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GNAS MUTATIONS AND HETEROTOPIC OSSIFICATION

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Abstract

GNAS is a complex imprinted gene encoding the alpha-subunit of the stimulatory heterotrimeric G protein (Gsα). *GNAS* gives rise to additional gene products that exhibit exclusively maternal or paternal expression, such as XLαs, a large variant of Gsα that shows exclusively paternal expression and is partly identical to the latter. Gsα itself is expressed biallelicaly in most tissues, although the expression occurs predominantly from the maternal allele in a small set of tissues, such as renal proximal tubules. Inactivating mutations in Gsα-coding *GNAS* exons are responsible for Albright's hereditary osteodystrophy (AHO), which refers to a constellation of physical and developmental disorders including obesity, short stature, brachydactyly, cognitive impairment, and heterotopic ossification. Patients with Gsα mutations can present with AHO in the presence or absence of end-organ resistance to multiple hormones including parathyroid hormone. Maternal Gsα mutations lead to AHO with hormone resistance (i.e. pseudohypoparathyroidism type-Ia), whereas paternal mutations cause AHO alone (i.e. pseudo-pseudohypoparathyroidism). Heterotopic ossification associated with AHO develops through intramembranous bone formation and is limited to dermis and subcutis. In rare cases carrying Gsα mutations, however, ossifications progress into deep connective tissue and skeletal muscle, a disorder termed progressive osseous heteroplasia (POH). Here I briefly review the genetic, clinical, and molecular aspects of these disorders caused by inactivating *GNAS* mutations, with particular emphasis on heterotopic ossification.

The *GNAS* complex locus

GNAS encodes the alpha-subunit of the stimulatory G protein, a signaling protein necessary for the actions of numerous hormones, neurotransmitters, and autocrine/paracrine factors [1–3]. Upon activation by one of many cell surface G protein-coupled receptors, Gsα stimulates membrane-bound adenylyl cylases and, thereby, leads to the generation of second messenger cAMP. Gsα also stimulates other "effectors", such as Src tryrosine kinase, but the stimulation of adenylyl cyclase by Gsα takes place in a wide range of tissues, is the most extensively studied Gsα action, and could well be the most important cellular role of this

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ubiquitously expressed protein. In addition to Gsα, *GNAS* gives rise to several coding and non-coding transcripts that show parent-of-origin specific expression (Fig. 1). The maternal *GNAS* allele leads to transcripts encoding the neuroendocrine secretory protein-55 (NESP55), which belongs to the chromogranin family of proteins. NESP55 uses a unique promoter and a first exon that splices onto exon 2–13 that encode Gsα. From the paternal *GNAS* allele originates a large Gsα variant termed extra-large Gα (XLαs) and the so-called A/B transcript (also known as 1A or 1′), both of which also use unique promoters and first exons that splice onto Gsα exons 2–13. In the NESP55 transcript, sequences derived from exons 2–13 are located within the 3′-untranslated region, whereas in the XLαs transcript these sequences are within the coding region. Thus, the XLαs protein is partly identical to the Gsα protein. The A/B transcript is considered to be non-coding and regulate imprinting within *GNAS*; however, it has been suggested that the translational product of A/B mRNA is an amino-terminally truncated form of Gsα that antagonize the latter [4]. A non-coding antisense transcript (*GNAS*-AS1), which acts as a negative regulator, in cis, of the NESP55 expression, is also derived from the paternal *GNAS* allele.

