The Role of Prokineticins in the Pathogenesis of Hypogonadotropic Hypogonadism

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Abstract

The prokineticin system comprises two multifunctional secreted proteins, prokineticin-1 (PROK1) and prokineticin-2 (PROK2), and their cognate G protein-coupled receptors. The prokineticins were originally identified as endogenous regulators of gastrointestinal motility. Currently, these bioactive peptides are involved in a wide spectrum of biological functions, including angiogenesis, neurogenesis, circadian rhythms, nociception, hematopoiesis and immune response. Mice homozygous for null mutations in *Prokr2* or *Prok2* recapitulate the human phenotype of Kallmann syndrome, exhibiting severe atrophy of the reproductive system and hypoplastic olfactory bulbs. Indeed, the evidence from several naturally inactivating mutations in the *PROK2* and *PROKR2* genes in patients with Kallmann syndrome and normosmic hypogonadotropic hypogonadism also indicate the essential role of PROK2 in olfactory bulb morphogenesis and GnRH secretion in humans.

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Introduction

The prokineticin system comprises two closely related proteins, prokineticin-1 (PROK1; also called EG-VEGF) and prokineticin-2 (PROK2; also called Bv8), and their cognate G protein-coupled receptors, PROKR1 and PROKR2 [1, 2]. Prokineticins are cysteine-rich secreted proteins that were originally identified as potent agents mediating gut motility in the digestive system, but were later shown to promote diverse biological functions, including normal development of the olfactory bulb and sexual maturation [1].

The mature forms of PROK1 and PROK2 consist of 86 and 81 amino acids, respectively [1]. These proteins are approximately 50% homologous to each other and share the identical amino terminal hexapeptide (AVITGA), which is crucial for their biological activities [2]. Both contain carboxyl-terminal cysteine-rich domains that form five disulfide bridges with conserved spacing. Substitutions, deletions and insertions to the conserved amino-terminal hexapeptide or mutations affecting selected cysteine residues in the carboxy-terminal domain result in prokineticins without biological activity [2].

PROK1, an angiogenic mitogen for endocrine gland vascular endothelium [3], is encoded by a small gene mapped on chromosome 1 (NCBI Gene ID: 84432). PROK2 is encoded by the *PROK2* gene on 3p13 and contains 4 exons (NCBI Gene ID: 60675). The most active form of PROK2 with 81 amino acids is encoded by the exons 1, 2 and 4. Molecular cloning analysis has further identified a long form of the PROK2 peptide, PROK2L, with 21 additional amino acids, encoded by all 4 exons of *PROK2* [4]. PROK1 and PROK2 are expressed in the adrenal gland, brain, testis, intestinal tract, heart, bone marrow and peripheral blood [5]. PROK1 is expressed in the ovary, uterus and placenta in response to hormonal changes across the menstrual cycle and during pregnancy [5, 6]. PROK2 exhibits circadian rhythmic expression in the suprachiasmatic nucleus [7, 8].

Prokineticin receptors belong to the family of G protein-coupled receptors. PROKR1 and PROKR2 show strong similarity in their primary structure (87% homology) [9–11]. PROK1 and PROK2 can bind and activate both receptors, although they exhibited a slightly higher binding affinity for PROKR2 when compared with PROKR1. PROK2 has the highest affinity for both receptors [9–11]. PROK2L displays strong receptor selectivity for PROKR1 over PROKR2 [2, 4]. Activation of prokineticin receptors, which couple to Gq protein, leads to mobilization of calcium, stimulation of phosphoinositide turnover and activation of p42/p44 MAPK signaling pathways [9–11]. The prokineticin receptors can also couple to Gi and Gs proteins [4, 12]. PROKR1 shows preferential distribution in the peripheral tissues, whereas PROKR2 shows relatively localized distribution in the central nervous system [9].

Prok2 and *Prokr2* are expressed in the ependymal and subependymal layers of the olfactory bulbs, preoptic area and median eminence in mice [13, 14]. It has been demonstrated that the prokineticins play an essential role in olfactory bulb morphogenesis [14], and this system may also be involved in the migration/survival of GnRH neurons.

