immutopics, Inc. catalog no. #60-2300). Plasma calcium levels were measured using a Calcium detection kit (Abcam, catalog no. ab102505).

SHPT cell culture
Human parathyroid tumor cell line sHPT-1 was established by Björklund et al., as previously described (39). sHPT-1 cells were cultured and expanded in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, catalog no. 11965-092) containing 1% penicillin/streptomycin and 10% FBS. After reaching 70-80% confluency, cells were cultured and starved overnight in DMEM supplemented with 1% penicillin/streptomycin and 1% FBS. They were subsequently treated with Sema3d conditioned medium or recombinant Sema3d (Abnova, catalog no. H00223117-P01) in desired concentrations at indicated time points with appropriate controls. For the proliferation assay, sHPT-1 cells were treated with or without recombinant Sema3d at 10 nM for 48 hours. In another experiment, sHPT-1 cells were stimulated with NSC 228155 (100 µM) or in combination with 10 nM of recombinant Sema3d for 24 hours. After treatment, cells were fixed with 4% PFA for 30 min at room temperature and evaluated for cell proliferation by Ki-67 (eBioscience, catalog no. 14-5698-82) immunofluorescence staining. Briefly, cells were fixed and washed with PBS and incubated for 2 hours at room temperature with Ki-67 antibody diluted at a concentration of 5 µg/ml in PBS containing 2% BSA. After washing with PBS, cells were incubated for 30 min at room temperature with goat anti-rat IgG, Alexa Fluor 586 (Invitrogen, catalog no. A-11077). The cells were then washed again and incubated with DAPI for nuclear staining visualized with fluorescence microscopy (Olympus IX71S1F3).
**EGFR activation assay**

sHPT-1 cells were incubated with either DMSO as vehicle or NSC 228155 compound (Calbiochem, catalog no. 5.30536.0001CN) for 30 minutes at 25, 50, 100, 200 and 300 µM and analyzed for EGFR members by western blot. In another experiment, sHPT-1 cells were treated with either NSC 228155 (100 µM) or in combination with recombinant Sema3d (5 nM, 10 nM, and 15 nM) for 30 minutes and analyzed for EGFR with western blot analysis.

**Mice and erlotinib**

Twelve weeks old Sema3d−/− mice were randomly assigned into 2 treatment groups: vehicle (DMSO) and Erlotinib (10 mg/kg body weight) (Selleckchem, catalog no. S1023). Treatments were administered every 2 days by intraperitoneal injection. After 4 weeks of treatment, the mice were sacrificed and serum and parathyroid tissue were collected for histologic and molecular analyses.

**Preparation of Sema3d conditioned medium**

HEK293T cells were cultured and expanded in DMEM (Gibco, catalog no. 11965-092) supplemented with 1% penicillin/streptomycin and 10% FBS. HEK293T cells were transfected with either Sema3d pAP-Tag4 or empty vector plasmid using the FuGENE® 6 Transfection Reagent (Promega, catalog no. E2691) according to the manufacturer’s protocol. After 24 hours, the medium was changed to DMEM (Gibco, catalog no. 11965-092) supplemented with 1% penicillin/streptomycin and 0.5% FBS. The cells were then incubated for 72 hours and the conditioned medium was collected. The medium was filtered using a 0.2 µm syringe filter and centrifuged at 5000 rpm for 5 min. Alkaline phosphatase (AP) activity in the conditioned medium was measured with colorimetric assay as described previously (30). The conditioned media was then used for the experiments.

**Kinase Activation assay**

HEK293T cells were transfected with either plasmid expressing wild-type ErbB2, a wild-type EGFR or a kinase-inactive EGFR (K721A) using lipofectamine transfection reagent (Thermo Fischer, catalog no. 11668019) according to the manufacturer’s protocol. Following transient transfection, cells were incubated for 48 hours and starved for 4 hours with serum-free DMEM supplemented with 1% penicillin/streptomycin. Cells were then treated with/without 200 ng/ml EGF (PeproTech, catalog no. AF-100-15), and with/without 10nM recombinant Sema3d for 30 minutes. Cell lysates were collected for western blot analysis. Plasmids expressing wild-type EGFR and kinase-inactive EGFR (K721A) were kindly provided by Sara Sigismund from Pier Paolo Di Fiore’s laboratory (European Institute of Oncology, Milan, Italy). The plasmid expressing ErbB2 was a gift from Martin Offterdinger (Addgene plasmid # 40268) (66).

