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Imprint status of *M6P/IGF2R* and *IGF2* in chickens

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Abstract Genomic imprinting is a method of gene regulation whereby a gene is expressed in a parent-of-origin-dependent fashion; however, it is hypothesized that imprinting should not occur in oviparous taxa such as birds. Therefore, we examined the allelic expression of two genes in the chicken that are reciprocally imprinted in most mammals, *mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R)* and *insulin-like growth factor 2 (IGF2)*. Single nucleotide polymorphisms were identified in these genes, and cDNA was prepared from several tissues of embryos heterozygous for these polymorphisms. Both alleles of *M6P/IGF2R* and *IGF2* were expressed in all tissues examined by RT-PCR. Since the expression of these genes was independent of the parent from which they were inherited, we conclude that neither *M6P/IGF2R* nor *IGF2* are imprinted in the chicken.

Keywords Genomic imprinting · Biallelic expression · Chicken · *IGF2* · *M6P/IGF2R*

Introduction

Genomic imprinting is a phenomenon whereby gene expression is parent-of-origin dependent. It appears to have evolved over 100 million years ago (Killian et al. 2000) and the actively debated genetic conflict hypothesis pur-

ports that it resulted from a parental genetic “tug-of-war” to control maternal-dependent growth of mammalian offspring (Moore and Haig 1991). According to this hypothesis, growth of the offspring should be promoted by paternally expressed genes and limited by those that are maternally expressed. Furthermore, the duration and extent of prenatal growth and postnatal care should be major driving forces in the evolution of genomic imprinting.

The reciprocal imprinting of *mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R)* and *insulin-like growth factor 2 (IGF2)* in mice and the phenotypes of mice deficient in their expression provide support for the genetic conflict hypothesis. Paternally expressed *IGF2* encodes for a critical fetal mitogen, and mice deficient in this growth factor have a dwarf phenotype (DeChiara et al. 1990) while mice over-expressing *Igf2* are larger than normal (Leighton et al. 1995). The *M6P/IGF2R* in viviparous mammals binds IGF2 and phosphomannosyl glycoproteins through independent binding sites and limits the biological activity of IGF2 by targeting the growth factor for degradation in lysosomes (Nolan et al. 1990; Kornfeld 1992; Dahms et al. 1993; Killian et al. 2000). The *M6P/IGF2R* in mice is expressed only from the maternal allele (Barlow et al. 1991), and animals lacking *M6P/IGF2R* accumulate excess IGF2, have somatic overgrowth and die perinatally (Lau et al. 1994; Wang et al. 1994; Ludwig et al. 1996; Nolan and Lawlor 1999).

The genetic conflict hypothesis also predicts that genes that regulate embryonic development would not be imprinted in oviparous taxa because in these animals genes expressed during embryogenesis cannot influence the amount of resources they receive from the mother (Haig and Graham 1991). However, recent work in the chicken examining the imprinted status of *IGF2* has yielded conflicting results. Koski et al. (2000) reported that *IGF2* was expressed monoallelically in some embryos from either paternal or maternal alleles. At the same time O'Neill et al. (2000) reported that *IGF2* was expressed in chick embryos in a strictly biallelic manner. Therefore, we re-examined the allelic expression of chicken *IGF2* and examined expression of chicken *M6P/IGF2R* for the first

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time, and discovered that expression was biallelic for both genes. Our findings are consistent with the predictions of the genetic conflict hypothesis.

Materials and methods

Fertile chicken eggs (White Leghorn or Barred Plymouth Rock strains) were generated at North Carolina State University Department of Poultry Science by artificial insemination of females and were incubated at 37.5°C and 60% relative humidity. Three-day-old embryos were dissected free of membranes, frozen immediately on dry ice and stored at -80°C. Individual tissues (i.e. liver, lungs, heart, skeletal muscle and brain) were removed from 17-day-old embryos and treated similarly. Blood was withdrawn from adult chickens into heparinized syringes and frozen at -80°C.

