Growth and Growth Factors

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When we were asked to select papers for this year's chapter on *Growth and Growth Factors*, it felt as if we had only just finished last year's selection, and that it would be a challenge to find papers that were of comparable quality to the previous 2 years. It was only when we prioritized the eligible papers that we realized that once again there was far too much new high-quality material in the field of growth and growth factors to include in the chapter. Examples include a paper on the IGF-independent involvement of IGFBP4 in cardiac development and work on the choice of signalling pathways by specific activation of the growth hormone receptor.

We have clearly entered the era of genome-wide associations, with two further papers on associations and height, in this chapter and in the chapter on *Population genetics, pharmacogenomics*, that follow up the work which we discussed last year [1–5]. Another recurring theme this year is imprinting and disorders thereof – imprinting has made the translational jump into clinical pediatric endocrinology, with major advances in our understanding of complex and heterogeneous conditions such as Beckwith-Wiedemann syndrome, Silver-Russell syndrome and Prader-Willi syndrome.

New genome-wide associations

Meta-analysis of genome-wide scans for human adult stature identifies novel loci and associations with measures of skeletal frame size

Soranzo N, Rivadeneira F, Chinappen-Horsley U, Malkina I, Richards JB, Hammond N, Stolk L, Nica A, Inouye M, Hofman A, Stephens J, Wheeler E, Arp P, Gwilliam R, Jhamai PM, Potter S, Chaney A, Ghorri MJ, Ravindrarajah R, Ermakov S, Estrada K, Pols HA, Williams FM, McArdle WL, van Meurs JB, Loos RJ, Dermitzakis ET, Ahmadi KR, Hart DJ, Ouweland WH, Wareham NJ, Barroso I, Sandhu MS, Strachan DP, Livshits G, Spector TD, Uitterlinden AG, Deloukas P

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PLoS Genet 2009;5:e1000445

Background: Recent genome-wide association (GWA) scans have identified several independent loci affecting human stature. This study analyzes their contribution through the different skeletal components of height.

Methods and Results: GWA scans in 2 large cohorts were performed. Seventeen genomic regions associated with height were identified. Two other regions were entirely novel (in CATSPER4 and in TMED10) and two had previously been described with weak statistical support (in NPR3 and JAZF1). One locus (at GNA12) identifies the first height eQTL. The contribution of height loci to the upper- (trunk) and lower-body (hip axis and femur) skeletal components of height were assessed and evidence was obtained for several loci associated with trunk length (most significant for GPR126 and LCORL), hip axis length (LCORL and UQCC), and femur length (PRKG2 and HIST1H1D). Finally, conditional analyses were used to explore a possible differential contribution of the height loci to these different skeletal size measurements.

Conclusions: This study has validated four novel loci controlling adult stature, and has successfully assessed the contribution of genetic loci to specific skeletal components of height.

In this large study, the authors searched for correlation of height-related SNPs, found in previous GWA studies [2–5], with spine length, femur length and hip axis length (femoral greater trochanter

to inner pelvic rim distance which correlates with total frame size), which is of interest since the regulation of growth varies for these skeletal components. Interestingly, apart from *SOCS2*, no genes from the GHRH-GH-IGF1 axis show major associations with height, possibly reflecting the contribution of non-endocrine processes to growth. Two novel loci associated with total height were found in *CATSPER4* (a cation channel, 0.45 cm/allele) and *TMED10* (involved in cellular protein trafficking, 0.34 cm/allele). The strongest association signals were at *HGMA2* and *UQCC* which confirms previous GWA data. *HGMA2* is of interest not only because it is involved in proliferation but overexpression results in pituitary adenomas, and *UQCC* is involved in the FGF pathway, well known for its involvement in bone development. The first quantitative trait locus described is in *GNA12*, a gene involved in modulating signaling pathways of G-protein-coupled receptors. *NPR3* encoding for natriuretic peptide receptor, which plays a key role in bone development and growth [6] and the mutation of which results in acromesomelic dysplasia type Maroteaux, was unexpectedly mostly associated with trunk height. Other genes associated specifically with trunk length, leg length or hip axis length are involved in multiple processes like G-protein-coupled receptor signaling, proliferation and chondrogenesis.

This and other GWA studies give a wealth of data and are paramount in unraveling the physiology and genetics of growth, and may help to find gene abnormalities in children with short stature. They also emphasize that frequent recording of height and other auxological parameters in short children may pay off! One thing to remember though is that these studies do not prove a relation between the SNP and the gene that it lies in or is in close proximity, especially when a SNP is in between two genes, since DNA elements can regulate genes at a long distance.

New mutations – with imprinting issues

Prader-Willi phenotype caused by paternal deficiency for the HBII-85 C/D box small nucleolar RNA cluster

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Nat Genet 2008;40:719–721

Background: Prader-Willi syndrome (PWS) is characterized by the presence of neonatal hypotonia, feeding difficulties, failure to thrive in infancy, hyperphagia and severe obesity in childhood, hypogonadism, developmental delay, small hands and feet and a tendency to pick skin. It maps to chromosome 15q11–q13 and is caused by deficiency of one or more paternally expressed imprinted transcripts within this region, including *SNURF-SNRPN* and multiple small nucleolar RNAs (snoRNAs). This may be due to large interstitial deletions, imprinting defects or maternal uniparental disomy 15. Balanced chromosomal translocations that preserve expression of *SNURF-SNRPN* and centromeric genes but separate the snoRNA HBII-85 cluster from its promoter also cause PWS.

Methods: A de novo paternally inherited microdeletion within 15q11–q13 was identified in a boy with several features of PWS by array-based comparative genomic hybridization (array-CGH) using a BAC array, followed by FISH analysis. DNA methylation analysis of the PWS-imprinting center and a karyotype revealed no abnormalities. Subsequently a combination of high-resolution oligonucleotide-based array CGH and quantitative real-time PCR was used to define the deletion more precisely.

Results: A deleted segment of just under 175 kB was identified and contained the entire HBII-85 snoRNA cluster as well as approximately half the HBII-52 cluster and one of the two copies of HBII-438.

Conclusion: Since previous studies showed that a paternally inherited microdeletion of the entire HBII-52 cluster was not associated with PWS, the authors suggested that deficiency of HBII-85 snoRNAs causes the key characteristics of the PWS phenotype, although some atypical features in the proband suggest that other genes in the region may make more subtle phenotypic contributions.

Prader-Willi syndrome is associated with significant morbidity and mortality and is extremely difficult to manage. It is an imprinted disorder with the loss of paternally expressed genes at 15q11–13. It is

considered a contiguous gene syndrome resulting from deletion of the paternal copies of the imprinted *SNRPN* and *necdin* genes, and possibly other genes within the chromosome region. This paper has implicated the non-coding *HBII-85* small nucleolar RNAs (snoRNAs) in the etiology of the syndrome. This observation is all the more important as PWS is the first human disorder to be associated with loss of expression of noncoding snoRNAs. However, the commentary by Peters [7] suggests a degree of caution in that the data are based on a single patient with a microdeletion, with some atypical features including relatively tall stature and a head circumference above the normal range. The *HBII-85* cluster is made up of *C/D* box snoRNAs that are predominantly expressed in the brain. The role of most *C/D* box sno-RNAs is to base pair with their target sequences in ribosomal RNA (rRNA) or small nuclear RNA (snRNA) and to methylate the ribose 2'-hydroxyl group of specific nucleotides. To date the targets of the *HBII-85* remain unidentified; it would now be highly important to identify these so that work could begin on developing potential therapies for this debilitating condition.

Germline mutation in *NLRP2* (*NALP2*) in a familial imprinting disorder (Beckwith-Wiedemann Syndrome)

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PLoS Genet 2009;5:e1000423

Background: Beckwith-Wiedemann syndrome (BWS) is a fetal overgrowth syndrome characterized by prenatal and postnatal overgrowth, macroglossia and anterior abdominal wall defects and other variable features such as organomegaly, hemihypertrophy, neonatal hypoglycemia and embryonal tumors. It is a human imprinting disorder resulting from the deregulation of a number of genes, including *IGF2* and *CDKN1C*, a candidate tumor suppressor that negatively regulates the cell cycle, in the imprinted gene cluster on chromosome 11p15.5. Most cases are sporadic and result from epimutations at either of the two 11p15.5 imprinting centers (IC1 and IC2). 5–10% of sporadic BWS cases have hypermethylation of the *H19* DMR at IC1 resulting in biallelic expression of *IGF2*. IC2 is a DMR located in intron 10 of the *KCNQ1* gene and is known as KvDMR1; loss of methylation at this locus is observed in up to 50% of sporadic BWS and is associated with biallelic expression of *KCNQ10T1* and silencing of maternal *CDKN1C* expression. Most BWS patients with IC2 methylation defects appear to have an epimutation of unknown cause. However, rare familial cases may be associated with germline 11p15.5 deletions causing abnormal imprinting in cis. The authors report a consanguineous family with 2 children affected with BWS and an IC2 epimutation; affected siblings had inherited different parental 11p15.5 alleles excluding an in cis mechanism.

