INVITED REVIEW

Parental genomic imprinting in endocrinopathies

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Abstract

Genomic imprinting is the phenomenon whereby some genes preferentially produce mRNA transcripts from the gene copy derived from the parent of a specific sex. It has been implicated in a number of human diseases (most of them of endocrine interest), such as Prader–Willi/Angelman syndromes, Silver–Russell syndrome, Beckwith–Wiedemann syndrome, transient neonatal diabetes, the focal form of nesidioblastosis, and pseudohypoparathyroidism. Involvement of imprinted genes affecting birth weight and causing susceptibility to type 1 diabetes is under investigation. Recent knowledge about the varied molecular mechanisms involved will be outlined.

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Introduction

The phenomenon of parental imprinting was first discovered in mammals in 1984, when embryos derived from zygotes artificially reconstituted with two paternal or two maternal nuclei failed to develop normally, indicating that the genetic material derived from sperm or egg were not equivalent (1). The best explanation for this phenomenon would be that the two copies of certain genes are expressed differentially, depending on the sex of the parent from whom each was inherited. The first mammalian gene found to have such parent-of-origin dependent control was *IGF2*, a gene encoding insulin-like growth factor II (IGF-II), a peptide of endocrine interest. In most tissues only the paternally derived *IGF2* copy is transcriptionally active, while the maternal one is completely silent (2). Imprinting also occurs in humans, and the first human gene demonstrated to be imprinted was also *IGF2* (3).

The ability of the offspring to distinguish parental origin requires some molecular modification (imprint) of the DNA or associated chromatin, as it passes through the male or female gamete. Such modification must be maintained through DNA replication in the somatic cells of the offspring, it must be reversible from generation to generation (as when a male transmits an allele inherited from his own mother, or a female from her father), and it must be capable of influencing transcription. DNA methylation fulfills all these criteria, and parental sex-specific methylation has been found on or near most imprinted genes and shown to be necessary for imprinting (4). One remarkable feature of imprinted genes is that most of them are physically

linked in clusters with other imprinted genes. Specific DNA sequences identifying some of these clusters as targets of the imprinting machinery of the gamete have been identified and termed imprinting centers or imprinting control elements. The total number of imprinted genes in humans is estimated to be less than 200.

Imprinting may cause disease if disrupted: aberrant expression of the normally silent allele would result in a double gene dosage, while aberrant repression of the normally active allele will result in deficiency. The latter is the case in Prader–Willi syndrome, while the former applies to the etiology of Silver–Russell syndrome, transient neonatal diabetes, pseudohypoparathyroidism type 1B and, almost certainly, Beckwith–Wiedemann syndrome. In the absence of disruption of normal imprinting, the phenomenon can simply modify the inheritance pattern and cause a recessive disorder to behave as dominant with inheritance dependent on the sex of the transmitting parent. This explains parental-origin effects in the genetics of the focal form of persistent hyperinsulinemic hypoglycemia of the newborn and, possibly, pseudohypoparathyroidism type 1A.

The purpose of this review is to describe endocrinopathies due to affected imprinted genes and outline the known or hypothesized mechanism involved.

Prader–Willi and Angelman syndromes

The first hint that imprinting may be involved in endocrine-related disorders came from the puzzling observation that a chromosome 15q12 deletion seen in

