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Investigating the Ancestral and Novel Functional Roles of Insl3: the Gene Structure Parameters and Bioinformatics as Key Tools in Evoutionary Biology Research


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INTRODUCTION & AIM

Insulin-like 3 (INSL3), also known as relaxin-like factor (RLF), is a member of the insulin-IGF-relaxin peptide superfamily (growth factors and hormones) that is synthesized mainly in somatic cells of the gonads, in testicular Leydig cells and in the ovarian follicular theca cells as a pre-prohormonal precursor containing a signal peptide linked to B-C-A domains (see Fig.1A). INSL3 exerts its biological activity through its receptor, RXFP2.

INSL3 is processed by an unidentified convertase to produce mature INSL3, a heterodimer (A-B) linked by two interchain and one intrachain disulfide bond, as shown in Fig. 1A. These disulfide bonds are crucial for maintaining the characteristic conformation and biological activity of insulin-like molecules. INSL3 is encoded by a relatively small gene consisting of two exons and one intron (see Fig 1B), similar to the genes coding for insulin and relaxins [1].

INSL3 cDNA sequences have been isolated from several Vertebrate species. Fig.1C shows the Danio rerio insl3 gene structure, including its nucleotide sequence and the predicted amino acid sequence as determined in our previous studies [2].

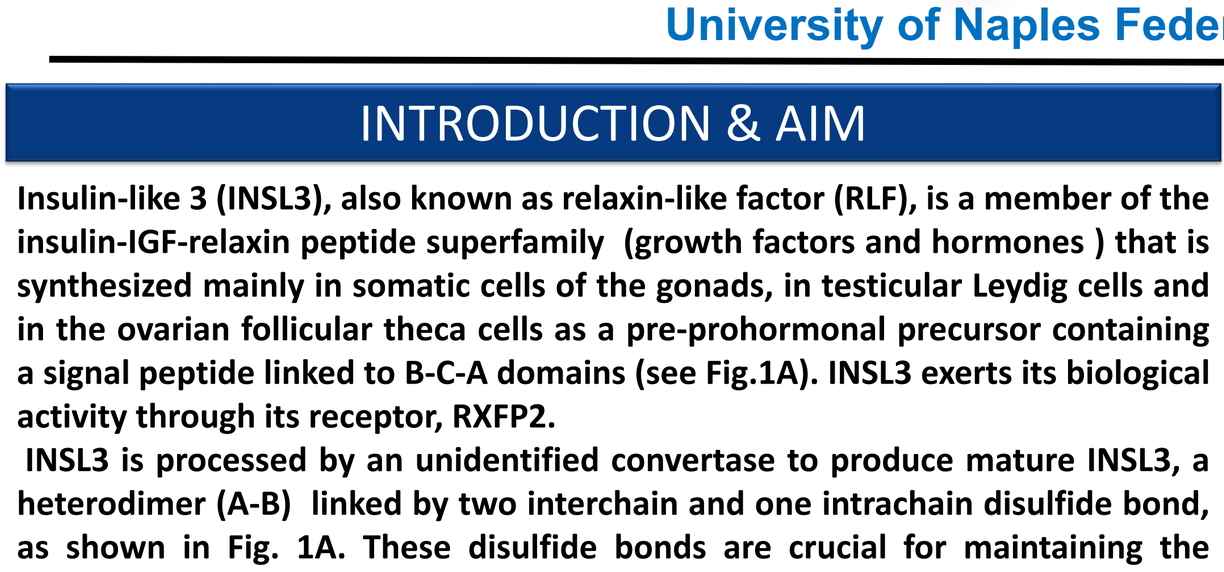


Fig.1

METHOD

Bioinformatics Sequence Analysis

To identify binding sites for potential transcription factors, the 35-bp region of the mouse *Insl3* promoter was analyzed using the bioinformatic tools TFbind (https://tfbind.hgc.jp/, last accessed 30 January 2026).