DNA and total RNA were isolated from whole adult blood or from embryos or tissues using DNA-STAT 120 or RNA-STAT-60 according to the manufacturer's instructions (Tel-test, Friendswood, Tex.). RNA (1–5 µg) was reverse transcribed using Superscript II and oligodT primers, according to the manufacturer's instructions (Life Technologies, Grand Island, N.Y.), and 1/40 of the cDNA obtained was used as a template for PCR amplification. PCR was routinely performed in a 30-µl reaction volume using 1.5 U platinum Taq DNA polymerase (Life Technologies, Baltimore, Md.), 15 pmol primers, 1.5 mM MgCl₂, and 100 µM dNTPs (94°C×15 s, 65°C×5 s, and 72°C×60 s for 35 cycles). Amplification products were purified from agarose gels using GenElute spin columns (Sigma, St Louis, Mo.). They were sequenced manually using radiolabeled terminator cycle sequencing (USB Corporation, Cleveland, Ohio) or with an automated ABI 377 sequencer (PE Biosystems, Foster City, Calif.) using the manufacturer's BigDye terminator cycle sequencing kit.

Polymorphic sites were identified in the chicken *M6PR/IGF2R* using genomic DNA amplified with primers designed to putative exon 48, M6PR48F2 (5'-gggaaggagagaa-agatgtcatggttg) and M6PR48R1 (5'-ggaataccacgtcatcttttttg-aaattgc); the amplified fragment was sequenced using the ABI 377 sequencer (PE Biosystems, Foster City, Calif.). For analysis of allelic expression of the *M6PR/IGF2R*, cDNA was amplified using M6PR48R1 and a primer located in exon 47 of the gene, M6PR47F1 (5'-ctgcagaagaacatcggtgttc); the amplified fragment was sequenced using M6PR48R1. Chicken *M6PR/IGF2R* intron 2 was amplified using primers Int2F (5'-tgtggaagatcaagtgcagtct) and Int2R (5'-ggtgctcactgccttgctgg), designed with reference to sequences in exon 2 and exon 3, respectively (Zhou et al. 1995). The Expand Long Template PCR system (Roche Boehringer Mannheim, Indianapolis, Ind.) was used with buffer 3 and amplification conditions of 94°C×10 s, 61°C×20 s, and 68°C×4 min for 30 cycles. The amplified fragment was sequenced using intron-specific primers (sequences available from the authors by request) by a combination of manual and automatic sequencing, and was analyzed by Grail informatics (<http://compbio.ornl.gov/grail-bin/EmptyGrail-Form>). The complete sequence of intron 2 of the chicken *M6PR/IGF2R* is deposited in GenBank (Accession No. AF305581).

To identify single nucleotide polymorphisms in chicken *IGF2*, a portion of the gene was amplified using primers IGF2F2 (5'-cctccacgacctcaacaag) and IGF2R2 (5'-tcccc-aggagatcacaaatcgag) and sequenced using the ABI 377 sequencer. These primers span an intron and were also used to amplify *IGF2* cDNA. Allelic expression was analyzed by manual sequencing with the primer IGF2R5 (5'-ccgctgcgagctctcttc).

Results and discussion

Imprint status of *IGF2* in chickens

IGF2 has been highly conserved in vertebrate evolution and is imprinted in all investigated eutherian and marsu-

pial mammals with only the paternal allele being expressed (DeChiara et al. 1990; Rainier et al. 1993; Overall et al. 1997; Feil et al. 1998; O'Neill et al. 2000). Tissue-specific biallelic expression of *IGF2* does occur, however, in mouse and human brain (Hu et al. 1995; Pham et al. 1998) and adult human liver (Vu and Hoffman 1994). Allelic expression of *IGF2* has also been analyzed in the chicken, with independent studies generating conflicting results (Koski et al. 2000; O'Neill et al. 2000). Further analysis of *IGF2* expression in this species is therefore required.

We identified a T to C transition in exon 3 of the *IGF2* coding sequence of White Leghorn chickens (position 936 of Accession no. AF218827; position 943 of Accession no. S82962). Amplified genomic DNA prepared from skeletal muscle of 17-day-old chicken embryos (*n*=18) identified six individuals heterozygous for this single nucleotide polymorphism (SNP). RNA and cDNA were then prepared from liver, lungs, heart, skeletal muscle and brain of these informative embryos. Both *IGF2* alleles were found to be expressed in all tissues examined (Fig. 1). We also found biallelic *IGF2* expression in heterozygous 3-day-old embryos (*n*=17, data not shown). These results are consistent with the conclusion of O'Neill et al. (2000) that *IGF2* is not imprinted in the chicken and agree with the prediction of the genetic