Methods: Methylation status of IC1 and IC2 was determined. Linkage studies were performed with a genome-wide linkage scan using the Affymetrix 250k SNP microarray.

Results: Molecular analysis revealed loss of maternal allele KvDMR1 (IC2) methylation in both affected children, but not in an unaffected sibling. Using a positional-candidate gene approach, they found that the mother was homozygous for a frameshift mutation in exon 6 of *NLRP2*. Analysis of methylation levels at the TND (6q24), *SNRPN* (15q13) and *PEG1* (7q32) differentially methylated regions (DMRs) revealed that both affected siblings had normal methylation levels at the TND and *SNRPN* DMRs, but child 2 demonstrated partial loss of methylation at the *PEG1* DMR.

Conclusions: While germline mutations in *NLRP7* have previously been associated with familial hydatidiform mole, this is the first description of *NLRP2* mutation in human disease and the first report of a trans mechanism for disordered imprinting in BWS. These observations are consistent with the hypothesis that *NLRP2* plays a previously unrecognized role in establishing or maintaining genomic imprinting in humans, in keeping with previous data suggesting that mutations in *NLRP7* can cause imprinting disorders in which epimutations at imprinted loci result from a *trans* effect.

This is potentially a highly important study that sheds a novel insight on the etiology of BWS, a disorder of imprinting. The familial case with an IC2 epimutation, with a homozygous mutation in the maternal *NLRP2* gene, reveals a role for this gene in the regulation of genomic imprinting in humans. Of note, homozygous inactivating mutations in the related gene *NLRP7*, which incidentally does not

have a rodent homolog, are associated with abnormal imprinting and recurrent hydatidiform moles [8]. *NLRP2* and *NLRP7* encode members of the NLRP (nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain) family of CATERPILLER proteins, of which 14 members have been identified and are encoded by two gene clusters on 11p15 and 19q13.4 (including *NLRP2* and *NLRP7*). Some NLRP proteins are components of the inflammasome that is implicated in sensing of, and inflammatory reaction to, extracellular pathogens and intracellular noxious compounds. It is important to note that the 2 family members who were homozygous for a *NLRP2* truncating mutation in this study did not show any evidence of an immune or autoinflammatory disorder. However, most NLRP family proteins are widely expressed, including the immune system, in human oocytes, and in embryos at an early stage of development. In particular, *NLRP2* and *NLRP7* expression progressively decreases from oocytes to day-3 embryos, and then increases sharply on day 5, suggesting a similar role for the 2 genes in early development/imprinting establishment. Interestingly, the methylation defects in FHM are specific for imprinted loci, and DNA methylation at non-imprinted genes and genes subject to X-inactivation is unaffected. It would appear that *NLRP2* mutations have a less severe effect on imprinting than *NLRP7* inactivation. Further study of these genes will shed further light on their role in human development and imprinting.

Severe intrauterine growth retardation and atypical diabetes associated with a translocation breakpoint disrupting regulation of the insulin-like growth factor 2 gene

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J Clin Endocrinol Metab 2008;93:4373–4380

Background: *IGF2* is an imprinted gene (predominantly transcribed from the paternally inherited allele), which plays an important role in fetal growth in mice, although no mutations have to date been identified in humans with IUGR. *IGF2* gene expression is regulated by a complex system of enhancers and promoters that determine tissue-specific and development-specific transcription.

Methods: A woman of short adult stature (height –3 SDS), born with severe intrauterine growth retardation (birth weight –5.4 SDS), atypical diabetes secondary to insulin resistance, a body mass index of 21.1 at the age of 23 years and lactation failure, was found to harbor a balanced chromosomal translocation t(1;11) (p36.22; p15.5). Both chromosomal break points were identified using fluorescent in situ hybridization. Sequence, methylation, and expression of the *IGF2* gene were examined.

Results: The 11p15.5 break point mapped 184 kb telomeric of the *IGF2* gene on the paternally derived chromosome 15p. High-density array CGH confirmed the balanced nature of the translocation. *IGF2* gene sequence and methylation were normal, but expression of the gene was reduced in her mesoderm-derived lymphoblast cells. Hyperinsulinemic, euglycemic clamp studies were performed with glucose tracers, and revealed marked hepatic and total insulin resistance. MRI of the thorax, pelvis and abdomen were performed and revealed an excess of subcutaneous fat.

Conclusion: A breakpoint was identified 184 kb upstream of the paternally derived *IGF2* gene, in a gene-poor region that contains nonskeletal muscle mesodermal-specific enhancers of *IGF2*, with other mesoderm-specific enhancers located between +188 and +273 kb. In the mouse, enhancers of *IGF2* gene expression are located up to 260 kb telomeric to the gene, and this suggests that the breakpoint separates the gene from some telomeric enhancers, and results in reduced expression in some mesoderm-derived adult tissues causing intrauterine growth retardation, short stature, lactation failure, and insulin resistance with altered fat distribution. The authors concluded that her phenotype resulted from disruption of her paternally derived *IGF2* gene because her daughter who inherited the identical translocation had normal birth weight (0.01 SDS at 29 weeks gestation).

IGF2 has been shown to play an important role in the regulation of intrauterine growth in animal studies. *IGF2* gene expression is regulated by a complex system of enhancers and promoters that determine tissue-specific and development-specific transcription. The gene is also imprinted so that in utero and in most tissues post-natally, only the paternal allele is expressed. To date, no human mutations in the *IGF2* gene have been described. However, maternal demethylation of the region containing the *IGF2* gene is associated with overgrowth as part of the Beckwith-Wiedemann syn-

drome, whereas paternal demethylation of the IGF2 gene regulatory region is associated with intra-uterine growth restriction and short stature in some cases of Silver-Russell syndrome. In this article, the authors describe a chromosomal translocation that would separate the *IGF2* gene from its enhancers and is associated with the phenotype of IUGR and diabetes mellitus, which does not fit within either the type 1 or type 2 disease categories. This interesting paper highlights the critical role of IGF2 in pre- rather than postnatal growth in humans, and also highlights its potential role in the regulation of insulin secretion. The authors comment that the diabetes is probably a combination of insulin insensitivity due to altered body fat distribution as well as probable β -cell failure.

New mutations – without imprinting issues

Growth hormone deficiency and splicing fidelity: two serine/arginine-rich proteins, ASF/SF2 and SC35, act antagonistically

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Background: The majority of mutations that cause isolated growth hormone deficiency type II are the result of aberrant splicing of transcripts encoding human growth hormone, leading to skipping of exon 3 with the production of a 17.5-kDa protein that acts as a dominant negative to block secretion of the full-length 22-kDa form of hGH generated from unaffected alleles. The most frequent mutations include those at the donor splice site of IVS3, as well as mutations in a purine-rich sequence at the beginning of exon 3 known as an exonic splice enhancer (ESE)1 and an intronic splice enhancer in IVS3. The authors had previously identified a splicing regulatory element in exon 3 [exonic splicing enhancer 2 (ESE2)], but the molecular mechanism by which this element prevents exon skipping is unknown. The aim of this study was to better understand the molecular mechanisms underlying correct splicing of the *GH1* gene, and to therefore understand the pathogenesis of a mutation in ESE2 (A1338G) that is associated with IGHD II.

Methods: Plasmid constructs of the wild-type *GH1*, A1338G *GH1*, and exon 3 deletion mutants were constructed and in vivo splicing assays performed using GH3 (rat somatotroph) cells. In vitro splicing assays were performed. Baculovirus-expressed ASF/SF2, SC35, and SRp20 were purified from infected Sf9 cells. RNA affinity chromatography was performed.

Results: The authors showed that two members of the serine/arginine-rich (SR) protein superfamily (ASF/SF2 and SC35) bind to ESE2 and act antagonistically to regulate exon 3 splicing. ASF/SF2 activates exon 3 inclusion, but SC35, acting through a region just downstream of ESE2, can block such activation. These findings explain the disease-causing mechanism of a patient's mutation in ESE2 that creates a functional SC35-binding site that then acts synergistically with the downstream SC35 site to produce pathological levels of exon 3 skipping.

