The Loss of Adipokine Genes in the Chicken Genome and Implications for Insulin Metabolism

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Abstract

Gene loss is one of the main drivers in the evolution of genomes and species. The demonstration that a gene has been lost by pseudogenization is truly complete when one finds the pseudogene in the orthologous genomic region with respect to active genes in other species. In some cases, the identification of such orthologous loci is not possible because of chromosomal rearrangements or if the gene of interest has not yet been sequenced. This question is particularly important in the case of birds because the genomes of avian species possess only about 15,000 predicted genes, in comparison with 20,000 in mammals. Yet, gene loss raises the question of which functions are affected by the changes in gene counts. We describe a systematic approach that makes it possible to demonstrate gene loss in the chicken genome even if a pseudogene has not been found. By using phylogenetic and synteny analysis in vertebrates, genome-wide comparisons between the chicken genome and expressed sequence tags, RNAseq data analysis, statistical analysis of the chicken genome, and radiation hybrid mapping, we show that resistin, *TNFα*, and *PAI-1* (*SERPINE1*), three genes encoding adipokines inhibiting insulin sensitivity, have been lost in chicken and zebra finch genomes. Moreover, omentin, a gene encoding an adipokine that enhances insulin sensitivity, has also been lost in the chicken genome. Overall, only one adipokine inhibiting insulin sensitivity and five adipokines enhancing insulin sensitivity are still present in the chicken genome. These genetic differences between mammals and chicken, given the functions of the genes in mammals, would have dramatic consequences on chicken endocrinology, leading to novel equilibriums especially in the regulation of energy metabolism, insulin sensitivity, as well as appetite and reproduction.

Key words: **chicken, adipokines, insulin resistance.**

Introduction

Notable differences exist in the regulation of energy metabolism between chicken and mammals. In mammals, and in particular human, increased body mass index is frequently associated with a decrease in insulin sensitivity, leading to insulin and type II diabetes (Bray and Bellanger 2006; Hajer et al. 2008), and altered plasma profiles at higher leptin and lower adiponectin levels (Finucane et al. 2009; Barazzoni et al. 2012). Insulin circulates at comparable levels in chickens and mammals, but chickens are less sensitive to insulin action (Simon 1989) and glucose circulates in birds at much higher level than in mammals (2 g/l vs. 1 g/l, respectively). This refractoriness is particularly evident in adipose tissues (Dupont et al. 2012). In an experimental selection for fatness in chickens, no insulin resistance was observed, but only an imbalance in glucose versus insulin, with lower glucose and higher insulin levels (Touchburn et al. 1981; Simon and Leclercq 1982) and an increased activation of the insulin cascade in the liver (the major site of lipogenesis in chicken) of fat chickens (Dupont et al. 1999). Although insulin resistance can

Article

be induced by corticosterone treatment of chickens (Taouis et al. 1993, 1996), to our knowledge, there is no report of spontaneous insulin resistance or type II diabetes in this species. In mammals, insulin sensitivity is notably regulated by the action of adipokines, hormones secreted by adipose tissue, which are able to modulate the activity of the insulin receptor (Steppan et al. 2005; Mao et al. 2006; Wang et al. 2009; Jacques et al. 2012). Among the subset of analyzed adipokines, seven are known to enhance insulin sensitivity (leptin, omentin, visfatin, adiponectin, vaspin, chemerin, and apelin), whereas four others decrease insulin sensitivity (*IL6*, *TNFα*, *PAI-1* [*SERPINE1*], and *resistin*) (Fantuzzi 2005; Alessi and Juhan-Vague 2006; Chang et al. 2011; Kwon and Pessin 2013).

Here, we analyze the chicken genome in an attempt to prove the presence or absence of some of the genes important for the above mentioned functions. It is, however, difficult to demonstrate that a gene has been lost from a genome. Ideally, the presence of an inactivated copy of a gene, which is unable to lead to a functional protein because of the presence of premature stop codons or loss of open reading frame, and which is located in the orthologous genomic region with respect to active genes in other species, is considered sufficient evidence of gene loss. However, in some cases, the identification of such orthologous loci is difficult because chromosomal rearrangements tend to reorganize genomes and erase the signals of conservation in the locus of interest, particularly between species separated by long evolutionary distances. Alternatively, the gene of interest may be located in a genomic region that is not yet sequenced. Sequence coverage for the autosome chromosomes in chicken was estimated to be 98% and gene coverage 90–95% (Burt 2006). About 5–10% of the protein-coding genes are still missing from the Ensembl chicken gene set, with a very poor coverage of microchromosomes, particularly GGA16 and GGAW (Hiller et al. 2004; Griffin and Burt 2014). The genomes of avian species possess only about 15,000 predicted genes, whereas in mammals, a genome typically contains more than 20,000 genes (Hughes and Friedman 2008). If this reflects the true state of the chicken genome and is not due to sequencing artifacts (because several microchromosomes have not yet been sequenced in birds), this raises the question of which functions were affected by the changes in gene counts, either through gene family amplification in mammals or gene losses in birds.