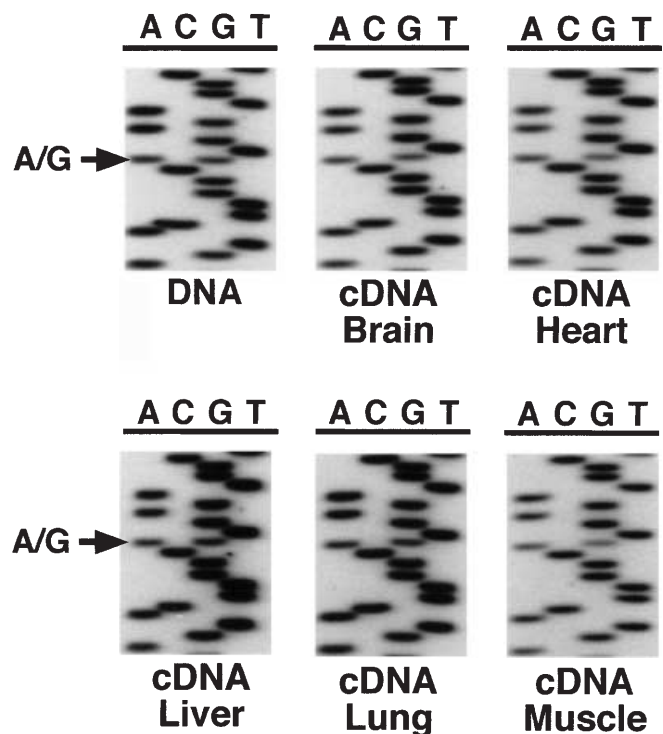


Fig. 1 Allelic expression of the chicken *insulin-like growth factor 2* (*IGF2*). Sequence analysis of genomic DNA amplified from skeletal muscle of a 17-day-old chicken embryo identifies an individual heterozygous for a T to C polymorphism (arrow, sequenced in reverse direction). Similar analysis of cDNA prepared from embryonic tissues of the same embryo demonstrates expression of both alleles. The analysis shown is representative of all informative embryos analyzed

conflict hypothesis that genes that regulate embryonic development would not be imprinted in members of oviparous taxa (Moore and Haig 1991).

Koski et al. (2000) also examined *IGF2* expression in chicken embryos and reported that 33% of chickens have monoallelic expression from either the paternal or maternal allele. They concluded that imprinting at the *IGF2* locus is a polymorphic trait. Thus, there is a significant discrepancy between these results and our observations and those of O'Neill et al. (2000). This discrepancy does not result from *IGF2* imprinting being dependent upon stage of development since we found biallelic *IGF2* expression in 3-day-old embryos, the same age studied by Koski et al. (2000). Each of the heterozygote embryos we examined was based on separate individual matings of an out-bred line of chickens and we did not find any evidence for polymorphic *IGF2* imprinting. There are several possible reasons for the observed difference in imprint status of chicken *IGF2*. Apparent monoallelic expression of a non-imprinted *IGF2* gene could result from the preferential PCR amplification of a single allele when template DNA/cDNA concentration is low. It could also result from genetic anomalies such as somatic mosaicism in the parents or loss of heterozygosity at the *IGF2* locus in embryos. Such anomalies do occur in the chicken and karyotypic mosaicism (Abdel-Hameed and Shoffner 1971) and triploidy (Ohno et al. 1963) can be compatible with viable postnatal life in this species. In addition parthenogenetic chickens can develop to maturity (Sarvela 1973). Rather than inferring the genotype of embryos from those of their parents, embryos should be accurately genotyped to guarantee that they are heterozygous at the investigated polymorphic locus. It may be significant that the embryos analyzed by Koski et al. (2000) were not reported as having been genotyped and that monoallelic expression was limited to the offspring of one specific male. Therefore, the random monoallelic expression described by Koski et al (2000) clearly does not constitute the parent-of-origin-dependent expression that is the hallmark of imprinted genes. Taken together with the results of the current study, the available evidence is most consistent with *IGF2* not being imprinted in chickens.