Animal Models

The generation of *Prok2* and *Prokr2* knockout (KO) mice demonstrated the importance of these genes in normal olfactory bulb development and sexual maturation. Both *Prok2* and *Prokr2* KO mice displayed defects in the olfactory bulb, with *Prok2* KO mice showing reduced olfactory bulb size, loss of normal olfactory bulb architec-

ture and accumulation of neuronal progenitors in the rostral migratory stream [14]. *Prokr2* KO mice showed olfactory bulb hypoplasia during early fetal development [15].

Several abnormalities in reproductive organs were also observed in the *Prokr2* KO mice, including severe atrophy of the testes, ovaries, uterus, vagina and mammary glands. Immunohistochemical analysis of *Prokr2*^{-/-} mice showed an absence of GnRH-immunoreactive neurons in the preoptic area and median eminence of the hypothalamus, which correlated with reduced mRNA levels of hypothalamic GnRH and pituitary gonadotropins as well as decreased plasma levels of testosterone and FSH [15]. Interestingly, GnRH neurons in *Prok2*^{-/-} mice were able to cross the cribriform plate to enter the central nervous system during embryonic development, but the neurons did not migrate into and populate the hypothalamus [16]. The remarkable phenotypic similarities between *Prokr2*^{-/-} and *Prok2*^{-/-} mice and Kallmann syndrome in humans inspired the hypothesis that inactivating mutations in the prokineticin system could lead to anosmia and hypogonadotropic hypogonadism in humans.

The rhythmic expression of PROK2 in the suprachiasmatic nucleus, the expression of PROKR2 in most primary target areas of the suprachiasmatic nucleus and the control of PROK2 expression/release by core clock genes [7] led to the evaluation of the circadian rhythms in *Prok2* and *Prokr2* KO mice. As anticipated, these mice exhibited disruption of circadian rhythms, with reduced rhythmicity of locomotor activity, body temperature and sleep [17–20].

Human *PROK2* and *PROKR2* Mutations

Kallmann syndrome is a complex developmental disorder combining congenital hypogonadotropic hypogonadism with anosmia or hyposmia. This phenotype is related to failure of embryonic migration of GnRH and olfactory neurons [21]. Kallmann syndrome is genetically heterogeneous [22]. Loss-of-function mutations in *KALI*, *FGFR1*, *FGF8* or *CHD7* underlie either X-linked recessive or autosomal dominant forms of the disease [23–28]. Human *PROK2* and *PROKR2* mutations were first described in 2006. Dodé et al. [29] studied a cohort of 192 patients with Kallmann syndrome, and mutations in *PROK2* and *PROKR2* were identified in 2 and 7% of the patients, respectively. Since then, approximately nine different mutations of *PROK2* and 24 mutations of *PROKR2* have been

reported in patients with variable degrees of GnRH deficiency and olfactory abnormalities (fig. 1a, b) [16, 29–36].

These mutations have been found in both male and female patients from a broad range of ethnic backgrounds [16, 29, 30, 34]. Complete and partial hypogonadotropic hypogonadism as well as delayed puberty have been associated with *PROK2* and *PROKR2* mutations. Additional clinical anomalies frequently described in patients with Kallmann syndrome caused by *KAL1* or *FGFR1* mutations were rarely described in patients with *PROK2* or *PROKR2* mutations. Five cases of high arched palate [30, 36], four cases of bimanual synkinesia [34, 36], three cases of hearing loss [34, 36], three cases of pectus excavatum [30, 34] and one case of hypodontia were described [36]. No cases of renal agenesis or cleft lip/palate were reported.

Prokineticins have been implicated in circadian rhythms, and it is speculated that patients with mutations in these genes may have circadian rhythm alterations. In one study, a Kallmann syndrome patient with a *PROK2* p.R73C mutation exhibited a severe sleep disorder, suggesting a potential contribution of the *PROKR2* signaling to physiological sleep-wake cycle [29]. However, in another recent study, patients with *PROKR2* or *PROK2* mutations did not show sleep abnormalities or circadian cortisol pattern alterations [36]. A more detailed investigation is necessary to determine whether mutations in *PROK2* or *PROKR2* lead to disruption in circadian rhythms in humans.