Multiple sequence alignment was performed using the CLUSTAL Omega multiple sequence alignment tool (version 1.2.4, last accessed October 2024, https://www.ebi.ac.uk/Tools/msa/clustalo/) and BLAST, https://blast.ncbi.nlm.nih.gov/

RESULTS & DISCUSSION

In this study, bioinformatics analyses of known insl3 genes are presented. The *insl*3 gene structure (see Fig.2) and exon length are both conserved among vertebrates, but intron length varies (see Fig.3). These findings are based on bioinformatics analyses conducted on known insl3 genes. The presence of at least two different kinds of *insl*3 transcripts in both adult tissue and developing *Danio rerio* embryos is demonstrated by RT–PCR. Using an in silico-lab method, I discovered that fish insl3 introns contain sequences, a conserved open reading frame that codes for a 3' mRNA maturation signal, and a peculiar A peptide domain. My theory that multiple transcripts could mature from the insl3 pre-mRNA (manuscript in preparation) is supported by this finding.

As illustrated in Figure 3, despite the observed variation in intron length, all insl3 genes that we have examined always have two exons that are homogeneous in length among fish, in contrast to the observed variation in intron length. Studies on the zebrafish INSL3 proximal promoter, as depicted in Fig.4 have identified the same point mutation on the binding site of an important regulator of promoter activity of mouse and human INSL3 that have negative effect on insulin-like 3 gene transcription in Leydig cells (see Fig.5). Since the gene coding for this factor is present in the zebrafish genome, the proposal suggests that the role of INSL3 in testis development could also arise and be gained from a point mutation in the NR4A1 binding site within its promoter. This would provide molecular evidence for a potential functional role-gain of insl3 in mammalian testis development.

Comparison of vertebrate insl3 gene structures

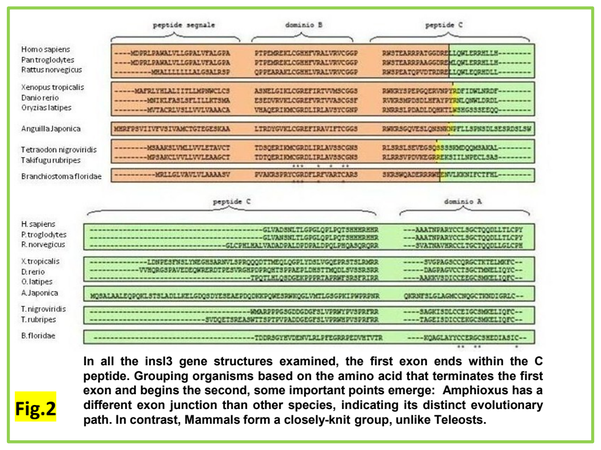


Fig.2 **In all the insl3 gene structures examined, the first exon ends within the C peptide. Grouping organisms based on the amino acid that terminates the first exon and begins the second, some important points emerge: Amphioxus has a different exon junction than other species, indicating its distinct evolutionary path. In contrast, Mammals form a closely-knit group, unlike Teleosts.**

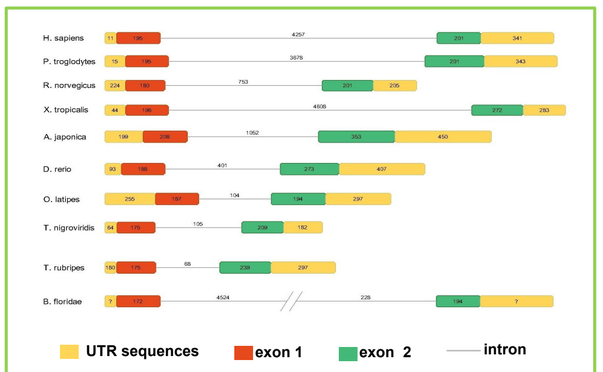


Fig3 **All insl3 genes are composed of two exons and one intron, and the length of the exons appears homogeneous in the genes examined, while Intron length tends to increase as one moves up the evolutionary hierarchy.**