The difficulty in concluding about the gene loss is illustrated by the leptin gene. Since the first publication on the cloning of a chicken leptin cDNA (Taouis et al. 1998), several studies have described the in vitro or in vivo regulation of its expression, as well as its biological roles in this species (Taouis et al. 2001; Dridi et al. 2005). However, these results led to a controversy that questioned whether the gene existed in the chicken genome at all (Sharp et al. 2008; Simon et al. 2009). We have previously brought forward several strong arguments for the loss of the leptin gene in the chicken genome resulting from the deletion of a 1-Mb genomic region (Pitel et al. 2010), without detectable presence of a pseudogene.

By using a phylogenetic and synteny analyses, genome-wide comparisons between the chicken genome and expressed sequence tag (ESTs), RNAseq data analysis, statistical analysis of the chicken genome, and radiation hybrid (RH) mapping, we show in this study that three genes encoding adipokines inhibiting insulin sensitivity (resistin, *TNFα*, and *PAI-1*) and one gene encoding an adipokine that enhances insulin sensitivity (omentin) have been lost in the chicken and zebra finch genomes.

Results

Identification of Adipokine-Encoding Genes Putatively Lost in Bird Genomes

We chose to study the evolution of 11 genes known to encode adipokines in mammals: Leptin, *IL6*, resistin, *TNFα*, *PAI-1*, adiponectin, omentin, apelin, visfatin, vaspin, and chemerin. By systematically analyzing phylogenetic trees of these genes in the Ensembl database, we found that five were present in fish and mammalian genomes but absent in birds: Leptin as shown previously (Pitel et al. 2010), resistin, *TNFα*, *PAI-1*, and omentin (fig. 1). In addition, leptin and resistin are also lost in all sauropsids. We thus systematically looked for their pseudogenes in syntenic regions and did not find any.

Characterization of Deleted Genomic Regions in Chicken—Loss of Resistin, TNFα, PAI-1, and Omentin Genes

We only describe here the approach for one locus, centered on the resistin gene, because we have applied the same procedure for the four loci (and similarly to the previous analysis of the leptin gene (Pitel et al. 2010). The human resistin gene is localized on chromosome 19, in a synteny block well conserved within mammals. Genes that are part of this human block (from 7.585 to 7.987 Mb) that contains the resistin gene is not annotated in the current chicken sequence assembly. More precisely, this block contains 26 genes, of which 15 do

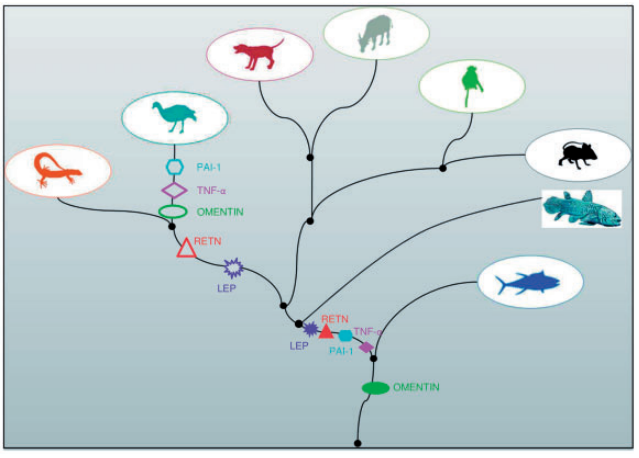

FIG. 1. The birth and loss of four adipokine genes studied here, as well as leptin (previously described by Pitel et al. 2010). Different genes are represented by different symbols in different colors. The birth of a gene is depicted by a solid symbol, and the loss of a gene is depicted by a hollow symbol.

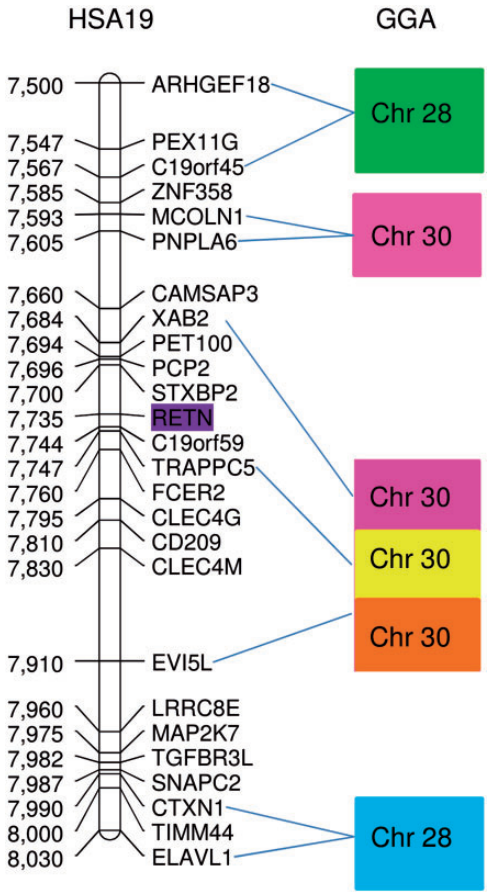

FIG. 2. Comparative map of the resistin genomic region. Conserved synteny between human chromosome 19 (HSA19) and the chicken (GGA) genome, obtained through RH mapping. The map is given in Mb, from assembly version GRCh37/hg19. The color corresponds to the gene fragments in supplementary table S1, Supplementary Material online.