Initially, a genotype/phenotype association of the *PROK2* and *PROKR2* mutations in Kallmann syndrome was exclusively based on the absence of the genetic variants in control groups and their localization in conserved amino residues [29]. Intriguingly, most of the described variants of *PROK2* and *PROKR2* were missense mutations found in the heterozygous state, and only nine of them (3 in *PROK2*, fig. 1a, and 6 in *PROKR2*, fig. 1b) were identified in the homozygous state [16, 30–32, 36]. Notably, segregation analysis of two homozygous deleterious mutations of *PROK2* (p.I55fsX1) and *PROKR2* (p.Y140X) revealed that asymptomatic relatives also carried the same mutations in the heterozygous state, indicating that haploinsufficiency was not sufficient to cause the hypogonadotropic hypogonadism phenotype [16, 30]. This finding was in agreement with the report that only homozygous null mice for *Prok2* or *Prokr2* exhibited abnormal phenotypes [15]. Therefore, the role of heterozygous mutations in *PROK2* and *PROKR2* remains unclear.

Definitely one of the major challenges in the genetic investigation of human prokineticin defects is to under-

stand their inheritance pattern. Undiscovered mutations in promoter or intronic regions of the *PROK2* or *PROKR2* genes in association with heterozygous mutations within the coding regions could explain some of the unresolved phenotype-genotype correlations. Another appealing possibility is the existence of digenic or oligogenic inheritance in some of these families with heterozygous *PROK2* and *PROKR2* mutations. Indeed, approximately 10% of the patients with heterozygous mutations of *PROK2* or *PROKR2* harbored mutations in other genes (table 1); however, the functional role of some of these genetic variants has not been well defined by in vitro studies [29, 33–36]. Alternatively, the presence of polymorphisms, epigenetic and environmental effects are other potential factors that should be considered.

A comparative phenotypic study involving 55 patients, including 42 men and 13 women with Kallmann syndrome who carried either monoallelic or biallelic mutations in *PROK2* or *PROKR2* was recently conducted by several groups of investigators in France [36]. Male patients carrying biallelic mutations generally had a less variable and more severe reproductive phenotype than patients carrying monoallelic mutations. A high prevalence of both micropenis and cryptorchidism, and a lower mean testicular volume were noted in male patients with biallelic mutations. Their hormonal profile was also different, with lower testosterone, basal FSH and GnRH-stimulated LH levels in patients with biallelic mutations. Interestingly, nonreproductive and nonolfactory clinical anomalies were restricted to Kallmann syndrome patients with monoallelic mutations [36].

In vitro Functional Studies of *PROK2* and *PROKR2* Mutants

In vitro functional studies of *PROK2* and *PROKR2* mutants have been reported, establishing the causal role of mutations described in these genes [16, 34, 37]. In a comprehensive study of 324 patients with either Kallmann syndrome or normosmic hypogonadotropic hypogonadism, five mutations in *PROK2* and ten mutations in *PROKR2* were identified (fig. 1a, b). The ability of *PROK2* mutant proteins to activate intracellular Ca^{2+} mobilization in cells expressing *PROKR2* was assessed using an aequorin-based luminescent assay. The *PROK2* mutations p.C34Y, p.R73C and p.I55fsX1 resulted in inactivation or markedly reduced activity of the peptide (table 2). The p.I55fsX1 is a frameshift mutation that introduces a premature stop codon, which results in the production of

Fig. 1. Mutations identified in PROK2 and PROKR2. The mutations identified in the heterozygous state are shown in red, the mutations identified in both homozygous and heterozygous states are shown in gray and the mutations identified only in the homozygous state are shown in green. **a** Peptide sequence of PROK2 (81

amino acids) with the signal peptide (27 amino acids, shown in light blue). Exon 1 includes the signal peptide and the amino acids shown in dark blue. Exon 2 is shown in pink and exon 4 in yellow. Exon 3 is not shown. No mutations have been identified in exon 3. **b** Schematic representation of PROKR2.

a truncated protein that lacks the cysteine-rich domains critical for biological activity. The mutations p.C34Y and p.R73C are predicted to disrupt disulfide bond formation. However, a p.I50M mutation was not shown to affect the function of the peptide (table 2). Indeed, both me-

thionine and isoleucine are neutral amino acids and, therefore, this amino acid change at codon 50 of the PROK2 does not result in a significant alteration of protein charge. A p.A24P mutation, located in the signal peptide, was not tested [34].