Imprint status of *M6P/IGF2R* in chickens

In viviparous mammals the *M6P/IGF2R* encodes for a receptor that binds both phosphomannosyl glycoproteins and IGF2 through independent binding sites (Kornfeld 1992; Dahms et al. 1993; Yandell et al. 1999). The platypus, chicken and *Xenopus* receptors lack the ability to bind IGF2, however (Clairmont and Czech 1989; Zhou et al. 1995; Killian et al. 2000). It has been proposed that IGF2 binding by *M6P/IGF2R* evolved in response to high levels of paternally produced IGF2 (Haig and Graham 1991), and that natural selection would have favored inactivating the paternal allele once the receptor acquired its ability to bind IGF2. According to this hy-

pothesis, *M6P/IGF2R* is predicted to be biallelically expressed in the chicken as it is in monotremes (Killian et al. 2000).

Allelic expression of the chicken *M6P/IGF2R* was determined with a G to A transition SNP at nucleotide position 8,637 in the 3'UTR (Accession No. U35037). Embryos heterozygous for the polymorphism were identified by sequencing of genomic DNA isolated from the skeletal muscle of 24 embryos. RNA was then extracted from several tissues of these informative embryos (5 White Leghorn, and 2 Barred Plymouth Rock). RT-PCR was used to amplify the region containing the polymorphism, and the amplified fragment was sequenced to identify expressed alleles. In all heterozygous embryos examined and in all tissues examined (i.e., liver, lungs, heart, skeletal muscle and brain) both alleles of *M6P/IGF2R* were expressed (Fig. 2). We also showed biallelic expression of *M6P/IGF2R* in blood of heterozygous adult chickens ($n=21$, data not shown). Therefore, we conclude that the chicken *M6P/IGF2R* is not imprinted. Thus, the *M6P/IGF2R*'s acquisition of IGF2 binding, and its resultant ability to limit embryonic growth by degrading IGF2, appears to be closely related to the evolution of imprinting at the *M6P/IGF2R* locus.

The molecular mechanism by which the imprinted status of the *M6P/IGF2R* is achieved and maintained

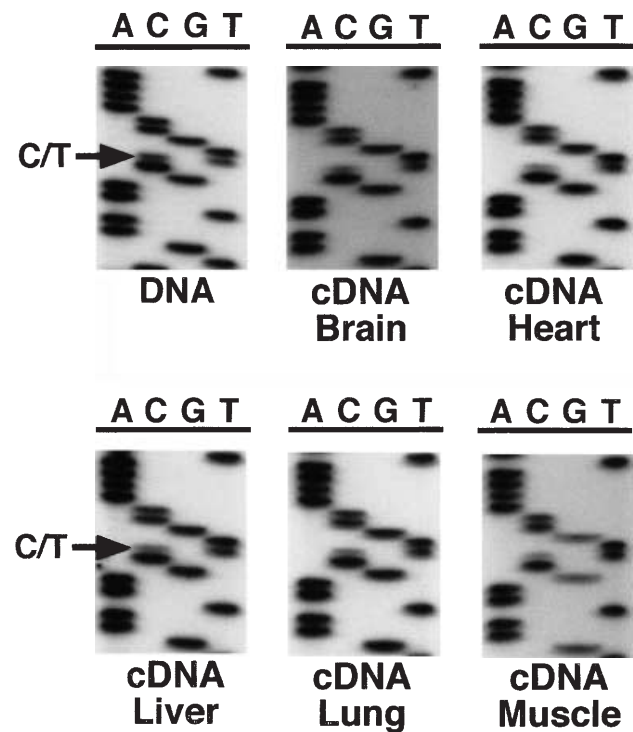


Fig 2 Allelic expression of the chicken mannose 6-phosphate/inulin-like growth factor 2 receptor (*M6P/IGF2R*). Sequence analysis of genomic DNA amplified from skeletal muscle of a 17-day-old chicken embryo identifies an individual heterozygous for a G to A polymorphism (arrow, sequenced in reverse direction). Similar analysis of cDNA prepared from embryonic tissues of the same embryo demonstrates expression of both alleles. The analysis shown is representative of all informative embryos analyzed

