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Research paper

Progressive erosion of the Relaxin1 gene in bovids

Loggan Malone [a], Juan C. Opazo [b], Peter L. Ryan [c,d], Federico G. Hoffmann [a,e,*]

[a] Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, Mississippi State University, MS 39762, USA
[b] Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile
[c] Department of Animal and Dairy Sciences, Facility for Organismal and Cellular Imaging (FOCI), Mississippi State University, MS 39762, USA
[d] Department of Pathobiology & Population Medicine, Mississippi State University, MS 39762, USA
[e] Institute for Genomics, Biocomputing, and Biotechnology, Mississippi State University, MS 39762, USA

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ABSTRACT

The relaxin/insulin-like (RLN/INSL) gene family is a group of genes that encode peptide hormones involved in a variety of physiological functions related to reproduction. Previous studies have shown that relaxin plays a key role in widening of the pubic bone during labor and in gamete maturation. Because of these functions, studying the evolution of RLN1, the gene encoding for relaxin, is relevant in livestock species, most of which belong in the group Laurasiatheria, which includes cow, pig, horse, goat, and sheep in addition to bats, cetaceans and carnivores. Experimental evidence suggests that cows do not synthesize relaxin, but respond to it, and sheep apparently have a truncated RLN1 gene. Thus, we made use of genome sequence data to characterize the genomic locus of the RLN1 gene in Laurasiatherian mammals to better understand how cows lost the ability to synthesize this peptide. We found that all ruminants in our study (cow, giraffe, goat, sheep and Tibetan antelope) lack a functional RLN1 gene, and document the progressive loss of RLN1 in the lineage leading to cows. Our analyses indicate that 1 – all ruminants have lost all key regulatory elements upstream of the first exon, 2 – giraffe, goat, sheep and Tibetan antelope have multiple inactivating mutations in the RLN1 pseudogene, and 3 – the cow genome has lost all traces of RLN1. The 5′ regulatory sequence plays a key role in activating expression, and the loss of this sequence would impair synthesis of mRNA. Our results suggest that changes in regulatory sequence preceded mutations in coding sequence and highlight the importance of these regions in maintaining proper gene function. In addition, we found that all bovids examined posses copies of the relaxin receptors, which explains why they are able to respond to relaxin despite their inability to produce it.

1. Introduction

The genes in the relaxin/insulin-like (RLN/INSL) gene family encode for peptide hormones related to insulin that are functionally involved in processes related to reproduction, neuroendocrine regulation, fibrosis and the promotion of wound healing (Bathgate et al., 2003, 2013; McGowan et al., 2008; McGowan et al., 2014; Park et al., 2005; Sherwood, 2004). The evolutionary history of this group of genes combines the effect of gene and genome duplications, gene loss and the differential retention of relatively old paralogs in generating differences in gene complement. In placental mammals, this gene family includes multiple RLN and INSL genes: RLN1 and its ape-specific duplicate RLN2, which encode for relaxin, a hormone that is mostly involved in reproduction; RLN3, which encodes for a neuropeptide involved in stress and metabolic control feeding among other processes; INSL3, which encode for a hormone involved with testis descent and female fertility; and the less well known INSL4, 5 and 6. The products of INSL4 and 6 have reproductive roles, whereas INSL5 is expressed in the gastrointestinal tract (see Bathgate et al., 2013 for a review of the functional role of these peptides). From an evolutionary standpoint, the emergence of the RLN/INSL gene family traces back to the common ancestor of vertebrates, and the early diversification of these genes has been linked to the two whole genome duplications that occurred early in vertebrate evolution (Hoffmann and Opazo, 2011; Yegorov and Good, 2012). After that, tandem duplications and the differential retention of the resulting duplicates account for the different complements of RLN/INSL genes found in the different vertebrate groups (Park et al., 2008; Good-Avila et al., 2009; Hoffmann and Opazo, 2011). This is particularly evident in the RLN1-INSL4-INSL6 cluster of placental mammals where different

* Corresponding author at: Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Box 9655, MS 39762, USA.
E-mail address: federico.g.hoffmann@gmail.com (F.G. Hoffmann).