Diseases caused by inactivating mutations within Gsα-coding *GNAS* exons

Albright's hereditary osteodystrophy and pseudohypoparathyroidism type-Ia

Consistent with the importance of Gsα in development and many physiological processes, genetic alterations that disrupt Gsα expression or activity result in a constellation of physical features including obesity, short stature, brachydactyly, cognitive impairment, and heterotopic ossification. Described originally by Albright et al [5], these features are now collectively termed Albright's hereditary osteodystrophy (AHO). Heterozygous inactivating mutations within one of 13 *GNAS* exons encoding Gsα are responsible for AHO [6, 7]. These physical features are often found in patients who also present with hypocalcemia unresponsive to exogenously administered parathyroid hormone [5]. These patients are resistant to the actions of PTH, particularly in the renal proximal tubule, and therefore, develop not only hypocalcemia, which is a consequence of reduced 1,25 dihydroxyvitamin D levels, but also hyperphosphatemia. Indicating PTH resistance, serum PTH levels are elevated in these patients despite these biochemical alterations [8]. Most AHO patients also display resistance to some other hormones whose actions occur through Gsα, including thyroid stimulating hormone, gonadotropins, and growth hormone releasing hormone [1–3, 9, 10]. Therefore, hypothyroidism, hypogonadism, and growth hormone deficiency are common hormonal abnormalities in these patients. This disorder of AHO with multihormone resistance is referred to as pseudohypoparathyroidism type-Ia (PHP-Ia). By definition, patients with PHP type-I display blunted excretion of urinary cAMP and phosphate in response to exogenously administered biologically active PTH. This is in contrast to PHP type-II, in which PTH-induced phosphate but not cAMP excretion is blunted [11]. Another subtype of PHP type-I is PHP-Ib, which describes patients who have PTH resistance without AHO features [12, 13]. PHP-Ib is caused by epigenetic defects within the *GNAS* complex locus [14–16]. This PHP-I subtype has recently been reviewed elsewhere [10, 17, 18].

Pseudo-pseudohypoparathyroidism

Certain patients with AHO do not show any evidence of hormone resistance. This disorder has been termed “pseudo-pseudohypoparathyroidism” (PPHP) [19]. Like PHP-Ia, PPHP is caused by heterozygous inactivating mutations within Gsα-coding *GNAS* exons. Patients with PHP-Ia and PPHP can be found in the same kindreds, but it is the parent-of-origin of the mutation that determines the outcome in the offspring [20]. When the mutation is inherited from a female obligate carrier (i.e. maternal), the individual develops PHP-Ia, i.e. AHO plus hormone resistance. In contrast, when a male obligate carrier transmits the same mutation (i.e. paternal), then the offspring develops PPHP, i.e. AHO alone. The parent-of-origin specific inheritance of the hormone resistance reflects the finding that Gsα expression is biallelic in most tissues but predominantly maternal in a small number of hormone-responsive tissues, including renal proximal tubule, thyroid, gonads, and anterior pituitary [21–25]. Due to their manifestation upon both maternal and paternal *GNAS* mutations, AHO features are considered to occur as a result of Gsα haploinsufficiency in various tissues, except for obesity and cognitive impairment, which develop predominantly upon maternal transmission of Gsα mutations [26, 27]. Investigations using mouse models suggest that the obesity related to *GNAS* mutations results from Gsα deficiency in the dorsomedial hypothalamus [28]. Recent studies have shown that both PHP-Ia and PPHP patients can present with intrauterine growth retardation; however, it appears that this finding is significantly more severe in those carrying paternal (PPHP) than maternal mutations (PHP-Ia) [29, 30], implicating a paternally expressed *GNAS* product in fetal growth. This product is likely XLαs, given that mice with XLαs ablation [31], as well as patients with either maternal uniparental disomy involving the chromosomal region including *GNAS* (20q13.32) or large paternal *GNAS* deletions, also have perinatal growth retardation [32–36].

Heterotopic ossifications in AHO

Heterotopic ossification found in the context of AHO is limited to dermis and subcutis and cause painful lesions that may require surgery [37–49]. Patients can show heterotopic ossification after minor trauma, but the lesions often appear to arise spontaneously. Heterotopic ossification could be the presenting feature of AHO during childhood or adulthood. In some cases, the lesion appears as a bony plate under the skin, hence termed plate-like osteoma cutis. Histological analysis of biopsy specimens clearly indicates the presence of mineralized bone tissue with marrow elements. No cartilage is detected, indicating that *GNAS*-related heterotopic ossification arises primarily from intramembranous bone formation. Unlike hormone resistance, as mentioned above, cutaneous or subcutaneous ectopic ossification can develop both after maternal and paternal transmission of the Gsα mutation [20]. Therefore, it is thought that these osseous lesions reflect Gsα haploinsufficiency and are not related to the abnormalities in serum calcium and phosphate levels.