Conclusion: Although the precedent for SR proteins acting as repressors is established, this is the first example of a patient mutation that creates a site through which an SR protein represses splicing.

IGHD Type II is due to dominant negative mutations in the *GH1* gene. Mutations in the donor splice site of IVS3 have been identified at IVS3 +1, 2, 3, 5, 6, and are the most frequent cause of the condition. Mutations in an exonic splice enhancer at the beginning of exon 3 (ESE1) have also been associated with IGHD II, as have mutations in an intronic splice enhancer. Additionally, a number of mis-sense mutations have been associated with the phenotype, and these include R77C, R183H, P89L and V110F. This paper describes the molecular mechanisms underlying splicing. Two members of the serine/arginine (SR) protein superfamily (ASF/SF2 and SC35) bind to ESE2 and act antagonistically to regulate exon 3 splicing. Through some elegant in vitro and in vivo studies, the authors have demonstrated that a heterozygous mutation previously described in the *GH1* gene ESE2 (A1338G) is associated with the creation of a binding site for SC35 that then acts synergistically with the downstream SC35-binding site to repress normal splicing.

The -202 A allele of insulin-like growth factor binding protein-3 (IGFBP3) promoter polymorphism is associated with higher IGFBP-3 serum levels and better growth response to growth hormone treatment in patients with severe growth hormone deficiency

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J Clin Endocrinol Metab 2009;94:588–595

Background: The response to recombinant human GH (rhGH) therapy in patients with a variety of disorders including GH deficiency (GHD), Turner syndrome and SGA remains highly variable. Genetic factors that influence the response to rhGH remain mostly unknown, although recent studies have suggested that a polymorphic variant of the gene encoding the GH receptor gene can influence the responsiveness to rhGH. A single-nucleotide polymorphism (SNP) in the IGFBP3 gene promoter (-202 A/C), believed to control basal IGFBP3 promoter activity, has been correlated to circulating concentrations of IGFBP3 in healthy adults, with the AA genotype being associated with highest concentrations of IGFBP3 and the CC genotype being associated with lower IGFBP3 concentrations. The aim of the study was to assess the influence of the polymorphism on circulating IGFBP-3 concentrations and growth response to rhGH therapy in children with GHD.

Methods: -202 A/C IGFBP3 genotyping (rs2854744) was performed using PCR amplification followed by restriction digest and was correlated with data of 71 children with severe GHD who remained prepubertal during the first year of rhGH treatment. Of these, 36 had attained adult height after a mean period of rhGH treatment of 8 ± 3 years. IGFBP-3 concentrations were measured at the start and near the end of the first year of treatment and first year growth velocity (GV) calculated during rhGH treatment.

Results: The distribution of patients among the different -202 IGFBP3 genotypes was 21% AA, 54% AC, and 25% CC. Clinical and laboratory data at the start of treatment were indistinguishable among patients with different -202 A/C IGFBP3 genotypes, although patients with one or two copies of the -202 C IGFBP3 allele had a tendency to lower IGFBP3 concentrations, but this did not reach statistical significance. Despite similar rhGH doses, AA patients had higher IGFBP3 concentrations and higher mean GV in the first year of rhGH treatment than patients with AC or CC genotypes (first year GV, AA = 13.0 ± 2.1 cm/year, AC = 11.4 ± 2.5 cm/year, and CC = 10.8 ± 1.9 cm/year; $p = 0.016$). Multiple linear regression analyses demonstrated that the influence of -202 A/C IGFBP3 genotype on IGFBP-3 concentrations and GV during the first year of rhGH treatment was independent of other variables. There was a tendency for better final height outcomes in patients with the AA -202 IGFBP3 genotype, although the difference did not reach statistical significance.

Conclusion: The -202 A allele of IGFBP3 promoter region is associated with increased IGFBP-3 concentrations and GV during rhGH treatment in prepubertal GHD children. Patients with at least one C allele had a mean GV that was 1.9cm/year lower than that of patients homozygous for the A allele. Although there was a tendency to improved final height in AA patients, this effect did not reach statistical significance.

The last few years have focused on the pharmacogenomics of rhGH treatment, with considerable debate generated with respect to the effect of a polymorphic variant of the GH receptor (d3-GHR) that results in deletion of exon 3, on the growth response to rhGH treatment. A number of papers have been published that show an improved growth velocity in patients who carry either a single or both d3-GHR alleles [9–11], whereas other studies fail to confirm these observations [12, 13]. The effects on growth velocity are generally small (e.g. 1.7 cm/year). It would appear likely that other genetic variants may also contribute to the marked variability in growth rate. GH promotes growth mainly via IGF-I; transport of the latter in serum is mediated mostly by IGF-binding protein-3 (IGFBP-3) and acid labile subunit. IGFBP-3 can also modulate the bioactivity of IGF-I. Twin studies have indicated that approximately 60% of the inter-individual variability in circulating levels of IGFBP-3 is genetically determined. Deal et al. [14] have identified a number of SNPs in the promoter region of *IGFBP3*. Of these the most relevant is a SNP (A–C nucleotide change) located at -202, upstream of the transcription start site. The AA genotype is associated with higher mean IGFBP-3 concentrations, and the latter decline with the presence of the C allele. In vitro studies have confirmed the higher promoter activity of the A allele in comparison to the C allele. In this article, the authors reveal that the

A allele is associated with an improved response with respect to both IGFBP3 and growth velocity during the first year of GH treatment in a cohort of prepubertal children with severe GHD. It will be important to investigate whether this finding holds true in other GHD cohorts, as well as children with Turner syndrome and SGA. In such children, slight differences in GH sensitivity are likely to be counterbalanced by a corresponding increase in GH secretion. Additionally, it would be important to follow these cohorts up with a view to obtaining longer-term data – in particular, does the presence of the A allele lead to an taller final height?

Mechanism of the year – how to twist it

An agonist-induced conformational change in the growth hormone receptor determines the choice of signalling pathway

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Nat Cell Biol 2008;10:740–747

Background: The growth and metabolic actions of growth hormone (GH) are believed to be mediated through the GH receptor (GHR) by JAK2 activation. The GHR exists as a constitutive homodimer, with signal transduction by ligand-induced realignment of receptor subunits.

Methods and Results: Based on the crystal structures, the authors identified a conformational change in the F'G' loop of the lower cytokine module, which results from binding of hGH but not the G120R hGH antagonist. Mutations disabling this conformational change cause impairment of ERK but not JAK2 and STAT5 activation by the GHR in FDC-P1 cells. This results from the use of two associated tyrosine kinases by the GHR, with JAK2 activating STAT5, and Lyn activating ERK1/2. The authors provide evidence that Lyn signals through phospholipase C', leading to activation of Ras. Accordingly, mice with mutations in the JAK2 association motif respond to GH with activation of hepatic Src and ERK1/2, but not JAK2/STAT5.

Conclusions: These data suggest that F'G' loop movement alters the signaling choice between JAK2 and a Src family kinase by regulating transmembrane domain realignment. This could explain attenuated ERK but not STAT5 signaling in some GH-resistant patients and suggest pathway-specific cytokine agonists.

The group of Mike Waters has once again performed an impressive piece of work that sheds novel insights into the function of the GHR. They suggest that separate tyrosine kinases associated with the GHR, namely Lyn and JAK2, are activated by different subunit alignments of the GHR dependent on the involvement of the F'G' loop within the GHR. They showed several years ago that GHR activation is initiated by a twist of the transmembrane domains of the 2 dimerized GHR molecules, brought about by GH binding [15]. This is believed to bring two GHR-associated JAK molecules in closer proximity, which results in activation of their kinase activity and subsequent further activation of the JAK-STAT5 pathway. Although it is known that GH can also activate other signaling pathways (MAPK-ERK1/2-RAS and PI3K pathways), it is unclear what triggers the activation of these other pathways and whether the pathways can be activated independently.

In this work, Rowlinson et al. compared GHR bound to hGH or to the G120R-hGH antagonist Pegvisomant which we know is unable to activate GHR signaling, and found a difference in the 3D structure in a 6 amino acid loop (F'G') of the ligand-GHR complexes. They continued to show that GHR F'G' loop mutations result in impaired ERK signaling, and a slightly impaired proliferation rate, but do not affect JAK-Stat signaling. Using siRNA they showed that the kinase involved in this pathway is not JAK2 but Lyn. Box 1 in the GHR is necessary for association of JAK2 with the GHR and mice with a mutated Box1 were therefore generated. These mice are indeed unable to activate the JAK-STAT pathway but able to activate ERK1/2, and no doubt we will soon hear about the growth phenotype of these mice.