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combinations of RLN1, INSL4 and INSL6 have been retained by different lineages, with several examples of additional duplications, some of which appear to be driven by natural selection (Arroyo et al., 2014, 2012a, b, c). From a functional standpoint, relaxin, encoded by the RLN1 gene in most mammals but by the RLN2 gene duplicate in apes is the best-known member of the family. The relaxin peptide is mainly associated with the reproductive system, whereas recent studies have also identified non-reproductive functions associated with fibrosis, wound healing, cardiac protection, allergic responses and cancer (see Sherwood, 2004; Bathgate et al., 2006 for reviews of the physiological role of relaxin). Because the relaxin peptide is encoded by the RLN1 gene in most mammals, with the noted exception of apes, we will use the RLN1 label for this group of genes from here onwards, and refer to the encoded peptide as relaxin.

Because of its involvement in parturition, the physiological role of relaxin has been intensely investigated in animal husbandry. Interestingly, experimental evidence suggests that many species in the family Bovidae, which includes antelopes, bison, buffalo, cattle, goats and sheep, do not synthesize a relaxin peptide but respond to it (Bagna et al., 1991; Roche et al., 1993; Wilkinson et al., 2005). Further research has shown that sheep posses a single RLN1-like pseudogene in their genomes that transcribes for a weakly expressed messenger RNA that cannot be processed properly and is unable to synthesize a functional peptide (Roche et al., 1993). However, a systematic assessment of the evolutionary fate of the RLN1 gene in ruminants is lacking. The suborder Ruminantia belongs to the order Cetartiodactyla, which includes eventoed ungulates (artiodactyls) and cetaceans, within the Laurasiatherian group of mammals. Thus, in this manuscript we make use of genome sequence data to characterize the locus of the RLN1-INSL4-INSL6 gene cluster in representative Laurasiatherian taxa to study the loss of the RLN1 gene in ruminants, explore potential explanations of this loss, and speculate on its physiological consequences. Briefly, our results indicate that the loss of a putatively functional RLN1 can be traced back to the common ancestor of giraffes and bovids, dated to ~29 million years ago, and document the progressive loss of this gene in the lineage leading to cows, probably triggered by changes in the 5′ upstream region.

2. Materials and methods

2.1. Sequence data and bioinformatic analysis

Our search strategy divided our efforts in two stages. In the first stage, we searched for traces of RLN/INSL genes in the RFLB locus in representative species of Laurasiatheria for which genomic data was available (Supplementary Table 1). We identified RLN1, INSL4 and INSL6 sequences by searching genomic sequence records in the Ensembl or NCBI (refse_genomic, htgs, and wgs) databases using BLAST (Altschul et al., 1990). In a second stage, we focused on a representative set of species that provided balanced taxonomic sampling to resolve the history of the RLN1 gene of ruminants, and where the RLN1-INSL4-INSL6 gene cluster was in relatively long fragments. The sampling for this second stage included representatives of most Laurasiatherian orders, with emphasis on cetartiodactyls. We included two carnivores (order Carnivora: dog, *Canis familiaris* and cat, *Felis domesticus*), one bat (order Chiroptera: flying fox, *Pteropus vampyrus*), two odd-toed ungulates (order Perissodactyla: horse, *Equus caballus* and rhino, *Ceratotherium simum*), and nine cetartyodactils. The latter represented four of the suborders of Cetartiodactyla: two from the suborder Tylopoda (alpaca, *Vicugna pacos* and domestic Bactrian camel *Camelus bactrianus*), one from the suborder Suina (pig, *Sus scrofa*), one from the suborder Cetacea (killer whale, *Orcinus orca*), and five from the suborder Ruminantia (giraffe, *Giraffa camelopardalis*; sheep, *Ovis aries*; goat, *Capra hircus*; Titetan antelope, *Pantholops hodgsonii*, and cow, *Bos taurus*). Representatives of the orders Eulipotyphlan and Pholidota were not included in these analyses because they are relatively distant from bovids and the corresponding genome assemblies are highly fragmentary. Conversely, despite the fragmentary nature of its genome, the giraffe was included because of its taxonomic position (Fig. 1).