Table 1. Eight patients who harbored *PROKR2* mutations associated with other gene defects

Patient	Mutations	Diagnosis	Functional consequence	References
1	PROKR2 p.L173R + KAL1 p.S396L	KS	PROKR2: ↓ signaling KAL1: not reported	[29, 36]
2	PROKR2 p.L173R + KAL1 p.E514K (polymorphism -rs28937309)	KS	PROKR2: ↓ signaling KAL1: altered function	[33]
3	PROKR2 p.V115M + PROK2 p.A24P	KS	PROKR2: ↓ signaling PROK2: not reported	[34]
4	PROKR2 p.S202G + GnRH1 p.R31C + FGFR1 p.I239T	nIHH	PROKR2: not reported GnRH1: not reported FGFR1: not reported	[35]
5	PROKR2 p.R85L + FGFR1 p.A604T	KS	PROKR2 and FGFR1: not reported	[36]
6	PROKR2 p.L173R + KAL p.R423X	KS	PROKR2: ↓ signaling KAL1: PTC	[36]
7	PROKR2 p.R268C + KISS1R p.A189T	KS	PROKR2: ↓ signaling KISS1R: not reported	[36]
8	PROKR2 p.V331M + GNRHR p.R240Q	KS	PROKR2: ↓ signaling GnRHR: not reported	[36]

KS = Kallmann syndrome; nIHH = normosmic isolated hypogonadotropic hypogonadism; PTC = premature termination codon.

Table 2. Functional effects of *PROK2* mutations as determined by in vitro studies

Mutation	Calcium mobilization	Potential mechanism	References
p.C34Y	no activity	disruption of disulfide bridges	[34]
p.I50M	similar to wild-type	does not alter the hydrophobic properties of the amino acids	[34]
p.I55fsX1	no activity	truncated protein	[16, 34]
p.R73C	60%↓	disruption of disulfide bridges	[34]

Some mutations identified in *PROKR2* were tested using experiments that measured cell surface expression, ligand binding and signal transduction through the activation of $G\alpha_q$, calcium mobilization (aequorin-based luminescence, Fluo-4 fluorescence) and MAPK pathway activation (Egr1-luciferase) [34, 37]. The amount of the receptor in the plasma membrane was quantified by ELISA in nonpermeabilized cells and compared to permeabilized cells. Mutations in the extracellular loops severely compromised signal transduction (table 3). These mutations most likely interfere with ligand binding, as shown in the binding study of the mutation p.Q210R located in the second extracellular loop [37]. The mutations in the

intracellular loops and in the carboxy-terminal domain affect the function of the receptor to variable degrees (table 3 and fig. 1b).

The exact mechanisms by which each of these mutations affect the function of the receptor are not known, but it is likely that they can interfere with G protein activation or with the correct transport of the receptor to the plasma membrane. This last hypothesis of impaired receptor trafficking is most likely for mutations in the transmembrane domains of the receptor, particularly for those changes that occur in amino acids that are highly conserved among species and among G protein-coupled receptors in general, such as p.L173R, p.W178S and

Table 3. Functional effects of *PROKR2* mutations as determined by in vitro studies