some patients with Prader–Willi syndrome (PWS) was always found on the paternal chromosome (5). The cardinal features of PWS include hypothalamic dysfunction with short stature and hypogonadism, obesity, characteristic facies and behavioral abnormalities. Neonatal hypotonia is a characteristic early sign. PWS affects males and females equally with an incidence of 1:12 000–20 000 live births. Approximately 70% of patients with PWS have a cytogenetically detectable deletion of 15q11–q13, which is always found on the paternally derived chromosome (5). The majority of the remaining 30% have two normal maternally derived chromosomes 15 (maternal uniparental disomy (UPD)). UPD is a rare phenomenon that arises when two independent events result in the rescue of a whole chromosome gain (that would otherwise result in lethal trisomy) by a loss of the same chromosome in the other gamete. Maternal UPD can be detected by the absence of paternal alleles at polymorphisms throughout the chromosome involved. That PWS can be caused by these two types of chromosomal aberrations suggests that the disease is due to loss of expression of one or more genes within 15q11–q13, normally expressed exclusively from the paternal chromosome. Several imprinted genes are in this region, including the paternally expressed small nuclear ribonucleoprotein polypeptide (SNRPN). This gene encodes a ribonucleoprotein involved in mRNA processing that may have brain specificity. A second highly conserved coding sequence of SNRPN is termed SNURF for the imprinting center and appears to be responsible for setting a complex pattern of differential methylation on the paternal vs maternal chromosome (6). In rare cases, no deletion of the region has been found in individuals with biparental inheritance of chromosome 15. In those cases, both chromosomes carry the methylation pattern specific for the maternal chromosome. Some of these cases are familial and due to mutations in the PWS imprinting center (PWS-IC), the DNA sequence that identifies the region as a target for imprinting (6, 7). In sporadic cases, where no such mutations are found, it can be presumed that the aberrant methylation of the paternal chromosome is due to stochastic failure of proper imprinting in a particular gamete (8) in the absence of a heritable mutation. We have recently presented direct evidence for such stochastic failure in the *Igf2-H19* region in normal mouse embryos (9). The specific gene involved in the Prader–Willi phenotype, and the mechanism of the neurodevelopmental/neuroendocrine disfunction remain unknown. However, understanding of the molecular pathology has allowed the development of a simple diagnostic test that detects DNA methylation (10). Normal DNA at the PWS-IC shows a pattern where both methylated and unmethylated DNA are present, while in PWS only the maternal methylation pattern is found, independently of whether the disease is due to a paternal deletion, maternal UPD,

or aberrant methylation of the paternal chromosome (Fig. 1).

Angelman syndrome (AS) is characterized by mental retardation, speech impairment, gait ataxia and a unique behavior with an inappropriate happy demeanor that includes frequent laughing. Unlike the PWS, there is no endocrine phenotype. Most deletions that cause PWS when paternally inherited result in AS when transmitted by the mother (11). AS is caused by loss of a gene with exclusive maternal expression in the same cluster of imprinted genes at the 15q11–q13 (AS/PWS) region. The E6-AP ubiquitin-protein ligase (*UBE3A*) gene is very likely the AS gene (12). *UBE3A* exhibits tissue-specific imprinting with preferential maternal expression in subregions of the brain in humans and mice (13).

Silver–Russell syndrome (SRS)

The main features of SRS are pre- and postnatal growth restriction and a characteristic small, triangular face. The growth problem is not related to deficiency of growth hormone or any known growth factor. It is probably a heterogeneous syndrome of varied molecular etiologies, as various modes of inheritance and abnormalities involving chromosomes 7, 8, 15, 17 and 18 have been described in SRS and SRS-like cases. Of interest in relation to imprinting are cases (up to 10% of SRS patients) with UPD of chromosome 7, which is always maternal. These cases are sporadic, consistent with UPD not being heritable (14, 15). The finding of a typical clinical phenotype in two familial cases with a small duplication, in both cases of maternal origin (16, 17), strongly suggests that SRS is due to a double dosage of a maternally expressed gene rather than deficiency of a paternally expressed gene. These cases have also allowed narrowing the location of this gene within 7p11.2–p13. More recently, a partial maternal isodisomy of 7q31–qter in a case with SRS features (18) has suggested a second, distinct locus on chromosome 7, increasing the complexity of the genetic heterogeneity in SRS. The involvement of imprinting in this locus is less clear, as isodisomy may cause disease by converting carrier status for a recessive disorder into homozygous disease state.

Of the several growth-related genes in the 7p11.2–p13 locus, only *GRB10*, the gene that encodes an adaptor protein involved in the signal transduction pathways for insulin and the insulin-like growth factors, has been demonstrated to be imprinted, both in humans and mice. *GRB10* imprinting is isoform specific in brain and muscle (19) but it is not clear how this might be related to the growth failure in SRS. We recently examined growth plate cartilage, the organ directly affected in growth failure, and have demonstrated that none of the *GRB10* isoforms is imprinted in it (20). This makes *GRB10* an unlikely candidate

Figure 1 Schematic representation of the different molecular pathologies on chromosome 11p that may result in PWS and of the expected findings on Southern blot of the differentially methylated region after digestion with a methylation-sensitive restriction endonuclease that does not cut methylated DNA. Normal DNA has both digested and undigested bands, one representing each gene copy while PWS patients, regardless of the type of molecular lesion, will show only the maternal (methylated) pattern. Chromosomes are identified as paternal (p) or maternal (m). UPD, uniparental disomy, where both chromosome 15 copies are derived from the mother.

for SRS, although indirect mechanisms could still be involved. An extensive search for mutations in cases without maternal duplication of chromosome 7 also failed to uncover point mutations of *GRB10* (21).