For these second set of species, we annotated the RLN and INSL genes in the RLN1-INSL4-INSL6 gene cluster manually by comparing exon sequences from previously characterized human and mouse reference genes to unannotated genomic sequences using the program Blast2seq (Tatusova and Madden, 1999). Putatively functional genes were characterized by an intact open reading frame with the canonical two exon/one intron structure typical of vertebrate RLN/INSL-like genes, whereas pseudogenes were identifiable because of their high sequence similarity to functional orthologs, with the presence of inactivating mutations and/or the lack of one of the exons. Because of the inherent difficulty associated with determining pseudogene boundaries, we combined synteny, BLAST and, when possible phylogenies to determine the putative orthology of pseudogenes.

2.2. Orthologous relationships

We performed phylogenetic analyses to infer genealogical relationships among the sequences annotated. To do so, sequences were aligned with Muscle (Edgar, 2004) via default parameter settings as implemented in MEGA6 version 6.06 (Tamura et al., 2013) and phylogenetic relationships were estimated by maximum likelihood (ML) based on the GTR model of nucleotide substitution, with rate variation among sites modeled following a discrete

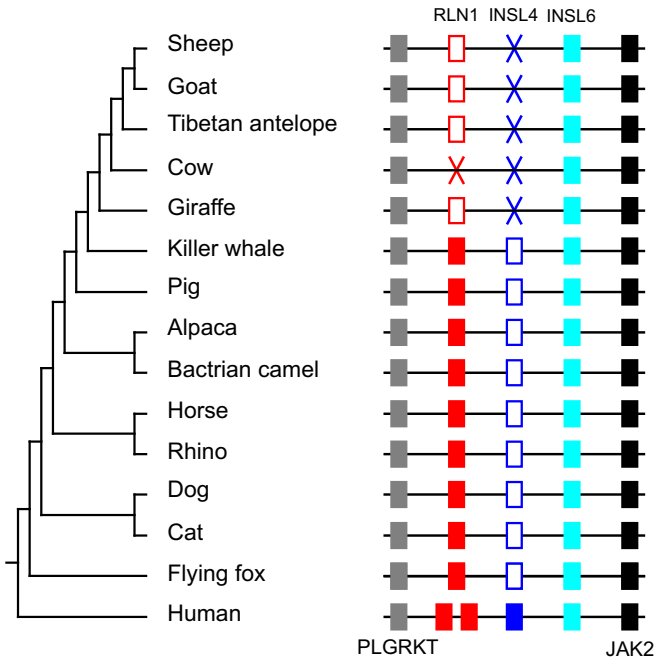

Fig. 1. Genomic structure of the RFLB in select Laurasiatherian mammals, with human as reference. Genes and distances among them are not drawn to scale, solid boxes denote putatively functional genes, white boxes denote pseudogenes, and the 'X' denotes a gene loss. The gene order in the giraffe is provided for reference, as in the current assembly the genes lie on separate contigs. Organismal relationships are taken from Meredith et al. (2011). Rhino, camel and cat, which are not included in the figure all have the RLN-INSL4 ps-INSL6 set of genes in their RFLB.

gamma distribution (Γ), and support for the nodes was estimated with 1000 bootstrap pseudoreplicates. To further explore patterns of sequence variation, dot plot pairwise comparisons between relevant sequences were graphed using Pipmaker (Schwartz et al., 2000), and promoter regions were predicted using FPROM (Solovyev et al., 2006). Visual assessments of conserved synteny were performed in the Genomicus browser (Louis et al., 2015).

3. Results and discussion

We surveyed publicly available genomic data from fourteen Laurasiatherian mammals, representing all orders other than Eulipotyphla and Pholidota, tracing back approximately 82 million years of evolution. The RLN1-INSL4-INSL6 gene cluster is defined based on conserved synteny across mammals as the genomic locus that included genes in the RLN/INSL gene family and is flanked by copies of Janus Kinase 2 (JAK2) on one side and of Plasminogen Receptor with a C-Terminal Lysine (PLGRKT, previously known as C9orf46) on the other. Most species in our study follow this pattern, with the exception of cases such as giraffe, where these genes fall on relatively short fragments.