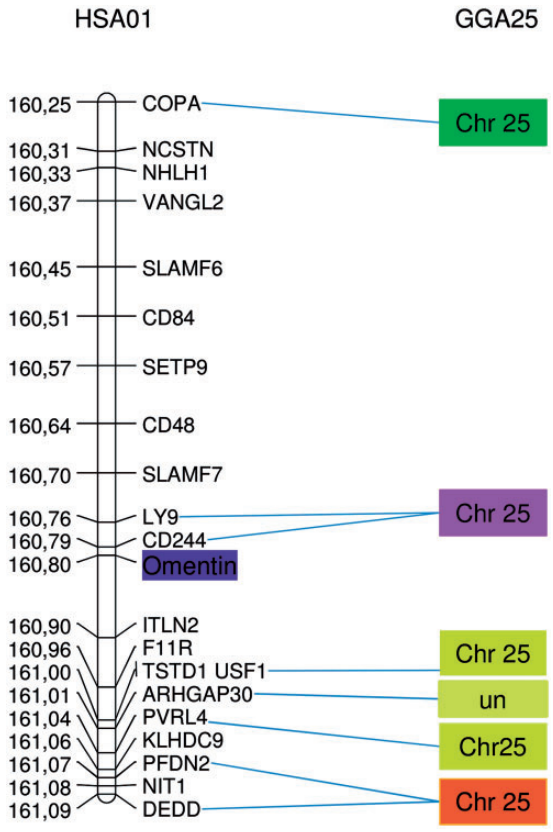

FIG. 3. Comparative map of the omentin genomic region. Conserved synteny between human chromosome 1 (HSA01) and the chicken (GGA) genome, obtained through RH mapping. The map is given in Mb, from assembly version GRCh37/hg19. The color corresponds to the gene fragments in supplementary table S1, Supplementary Material online. un, unlinked to *USF1* or *PVRL4*.

not match by reciprocal tBLASTn analysis on the chicken genome nor on chicken ESTs (supplementary table S1, Supplementary Material online). The four genes, *MCOLN1*, *PNPLA6*, *TRAPPC5*, and *EVI5L*, match with ESTs by reciprocal tBLASTn analysis and are annotated in the chicken genome but in unplaced contigs, whereas *XAB2* matched only chicken ESTs. To confirm that these five genes are not located near the chicken orthologs of gene at boundaries of this block in the human genome (*ARHGEF18*, *PEX11G*, and *C19Orf45* on the telomeric side and *CTXN1*, *TIMM44*, and *ELAVL1* on the centromeric side), experiments on RH panels were performed (fig. 2). On one side of the locus, *ARHGEF18* and *C19Orf45* are confirmed to be linked to markers on chicken chromosome 28. *CTXN1* and *ELAVL1* map near MNT-346, also a GGA28 (*Gallus gallus* chromosome 28) marker on the other side of the locus. Between these two blocks, we used the available sequences to design primer pairs amplifying five other genes (*MCOLN1*, *PNPLA6*, *XAB2*, *TRAPPC5*, and *EVI5L*). All the genes map to chicken chromosome 30 (see ChickRH web server: chickrh.toulouse.inra.fr), in two groups: *MCOLN1* and *PNPLA6*, and *XAB2*, *TRAPPC5*, and *EVI5L* (fig. 2). We thus show here that orthologs of the genes from the resistin locus on HSA chromosome 19 are not in a single conserved locus in the chicken genome, because the boundaries of the blocks are on chicken chromosome 28, whereas the rest of the block is separated into at least three independent blocks in the chicken genome on other chromosomes.

As shown in supplementary table S1, Supplementary Material online, similar genomic rearrangements of this region also seem to have occurred in the zebra finch genome, except for *EVI5L*, residing on zebra finch chromosome 8. Interestingly in lizard, the missing genes are less numerous.

As for the resistin example, we performed the same RH mapping experiments for markers located near the three other studied genes (*TNFα*, *PAI-1*, and omentin) in the human genome. In each case, the result confirmed the absence of synteny between the chicken and the human genomes in the region of interest (figs. 3–5).