Mutation	Calcium mobilization	MAPK activation	Binding assay	PROKR2 quantification by ELISA		Ref.
				permeabilized cells	nonpermeabilized cells	
Mutations that affect ligand binding						
p.Q210R	60%↓	N/A	95%↓	10%↓	10%↓	[37]
p.Y113H	80%↓	90%↓	N/A	N/A	N/A	[34]
p.V115M	80%↓	70%↓	N/A	N/A	N/A	[34]
Mutations that affect plasma membrane expression						
p.L173R	40–60%↓	95%↓	80%↓	50%↓	80%↓	[34, 37]
p.W178S	80%↓	no activity	no binding	60%↓	90%↓	[34, 37]
p.S188L	no activity	no activity	N/A	N/A	N/A	[34]
p.P290S	no activity	N/A	no binding	60%↓	90%↓	[37]
Mutations that affect G protein interaction						
p.R85C	40%↓	10%↓	60%↓	40%↓	40%↓	[34, 37]
p.R85H	40%↓	N/A	70%↓	40%↓	40%↓	[37]
p.R164Q	60%↓	90%↓	=	20%↓	20%↓	[34, 37]
p.R248Q	40%↓	=	N/A	N/A	N/A	[34]
p.R268C	20%↓	N/A	40%↓	20%↓	20%↓	[37]
p.V331M	40–60%↓	=	50%↓	30%↓	30%↓	[34, 37]
p.R357W	60%↓	=	N/A	N/A	N/A	[34]
Mutations that do not affect PROKR2 function						
p.M323I	=	N/A	=	30%↓	40%↓	[37]

Calcium mobilization experiments: aequorin-based luminescence assay or Fluo-4. MAPK activation experiments: Egr-1 luciferase activity. N/A: Not available; =: activity similar to wild-type.

p.P290S. These mutations resulted in reduced receptor levels in the plasma membrane [37]. Indeed, W178 and P290 are the most highly conserved amino acids in transmembrane domains 4 and 6, respectively. Notably, a dominant negative effect of any of these PROKR2 missense mutants on the wild-type receptor was not found in vitro [37].

Reversible Hypogonadotropic Hypogonadism due to PROKR2 Mutations

A homozygous PROKR2 mutation, p.V274D, was identified in a male Kallmann syndrome patient who had presented with absent puberty. After 3.5 years of treatment with hCG and testosterone, he presented with reversal of hypogonadism after discontinuing hormonal therapy. He maintained oligospermia and testosterone levels in the low normal range for 2 years without any treatment [32]. In vitro studies were not performed to assess the effect of this mutation on receptor function. If p.V274D is an inactivating mutation, then this report suggests that PROKR2 can also regulate reproduction be-

yond the stage of embryonic GnRH neuronal migration, as has been suggested for FGFR-1.

The presence of oligospermia in a patient with a homozygous PROKR2 mutation raises the issue of the role of the prokineticins in spermatogenesis. The long and short forms of PROK2 are expressed in human spermatocytes and PROKR2 is expressed in endothelial cells of the testis interstitium [38]. It is possible that the homozygous PROKR2 p.V274D mutation contributes directly to the oligospermia in this patient. In support of this possibility, a patient with normosmic hypogonadotropic hypogonadism and a heterozygous p.W178S PROKR2 mutation presented with LH and FSH levels suggestive of GnRH deficiency as well as primary gonadal resistance [34]. The precise roles of PROK2 and PROKR2 in reproduction need further study for a better understanding.

Conclusions

The inactivation of the PROK2 system leads to defective olfactory morphogenesis and hypogonadism in mice and humans. Several mutations in PROK2 and PROKR2

have been found in heterozygous, homozygous and compound heterozygous states in Kallmann syndrome and normosmic isolated hypogonadotropic hypogonadism, suggesting a complex mode of inheritance. A monogenic recessive mode of transmission has been demonstrated clearly, but in very few cases. Remarkably, many of the heterozygous mutations of *PROKR2* have also been identified in clinically unaffected individuals, raising the question of their actual contribution to the hypogonadotropic hypogonadism phenotype. Moreover, a dominant negative effect of the heterozygous mutations of *PROKR2* was not demonstrated by in vitro studies, arguing against a monogenic dominant transmission. Potential digenic and oligogenic transmissions have often been suggested;

however, further studies will be necessary to confirm the actual pathogenic role of heterozygous *PROKR2* mutations.

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