Once we located the RLN1-INSL4-INSL6 gene cluster, we annotated the RLN/INSL genes in the cluster and found that most species posses putatively functional copies of RLN1 and INSL6, with the exception of giraffe and all bovids, which only include a putatively functional copy of INSL6 in its genome (Fig. 1). In addition, we found several fragments similar to INSL4 and RLN1 in multiple species. Prior studies reported the presence of INSL4 pseudogenes in alpaca and dolphin (Arroyo et al., 2012a), and of a RLN1 pseudogene in sheep (Roche et al., 1993). Our bioinformatic searches revealed a much more widespread presence of both pseudogenes. We found INSL4-like pseudogenes in killer whale, pig, camel, horse, rhino, flying fox, cat, and dog, confirming that at least traces of an INSL4 gene were present in the last common ancestor of Laurasiatherian mammals. Thus, in combination with previous identification of INSL4 pseudogenes in other placental lineages (Arroyo et al., 2012a) our results confirm that that the INSL4 gene was originated in the common ancestor of the group, and that functional copies were only retained in catarrhine primates, the group that includes Old World monkeys and apes. In the case of RLN1, our analyses revealed that the presence of a pseudogene extended beyond sheep to also include goat, Tibetan antelope, and giraffe (Supplementary Table 1). Even though, the INSL6 sequence found on the current assembly of the domestic Bactrian Camel genome would not be functional, because of the preliminary stage of the assembly and the fact that other camels do posses putatively functional copies, it is considered as functional in the current study (Supplementary Table 1).

Phylogenetic analysis confirmed the orthology predictions derived from bioinformatic searches. The resulting tree resolved orthology for all gene and pseudogene sequences with relatively high confidence (Fig. 2), and within each paralog clade, relationships among the sequences did not deviate significantly from expected organismal relationships. The monophyly of the Laurasiatherian INSL4 pseudogenes suggest they all derive from an already inactive INSL4 copy in the last common ancestor of the group. The same can be said for the RLN pseudogenes from giraffe and bovids (sheep, goat, and Tibetan antelope), suggesting this gene lost its function early in the evolution of the suborder Ruminantia.

The phyletic distribution of the functional and non-functional copies of RLN1 indicates that the common ancestor of cetaceans and ruminants had a functional copy of the RLN1 gene, which was lost between the split of the giraffe and bovid lineages, dated to ~29 million years ago, and the split between artiodactyls and