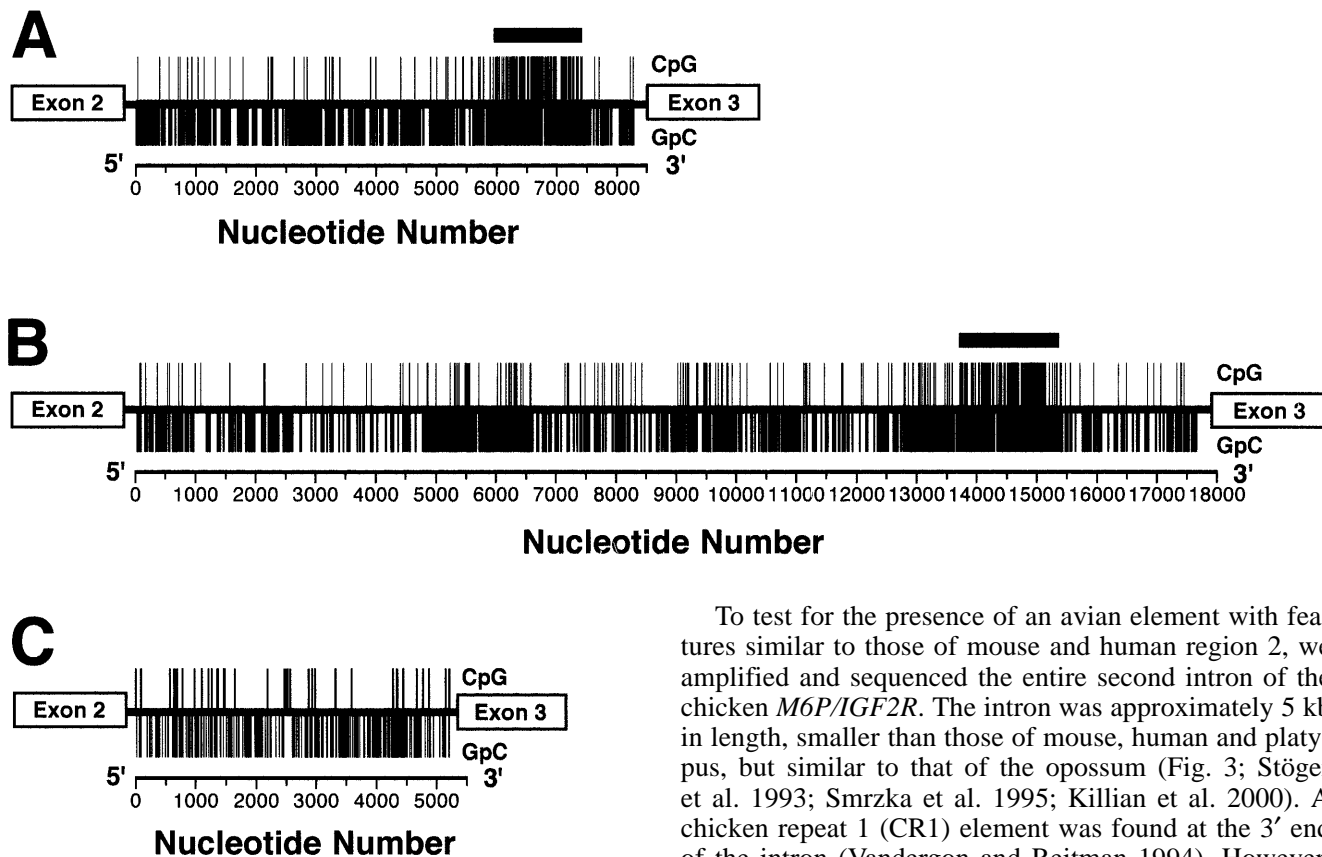


Fig. 3A–C Distribution of CpG and GpC dinucleotides in mouse, human and chicken *M6P/IGF2R* intron 2. **A** The positioning of CpG and GpC sites in the mouse *M6P/IGF2R* intron 2 reveals a 1.5-kb CpG island (Wutz et al. 1997) indicated by a solid horizontal bar. **B** The human *M6P/IGF2R* intron 2 also contains a CpG island (Smrzka et al. 1995). **C** Chicken intron 2 lacks a CpG island

is currently unclear. Monoallelic expression of the *M6P/IGF2R* in mice is purported to depend on differential methylation of a CpG island in the second intron (region 2; Stöger et al. 1993). Hybridization studies initially indicated that region 2 was present in primates, mice and birds (Stöger et al. 1993). Although a homologous element was reported in humans (Smrzka et al. 1995), the human *M6P/IGF2R* is expressed from both alleles (Kalscheuer et al. 1993; Ogawa et al. 1993). The relationship of mouse region 2 to that of the human is unclear, as there is no significant sequence homology between them. Nevertheless, they share a high level of organizational similarity with human region 2, also in intron 2, containing a CpG island with maternal-specific methylation. In contrast to initial indications that region 2 was widely conserved (Stöger et al. 1993; Smrzka et al. 1995), the platypus and opossum *M6P/IGF2R* do not have a region 2-like element (Killian et al. 2000). Since the opossum *M6P/IGF2R* is expressed only from the maternally inherited allele, either the mechanism of *M6P/IGF2R* imprinting in the opossum differs from that of the mouse, or region 2 may not be the primary regulator of imprinting at this locus.