Overall, our results show that the chicken genome have lost 5 out of 11 adipokines, omentin, *TNFα*, *PAI-1*, resistin (supplementary table S1, Supplementary Material online), and leptin (Pitel et al. 2010). Interestingly, the genes encoding the receptors of leptin (leptin receptor, *LEPR*: NP_989654.1), *TNFα* (tumor necrosis factor receptor type 1-associated DEATH domain protein, *TRADD*: XP_414067.1), and resistin

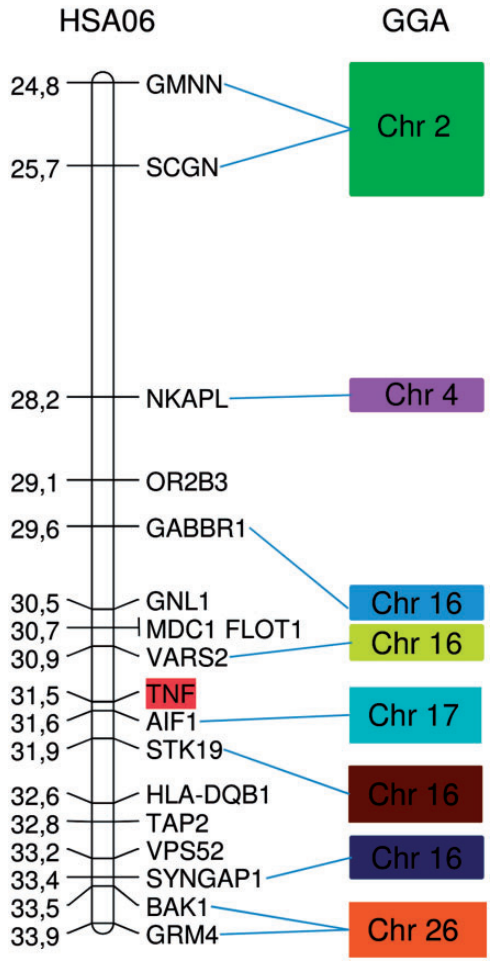

Fig. 4. Comparative map of the *TNF-α* genomic region. Conserved synteny between human chromosome 6 (HSA06) and the chicken genome, obtained through RH mapping. The map is given in Mb, from assembly version GRCh37/hg19. The color corresponds to the gene fragments in supplementary table S1, Supplementary Material online.

(adenylate cyclase-associated protein 1, *CAP1*: XP_003642632.1) are present in the chicken genome.

Characterization of Adipose Tissue RNA-Seq Data—No Transcript for Resistin, TNFα, PAI-1, and Omentin

We identified 14,385 genes in abdominal chicken adipose tissue with at least ten reads on average per bird among them 12,341 referenced genes, and 2,044 potentially new genes not yet annotated in the Ensembl reference genome. Among the referenced genes expressed, we observed visfatin and adiponectin with 1,204 and 4,247 reads per bird respectively, showing a relatively high level of expression in the adipose tissue of the 14-week-old chickens. In contrast, none of the 2,044 new genes expressed in the chicken adipose tissue matched by reciprocal tBLASTn analysis on human or mouse resistin, *TNFα*, *PAI*-1, or omentin gene sequences, further supporting the hypothesis that these genes are not present in the chicken genome. The de novo assembly of the RNA-Seq reads produced 57,875 contigs (total length: 85, 084,733 bp, N50:

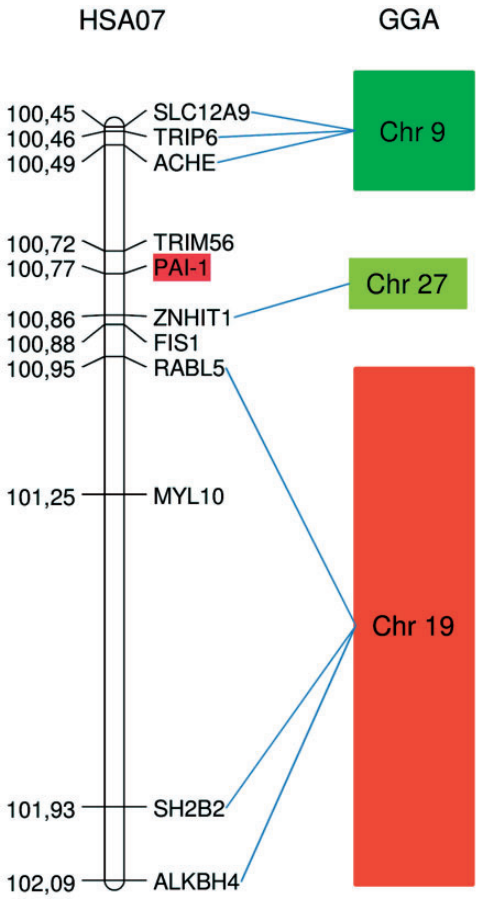

Fig. 5. Comparative map of the *PAI-1* (SERPINE1) genomic region. Conserved synteny between human chromosome 7 (HSA07) and the chicken (GGA) genome, obtained through RH mapping. The map is given in Mb, from assembly version GRCh37/hg19. The color corresponds to the gene fragments in supplementary table S1, Supplementary Material online.

3,550 bp). The bidirectional comparison of these contigs with chicken RefSeq transcripts shows that no transcripts corresponding to resistin, *TNFα*, *PAI-1*, and omentin were found with our RNA-Seq data after de novo assembly. The RNA-Seq results reinforce the conclusion that the genes encoding resistin, *TNFα*, *PAI*-1, and omentin were lost the chicken genome.