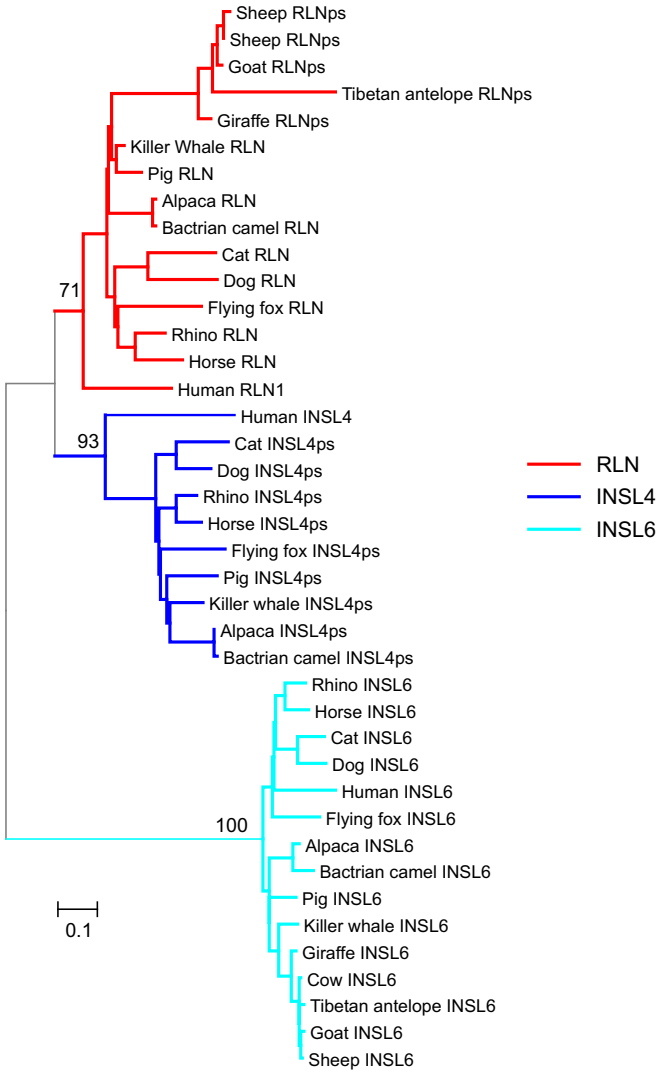

Fig. 2. Maximum-likelihood estimate of evolutionary relationships among the RLN, INSL4 and INSL6 sequences from representative Laurasiatherian mammals.

cetaceans, dated to ~56 million years ago. The RLN1 pseudogene includes two well-defined exons in sheep, goat and giraffe, but has been altogether lost in cow (Fig. 3). The current assembly of the Tibetan antelope genome appears to represent an intermediate state, with a well-defined exon 2 and an exon 1 which has greatly diverged. By contrast, the INSL4 pseudogenes in our study probably derive from a copy that was already non functional in the last common ancestor of Laurasiatherians. Despite the relative old age of these pseudogenes, they were clearly identifiable in bioinformatic searches. As expected, their sequences are highly divergent, many of them do not have start or stop codons, lack canonical splicing donor or acceptor sites, are shorter that the putatively functional human counterpart, and include multiple inactivating and frame shifting mutations. As expected given that the inactivation of the RLN1 pseudogene in bovids and giraffe is more recent in evolutionary time, the corresponding sequences are more similar to their functional counterparts.

We then explored the changes underlying the pseudogenization of the ruminant RLN1 copy by comparing the sequence corresponding to the exons, as well as the conceptual translation of the annotated sequences. These comparisons revealed the presence of multiple premature stop codons in goat, giraffe, sheep and Tibetan antelope (Supplementary Fig. 1). In most genes, the