To test for the presence of an avian element with features similar to those of mouse and human region 2, we amplified and sequenced the entire second intron of the chicken *M6P/IGF2R*. The intron was approximately 5 kb in length, smaller than those of mouse, human and platypus, but similar to that of the opossum (Fig. 3; Stöger et al. 1993; Smrzka et al. 1995; Killian et al. 2000). A chicken repeat 1 (CR1) element was found at the 3' end of the intron (Vandergon and Reitman 1994). However, the intron had no features characteristic of a CpG island. It did not have a high G+C content or any clustering of CpG dinucleotides (Fig. 3), and G/C boxes, a regular occurrence in CpG islands (Gardiner-Garden and Frommer 1987), are entirely missing from the sequence. Thus, although region 2 was reported to be present in birds (Stöger et al. 1993), our results demonstrate that chicken *M6P/IGF2R* intron 2 does not contain a CpG island analogous to those in mice and humans (Stöger et al. 1993; Smrzka et al. 1995). The absence of region 2 in the chicken, platypus and opossum, together with the lack of sequence homology between mouse region 2 and human region 2, suggest that such elements may have evolved independently in several mammalian orders. Analysis of the *M6P/IGF2R* in additional marsupial and eutherian mammals is necessary to clarify the involvement of region 2 in regulating imprinting of the *M6P/IGF2R*.

In conclusion, our demonstration that chicken *IGF2* and *M6P/IGF2R* are both expressed biallelically is consistent with the predictions of the genetic conflict hypothesis of imprint evolution (Moore and Haig 1991). Our data also support the idea that the reciprocal imprinting of these genes in viviparous mammals is closely related to their opposing effects on embryonic growth.