Chromosomal Rearrangements of the Region Containing Resistin Gene in Mammals Compared with Sauropsids

We took advantage of the Genomicus genome browser (Muffato et al. 2010) to provide a better understanding of the evolution of the organization of the genomic region that contains the resistin gene in mammals in comparison with chicken, zebra finch, and lizard. Thus, taking the human genome as a reference, we have focused on the 7.5–8 Mb region depicted in supplementary table S1, Supplementary Material online. In supplementary figure S1, Supplementary Material online, we propose a model of chromosomal evolution, with this region being divided into seven fragments defined by their gene content and gene order. The position of the corresponding genes in human, dog, chicken, zebra finch,

and lizard is presented in supplementary table S1, Supplementary Material online. The order of the seven fragments is well conserved from the ancestor of amniota to human and dog (mammals in general) and is subjected to dramatic rearrangements in lizard, with conserved fragments 2, 4, 5, and 6 (with a paracentric inversion of fragment 4 and an insertion within the fragment 2). In contrast, chicken and zebra finch only conserve fragments 1 and 7 (with paracentric inversion of fragment 1), one or two other fragments being inserted in between.

Discussion

In this study, we propose a methodological approach to demonstrate that a gene is lost by deletion of a genomic region. This approach combines reciprocal tBLASTn analysis of mammalian genes on the chicken genome and ESTs, with RH mapping of genes which are detected by reciprocal tBLASTn on the genome, but are not localized on a chromosome and ESTs. Among human genes present in the human genomic region "syntenic" with deleted chicken genomic locus, some have their chicken ortholog localized on another chromosome (or have not yet been localized) suggesting that several of these chicken genes have not been lost but rather have been "dispersed" in chicken genome. Concerning the resistin locus, there is no synteny conservation between human chromosome 19 and the chicken genome at the chromosome level, but there are several small synteny groups, mainly localized on chicken microchromosomes or unknown linkage groups, as previously observed (Morisson et al. 2007). The omentin gene maps to human chromosome 1 in a region with a high number of intrachromosomal rearrangements on chicken chromosome 25 (Douaud et al. 2008). Similarly, the *TNFα* gene maps to human chromosome 6, bearing the major histocompatibility complex. The homologous region in chicken is localized on GGA16, a microchromosome with very few sequence data when compared with other sequenced microchromosomes, due to compositional reasons, and already known showing many rearrangements compared with the human genome (Solinhac et al. 2010).

Our results also suggest that most but not all of the genes studied here, that have been lost in the chicken genome, have also been lost in zebra finch. This leads to the hypothesis that the loss of these genes may have occurred early in the evolution of birds. However, recent studies have reported presence of a leptin gene in several wild birds, including the zebra finch (Friedman-Einat et al. 2014; Huang et al. 2014; Prokop et al. 2014). Peregrine falcon genome sequence alignment showed high synteny alignment with human, mouse, and another falcon species (Friedman-Einat et al. 2014; Prokop et al. 2014). Friedman-Einat et al. (2014) also used the newly identified *LEP* sequences in the dove, Tibetan ground tit, zebra finch, and falcons as a query sequences for identification of *LEP* gene in the chicken, turkey, and duck genomes. However, no significant sequence similarity to *LEP* was found, which gives strong support that the *LEP* gene is missing from the chicken genome and in other domesticated poultry species. The presence of *LEP* gene in wild birds (such as falcon, rock dove, and zebra finch) (Friedman-Einat et al. 2014; Prokop et al. 2014; Huang et al. 2014) but not in domesticated birds (Friedman-Einat et al. 2014) suggests that *LEP* gene has been lost in the process of domestication.

Despite the high degree of interchromosomal conservation and conserved synteny between the human and chicken genomes (Burt et al. 1999; Groenen et al. 2000), intrachromosomal rearrangements are also common and associated with high recombination rates (Crooijmans et al. 2001; Volker et al. 2010). The rearrangements that occurred in the lizard genome were either quite similar, with fewer deleted genes than for birds (*TNFα* and *PAI-1*), or markedly different (resistin), suggesting that there is a species-specific chromosomal rearrangement and loss of genes during the evolution of sauropsids. The four loci studied are well conserved in mammals, at least in human and dog. Overall, our results confirm that the genome of sauropsids has undergone profound structural rearrangements after the divergence of amniota species.