This work suggests that the choice of multiple kinases (JAK, Lyn) facilitates a choice of signaling pathways. The ratio of Lyn and JAK may determine signaling, and this may be a means to allow for cell type-specific signaling. It also suggests that GH-induced MAP-ERK signaling plays a role in cell proliferation. For clinical medicine, this offers not only an explanation for cases of short stature with impaired ERK signaling but normal JAK-STAT signaling [16] but also opens the door to develop signaling pathway-specific drugs.

New concepts

IGFBP-4 is an inhibitor of canonical Wnt signalling required for cardiogenesis

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Nature 2008;454:345–349

Background: Some of the actions of IGFBPs have been reported to be independent of IGFs, but the precise mechanisms of IGF-independent actions of IGFBPs are largely unknown. A previously unknown function for IGFBP-4 as a cardiogenic growth factor is reported.

Methods and Results: IGFBP-4 enhanced cardiomyocyte differentiation in vitro and knockdown of *Igfbp4* attenuated cardiomyogenesis both in vitro and in vivo. The cardiogenic effect of IGFBP-4 was independent of its IGF-binding activity but was mediated by the inhibitory effect on canonical Wnt signaling. IGFBP-4 physically interacted with a Wnt receptor, Frizzled 8 (Frz8), and a Wnt co-receptor, low-density lipoprotein receptor-related protein 6 (LRP6), and inhibited the binding of Wnt3A to Frz8 and LRP6. Although IGF-independent, the cardiogenic effect of IGFBP-4 was attenuated by IGFs through IGFBP-4 sequestration.

Conclusions: IGFBP-4 is an inhibitor of the canonical Wnt signaling pathway required for cardiogenesis and provides a molecular link between IGF signaling and Wnt signaling.

This work set out to identify factors required for cardiogenesis. The experimental approach was extremely elegant, the experiments starting out to test conditioned cell media from various cell types for cardiomyocyte induction in a cardiomyocyte progenitor cell line in vitro. To then identify the cardiogenic factor, cDNA clones from the cell line were isolated and tested for their cardiomyocyte induction and thus IGFBP4 was identified.

The heart is the first organ to form during embryogenesis and abnormalities in this process result in congenital heart defects. The authors gave overwhelming evidence that IGFBP4 is involved in heart formation by inhibiting Wnt signaling. It does so after secretion, by physical direct binding to LRP6 and Frizzled-8, cell surface receptors for Wnt. Knockdown of *Igfbp4* in *Xenopus* embryos revealed the impact of IGFBP4 since 70% of the embryos had a small heart or no heart at all. Interestingly, IGFBP4 is expressed in the embryonic liver, just adjacent to the heart and likely affects the heart in a paracrine fashion. Conversely, the heart secretes factors that affect the liver in a paracrine fashion. IGFBP1, 2 and 6 had a similar, but more modest effect on Wnt inhibition. This suggests genetic redundancy and may account for the fact that *Igfbp4*-null mice lack a cardiac phenotype. The identification of IGFBP4 as a secreted modulator of heart development shows once again that IGFBPs have a life of their own, and will pave the way for further research into potential applications of IGFBPs.

Neurofibromin regulates somatic growth through the hypothalamic-pituitary axis

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Hum Mol Genet 2008;17:2956–2966

Background: Neurofibromatosis type 1 (NF1) is associated with short stature in 13–18% of patients. A significant proportion of patients have associated growth hormone deficiency (GHD). The mechanism whereby the condition leads to short stature remains unknown. Neurofibromin is thought to primarily regulate cell growth by modulating Ras activity.

Methods: To study the role of the neurofibromatosis-1 (*NF1*) gene in mammalian brain development, the authors generated mice in which *Nf1* gene inactivation occurs in neuroglial progenitor cells using the brain lipid-binding protein (BLBP) promoter.

Results: Nf1(BLBP)CKO mice exhibit significant reductions in body weight and anterior pituitary gland size. The authors further demonstrate that the small anterior pituitary size reflects loss of neurofibromin expression in the hypothalamus rather than the pituitary, leading to reduced growth hormone-releasing hormone, pituitary growth hormone (GH) and liver insulin-like growth factor-1 (IGF1) production, with a reduction in the number of proliferating cells within the anterior pituitary. Since neurofibromin both negatively regulates Ras activity and positively modulates cAMP levels, the authors examined the signaling pathway responsible for these abnormalities. BLBP-mediated expression of an activated Ras molecule did not recapitulate the body weight and hypothalamic/pituitary defects, and conditional expression of the Ras regulatory GAP domain of neurofibromin did not rescue the growth phenotype of Nf1(BLBP)CKO mice, suggesting that the hypothalamo-pituitary abnormalities associated with neurofibromin loss in the brain are not Ras-dependent. There was a 50% reduction in the levels of cAMP in Nf1(BLBP)CKO hypothalamic homogenates compared with littermate controls. Rolipram, a specific phosphodiesterase-4 inhibitor that elevates CREB activation in the brain and increases cAMP levels, resulted in a partial restoration of the body weight phenotype.

Conclusion: These data demonstrate a critical role for neurofibromin in hypothalamic-pituitary axis function, in part via the modulation of intracellular cAMP levels, and provide further insights into the short stature and GH deficits seen in children with NF1.

Short stature is a relatively common complication of NF1. In the largest study of pituitary function in children with NF1 and short stature, nearly 80% (15/19) of the children were found to have growth hormone deficiency in the absence of suprasellar abnormalities on neuroimaging. The NF1 gene, termed neurofibromin, functions as a negative regulator of Ras. Loss of *Nf1* activity results in increased Ras activity and downstream MAPK/Akt signaling. In this study, the authors show that neurofibromin regulates the function of the hypothalamo-pituitary axis, and that loss of *Nf1* in the brain leads to decreased GHRH, GH and IGF1 levels with anterior pituitary hypoplasia. It would appear that the growth retardation observed in *Nf1* mutant mice may be the result of a primary neuronal defect, since inactivation of the *Nf1* gene in embryonic neural stem/progenitor cells within the developing CNS was associated with a growth defect and anterior pituitary hypoplasia. Additionally, the reduction in cAMP levels in mice deficient in neurofibromin may in part account for the GH deficiency, as both cAMP and CREB are major regulators of hypothalamic development. cAMP is critical for hypothalamic neuronal maturation whereas brain-specific loss of CREB results in hypopituitarism and dwarfism. Hence this paper has identified for the first time a role for neurofibromin in the regulation of hypothalamic function and pituitary development in the mammalian CNS by modulating intracellular cAMP levels. Interestingly, despite the reduced body weights and organ sizes, the brain weights of Nf1 KO mice were unchanged. These disproportionate changes result in relative megalencephaly, similar to what has been reported for children with NF1. This could reflect increased numbers of glial precursors, oligodendrocytes or astrocytes. In support of this hypothesis, magnetic resonance imaging studies have found increases in both grey and white matter volumes in the brains of children with neurofibromatosis type 1.

Glutamate acting on N-methyl-D-aspartate receptors attenuates insulin-like growth factor-1 receptor tyrosine phosphorylation and its survival signaling properties in rat hippocampal neurons

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J Biol Chem 2009;284:855–861

Background: Excess glutamate may play an important role in neurodegenerative processes and neuronal survival. However, the underlying mechanism is unclear.

Methods and Results: Using hippocampal neuronal cell culture, the authors show that glutamate treatment or pretreatment attenuates the tyrosine phosphorylation of the insulin-like growth factor-1 (IGF-1) receptor and adapter proteins (IRS-1 and Shc) and the survival effect of IGF-1. The effect of glutamate was also evident on other molecules in the IGF1 signaling pathway like PI3K and Akt, as well as downstream targets GSK3 β and FOXO3a. The N-methyl-D-aspartate (NMDA) receptor, one of several glutamate receptors, was involved in this process, since the effect was blocked by antagonists of the NMDA receptor, but not by blockers of ionotropic or metabotropic glutamate receptors. In addition, treatment with NMDA inhibited the activation and phosphorylation of IGF-1 receptors and downstream targets induced by IGF-1.

Conclusions: Glutamate can block the effect of IGF-1 by decreasing IGF-1 receptor signaling and responsiveness, hence attenuating the survival properties of this trophic factor in neuronal cells. The results suggest a novel mechanism by which glutamate can reduce cell viability and induce neurotoxicity.