Beckwith–Wiedemann syndrome (BWS)

The main manifestation of BWS is fetal and post-natal overgrowth. Its major clinical features are macroglossia, macrosomia (sometimes asymmetrical), neonatal hyperinsulinism, abdominal wall defects and high risk of embryonal tumors (mostly Wilms'). Familial clustering accounts for approximately 15% of cases, with a clearly maternal transmission pattern, linked to 11p (22). Approximately 20% of cases demonstrate UPD (always paternal) for chromosome 11p15.5.

Deletions of this chromosomal region, always of maternal origin, are also found in non-syndromic, sporadic Wilms' tumors. In most of the remaining cases, the differential DNA methylation is lost, with both chromosomes showing methylation in the paternal pattern (23, 24). Thus the clinical features must be due to either a double dose of a paternally expressed gene, deficiency of a maternally expressed one, or a combination thereof. Indeed the paternally expressed fetal growth factor encoded by the *IGF2* gene is expressed from both copies and this can explain the overgrowth as well as contribute to the omphalocele (because of large viscera) and tumor susceptibility. The etiology of BWS, however, is likely to be more complex as the 11p15.5 domain involves a whole cluster of imprinted genes, subdivided to two major subgroups,

proximal and distal (the latter containing the *IGF2*, *INS* and *H19* genes) separated by non-imprinted genes (25). The *H19* gene, encoding an untranslated RNA of unknown function, is physically linked to *IGF2* (0.2 Mb downstream) and oppositely imprinted, expressed from the maternal copy only (26). *H19* is silenced on the paternal chromosome by methylation of its promoter. The same methylation appears to be necessary for expression of *IGF2* on the paternal chromosome, by inhibiting the binding of CTCF, an insulator protein that would otherwise prevent a 3' enhancer from activating *IGF2* transcription (27). The proximal cluster includes the maternally expressed *CDKN1C* gene that encodes a cyclin-dependent kinase inhibitor whose overexpression can inhibit growth by arresting cells in G1. Thus its silencing in paternal UPD or aberrant paternal methylation of the maternal chromosome could contribute to the overgrowth and tumor susceptibility. However, *Cdkn1c* knockout mice have no overgrowth phenotype although they do have abdominal wall defects closely paralleling the omphalocele of BWS, which makes silencing of *CDKN1C* (also referred to as *P57^{kip2}* in the human) the prime suspect for this phenotypic feature (28). Its role in the tumor susceptibility is less certain, as mutations of the maternal *CDKN1C* copy were not found in sporadic Wilms' tumors, despite exhaustive search (29). Another gene in this cluster, *KVLQT1*, which is mutated in the long QT syndrome, does not appear to have a role in BWS. In summary, a double dose of *IGF2* may by itself explain the overgrowth phenotype and contribute to tumor susceptibility. The abdominal wall defects are probably due to absence of *CDKN1C* expression, while the tumor susceptibility must be primarily due to loss of an as yet undefined tumor suppressor in the 11p15.5 locus, probably one with exclusive maternal expression. Alternatively, this tumor suppressor gene may not be imprinted and the maternal origin of deletions in the sporadic tumors explained by a permissive role, for tumorigenesis, of a closely linked paternally expressed gene that is co-deleted (and, therefore, would not be expressed if the deletion is paternal). If the latter is the case, *IGF2* is an excellent candidate for such a permissive role.