In contrast, the genes encoding the receptors of leptin, *TNFα*, and resistin are present in the chicken genome, increasing the list of orphan receptors. This result constitutes a novel example of a "break" between ligands and receptors during the course of species evolution (Markov et al. 2008). The leptin and *TNFα* receptors are functional, because heterologous ligands have biological effects on chicken in vitro and/or in vivo (Adachi et al. 2008; Takimoto et al. 2008). In vivo, one can hypothesize that the loss of some of these adipokines would be functionally compensated by paralogs. This is possible for adipokines that belong to families such as *TNFα*, which has two paralogs in the chicken genome, *FasL* and *TNFS15*. Lipopolysaccharide-induced *TNFα*, known to induce the expression of *TNFα* in mammals, is also able to induce the expression of chicken *TNFS15* in vitro (Hong et al. 2006). In the mouse, targeted inactivation of *TNFα* and its two mammalian close paralogs Lymphotoxin-a and -b (*Lta* and *Ltb*, absent in the chicken genome) showed that each paralog seems to play specific functions and has largely non redundant functions in vivo (Kuprash et al. 2002). Functional compensation may also be possible for *PAI-1* that has two paralogs in the chicken genome (*SERPINE2* and *SERPINE3*). In particular, it has been shown that *PAI-1*-deficient mice and humans have no spontaneous phenotype, likely because other inhibitors of the uPA/plasmin system mask the effect of *PAI-1* defect. In contrast to *TNFα* and *PAI-1*, omentin and resistin genes have one paralog in mammals (omentin-2 and resistin like, respectively) but none in chickens. So the consequence of their loss in the chicken genome remains to be elucidated.

Compared with mammals, chickens have much higher plasma glucose levels (2 g/l), despite the presence of hyperactive insulin (Hazelwood et al. 1968; Simon 1989). They are, however, less sensitive to the action of insulin relative to mammals. Nevertheless, spontaneous insulin resistance is not observed in this species. In mammals, insulin resistance and obesity are more apparent in adults. However, insulin resistance in adult, sexually mature chickens is not well known. Most of the studies of insulin sensitivity in chickens were performed on individuals after hatching up to 17 weeks of age (Simon and Leclercq 1982; Tokushima et al. 2003;

Dupont et al. 2008, 2009). However, Chou and Scanes (1988) showed that young male chickens were more sensitive to insulin than the adult male chickens of the same strains (White Leghorn). They also showed that there was no difference between young male chicks of broiler (meat-type breed) and White Leghorn (egg-type breed) strains (Chou and Scanes 1988).

Our results show that chicken genome have lost 5 out of 11 adipokines evaluated here, leptin (Pitel et al. 2010), omentin, *TNFα*, *PAI-1*, and resistin. Overall, out of 11 adipokines studied here, only 1 adipokine inhibiting insulin action, *IL-6*, and 5 adipokines enhancing insulin sensitivity, apelin, visfatin, vaspin, chemerin, and adiponectin, are still present in the chicken genome. This could lead to a profound change of endocrinological equilibrium, more permissive to insulin action in birds in comparison with mammals. Because the receptors of these lost adipokines are present in the chicken genome, it would be particularly interesting to treat in vivo chicken selected for fatness with one or several of human adipokines that reduce the sensitivity to insulin (*TNFα* and resistin) and to look for a possible effect on insulin resistance. It would also be interesting to inhibit one or several of the corresponding proteins in humans, which suffer from type II diabetes, and to look for a possible increase in insulin sensitivity and a reversion of the disease.

The loss of these adipokines may also have significant consequences on the regulation of reproduction. In mammals, adipokines appear to regulate the functions of the reproductive axis (Campos et al. 2008; Hausman et al. 2012; Landry et al. 2013; Dupont et al. 2014). In particular, leptin modulates mRNA expression of Kiss-1 (Tena-Sempere 2006; Luque et al. 2007; Ahn et al. 2012), which in turn stimulate GnRH and gonadotropin secretion (Caraty et al. 2012; Pinilla et al. 2012). In contrast to mammals, the female reproductive physiology of birds is characterized by the preovulatory release of LH, which relies on progesterone secreted by preovulatory follicles for the initiation of a GnRH peak prior to ovulation (Johnson and Leone 1985; Etches and Petitte 1990). Birds are the only species that have lost Kysspeptins (Um et al. 2010; Kanda and Oka 2013), and other mechanism, such as positive feedback by progesterone and not estrogens to induce the preovulatory surge of GnRH, seems to be central for the regulation of reproduction. Moreover, it is possible that birds maintain a tighter regulation of GnRH without the control by Kisspeptins or leptin due to environmental cues (e.g., photoperiod).

The regulation of appetite is also very different in chicken. In mammals, leptin partly exerts its anorexigenic effect via the increase and decrease of proopiomelanocortin and neuropeptide Y release, respectively, and ghrelin stimulates appetite. In chicken, in contrast to mammals, there are no leptin and Kiss system, and ghrelin inhibits food intake (Kaiya et al. 2013). It remains unknown whether other neuropeptides and/or circuitry replace leptin and Kiss peptide in functions in the hypothalamus.

Because several studies have shown that leptin and *TNFα* have biological effects on chicken in vivo and in vitro, some laboratories and/or companies have developed assays to measure the concentration of these peptides, as well as of omentin. Although several technical reports have pinpointed the fact that some antibodies (commercially available or produced by laboratories) are not well characterized (Saper 2005; Pradidarcheep et al. 2008) and recognize several molecules not related to the molecule of interest, the targets of antibodies against the product of missing genes remain to be identified.