These experiments link two pathways involved in neuronal death and survival. Glutamate is a neurotransmitter involved in multiple neuronal processes in the developing and adult brain, like neurogenesis, synaptogenesis and neurite outgrowth. It plays a major role in several neurodegenerative diseases as well as in hypoxic-ischemic neuronal death and is believed to impair neuron survival. In contrast, IGF1 has an established role in cell survival and is suggested to have neuroprotective effects. The experiments in this paper are straightforward and indeed show that glutamate, through the NMDA receptor rather than other glutamate receptors, impairs the activation of IGF-R signaling pathway and neuron survival in vitro, without affecting the number of IGF1-R. This offers a piece for the mechanistic puzzle of the actions of glutamate on neuron survival. However, the experiments do suggest that IGF1 treatment does not overcome glutamate-induced cell death. Current efforts to improve clinical outcome of brain injury focus on reducing glutamate exposure to injured neurons [17] as well as on manipulating the IGF1 pathway [18], and that approach seems justified.

The circadian clock components CRY1 and CRY2 are necessary to sustain sex dimorphism in mouse liver metabolism

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J Biol Chem 2009;284:9066–9073

Background: Hepatic enzyme activity is sexually dimorphic in mice and many other mammals including humans and is responsible for, e.g., sexually dimorphic steroid synthesis and drug metabolism. Hepatic enzyme activity however is also a noticeable target of circadian timekeeping. Whether the circadian clock itself contributes to sex-biased metabolism has remained unknown.

Methods: Mice with a deletion of the circadian regulators Cryptochromes Cry1 and Cry2 (Cry1/2 $^{-/-}$ mice) were used to study growth, growth hormone production and downstream effects of GH.

Results: Dimorphic liver metabolism is altered in double mutant Cry1/2 $^{-/-}$ male mice that lack a functional circadian clock. Expression of a number of sex-specific liver products, including several cytochrome P450 enzymes, are at levels close to those measured in normal females. In addition, body growth of Cry1/2-deficient mice is impaired, also in a sex-biased manner, and this phenotype goes along

with an altered pattern of circulating growth hormone (GH) in mutant males, specifically. Hormonal injections that mimic male GH pulses reverse the feminized gene expression profile in the liver of *Cry1/2*^{-/-} males.

Conclusions: This work suggests that the 24-hour clock paces the dimorphic ultradian pulsatility of GH that is responsible for sex-dependent liver activity. Circadian timing, sex dimorphism, and liver metabolism are thus finely interconnected.

Many physiological processes have a circadian rhythm and are regulated by our body clock. This body clock has a central master circadian pacemaker, residing in the suprachiasmatic nucleus, and a peripheral component, located in peripheral cells like adipocytes, myocytes and hepatocytes and dictates a circadian rhythm in these cells. As a result, transcription of genes involved in processes like gluconeogenesis, nitrogen metabolism and drug metabolism are circadian, and this probably allows anticipation of, for example, nutrient availability. The timing mechanism of both the central and peripheral clocks consists of a transcriptional/translational feedback mechanism of 'clock genes' consisting of the negative regulators Period (Per) and Cryptochrome (Cry), and the positive regulators Clock and Bmal.

Hepatic enzyme activity is sexually dimorphic in mice and humans and the sexual dimorphism is partly dependent on sexually dimorphic GH-secretory patterns and JAK-STAT signaling.

The central and peripheral clocks are disturbed in *Cry1/2* null mice and the authors therefore used this model to establish whether the circadian rhythm and GH secretion interact in the regulation of sexually dimorphic hepatic metabolism. Interestingly, they found that indeed interference in the circadian clock disturbs sexually dimorphic hepatic P450 cytochrome enzyme expression but also GH secretion pattern and body growth. Reintroduction of a pulsatile (male) GH secretory pattern re-masculinized some of the hepatic enzyme expression. This suggests that the circadian rhythm is a major regulator of GH secretory patterns thereby affecting growth and hepatic metabolism. For more on peripheral circadian clock, see *Editors' choice*.

Important for clinical practice – tall tales and more imprinting

Treatment of pituitary gigantism with the growth hormone receptor antagonist pegvisomant

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J Clin Endocrinol Metab 2008;93:2953–2956

Background: Pituitary gigantism is a rare disorder characterized by excessive secretion of GH from a somatotroph pituitary tumor. Treatment of pituitary gigantism is complex, entailing the use of surgery, radiotherapy and medical therapy in the form of somatostatin analogs or dopamine agonists, and the results are usually unsatisfactory. The authors of the study describe the results of therapy of 3 children with pituitary gigantism by a GH receptor antagonist, pegvisomant.

Methods: This was a descriptive case series of up to 3.5 years duration at a university hospital. Patients included 3 children (1 female, 2 males) with pituitary gigantism whose GH hypersecretion was incompletely controlled by surgery, somatostatin analog, and dopamine agonist, and who were then treated by the administration of pegvisomant. Plasma IGF-I and growth velocity were measured on the pegvisomant.

Results: In all 3 children, pegvisomant rapidly decreased plasma IGF-I concentrations and growth velocity. Statural growth fell into lower growth percentiles and acromegalic features resolved. Pituitary tumor size did not change in 2 children but increased in 1 boy despite concomitant therapy with a somatostatin analog.

Conclusions: Pegvisomant may be an effective modality for the therapy of pituitary gigantism in children. Titration of the dose is necessary for optimal efficacy, and regular surveillance of tumor size is mandatory.

This important study adds another therapeutic agent that can be used to treat a rare but potentially devastating disorder, namely pituitary gigantism. Pituitary gigantism can be extremely difficult to treat, often necessitating a combination of surgery, radiotherapy and medical treatment in the form of somatostatin analogs. This study describes the use of a GH antagonist, pegvisomant, in 3 children with pituitary gigantism in whom surgery had failed to cure the excess GH secretion. Initiation of pegvisomant therapy led to a reduction in height velocity with a concomitant reduction in IGF-I concentrations, along with improvement in clinical features such as soft tissue hypertrophy and acromegaly features. In 2 of the 3 patients, the tumor size remained stable whereas in the 3rd patient it increased, but then stabilized following the cessation of pegvisomant. Pegvisomant binds to the GH receptor and prevents dimerization and subsequent receptor-mediated activity, thus reducing GH-dependent IGF-I generation. One of the drawbacks is an elevation in GH concentrations due to removal of IGF-I-mediated negative feedback. Additionally, an increase in tumor size may ensue on pegvisomant treatment, necessitating the combined use of a somatostatin analog. Hence, although the prompt and sustained improvement in clinical features makes pegvisomant therapy attractive, tumor size must be carefully monitored on therapy.

Clinically distinct epigenetic subgroups in Silver-Russell syndrome: the degree of H19 hypomethylation associates with phenotype severity and genital and skeletal anomalies

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J Clin Endocrinol Metab 2009;94:579–587

Background: Silver-Russell syndrome (SRS) is characterized by severe fetal onset growth restriction and variable dysmorphism including relative macrocephaly, triangular facies, skeletal asymmetry and hemi-hypertrophy, and clinodactyly. Maternal uniparental disomy of chromosome 7 (matUPD7) has been identified in 5–10% of SRS patients. Recently, the H19 imprinting control region (ICR), located on chromosome 11p15.5, has been reported to be hypomethylated in 20–65% of SRS patients.

Methods: The authors investigated the methylation status of 11p15.5 ICRs (H19 and KCNQ1OT1) in 42 SRS patients including 7 matUPD7 patients, and 90 small-for-gestational age (SGA) children without SRS to clarify the relationship between phenotype and H19 methylation status. Clinical data were evaluated from patient records, and 7 hypomethylated patients were clinically and radiologically reexamined.

Results: Hypomethylation of H19 ICR was found in 62% of SRS patients but in no SGA children. A clinical severity score demonstrated strong correlation between the hypomethylation level and phenotype severity. Hypomethylation related to a more severe SRS phenotype, in which asymmetry and micrognathia were especially more common. Extremely hypomethylated patients (<9%) had severe pre- and postnatal growth retardation, relative macrocephaly, asymmetry and 5th finger clinodactyly. Additionally, genital abnormalities were present in the majority: congenital aplasia of the uterus and upper vagina, equivalent to the Mayer-Rokitansky-Küster-Hauser syndrome in females, and cryptorchidism and testicular agenesis in males. In the moderately H19 hypomethylated individuals (9–35%), although many of the classical features were present, the patients were not significantly smaller at birth or 2 years, although they tended to be thinner and shorter at 2 years. Skeletal abnormalities such as abnormally high lumbar vertebrae, lumbar hypomobility, elbow subluxations, and distinct hand and foot anomalies, were more likely in the more severely hypomethylated patients. On the other hand, none of the matUPD7 patients showed a triangular face, and they had a lower incidence of asymmetry, 5th finger clino- and brachydactyly, cryptorchidism, toe syndactyly, muscle hypotonia, crowded teeth and a high arched palate. However, matUPD7 patients presented more frequently with speech delay, feeding difficulties, a high-pitched voice, low set/abnormal ears and excessive sweating.