The pathogenesis of *GNAS*-related heterotopic ossification involves aberrant differentiation of mesenchymal stem cells or early progenitors located in the dermis or subcutaneous fat. Role of Gsα has been studied in mesenchymal stem cells with respect to osteogenic differentiation. In human mesenchymal stem cells, reduction of Gsα protein levels has been

shown to cause osteogenic differentiation, while inhibiting the formation of adipocytes [50, 51]. In addition, Runx2, a key regulator of osteoblast-specific gene expression, appears to suppress Gsα expression [52], suggesting that Gsα actions oppose osteogenic differentiation. In addition, ablation of Gsα in progenitor cells that express osterix, a marker of early osteoblasts, leads to reduced number of osteoprogenitors but enhanced osteoblast differentiation [53]. Accordingly, activating Gsα mutations are found in fibrous dysplasia of bone, a disorder characterized by intramedullary accumulation of fibrous tissue containing poorly differentiated early osteoblasts [54]. Demonstrating a direct role of Gsα deficiency in the development of heterotopic ossification, Huso et al have found ectopic bone formation in dermis and subcutaneous tissue of adult mice in which *Gnas* exon 1 is ablated [55]. Histological analyses revealed mineral deposits and bone marrow elements located around hair follicles, and these findings were confirmed by further imaging studies. As in patients with PHP-Ia and PPHP, the subcutaneous lesions were observed regardless of the parental origin of the genetic manipulation. Cheeseman et al obtained similar findings independently, revealing heterotopic ossification in another mouse model in which the *Gnas* locus has a loss-of-function missense mutation in exon 6 [56]. Additional investigations using mice with paternal ablation of *Gnas* exon 1 have demonstrated that loss of one Gsα allele enhances osteogenic differentiation of stromal cells derived from adipose tissue while inhibiting their adipogenic differentiation [57, 58].

Progressive Osseous Heteroplasia

Progressive osseous heteroplasia (POH) is a disorder in which patients display severe extraskeletal ossifications that involve deep connective tissue and skeletal muscle [59–61]. The lesions mostly start during infancy as cutaneous or subcutaneous ossification and subsequently become invasive. Patients often develop severe ankylosis of affected joints, which restrict growth. Few patients with POH demonstrate AHO features and, consistent with the occasional co-existence of these two sets of clinical defects, heterozygous inactivating Gsα mutations have been identified as a cause of POH [62–64]. Several of the identified *GNAS* mutations in POH are identical to those found in PHP-Ia/PPHP kindreds in whom no progressive and invasive heterotopic ossification is present [63, 64]. The severity of the heterotopic ossification in these patients can be comparable to that of fibrodysplasia ossificans progressiva (FOP) [65, 66]. Unlike in the latter, however, the heterotopic bone in POH is formed primarily through intramembranous ossifications, i.e. similar to ossifications seen in AHO. Nevertheless, chondrocyte clusters have been documented in the histopathological analysis of a POH patient in whom existence of a *GNAS* mutation was confirmed [67].

Disease mechanisms leading to POH

Since the same *GNAS* mutations can cause either AHO or POH, it appears that genetic background, epigenetic events, or environmental factors contribute to the severity of heterotopic ossifications. It is important to note that in most POH patients, the heterotopic bone formation appears to be the isolated clinical finding, i.e. other typical AHO features are seemingly absent [64, 68]. Furthermore, most, but not all, *GNAS* mutations that lead to POH are inherited from male obligate carriers or occur de novo on the paternal allele [30, 64, 67,

69]. In fact, as shown previously, the same *GNAS* mutation in a three-generation kindred led to POH upon paternal transmission but typical AHO features upon maternal transmission [64]. These observations suggest that important distinctions exist between the molecular and genetic mechanisms governing POH and AHO.