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References

- Abdel-Hameed F, Shoffner RN (1971) Intersexes and sex determination in chickens. *Science* 172:962–964
- Barlow DP, Stöger R, Herrmann BG, Saito K, Schweifer N (1991) The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature* 349:84–87
- Clairmont KB, Czech MP (1989) Chicken and *Xenopus* mannose 6-phosphate receptors fail to bind insulin-like growth factor II. *J Biol Chem* 264:16390–16392
- Dahms NM, Brzycki-Wessell MA, Ramanujam KS, Seetharam B (1993) Characterization of mannose 6-phosphate receptors (MPRs) from opossum liver: opossum cation-independent MPR binds insulin-like growth factor-II. *Endocrinology* 133:440–446
- DeChiara TM, Efstratiadis A, Robertson EJ (1990) A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* 345:78–80
- Feil R, Khosla S, Cappai P, Loi P (1998) Genomic imprinting in ruminants: allele-specific gene expression in parthenogenetic sheep. *Mamm Genome* 9:831–834
- Gardiner-Garden M, Frommer M (1987) CpG islands in vertebrate genomes. *J Mol Biol* 196:261–282
- Haig D, Graham C (1991) Genomic imprinting and the strange case of the insulin-like growth factor II receptor. *Cell* 64:1045–1046
- Hu JF, Vu TH, Hoffman AR (1995) Differential biallelic activation of three insulin-like growth factor II promoters in the mouse central nervous system. *Mol Endocrinol* 9:628–636
- Kalscheuer VM, Mariman EC, Schepens MT, Rehder H, Ropers H-H (1993) The insulin-like growth factor type-2 receptor gene is imprinted in the mouse but not in humans. *Nat Genet* 5:74–78
- Killian JK, Byrd JC, Jirtle JV, Munday BL, Stoskopf MK, MacDonald RG, Jirtle RL (2000) *M6P/IGF2R* imprinting evolution in mammals. *Mol Cell* 5:707–716
- Kornfeld S (1992) Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu Rev Biochem* 61:307–330
- Koski LB, Sasaki E, Roberts RD, Gibson J, Etches RJ (2000) Monoallelic transcription of the insulin-like growth factor-II gene (*Igf2*) in chick embryos. *Mol Reprod Dev* 56:345–352
- Lau MM, Stewart CE, Liu Z, Bhatt H, Rotwein P, Stewart CL (1994) Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes Dev* 8:2953–2963
- Leighton PA, Ingram RS, Eggenschwiler J, Efstratiadis A, Tilghman SM (1995) Disruption of imprinting caused by deletion of the *H19* gene region in mice. *Nature* 375:34–39
- Ludwig T, Eggenschwiler J, Fisher P, D'Ercole AJ, Davenport ML, Efstratiadis A (1996) Mouse mutants lacking the type 2 IGF receptor (*IGF2R*) are rescued from perinatal lethality in *Igf2* and *Igf1r* null backgrounds. *Dev Biol* 177:517–535
- Moore T, Haig D (1991) Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet* 7:45–49
- Nolan CM, Lawlor MA (1999) Variable accumulation of insulin-like growth factor II in mouse tissues deficient in insulin-like growth factor II receptor. *Int J Biochem Cell Biol* 31:1421–1433
- Nolan CM, Kyle JW, Watanabe H, Sly WS (1990) Binding of insulin-like growth factor II (IGF-II) by human cation-independent mannose 6-phosphate receptor/IGF-II receptor expressed in receptor-deficient mouse L cells. *Cell Regul* 1:197–213
- Ogawa O, McNoe LA, Eccles MR, Morison IM, Reeve AE (1993) Human insulin-like growth factor type I and type II receptors are not imprinted. *Hum Mol Genet* 2:2163–2165
- Ohno S, Kittrell WA, Christian LC, Stenius C, Witt GA (1963) An adult triploid chicken (*Gallus domesticus*) with a left ovotestis. *Cytogenetics* 2:42–49
- O'Neill MJ, Ingram RS, Vrana PB, Tilghman SM (2000) Allelic expression of *IGF2* in marsupials and birds. *Dev Genes Evol* 210:18–20
- Overall M, Bakker M, Spencer J, Parker N, Smith P, Dziadek M (1997) Genomic imprinting in the rat: linkage of *Igf2* and *H19* genes and opposite parental allele-specific expression during embryogenesis. *Genomics* 45:416–420
- Pham NV, Nguyen MT, Hu JF, Vu TH, Hoffman AR (1998) Dissociation of *IGF2* and *H19* imprinting in human brain. *Brain Res* 810:1–8
- Rainier S, Johnson LA, Dobry CJ, Ping AJ, Grundy PE, Feinberg AP (1993) Relaxation of imprinted genes in human cancer. *Nature* 362:747–749
- Sarvela P (1973) Adult parthenogenetic chickens. *Nature* 243:171
- Smrzka OW, Fae I, Stöger R, Kruzbauer R, Fischer GF, Henn T, Weith A, Barlow DP (1995) Conservation of a maternal-specific methylation signal at the human IGF2R locus. *Hum Mol Genet* 4:1945–1952
- Stöger R, Kubicka P, Liu CG, Kafri T, Razin A, Cedar H, Barlow DP (1993) Maternal-specific methylation of the imprinted mouse *Igf2r* locus identifies the expressed locus as carrying the imprinting signal. *Cell* 73:61–71
- Vandergon TL, Reitman M (1994) Evolution of chicken repeat 1 (CR1) elements: evidence for ancient subfamilies and multiple progenitors. *Mol Biol Evol* 11:886–898
- Vu TH, Hoffman AR (1994) Promoter-specific imprinting of the human insulin-like growth factor-II gene. *Nature* 371:714–717
- Wang ZQ, Fung MR, Barlow DP, Wagner EF (1994) Regulation of embryonic growth and lysosomal targeting by the imprinted *Igf2/Mpr* gene. *Nature* 372:464–467
- Wutz A, Smrzka OW, Schweifer N, Schellander K, Wagner EF, Barlow DP (1997) Imprinted expression of the *Igf2r* gene depends on an intronic CpG island. *Nature* 389:745–749
- Yandell CA, Dunbar AJ, Wheldrake JF, Upton Z (1999) The kangaroo cation-independent mannose 6-phosphate receptor binds insulin-like growth factor II with low affinity. *J Biol Chem* 274:27076–27082
- Zhou M, Ma Z, Sly WS (1995) Cloning and expression of the cDNA of chicken cation-independent mannose-6-phosphate receptor. *Proc Natl Acad Sci USA* 92:9762–9766