Conclusions: This study reports a dose-response relationship between the degree of H19 hypomethylation and phenotype severity in SRS, suggesting that 35% of the variation in clinical severity was explained by the degree of H19 hypomethylation. The authors report for the first time the association of specific anomalies of the spine, elbows, hands and feet, and genital defects in SRS with severe H19 hypomethylation. Classical SRS features were found in H19 hypomethylation and milder symptoms in maternal uniparental disomy of chromosome 7, thus distinguishing two separate clinical and etiological subgroups.

It is now clear that Silver-Russell syndrome is a highly heterogeneous condition. This and previous studies [19] have revealed possible (epi)genotype-phenotype correlations. H19 ICR hypomethylation appears to be the most frequent genetic abnormality identified in patients with SRS, and is associated with a more severe phenotype than matUPD7. Within the hypomethylation cohort, the degree of methylation seemed to correlate with the phenotype, the most severe degree of hypomethylation being associated with the asymmetry, skeletal and urogenital abnormalities. It remains to be established whether long-term outlook will also correlate with the genotype of these patients, and only careful long-term follow-up will allow us to answer such questions.

New hope – inhibition of IGF1 signaling

Characterization of inhibitory anti-insulin-like growth factor receptor antibodies with different epitope specificity and ligand-blocking properties: implications for mechanism of action in vivo

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J Biol Chem 2009;284:10254–10267

Background: Antibodies directed against the type 1 insulin-like growth factor receptor (IGF-1R) have recently gained significant momentum because of their potential use in cancer treatment. These antibodies inhibit ligand-mediated activation of IGF-1R and the resulting down-stream signaling cascade.

Methods and Results: The authors generated a panel of antibodies against IGF-1R and screened them for their ability to block the binding of IGF-1 or IGF-2. Four distinct inhibitory classes were found as follows: (1) allosteric IGF-1 blockers, (2) allosteric IGF-2 blockers, (3) allosteric IGF-1 and IGF-2 blockers, and (4) competitive IGF-1 and IGF-2 blockers. The epitopes of representative antibodies from each of these classes were mapped using a purified IGF-1R library containing 64 mutations. Most of these antibodies bound overlapping surfaces on the cysteine-rich repeat and L2 domains. One class of allosteric IGF-1 and IGF-2 blockers was identified that bound a separate epitope on the outer surface of the FnIII-1 domain. The dual IGF blockers inhibited ligand binding using a spectrum of mechanisms ranging from highly allosteric to purely competitive. The data also suggest that IGF-1R uses disorder/order within its polypeptide sequence to regulate its activity. The activity of representative allosteric and competitive inhibitors on H322M tumor cell growth in vitro was reflective of their individual ligand-blocking properties.

Conclusions: Many of the clinically used antibodies likely adopt one of the inhibitory mechanisms described, and the outcome of future clinical studies may reveal whether a particular inhibitory mechanism leads to optimal clinical efficacy.

Apart from regulating normal growth, IGF1 and IGF2 play a role in tumor formation, at least partly due to their actions on proliferation, cell survival and angiogenesis through IGF1R. Expression of IGF1/IGF2 or IGF1R is increased in many human malignancies, for example in tumors of the breast, lung, gastrointestinal tract, prostate and bladder [20]. Antibody-mediated inhibition of IGF1-R activity may therefore offer a new therapeutic strategy, and this idea has been taken forward in trials. However, activity and toxicity of antibodies differ, likely due to their specific inhibitory effect on the IGF1/IGF2-IGF1R interaction. This study has evaluated the mechanism of action for a large number of antibodies. Four different classes of action were found: allosteric inhibition of IGF1 binding, allosteric inhibition of IGF2 binding, allosteric inhibition of both IGF1 and IGF2 binding and competitive inhibition of IGF1 and IGF2 binding. Allosteric inhibition refers to a decreased affinity of a ligand due to action on a site different from the ligand-binding site, and is likely due to a conformational change of the IGF1R but does not block IGF1 or IGF2 action completely. Competitive inhibition however can block action completely and differentiation between these categories is therefore relevant. The

authors showed that the in vitro inhibition of tumor growth indeed reflected the individual ligand-blocking properties for some antibodies tested. Using a panel of 64 mutated IGF1-R molecules, binding domains of many antibodies were determined.

This work offers a range of new well-characterized IGF1-R antibodies. It also offers a platform to determine the molecular action of IGF-R antibodies and suggest that most antibodies will fit in one of the four classes. Determining the molecular action of an antibody may allow for the prediction of their clinical actions and this work may therefore help to advance the field of cancer treatment.

New mechanism – for an old question

Growth hormone-releasing hormone as an agonist of the ghrelin receptor GHS-R1a

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Proc Natl Acad Sci USA 2008;105:20452–20457

Background: Ghrelin synergizes with growth hormone-releasing hormone (GHRH) to potentiate growth hormone (GH) response through a mechanism not yet fully characterized. Additionally, ghrelin is implicated in energy homeostasis, and may contribute to the metabolic syndrome. This study was conducted to analyze the role of GHRH as a potential ligand of the ghrelin receptor, GHS-R1a.

Methods: HEK293 cells were stably transfected with GHS-R1a (HEK-GHSR1a) conjugated with enhanced green fluorescent protein fluorophore. Membrane-binding assays were performed using HEK-293 and HEK-GHSR1a cells. Measurement of intracellular cAMP and inositol phosphate and chemical cross-linking assays were performed.

Results: hGHRH(1–29)NH(2) (GHRH) induces a dose-dependent calcium mobilization in HEK293 cells stably transfected with GHS-R1a, an effect not observed in wild-type HEK293 cells. This calcium rise is also observed using the GHRH receptor agonists JI-34 and JI-36. HEK-GHSR1a membranes showed a higher binding capacity of ^{125}I -GHRH that was displaced by unlabelled GHRH, as well as ghrelin. The presence of increasing amounts of GHRH cooperatively exerted a significant and dose-dependent increase in the ^{125}I -ghrelin-binding capacity in HEK-GHSR1a cells. Radioligand binding and cross-linking studies revealed that the calcium response to GHRH is mediated by the ghrelin receptor GHS-R1a. GHRH activates the signaling route of inositol phosphate and potentiates the maximal response to ghrelin measured in inositol phosphate turnover. In addition, confocal microscopy in CHO cells transfected with GHS-R1a tagged with enhanced green fluorescent protein shows that GHRH activates GHS-R1a endocytosis. Furthermore, the selective GHRH-R antagonists, JV-1-42 and JMR-132, act also as antagonists of the ghrelin receptor GHS-R1a.

Conclusion: The study findings suggest that GHRH interacts with the ghrelin receptor GHS-R1a and, in consequence, modifies the ghrelin-associated intracellular signaling pathway. This interaction may represent a form of regulation, which could play a putative role in the physiology of GH regulation and appetite control.

From its very early research the GH secretagogues showed synergism with GHRH in the release of GH [21]. This paper describes a possible mechanism whereby GHRH and ghrelin synergistically release GH. GHRH interacts with the ghrelin receptor GHS-R1, and allosterically enhances ghrelin action, displaying positive binding cooperation. Binding of GHRH to GHS-R1 then activates the signaling route of inositol phosphate and this in turn leads to a rise in intracellular calcium. Finally, GHRH leads to endocytosis of GHS-R1. The complete elucidation of this process will aid in understanding the interaction between GHRH and ghrelin and its impact on GH secretion and appetite control, and hence in understanding regulation of growth in terms of both height and weight.

The hormonal action of IGF1 in postnatal mouse growth

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Proc Natl Acad Sci USA 2008;105:19378–19383

Background: IGF1 is secreted by the liver and circulates in plasma. However, whether it acts systemically as a classical hormone to stimulate growth has been disputed since a conditional, liver-specific *Igf1* gene knockout in mice (LID mice) significantly reduced the level of serum IGF1, but did not affect average body weight. Because alternate interpretations of these negative data are tenable, the Efstratiadis group addressed genetically the question of systemic IGF1 action by using a positive experimental strategy based on the *cre/loxP* recombination system.