**RNA extraction and quantitative RT-PCR**

Parathyroid glands were dissected from control and Sema3d−/− mice. Since only ~40% of the Sema3d−/− mice had parathyroid hyperplasia, we decided to use hyperplastic (enlarged) parathyroid tissue from Sema3d−/− mice. Total RNA was isolated glands using TRIzol (Life Technologies, catalog no. 15596-018). RNA was reverse-transcribed using random hexamers and the SuperScript III First-Strand Synthesis system (Life Technologies, catalog no. 18080-051). Gene expression was measured by quantitative RT-PCR (ABI PRISM 7900 or ViiA7 Real-Time PCR System) using the Power SYBR Green master mix (Life Technologies, catalog no. 4368702). Both signals and relative gene expression were normalized to corresponding glyceraldehyde-3-phosphate dehydrogenase (Gapdh) controls. PCR conditions and primer set sequences are available upon request.

**Western blot analyses**

Western blots were performed as described previously (61,67). Briefly, cells were washed twice with cold DPBS (Lonza, catalog no. 17-512F) and lysed with RIPA buffer (Thermo Scientific, catalog no. 89901) containing 1:100 diluted protease and phosphatase inhibitor cocktail (Sigma). The cell lysates were centrifuged at 13000 rpm for 10 min at 4°C and the supernatant was collected for immunoblot analyses. Total protein concentration was determined with Pierce BCA protein assay kit (Thermo Scientific, catalog no. 23225) following manufacturer's instructions. Approximately 20-30 µg of total protein samples were separated by SDS-PAGE and transferred to nitrocellullose membrane using the Trans-Blot Turbo system (Bio-Rad). Membranes were then blocked with 2-5% BSA in TBS containing 0.1% Tween (TBST) and subsequently incubated with primary antibodies diluted in TBST containing 2-5% BSA for overnight at 4°C. Blots were then washed in...
TBST and incubated for 1.5 h at room temperature probed with the appropriate horseradish peroxidase-linked secondary antibodies (Santa Cruz). Immunoreactive bands were detected by chemiluminescence (Hiss GmbH, catalog no. 16026) using Gel Doc XR+ System (Bio-Rad). All experiments were repeated three times and quantified. Primary antibodies used for immunoblot analyses were: pAkt (Cell signalling, Catalog no. 4060S), Akt (Cell signalling, Catalog no. 9272S), pErk1/2 (Cell signalling, Catalog no. 9101S), Erk1/2 (Cell signalling, Catalog no. 9102S), pErB2 (Cell signalling, Catalog no. 2247), ErbB2 (Cell signalling, Catalog no. 2242), CyclinD1 (Santa Cruz, Catalog no. sc-8396), pEGFR-Y1045 (Cell signalling, Catalog no. 2237), pEGFR-Y992 (Cell signalling, Catalog no. 2235), EGFR (Cell signalling, Catalog no. 4267), Vinculin (Sigma, Catalog no. V9131), GAPDH (Santa Cruz, Catalog no. sc-20357) and β-actin (Santa Cruz, Catalog no. sc-47778).

**Statistical Analyses**

Statistical analyses were performed using two-tailed Student's t-test. Data were expressed as mean ± standard deviation (SD). Differences were considered significant with the p-value < 0.05. One-way analysis of variance (ANOVA) was used to assess statistical differences between groups. Significant ANOVA results were further analyzed by Tukey’s multiple comparisons test (*, p<0.05; **, p<0.01; ***, p<0.001; NS, not significant).

**Author contributions**

A.S., M.M.M., D.M.C., A.K.A., S.K.B., and M.K.S. designed, performed experiments and analyzed the data. M.K.S. oversaw the entire project, designed experiments, analyzed data, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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**Conflict of interests**

The authors declare no conflict of interest.