Based on the finding that most *GNAS* mutations causing POH are paternal, it appears plausible that the deficiency of a *GNAS* product showing exclusive paternal expression contributes to the molecular pathogenesis of POH. A paternal *GNAS* product that is partly identical to Gsα is XLαs [70], which, when overexpressed, can mimic the action of Gsα with respect to cAMP signaling [71–73]. XLαs is expressed in various tissues, with abundant expression in central nervous system, neuroendocrine tissues, and muscle [74, 75]. XLαs expression has also been detected in bone and osteoprogenitor cells [57, 76, 77]. Strikingly, Pignolo et al have determined that XLαs and Gsα mRNA levels are comparable in mouse soft tissue stromal cells differentiated toward the osteogenic lineage [57]. Upon reviewing a large cohort of patients with POH, Adegbite et al revealed that, with the exception of one mutation, all Gsα mutations are located within exons shared by Gsα and XLαs [68]. It has also been shown that, despite the knowledge that exon 1 is specific for Gsα, expression level of XLαs mRNA is significantly lower in differentiated soft tissue stromal cells from mice heterozygous for paternal ablation of *Gnas* exon 1 than wild-type mice [57]. Based on these findings, it appears likely that, when the Gsα mutation is on the paternal allele, a more severe deficiency of cAMP signaling is encountered than that is observed upon Gsα haploinsufficiency alone, thus resulting in a POH-like phenotype. Considering that POH develops only in a small subset of patients carrying paternal *GNAS* mutations, the effect of XLαs deficiency in this regard may vary among individuals, perhaps due to variation in XLαs expression levels or activity.

Nevertheless, studies using mouse models have largely failed to confirm that XLαs has a physiological role as a stimulator of cAMP generation; in fact, agonist-induced cAMP response appears more exuberant in certain XLαs knockout tissues, such as brown adipose tissue and renal proximal tubules [31, 78]. Moreover, our group has recently shown, by studying mouse renal proximal tubules during early development, that XLαs knockout mice demonstrate deficiency of PLC/PKC signaling rather than cAMP signaling [78]. In addition, POH is also observed in few cases carrying maternal Gsα mutations [79, 80]), indicating that XLαs deficiency is not necessary for the development of invasive heterotopic ossification. Thus, it remains to be determined whether XLαs deficiency contributes, at least in some cases, to POH development, and if so, whether this effect occurs due to diminished PLC/PKC and/or cAMP signaling.

Recently, several patients have been analyzed systematically regarding their clinical phenotypes, revealing that the POH lesions predominantly show a dermomytomal distribution and a significant lateralization bias [81]. This study also showed that a severe loss of Gsα activity (via injection of dominant negative Gsα) in chick somitic cells is sufficient to cause POH-like formations. These findings led to a novel hypothesis that the pathogenesis of this disorder entails aberrant differentiation of progenitor cells of somitic origin, which may sustain severe Gsα deficiency due to a possible loss of heterozygosity at the *GNAS* locus, i.e. inactivation of the intact *GNAS* allele [81]. Accordingly, homozygous

ablation of Gsα in different mouse models leads to invasive heterotopic ossification [82], unlike the subcutaneous heterotopic ossification observed in mice with heterozygous Gsα ablation [55, 56]. Recently, Happle has presented a strong argument for this hypothesis, proposing that POH should be accepted as a disorder of type-2 segmental mosaicism rather than a mendelian disorder [83]. Type-2 segmental mosaicism refers to a genetic phenomenon in which loss of heterozygosity is acquired during early development on top of a germ-line inactivating mutation. This is a plausible hypothesis that accommodates most of the clinical and genetic observations in POH; however, the significant tendency of the *GNAS* mutations to be paternal and the existence of few familial cases are difficult to explain. POH might also arise from a similar but not identical genetic mechanism, in which, instead of severe Gsα deficiency caused by loss of heterozygosity, a second mutational hit takes place in another essential gene in the same molecular pathway. Genetic analysis of the affected tissues in POH patients will be required to dissect out these hypotheses.

Cellular and molecular mechanisms underlying *GNAS*-related heterotopic ossification

Extensive extraskeletal ossifications that resemble POH lesions have been obtained in mice upon deletion of both *Gnas* alleles through the use of various Cre lines that are active in mesenchymal tissues, including Prx1-Cre (limb bud development), Dermo1-Cre (mid-gestational mesodermal tissues including mesenchyme-derived chondrocytes and osteoblasts), or Ap2α-Cre (embryonic face and limb mesenchyme) [82]. In addition, a POH-like severe phenotype was also observed upon the use of a renin-Cre transgenic mouse model; however, this outcome resulted from an unexpected activation of Cre in an as-yet-unidentified progenitor cell in soft tissues, including skin and developing limb [84]. For a better understanding of the pathophysiology and effective therapies, it is important to identify the specific lineage of mesenchymal stem cells critical for *GNAS*-related heterotopic ossification.