Methods: Bitransgenic mice were generated that carry in an *Igf1* null background a dormant *Igf1* cDNA placed downstream of a transcriptional 'stop' DNA sequence flanked by *loxP* sites ('floxed') and also a *cre* transgene driven by a liver-specific promoter. The *Igf1* cDNA, which was inserted by knock-in into the mutated and inactive *Igf1* locus itself to ensure proper transcriptional regulation, was conditionally expressed from cognate promoters exclusively in the liver after Cre-mediated excision of the floxed block.

Results: 6 different groups of mice were generated. If U = ubiquitous IGF1 expression, L = liver IGF1 expression, and number = number of alleles, groups were 1xU, 2xU, 1xU/1xL, 2xL (=LIP), Null and WT. LIP mice had larger weights and bone size than Null mice from 25 days of age.

Conclusions: The authors conclude that endocrine IGF1 plays a very significant role in mouse growth, as its action contributes approximately 30% of the adult body size and sustains postnatal development, including the reproductive functions of both mouse sexes.

This paper describes an elegantly designed labor-intensive experiment aimed to answer a specific question. The authors generated *Igf1* null mice that express *Igf1* only in the liver ('LIP' mice for 'Liver IGF1 Producers'), but to ensure that IGF1 was produced in a physiological amount and pattern, they used a genetic approach so that the transgene IGF1 was transcribed under the control of the endogenous IGF1 locus, but in the liver only. As controls, mice were used in which IGF1 should be expressed under its own locus in all tissues. Mice with a gradation of ubiquitous and hepatic IGF1 production (homozygous or heterozygous) were thus available, as well as complete *Igf* null mice and normal WT mice.

LIP mice were heavier than Null mice from 25 days of age and had larger tibial length at 10 weeks of age. The hierarchy for weight was (% of normal): 2xU 70%, 1xU/1xL 61%, 1xU 54%, LIP 50%, Null 34%, but, as in other experiments, the effect on tibial growth was much smaller (Null 67% of normal). Do the results justify the conclusion that endocrine IGF1 contributes 30% to adult body size though? There are a few unavoidable complications. The efficiency of recombination necessary for *Igf1* transgene expression is beyond control and was low prenatally in LIP mice, so that liver-specific IGF1 production only started postnatally and plasma IGF1 was only 44% of normal at 10 weeks of age. In 2xU mice, the plasma IGF1 concentration ranged from 30% of normal around birth to 50% in adult life and mice were indeed dwarfed. This low level prenatal IGF1 production makes it difficult to compare the control mice to LIP mice or normal mice. High perinatal mortality in LIP and Null mice (90%) meant that only very few mice were available for growth assessment ($n = 3/\text{group}$), and these mice also lost weight after weaning possibly due to feeding difficulties. Interpretation of the data is further complicated by testosterone deficiency in the Null mice.

In conclusion, male LIP mice are larger than male IGF Null mice but how much is being contributed by a deficiency of GH, IGF1, testosterone, malnutrition or catch-up growth, or even by altered local IGF1 sensitivity, remains difficult to assess.

Autocrine IGF-1 action in adipocytes controls systemic IGF-1 concentrations and growth

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Diabetes 2008;57:2074–2082

Background: IGF1 and IGF1 receptor (IGF-1R) have been implicated in the regulation of adipocyte differentiation and lipid accumulation in vitro.

Methods: To investigate the role of IGF-1 receptors in vivo, the authors have inactivated the *Igf1r* gene in adipose tissue (IGF1R-aP2Cre mice) using conditional gene targeting strategies.

Results: IGF1R-aP2Cre mice had increased adipose tissue mass with more and larger adipocytes with increased lipid accumulation in epigonadal but not subcutaneous fat pads. The IGF1R-depleted adipocytes showed increased insulin receptor mRNA content but whole body insulin and glucose tolerance was unaffected. Surprisingly, IGF1R-aP2Cre mice exhibited markedly increased somatic growth in the presence of elevated IGF1 serum concentrations and IGF1 mRNA expression in liver and adipose tissue. IGF1 stimulation of wild-type adipocytes significantly decreased IGF1 mRNA expression, as expected, but IGF1 mRNA increased in IGF1R-deficient adipocytes upon IGF1 stimulation.

Conclusions: IGF1R signaling in adipocytes is not crucial for the development and differentiation of adipose tissue in vivo. The authors concluded that a negative IGF-1R-mediated feedback mechanism of IGF1 on its own gene expression exists in adipocytes, indicating an unexpected role for adipose tissue IGF1 signaling in the regulation of IGF1 serum concentrations in control of somatic growth. Bartke and Kopchick [22], however suggest, in a letter to the editor, the involvement of altered GH secretion.

This group has taken the reverse strategy to the Efstratiadis group to study the role of IGF1 and created mice that have a tissue-specific deletion of IGF1R. Surprisingly, the adipocyte IGF1R^{-/-} mice showed, apart from increased size of epigonadal fat pads, increased growth. IGF1 mRNA was almost doubled in adipose tissue and liver, systemic IGF1 and IGFBP3 concentrations were increased (20%), and a late onset increase in weight and length (15%) was noted. Since adipocyte IGF1 content was increased, the authors hypothesized that adipose IGF1 signaling regulates serum IGF1 concentration and growth. However, the increase in growth, IGF1 and IGFBP3 production may well reflect increased GH secretion. This is the first of a few models with tissue-specific IGF1R deletion [23, 24] that shows such a marked effect on growth and systemic IGF1 concentrations. This mouse model therefore may not give as much insight as hoped in the (endocrine) effect of IGF1 on adipocytes, but, given the known adverse effect of obesity on GH secretion, may be of great importance in studying the role of adipose tissue in the feedback control of GH secretion.

Brain IGF-1 receptors control mammalian growth and lifespan through a neuroendocrine mechanism

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PLoS Biol 2008;6:e254

Background: Mutations that decrease insulin-like growth factor (IGF) and growth hormone signaling limit body size and prolong lifespan in mice. In vertebrates, the GH-IGF1 axis is controlled by neuroendocrine hormones. Hormone-like regulations discovered in nematodes and flies suggest that IGF signals in the nervous system can determine lifespan, but it is unclear whether this applies to higher organisms.

Methods: Using conditional mutagenesis in the mouse and a Nestin-Cre promoter to target the brain but not pituitary, mice with a brain-specific *Igf1r* deletion were created.

Results: Partial inactivation of *Igf1r* in the embryonic brain selectively inhibited GH and IGF-I pathways after birth. This caused growth retardation, smaller adult size and metabolic alterations, and led to delayed mortality and longer mean lifespan.

Conclusions: Brain IGF1R regulates somatotrophic development. Early changes in neuroendocrine development can durably modify the life trajectory in mammals. The underlying mechanism appears to be an

adaptive plasticity of somatotrophic functions allowing organisms to decelerate growth and preserve resources, and thereby improve fitness in challenging environments. The results also suggest that tonic somatotrophic signaling entails the risk of shortened lifespan.

Kappeler et al. aimed to study the neuronal control of aging through insulin-like signals, and therefore created brain-specific IGF1R null mice. In Yearbook 2008 we discussed the relation between reduced activity of the GH/IGF1 pathway and increased lifespan in a range of species as well as an extensive study of a cohort of centenarians that may suggest a reduced IGF1 action in some of these individuals [1, 25]. Interestingly, brain Igf1r^{-/-} mice are born small but have a similar phenotype as described for the adipose tissue-specific Igf1r deletion: increased (30–40%) serum IGF1 concentrations from the age of 4 weeks and late onset (4 months) increased growth. However, lifespan was unaffected in these homozygous mice and the authors therefore chose to focus on the heterozygous mice that, indeed, on average have an increased lifespan (approx. 10%). They have a mild growth defect (10%) from the age of 3 weeks. The authors showed very nicely that this was due to a specific hypothalamic-pituitary somatotrophic dysfunction and that the mice had low hypothalamic GHRH, low Pit1 expression, small pituitaries with low GH content and low systemic IGF1 and ALS concentrations as well as the metabolic consequences of GH deficiency; enlarged adipose tissue and impaired glucose tolerance. The increased average lifespan was mostly due to a lower mortality rate at middle age, but mortality rate at higher age and maximum age reached was not different from controls. These mice show that the IGF1 (feedback) action in the brain regulates the GHRH-GH-IGF axis and suggest that deficient IGF1 action results in protection from death at an early age, at least in mice. Whether the downstream GH/IGF1 deficiency or the neuroendocrine environment is responsible for this protection remains a question. Relative IGF1 deficiency/resistance has been suggested to underlie the regulation of lifespan in some other species and is indeed the common factor between these mice and some centenarians. The question as to why GH deficiency in humans does not seem to enhance lifespan remains unanswered [26]. In the mean time, these mice add nicely to the collection of models for GH deficiency.