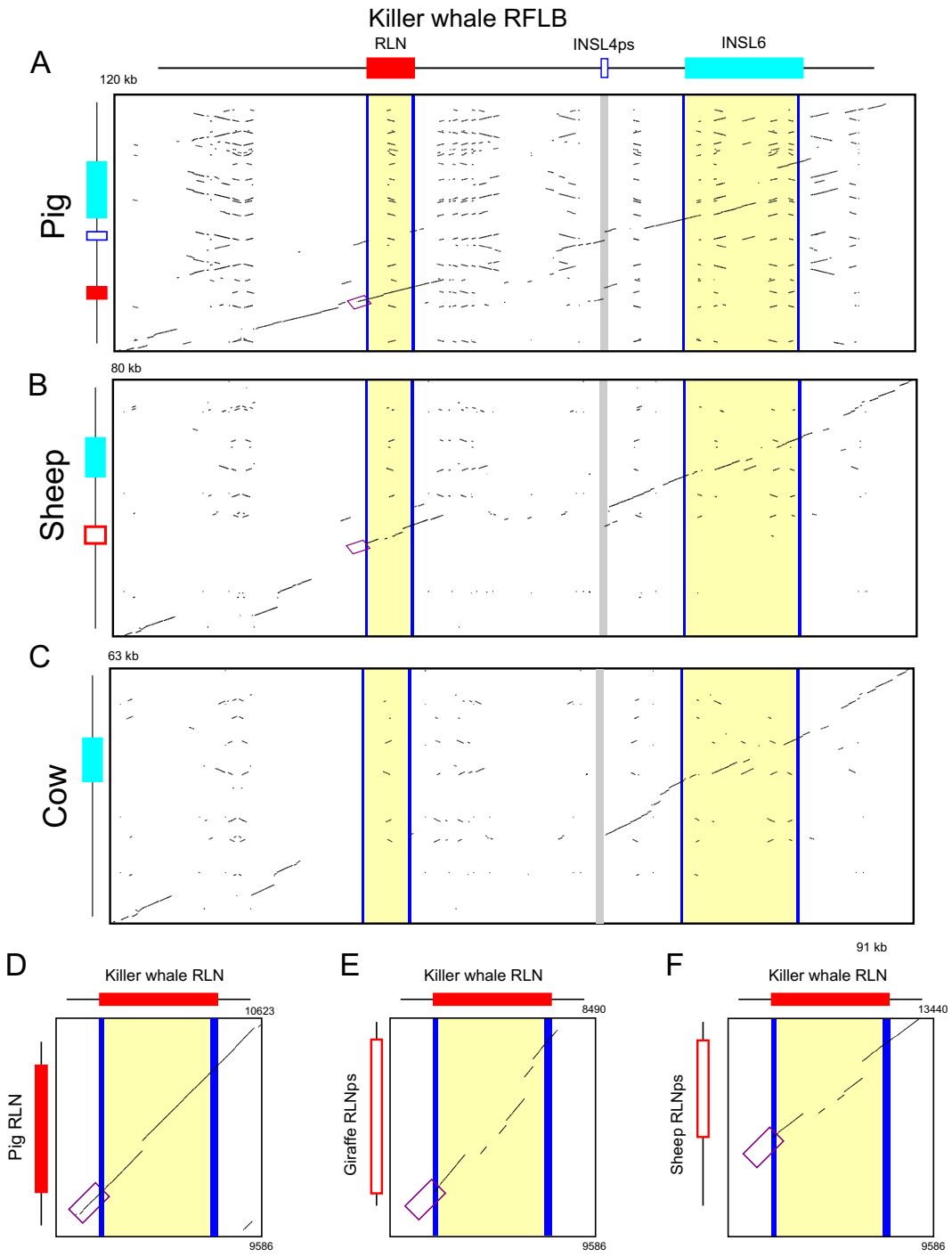

Fig. 3. Panels A–C: Pairwise dot plot comparisons of a genomic fragment spanning the RFLB. Panels D-F: Pairwise dot plot comparisons of the RLN gene plus 2000 nucleotides upstream and downstream, in killer whale vs pig, (divergence: 64 mya, panels A and D), killer whale vs giraffe (divergence: 56 mya, panels B and E), and killer whale vs sheep (divergence: 56 mya, panels C and F). Blue shading for exons, yellow shading for introns and gray shading for the INSL4 pseudogene. The purple rectangle identifies the region immediately upstream of the start codon of the RLN gene. Divergence time estimates were obtained in the TimeTree databases (Hedges et al., 2015). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

non-coding sequence immediately upstream of the start codon plays a key role in *cis* regulation of gene expression and changes in this region could have profound effects in gene expression. We first used FPROM to search for promoters in the 1000 nucleotides upstream of the start codon. As expected, we found that all species with a putatively functional RLN1 gene were predicted to have promoter and a TATA box approximately 150 nucleotides upstream of the start codon. Species with pseudogenes, on the other hand, possessed neither (Table 1). In fact, pairwise sequence comparisons of this region reveal that the killer whale region upstream of the RLN1 gene is more similar to the corresponding region in pig, rather than giraffe or sheep, despite the phylogenetic affinities of cetaceans with ruminants (Fig. 3). Thus, our results suggest that, in addition to coding sequence mutations, changes in the 5′ regulatory sequence in the common ancestor of giraffe and bovids were prob-

Table 1
Bioinformatic prediction of promoter and TATA box sites in 1000 nucleotides upstream of the start codon.

Species	Promoter Position	TATA box Position
Alpaca	892	862
Pig	895	866
Killer whale	894	862
Giraffe	–	–
Sheep	–	–
Goat	–	–
Tibetan antelope	–	–

ably also involved in the loss of a functional RLN1 gene in this group.