Transient neonatal diabetes

Transient neonatal diabetes (TND) is a rare disease, manifesting in the early neonatal period with hyperglycemia, glycosuria and severe dehydration, but ketosis, if present, is mild indicating partial insulin deficiency. The need for insulin replacement therapy disappears within days or weeks but patients are predisposed to develop mild insulin-deficient diabetes later in life (30). Several sporadic cases of TND with paternal UPD on chromosome 6 have suggested

either a double dose of a paternally expressed imprinted gene on chromosome 6, or deficiency of a maternally expressed one (31). Familial cases are less common but in these families the transmission is always paternal and is associated with duplications in 6q24 (31), a pattern that points to double dose of a paternally expressed gene as the cause of TND. Molecular exploration of such families with small duplications has allowed us to narrow the region down to 0.5 Mb (32) that includes *ZAC*, encoding a recently isolated zinc finger protein inducing apoptosis and cell arrest. As *ZAC* is imprinted and expressed only from the paternal copy, it seems to be the ideal candidate for TND gene (33).

The focal form of persistent hyperinsulinemic hypoglycemia of infancy

Persistent hyperinsulinemic hypoglycemia of infancy (PHHI) is characterized by unregulated secretion of insulin that cannot be down-regulated in the face of severe hypoglycemia. There are two histopathological types, a focal adenomatous hyperplasia of islet cells of the pancreas and a diffuse form. Most cases of the classical diffuse form are inherited as autosomal recessive and are due to mutational inactivation of one of two closely linked genes, encoding components of potassium channels involved in the regulation of insulin release (34). These two genes are *SUR* (encoding the sulfonylurea receptor, whose inactivation by this class of drugs results in insulin release) and *KIR6.2*. They are closely linked and map to 11p14, about 30 Mb away from the imprinted cluster of genes implicated in BWS (35; Fig. 2). Neither is imprinted, consistent with the recessive, biparental inheritance pattern. For this reason it was surprising to find that patients with PHHI due to adenoma (usually sporadic) are heterozygous for a point mutation in *SUR*, which is always on the paternal chromosome (36). The maternal allele is normal in these patients' germline, but clonally lost in the adenoma cell lineage, through a large deletion that involves the nearby imprinted cluster on 11p15.5 (37). Taken together these observations invite us to propose that the generation of the adenoma requires two molecular events. First, inactivation of both copies of *SUR*, one by a germline mutation, the other by a somatic one, in the classical Knudsen two-hit fashion. Since this occurs in only one cell, it is not sufficient to cause hyperinsulinism. If, however, the somatic mutation is a deletion large enough to involve the maternally expressed 11p15.5 gene(s), whose loss is responsible for the susceptibility to Wilms' and other embryonal tumors in BWS (or in non-syndromic embryonal tumors with an 11p15.5 deletion, referred to as the *WT2* locus), then the cell in which this somatic event occurred gains a growth advantage that results in clonal expansion and adenoma (explained in Fig. 2).

Figure 2 Proposed explanation for the paternal origin of germ-line mutations of patients with the focal form of neonatal hyperinsulinism. It can be postulated that two events are required for adenoma formation: loss of a maternally expressed tumor suppressor at 11p15.5 (also involved in Wilms, and other embryonal tumors) through a mitotic accident in a single beta cell is necessary but not sufficient to cause clonal expansion of that cell into a tumor. A second hyperplasia-inducing stimulus is needed, in the form of loss of both copies of *SUR* (or its closely linked molecular partner, *KIR6.2*). For this to happen in *SUR* mutation carriers, only a single additional event is needed. Such an event may be a point mutation or a large chromosomal deletion. (A) If this event happens in a normal person, there are no consequences. (B) If the second event inactivating *SUR* is a point mutation in a carrier, the 11p15.5 locus is intact and tumor does not occur. Insulin hypersecretion from a single cell has no consequences. (C) If the second event is a large deletion in a carrier of a maternal *SUR* mutation, the loss of the paternal copy of the 11p15.5 tumor suppressor has no consequences, as this copy is normally inactive. (D) Loss of the second (maternal) *SUR* copy in a carrier of a paternal mutation through a large deletion is the *only* situation in which both requirements for tumorigenesis can result from a *single somatic mutation*. Clonal expansion of that beta cell results in a hypersecreting tumor.

Pseudohypoparathyroidism and Albright hereditary osteodystrophy

Pseudohypoparathyroidism (PHP) is due to resistance to the action of parathormone (PTH). It manifests as hypocalcemia and hyperphosphatemia in the face of elevated plasma PTH and resistance to exogenous PTH (38). It often occurs in combination with resistance to the action of other peptide hormones and Albright's hereditary osteodystrophy (AHO), a morphological phenotype characterized by short stature, round facies, brachydactyly and short fourth metacarpal bone.