Review – going up and down the slopes

Motivations and methods for analyzing pulsatile hormone secretion

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Endocr Rev 2008;29:823–864

Overview: Endocrine glands communicate with remote target cells via a mixture of signal exchange both continuous and intermittent. Continuous signaling allows slowly varying control, whereas intermittency permits large rapid adjustments. The control systems that mediate such homeostatic corrections operate in a species-, gender-, age-, and context-selective fashion. Mechanisms of adaptive interglandular signaling in vivo are becoming more clear. Principal goals are to understand the physiological origins, significance, and mechanisms of pulsatile hormone secretion. Key issues for analysis of pulsatile secretion are: (1) to quantify the number, size, shape, and uniformity of pulses, nonpulsatile (basal) secretion, and elimination kinetics; (2) to evaluate regulation of the axis as a whole, and (3) to reconstruct dose-response interactions without disrupting hormone connections.

Conclusion: This review focuses on the motivations driving and the methodologies used for such analyses but also reviews the knowledge of pulsatile endocrine systems.

This is an in-depth review from the leaders in the field, and is a must for those involved in pulse analysis, but of interest for anyone who would like to broaden their understanding of the mechanisms and consequences of pulsatile hormone secretion and the potentials and pitfalls of methods analyzing secretion. It discusses not only the classical intermittently secreted hormones like GH and

LH/FSH but also hormones that are secreted in a shorter timeframe like oxytocin, glucagon, insulin and PTH. Mechanisms of pulse generation, implications of pulsatile signals and altered pulsatility in pathology are discussed. Methodologies for pulse analysis, including their application and pitfalls are then described in great detail. The concept of Approximate Entropy (ApEn) analysis, which assesses orderliness of secretion, and its advantages (for example the small amount of data required) are discussed as well as methods to assess synchrony of hormone secretion. By putting emphasis on the application of the analytical methods, this review gives great insight into the regulation of the pulsatile secretion of multiple hormones.

Food for thought – or hot coffee?

Maternal caffeine intake during pregnancy and risk of fetal growth restriction: a large prospective observational study

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BMJ 2008;337:a2332

Background: There is evidence that excessive caffeine intake is associated with lower birth weight, but previous studies have been inconclusive. Maternal caffeine intake is difficult to assess and confounders such as smoking often exist.

Methods: A prospective longitudinal observational study including 2,635 low-risk pregnant women in the UK. Total caffeine intake from 4 weeks before conception and throughout pregnancy was quantified with a validated caffeine assessment tool. Caffeine half-life was determined by measuring caffeine in saliva after a caffeine challenge. Smoking was assessed by measuring salivary cotinine concentrations.

Results: Caffeine consumption throughout pregnancy was associated with an increased risk of fetal growth restriction (odds ratios 1.2 (95% CI 0.9–1.6) for 100–199 mg/day, 1.5 (1.1–2.1) for 200–299 mg/day, and 1.4 (1.0–2.0) for >300 mg/day compared with <100 mg/day; test for trend $p < 0.001$). Mean caffeine consumption decreased in the first trimester and increased in the third. The association between caffeine and fetal growth restriction was stronger in women with a faster compared to a slower caffeine clearance (test for interaction, $p = 0.06$).

Conclusion: Caffeine consumption during pregnancy was associated with an increased risk of fetal growth restriction and this association continued throughout pregnancy. It would be sensible to advise to reduce caffeine intake before conception and throughout pregnancy.

In 2001, the Committee on Toxicity of Chemicals in Food in the UK concluded that caffeine intake >300 mg/day may be associated with low birth weight and spontaneous miscarriage, but that the evidence was inconclusive. That caffeine has an effect on the placenta is clear though – ingestion of 200 mg caffeine (approx. 2 cups of coffee) reduces placental blood flow by 25%. The current study aimed to study the effect of caffeine during pregnancy in more detail, and found a relation between caffeine intake and growth restriction (birth weight <10th centile), and is interesting for several reasons. Firstly, the authors claim to have estimated caffeine intake more accurately by taking brand, portion size, mode of preparation and caffeine intake through all dietary sources into account. Sixty-five percent of caffeine intake was from tea and the remainder from coffee, cola and chocolate. Secondly, a 'caffeine challenge test' was performed, and caffeine half-life was calculated. Thirdly, approximately half of the participants reduced their coffee intake spontaneously to <100 mg/day from the first trimester.

After adjustment for smoking, the effect of maternal caffeine intake (>200 mg/day) equaled a 60- to 70-gram reduction in birth weight. The presence of a dose-response relation and an effect of effi-

ciency of caffeine clearance support the authors' conclusion that caffeine intake increases the risk of fetal growth restriction. One double-blind study showed that reducing caffeine intake of regular coffee drinkers did not affect fetal birth weight, but the CARE study group argues that this intervention may have been too late in pregnancy to result in an effect.

Certainly, the last word on caffeine consumption during pregnancy has not been said but as the authors suggest it may be sensible to avoid excessive caffeine intake during pregnancy, as half of pregnant women do already.

Temperature regulates limb length in homeotherms by directly modulating cartilage growth

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Proc Natl Acad Sci USA 2008;105:19348–19353

Background: Allen's Rule documents a century-old biological observation that strong positive correlations exist among latitude, ambient temperature, and limb length in mammals. Although genetic selection for thermoregulatory adaptation is frequently presumed to be the primary basis of this phenomenon, research has shown that appendage outgrowth is also markedly influenced by environmental temperature. Appendages (ears, limbs and tails) of animals living in cold geographical regions are consistently shorter than those of closely related counterparts occupying warmer climes. Alteration of limb blood flow via vasoconstriction/vasodilation is the current default hypothesis for this growth plasticity.

Methods: Outbred mice were housed continuously at cold (7°C), control-intermediate (21°C) or warm (27°C) ambient temperatures from weaning through maturity (3.5–12 weeks age). Two intermediate end points were evaluated (4.5 and 6.5 weeks) to assess temperature effects during ontogeny. Regional organ and bone perfusion was quantified by measuring relative bone blood flow (BBF) to the hindlimb bones and tail base of juvenile mice using fluorescent microspheres. Additionally, the authors evaluated the growth of neonatal mouse metatarsals in culture maintained at cold (32°C), control (37°C), or warm (39°C) temperatures, holding all other factors constant.

Results: The authors show that the ears, limbs and tails of warm-reared mice were significantly longer than those of siblings raised in the cold, but without any change in body mass. The authors show that tissue perfusion does not fully account for differences in extremity elongation in mice. Mice raised at the coldest temperature had reduced femur, tibia and hindpaw BBF at 4.5 weeks of age, and reduced tibia, paw and tail BBF at 6.5 weeks. However, although the highest growth rates were found in the warmest reared animals, these animals did not also exhibit the correspondingly highest predicted BBF. They show that peripheral tissue temperature closely reflects housing temperature *in vivo*, and they demonstrate that chondrocyte proliferation and extracellular matrix volume strongly correlate with tissue temperature in metatarsals cultured without vasculature *in vitro*. Warm metatarsals showed significantly greater growth.

Conclusions: The data suggest that environmental temperature may modulate extremity growth by inducing physiological responses in peripheral tissue temperature, rather than by affecting vascular nutrient delivery. Such temperature lability may then impact extremity size via its direct effect on cell proliferation and matrix production in cartilage. Taken together, these data suggest that vasomotor changes likely modulate extremity growth indirectly, via their effects on appendage temperature, rather than vascular nutrient delivery. When combined with classic evolutionary theory, especially genetic assimilation, these results may substantially impact our understanding of phenotypic variation in living and extinct mammals, including humans.

This fascinating paper raises important questions. The genetic contribution to growth is undisputed, yet poorly understood. Although the role of the environment in regulating growth is unclear, certain environmental factors have been associated with poor growth. These factors include exposure of a fetus to smoking and alcohol, and postnatal child abuse or neglect. This study now proposes a role for environmental temperature in determining extremity growth. Mice reared in warmer conditions had significantly longer ears, limbs and tails than those raised in the cold, and this effect was also observed in neonatal mouse metatarsals grown in culture. These effects were largely due to increased chondrocyte proliferation and matrix volume in warmer conditions. These data may explain the dis-

proportionately shortened long bones in cold-dwelling humans such as Neanderthals, and the relative reduction in tibial length as compared with femoral length that is observed in decreasing environmental temperatures. The contribution of genotype to this process, and its interaction with the environmental temperature, remains to be established.

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