Bioinformatics analysis of *Danio rerio* insl3 proximal promoter

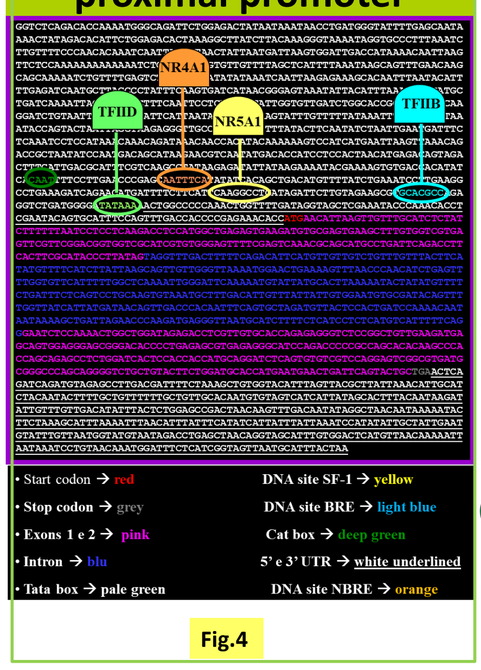
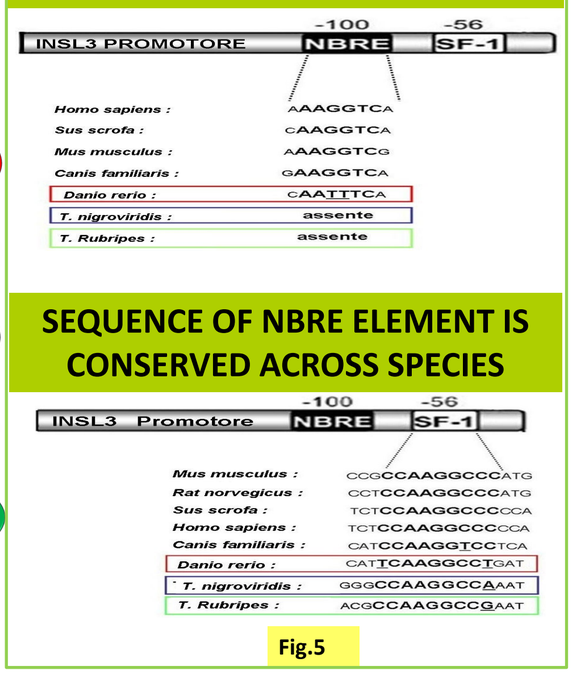


Fig.4

COMPARISON OF DNA *CONSENSUS* SEQUENCE BINDING SITE (SF-1) FOR NR5A1



SEQUENCE OF NBRE ELEMENT IS CONSERVED ACROSS SPECIES

Fig.5

CONCLUSION

The absence of INSL3 or its receptor, RXFP2, leads to cryptorchidism in mice, underscoring the crucial role of INSL3 in testicular descent during fetal development in most mammals. Research has also been conducted on INSL3 in non-mammalian vertebrates, including fish. Since testicular descent is unique to mammals, studying non-mammalian vertebrates allows for the exploration of other biological functions of INSL3. The expression of the gene coding for INSL3 (insl3) is conserved and present in teleost fish, such as zebrafish (Danio rerio). In zebrafish, insl3 transcript levels significantly rise in response to follicle-stimulating hormone (FSH), but not to luteinizing hormone (LH) [3]. Bioinformatics analyses presented here were performed on nearly all known insl3 genes and cDNA sequences from the database, revealing that the insl3 gene structure is conserved among fish and various non-mammalian vertebrate species, although intron lengths differ. In silico results also provide evidence for a gain-of-function role of a single copy of the insl3 gene in mammalian testis development. The zebrafish insl3 proximal promoter reveals the presence of the same point mutation in the binding site of a key transcription factor, which affects promoter activity of mouse and human INSL3. This mutation negatively impacts the transcription of the insulin-like 3 gene in Leydig cells. Since the gene for the NR5A1 binding factor is present in the zebrafish genome, the proposed hypothesis suggests that the key role of the new Insl3 in testicular descent and in testis development might also arise from a point mutation in its DNA binding site on the promoter.

FUTURE WORK / REFERENCES

It is important to explore and clarify the precise mechanisms through which INSL3 promotes the proliferation and differentiation of spermatogonia, as this knowledge could significantly enhance our understanding of spermatogenesis.

[1]Esteban-Lopez M, Agoulnik AI. Diverse functions of insulin-like 3 peptide. J Endocrinol. 2020 Oct 1;247(1):R1-R12. doi: 10.1530/JOE-20-0168. PMID: 32813485; PMCID: PMC7453995.

[2] Donizetti, A.; Calicchio, M.; Romano, M.Z.; Rosati, L.; Turco, M.; Carrese, A.M.; del Gaudio, R.; Ferrandino, I.; Aniello, F. Expression of Insl3 Protein in Adult Danio rerio. Int. J. Mol. Sci. 2024, 25, 5419. https://doi.org/10.3390/ijms25105419

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