The combination is termed PHP type 1A, while type 1B refers to the occurrence of PTH resistance alone, without resistance to other hormones or the AHO phenotype. The PTH receptor is normal in both disorders (39). Both PHP1A and 1B are inherited as autosomal dominant and, in pedigree linkage analysis, both map to the same locus on chromosome 20q13.3 (40). Of interest to the study of imprinting, both are transmitted only from the mother (40, 41). Different members of the same PHP1A pedigree may have the full PHP1A, or the AHO phenotype alone without PTH resistance (pseudo-pseudohypoparathyroidism), indicating that the same mutation may or may not cause PTH resistance depending on other genetic or environmental

factors. However, members of PHP1B pedigrees never have AHO or multiple hormone resistance, indicating a distinct type of mutation in the same locus (40). The cause of PHP1A was elucidated when inactivating mutations were found on the maternal copy of the *GNAS1* gene which encodes an α subunit of the activating G-protein complex ($G_s\alpha$), which couples receptors with seven transmembrane domains (including PTHr) to adenylate cyclase (42). Affected individuals have ~50% of the $G_s\alpha$ protein level and activity, which would explain the dominant inheritance on the basis of haploinsufficiency. There is no obvious explanation for the maternal inheritance because, although *GNAS1* is imprinted with exclusive maternal expression in rodents (43) $G_s\alpha$ is expressed from both alleles in all human tissues studied (44), including isolated renal cortex (45). There is also no explanation for the genetic mapping of PHP1B to the *GNAS1* locus at 20q13.3, despite the fact that no inactivating $G_s\alpha$ mutations have been found in this variant (46).

An attractive explanation for both of these paradoxes is offered by the recent discovery that transcripts derived from an alternative transcription start site which replaces exon 1 of *Gas* with an alternative coding exon, termed 1A, is imprinted in humans with exclusive *paternal* expression (47). This alternative protein lacks part of the binding site for the β and γ subunits of the G-protein heterotrimer while it contains the receptor-interaction site and may therefore act as a dominant-negative inhibitor of $G_s\alpha$. Liu *et al.* (47) reported maternal-specific methylation of this alternative transcription site, likely the underlying mechanism of its normal maternal silencing. Interestingly, all 13 PHP1B patients studied by these authors displayed biallelic expression of 1A- $G_s\alpha$ and lack of methylation on both alleles (47). In other words, both chromosomes behave as if of paternal origin, likely because of a heritable mutation that disrupts normal imprinting. This gives rise to the prediction that there will be sporadic cases of PHP1B with paternal UPD. Such a case was, indeed, recently described (48). The resulting double dose of 1A- $G_s\alpha$, a putative dominant negative inhibitor of the otherwise normal and normally expressed $G_s\alpha$, offers a plausible explanation for the compromise in function resulting in the PHP1B phenotype, as proposed by Zheng *et al.* (45). Furthermore, parental imprinting of 1A- $G_s\alpha$ may also explain the maternal inheritance of PHP1A. A *GNAS1* mutation on the paternal chromosome, inactivating both $G_s\alpha$ and 1A- $G_s\alpha$, would moderate the effects of the $G_s\alpha$ haploinsufficiency by removing 1A- $G_s\alpha$, its physiologic inhibitor.

Figure 3 illustrates the above reasoning, but the whole picture may be more complex. Upstream of 1A, there are two additional alternative imprinted first exons. The paternally expressed XL, resulting in an extra large form that may play a role similar to 1A, and the maternally expressed NESP55, which splices

out of frame and produces a different protein, not involved in PHP.