This work has implications for comparative physiology and endocrinology. In birds, the absence of several genes reported to control the energetic metabolism of mammals, could explain some species specificities, such as hyperglycemia and relative insensitivity to insulin action. This basic information could contribute to a better understanding of the physiology of metabolic regulations across species and of physiopathological conditions such as human type II diabetes.

Materials and Methods

General Methodological Approach to Study Gene Loss

To look for a possible loss of genes resistin (*RETN*), *PAI-1*, omentin (*ITLN1*), and *TNFα* in the chicken genome, we performed reciprocal tBLASTn analyses http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed July 23, 2014) on the sequence of the chicken genome and on chicken ESTs with the human protein sequence as a query (fig. 6). We also used the dog protein sequence as a control. We searched for possible pseudogenes and inferred the presence of a pseudogene only if a match was found by tBLASTn analysis in the syntenic locus in comparison with the other species of interest, with a stop codon or an indel in the sequence identified by the similarity search (Meslin et al. 2012).

We performed reciprocal tBLASTn analyses on the chicken genome using the following general parameters: Expect threshold 10, word size 3; scoring matrix: BLOSUM62; gap cost: existence 11, extension 1; compositional adjustments: conditional compositional scorematrix adjustments; and filter: low complexity regions. To assess whether a given alignment constitutes evidence for homology and is not due to a chance, we checked the statistics of sequence similarity scores. The statistics for positive reciprocal best hits was as follows: Max scores ranged between 63 and 554; *E* value ranged between 0 and 9e-156; and % identity ranged between 47% and 95%. We have also checked sequences of short length with high *E* value to search for possible pseudogenes (Meslin et al. 2012). The number of *G. gallus* ESTs screened was 600,434 (dbEST release 130101) from all available tissues.

First, we ran reciprocal tBLASTn analyses on the chicken genome (fig. 6, step 1) with two possible outcomes: Match or no match. In case we obtained a positive reciprocal match, that is, two genes (in our study a human and a chicken gene), each in different genome, find each other as their best respective hit, the gene was localized on a known or unknown chromosome. Furthermore, if the outcome was "no match," we performed reciprocal tBLASTn analyses on chicken ESTs (fig. 6, step 2) for genes that are annotated in human but not in the chicken genome (no match). If no positive reciprocal tBLASTn sequence (no match) was found in ESTs, we tested

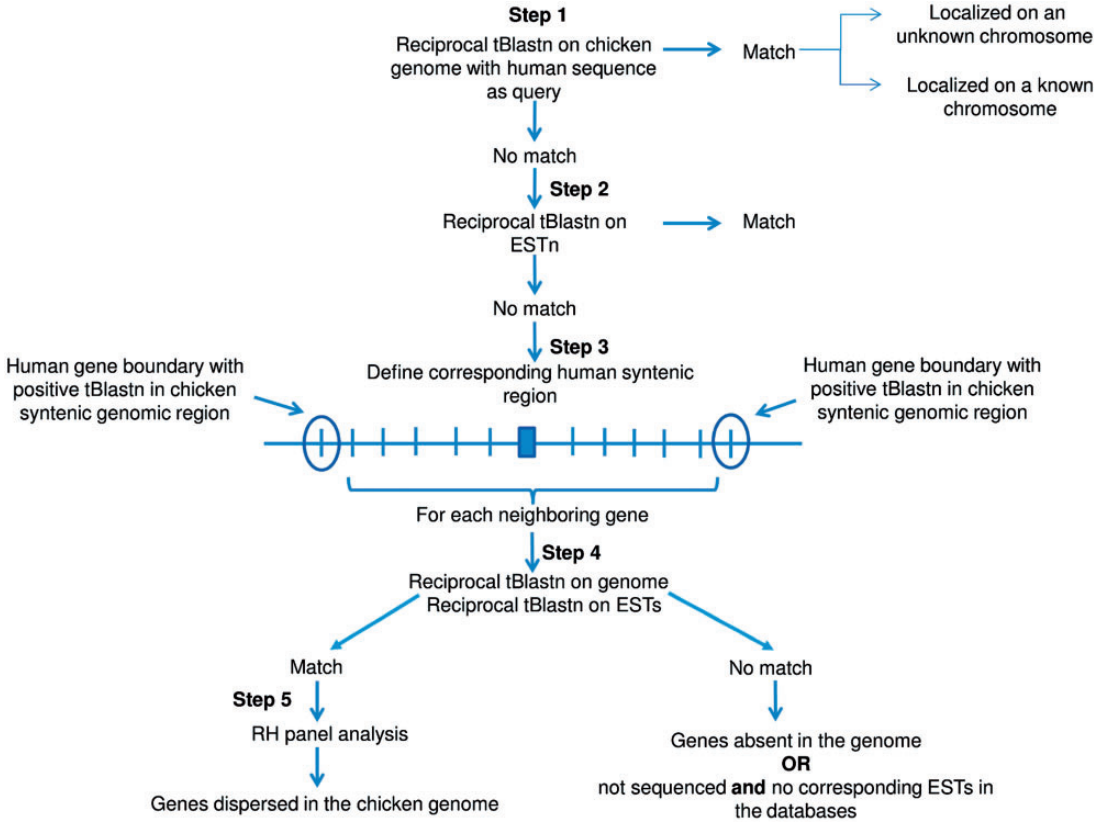

FIG. 6. Methodological approach to study gene loss.