Gene loss is emerging as an important factor in generating novel gene combinations that in turn can underlie the emergence of novel phenotypes (see Albalat and Cañestro, 2016 and references therein). In this case, our results explain why the search for a RLN peptide in bovids was not successful, and indicate that the RLN1-INSL4-INSL6 cluster of bovids and giraffe has reverted to the ancestral single-gene condition found in marsupials and egg-laying mammals. The interesting twist is that rather than retaining a copy of RLN1, they have retained a copy of the INSL6 paralog, whose function is not well understood. In vivo, the relaxin peptide binds to two closely related G protein-coupled receptors, relaxin family peptide receptors 1 and 2 (RFXP1 and 2) to activate downstream signaling pathways (see Bathgate et al., 2013, and Yegorov et al., 2014, for reviews). Despite the absence of a functional RLN1 gene in cow and sheep, we did find putatively functional copies of the relaxin receptors, RXFP1 and RXFP2 in the genomes (ENSBTAG00000010306 and ENSBTAG00000015132 in the case of the cow, and (ENSOARG00000004733 and ENSOARG00000011653 in the case of the sheep (Supplementary Table 2). In fact, cow and sheep posses putatively functional copies of the 4 RXFP receptors described for mammals (Supplementary Table 2). Thus, we suspect the relaxin signaling pathway remains functional, which explains why cattle respond to pig relaxin, even though they cannot actually produce it (Bagna et al., 1991).

Even though relaxin plays very important roles in parturition in most mammals, ruminants have been living without it for several million years. This is remarkable, given that in the closely related pig, relaxin is essential for normal parturition and delivery of live piglets and when deficiencies arise at term the incidence of prolonged delivery and a high incidence of stillbirths arises (Nara et al., 1982 and Cho et al., 1998). Relaxin or relaxin receptor deficiencies (e.g., RXFP1 receptor) in rodents may also lead to incomplete softening of the pubic symphysis, cervical growth and ripening resulting in prolonged labor and increased incidence of stillbirths (Hwang et al., 1989, and Krajnc-Franken et al., 2004). Thus, the known actions of relaxin on cervical dilation and facilitation of delivery in other species has prompted the clinical use of relaxin to reduce the incidence of dystocia and stillborn deliveries in dairy and beef heifers. A number of studies have explored the efficacy of purified porcine relaxin on parturition outcomes in heifers with mixed results. Some studies observing a positive outcome including earlier calving, increased cervical dilatations, pelvic area expansion and decreased incidence of dystocia in dairy heifers (Bagna et al., 1991) and retained placentas in beef heifers (Musah et al., 1986), but the same effects were not as apparent in some beef heifers studies (Smith et al., 1996, 1997). However, discrepancies in results may be attributable, in part, to mode of administration. Given that calving difficulties in beef and dairy heifers remains challenging due to economically-driven breeding practices, it may be worth revisiting the therapeutic use of relaxin in facilitating parturition in heifers by considering the development of not only relevant synthetic relaxin hormone preparations from species more closely related to the bovine, but also exploring more appropriate delivery strategies of the hormone to the intended target sites.

The absence of a native relaxin peptide would leave the receptor available for competing but probably less specific factors, creating opportunities for novel interactions with the potential to generate new signaling pathways that could lead to biological innovations. Specifically, we speculate that there is a certain level of redundancy in the relaxin signaling pathway of mammals, so that other members of the RLN/INSL gene family could compensate for the loss of the RLN1 gene. Thus, this group of mammals could be considered as natural RLN1 knockout experiments provided by the evolutionary process (Albertson et al., 2009) that offer great opportunities to better understand the role of relaxin in mammalian physiology, and evaluate how gene loss can also promote biological innovation. Recently a similar case of gene loss in ruminants showed that the lack of upper jaw incisors in this group could be explained by the lack of the gene (gremlin 2) that possesses a role in tooth development (Opazo et al., 2017).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2017.07.011.

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