The activation of the hedgehog signaling pathway has recently been documented in osteoblasts and progenitor cells in POH lesions [82]. It was also shown that Gsα ablation causes severe heterotopic ossification through activation of hedgehog signaling [82]. Furthermore, in a limb-bud culture model, small molecule inhibitors of Gli transcription factors– which mediate hedgehog signaling – suppress the expression of hedgehog target genes induced by Gsα deficiency [82]. Hence, this signaling pathway, which is a key player in skeletal development and osteogenic differentiation [85], may serve as a potential therapeutic target for POH. It is uncertain whether hedgehog signaling also plays a role in the dermal and subcutaneous ossification seen in patients with AHO. Liu et al have found that various early and mature osteoblast markers, such as Msx2, are expressed at modestly higher levels in adipose-derived stromal cells from mice heterozygous for paternal *Gnas* exon 1 ablation than in those from wild-type mice [58]. Under in vitro adipogenic conditions, the mutant cells showed impaired differentiation into adipocytes and retained higher expression of the osteoblast markers. While these findings indicate a shift in the commitment of the mutant stromal cells toward the osteoblast lineage, the hedgehog pathway has not been scrutinized in this setting. It is conceivable that hyperactive hedgehog

signaling is unique to POH and presents a precondition for the lesions to become progressive. Further investigations are needed to elucidate the underlying molecular mechanisms.

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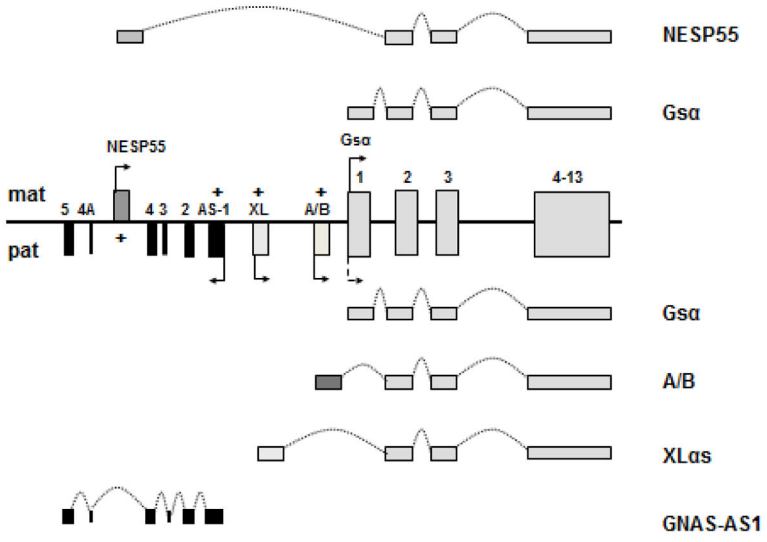

Figure 1. The *GNAS* complex locus

Located on chromosome 20q13.32 (GRCh38/hg38 chr20:58818918-58911196), *GNAS* encodes the ubiquitous signaling protein Gsα through the use of exons 1–13. Although Gsα is expressed biallelically in most tissues, paternal Gsα allele is silenced in some tissues (dotted arrow). Additional transcripts include the maternally expressed NESP55 and the paternally expressed XLαs and A/B (also referred to as 1A or 1′). These additional transcripts use individual promoters and first exons that splice onto exons 2–13 of *GNAS*. *GNAS*-AS1 transcript is a non-coding transcript derived from the paternal *GNAS* allele. Boxes and connecting lines depict exons and introns, respectively. Maternal (mat) and paternal (pat) *GNAS* products are illustrated above and below the gene structure, respectively, and the splicing patterns indicated by dotted curves. “+” indicates the regions of CpG methylation. Arrows indicate direction of transcription.