the hypothesis that the gene of interest, and possible its neighboring genes, were deleted from the genome. For this purpose, we defined the corresponding human syntenic region, centered on the gene of interest and bordered by the first neighboring genes for which chicken orthologs exist and were localized in a syntenic region of the chicken genome (fig. 6, step 3). For each of these genes, we systematically performed reciprocal tBLASTn analysis against the chicken genome and chicken ESTs (fig. 6, step 4). Most frequently, both tBLASTn analyses were negative (no match), and the genes were predicted to be deleted with the block. For some genes, reciprocal positive matches were found against ESTs, the gene being either localized in the chicken genome (in a non syntenic region), or predicted to be on an "unknown chromosome." In this case, experiments on chicken RH panels were conducted to verify if these genes were localized within the syntenic region (fig. 6, step 5).

Finally, we analyzed an RNA-Seq data set generated from adipose tissue and characterized by high sequencing coverage (300 millions of mapped reads from 8 birds; see below) to model new genes absent from the Ensembl database to verify that these genes are not expressed in this tissue.

RH Panel Analysis

RH mapping was performed for markers located near the genes of interest in the human genome. Polymerase chain reaction (PCR) amplifications were carried out for each marker with specific primers (supplementary table S2, Supplementary Material online) in 15 µl reactions containing 25 ng DNA from the chickRH6 panel (Morisson et al. 2002), 0.4 µM of each primer, 0.25 units Taq polymerase (GoTaq, Promega), 1.5 mM MgCl2, and 0.2 mM dNTP on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). The first 5-min denaturation step was followed by 35 cycles, each consisting of denaturation at 94 °C for 30 s, annealing at Tm for 30 s and elongation at 72 °C for 30 s. PCR products were analyzed on 2% agarose gels, electrophoresed in 1× TBE buffer, and visualized by staining with ethidium bromide. Mapping of the markers on the RH panel was performed through the ChickRH server (http://chickrh.toulouse.inra.fr/, last accessed July 21, 2014). Distances and two-point LODs were calculated through the Carthagene software (de Givry et al. 2005). Maps were drawn with MapChart 2.0 (Voorrips 2002).

RNA-Seq Analysis

RNA-Seq data sequencing and analysis were performed from abdominal adipose tissue of 8 male and female chickens of 14 weeks of age with 40 million of reads expected per bird, described by Roux P-F, Frésard L, Leroux S, Klopp C, Martin P, Désert C, Fabre S, Esquerre D, Dehais C, Djari A, Zerjal T, Gourichon D, Pitel F, Lagarrigue S (unpublished data). To check if the genes resistin, *PAI-1*, omentin, and *TNFα* can be found in the adipose chicken transcriptome, all the resulting bam files were merged to produce a unique reference alignment on which the discovery of new genes and transcripts was performed using CUFFLINKS 2.0.0 (Trapnell et al. 2010). The resulting GTF file was used to extract the fasta

sequence of the newly discovered transcripts to annotate and quantify them for each bird. In addition, an RNA-Seq subset was assembled de novo (26, 828,416 reads pairs from adipose tissue from one individual bird, because of the high calculation resources required). The assembly produced 57,875 contigs (total length: 85,084,733 bp, N50: 3,550 bp). The new transcripts discovered from the first approach and the contigs obtained by the de novo assembly were aligned on the human RefSeq set using BLASTx (E-value < 1e-3) ftp://ftp.ncbi.nlm.nih.gov/refseq/H_sapiens/mRNA_Prot/human.rna.fna.gz 65.4 MB 06/01/14 17:36:00), and the RefSeq set was aligned on the new transcripts discovered and contigs using tBLASTn (E-value < 1e-3). For the five sought proteins, the ranks of the hits in both alignments were compared with find the possible best reciprocal BLAST hits.

Multispecies Comparative Map

Genome fragments were defined according to their gene content and order. Their localization in human, dog, chicken, zebra finch, and lizard was determined from the National Center for Biotechnology Information genome browser (http://www.ncbi.nlm.nih.gov/, last accessed July 23, 2014) using the most recent assemblies (Build 36.3 for *Homo sapiens*, Build 3.1 for *Canis familiaris*, Build 4.0 for *G. gallus*, Build 2.1 for *Taeniopygia guttata*, and Build 1.1 for *Anolis carolinensis*). Comparative mapping results based on gene content were also confirmed by comparative sequence analysis of the region of interest available from the Ensembl (version 71–74) (http://www.ensembl.org, last accessed July 23, 2014) and Genomicus (http://www.genomicus.biologie.ens.fr/genomicus-72.01/cgi-bin/search.pl, last accessed July 23, 2014) genome browsers.

Supplementary Material

Supplementary tables S1 and S2 and figure S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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