Imprinting and type 1 diabetes

Genetic susceptibility plays a major role in the etiology of type 1 (autoimmune) diabetes, and is a polygenic trait. It has been recognized that the risk of inheriting type 1 diabetes from an affected father is 2–3 times higher than from an affected mother (49), but it is far from clear whether this is due to imprinted genes or to effects of the intra-uterine environment of a diabetic pregnancy. Evidence of parent-of-origin effects has been found in some linkage studies, with loci involving unknown genes (50). Of the known type 1 diabetes genes, the class II HLA complex (*IDDM1*) which accounts for almost half of the genetic susceptibility, shows classical Mendelian inheritance with no evidence of imprinting effects. The *IDDM2* locus, on the other hand, which includes the insulin and *IGF2* genes at the 11p15 imprinted region, was reported to be involved in diabetes linkage mostly through paternal inheritance (51). Our results from association studies, using the haplotype relative risk (HRR) method, seem to be consistent with this. We found that the HRR conferred by paternal chromosomes was higher than the corresponding maternal value (52). However, functional evaluation of the two genes whose expression could be modulated by the repeat polymorphism involved in the *IDDM2* locus has shown that insulin, not *IGF2*, is likely the gene involved (53–56). Insulin is normally not imprinted in the pancreas or thymus (57) but there is evidence that it is imprinted in the yolk sack (58), an expression site where the biological role of insulin is unknown but might have effects on insulin-specific autoimmunity later in life. In the thymus, where insulin is expressed at low levels for purposes of immune tolerance (53), one of the two alleles is occasionally silenced (56). Lower levels of insulin expression in the thymus could compromise central immune tolerance to this very important diabetes auto-antigen (59). Indeed, we have recently demonstrated that alleles associated with this silencing strongly predispose to diabetes (56). Whether these alleles silence thymic insulin through a(n) (allele-restricted) parental imprinting mechanism is currently a question under investigation.

Imprinting and human reproduction

There are some questions regarding imprinting effect in assisted reproduction. For example, whether the immature spermatozoa prepared for intracytoplasmic sperm injection have the appropriate molecular imprints in place. This was reported recently to be the same as in controls (60). Abnormal birth weight in cloning from somatic cells has raised the same question about

Figure 3 Proposed explanation for the genetics of pseudohypoparathyroidism type 1A and 1B (according to the scheme proposed by Zheng *et al.* (45)). Both diseases are due to decreased activity of the α subunit of the stimulatory G-protein ($Gs\alpha$), encoded by transcripts of the *GNAS1* gene originating from exon 1. Transcription from this exon is not imprinted in humans but an alternative upstream promoter (exon 1A) is known to be active only on the paternal copy. It produces the closely related protein $Gs\alpha$ -1A which may act as a competitive inhibitor (dominant negative). Shaded boxes indicate exon silenced by imprinting. (1) Normal G-protein function depends on a balance between levels of $Gs\alpha$ (produced from two *GNAS1* copies) and the inhibitor $Gs\alpha$ -1A (from the paternal copy only). (2 and 3) PHP1-1A is known to be due to mutational inactivation of the maternal *GNAS1* copy, which halves $Gs\alpha$ levels and tips the balance in favor of the inhibitor. If the paternal *GNAS1* copy is inactivated by the same mutation the inhibitor is also lost, which explains the absence of phenotype (maternal inheritance of PHP1A). (4) In PHP-1B there is no protein-altering mutation but both copies of $Gs\alpha$ -1A are known to be expressed (loss of imprinting). Excess inhibitor causes the loss of function responsible for 1B. Specificity of the inhibitor function for PTHr is a putative explanation for the absence of resistance to other hormones in 1B.

correct imprinting in fetuses resulting from somatic cell cloning (61). This question was answered in a recent report, which demonstrates parent-of-origin appropriate expression in a large number of imprinted genes in cloned mice, suggesting that the imprint received in the gametes remains unaltered throughout the life of the somatic cell lineages (62).

IGF-II, the *IGF2* peptide product, exerts its mitogenic effects by binding to the IGF-I receptor. It also binds to a specific non-signaling, inactivating receptor, encoded by the *IGF2R* gene. Loss of *IGF2R* function results in fetal macrosomia, presumably due to decreased IGF-II inactivation. The mouse homologue, *Igf2r*, is imprinted but expressed only from the maternal copy (63). This reciprocal imprinting of two genetically unlinked

genes encoding a functional pair of proteins has prompted the hypothesis that imprinting is involved in the regulation of fetal growth (64).

Pursuing this hypothesis, we studied the effect of genetic variation in two known imprinted genes, namely *GRB10* and *IGF2R*, on birth size. We did not find evidence that common variants of either *IGF2R* or *GRB10* are important in intra-uterine growth variability within the normal range (65).

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