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Figure 1. Sema3d expression in the developing third pharyngeal pouch. (A) Bright field and direct GFP fluorescence in E9.5 Sema3d<sup>GFP</sup>Cre<sup>+/−</sup> mouse embryos. (B) Whole mount X-gal staining of E9.5 and E10.5 Sema3d<sup>GFP</sup>Cre<sup>+/−</sup>;R26R<sup>lacZ</sup> embryos. pp1, pharyngeal pouch 1; pp2, pharyngeal pouch 2; pp3, pharyngeal pouch 3; H, Heart.
Figure 2. Primary hyperparathyroidism in Sema3d−/− mice. (A, B) Morphological and histological examination of parathyroid glands from 6 (n=12 per genotype) and 12 months old (n=15 per genotype) control and Sema3d−/− mice. (C) GCM2 immunohistochemistry on parathyroid sections from 12 months old control and Sema3d−/− mice. (D) Quantification of parathyroid area in 12 months old control and Sema3d−/− mice (n=12). (E) Serum PTH levels in 12 months old control (n=8) and Sema3d−/− mice (n=10). (F) Serum calcium levels in 12 months old control (n=7) and Sema3d−/− mice (n=8). (G) Real-time qPCR for PTH, Gcm2, and CaSR on RNA isolated from the micro-dissected parathyroid gland of 6 months old control (n=3) and Sema3d−/− mice (n=3). Fold changes in gene expression are presented. T, thyroid; PT, parathyroid. Scale bars are 200µm.
Figure 3. Sema3d inhibits parathyroid cell proliferation in vivo and in vitro. (A) Immunohistochemistry for Ki67 on parathyroid sections from P0 and 6 months old control (n=5) and Sema3d−/− (n=5) mice. High magnification images show nuclear Ki67 staining. Scale bars are 50 µm. (B) Real-time qPCR for cell cycle regulators such as Ccdn1, Ccdn1, Cdk4, Cdkn2c, Cdkn2a, Cdkn1b and Cdkn1c on RNA isolated from the micro-dissected parathyroid gland of 6 months old control (n=3) and Sema3d−/− mice (n=3). (C) Immunostaining for Ki67 on sHPT cells treated with control or recombinant Sema3d for 48 hrs. Nuclei were visualized by DAPI staining (blue), and the percentage of Ki67 positive cells were quantified. T, thyroid; PT, parathyroid.
Figure 4. Sema3d inhibits parathyroid cell proliferation by decreasing EGFR signaling pathway. (A) Western blot and quantification for EGFR signaling pathway molecules from sHPT cells incubated with control or Sema3d condition media for 30 min. (B-D) Western blot and quantification for EGFR signaling pathway molecules from sHPT cells treated with 4 nM recombinant Sema3d for 0, 5, 15 and 30 mins (B), or sHPT cells treated with 0, 0.1, 1 and 10 nM recombinant Sema3d for 30 mins (C, D). *, p<0.05; **, p<0.01; ***, p<0.001; NS, not significant (one-way ANOVA between groups, post hoc multiple comparisons, Tukey’s test). (E) EGFR signaling in cells expressing mixed wild type and kinase-inactive EGFR/ErbB2 heterodimers. HEK293T cells expressing the indicated pairs of EGFR and ErbB2 receptors were treated with recombinant EGF with and without recombinant Sema3d for 30 mins. Cell lysates were prepared for western blots to detect pEGFR, EGFR, pErbB2, ErbB2 and beta-actin levels. Western blots were quantified for pEGFR and pErbB2 relative to beta-actin levels. Six bars in the graph represent 6 conditions labelled above the blots. Experiments were repeated three times and quantified.
**Figure 5. Sema3d inhibits chemically induced EGFR signaling pathway.** (A) Optimizing NSC 228155 concentrations to activate the EGFR signaling pathway in sHPT cells. Western blot for pEGFRs from sHPT cells incubated with increasing concentrations of NSC 228155 for 30 mins. (B) Western blot and quantification for pEGFR and Cyclin D1 from sHPT cells incubated with 100 µM of NSC 228155 with or without increasing amount of recombinant Sema3d (5, 10 and 12 nM) for 30 mins. *, p<0.05; **, p<0.01; ***, p<0.001 (one-way ANOVA between groups, post hoc multiple comparisons, Tukey’s test). (C) Immunostaining for Ki67 on sHPT cells treated with either control or NSC 228155 (100 µM) or NSC 228155 (100 µM) together with 10 nM recombinant Sema3d for 24 hrs. Nuclei were visualized by DAPI staining (blue) and the percentage of Ki67 positive cells were quantified. Experiments were repeated three times and quantified. ***, p<0.001 (one-way ANOVA between groups, post hoc multiple comparisons, Tukey’s test).
Figure 6. Inhibition of EGFR signaling in Sema3d mutants can partially rescue the parathyroid hyperplasia phenotype. (A) Immunohistochemistry for pEGFR on parathyroid sections from 6 months old control and Sema3d−/− mice. Scale bars are 200 µm. (B) Immunohistochemistry for pEGFR on parathyroid sections from Sema3d−/− mice treated either with either vehicle (n=5) or Erlotinib (n=5). Scale bars are 200 µm. (C) Decreased parathyroid area in Sema3d−/− mice treated with EGFR inhibitor, Erlotinib (n=12), compared with vehicle (DMSO) treated controls (n=12). (D) Immunohistochemistry for Ki67 on parathyroid sections from Sema3d−/− mice treated either with vehicle (n=5) or Erlotinib (n=5). High magnification images show nuclear Ki67 staining. Scale bars are 50 µm. (E) Serum PTH levels in Sema3d−/− mice treated either with vehicle (n=12) or Erlotinib (n=12). T, thyroid; PT, parathyroid.
Deficiency in the secreted protein Semaphorin3d causes abnormal parathyroid development in mice
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