**Scientific Abstract - The origin of the intermediate mesoderm and the nephric system in vertebrates: A comparative developmental approach**

Understanding the origins of different body plans requires knowledge of how genes and genetic pathways that control embryonic development have evolved. The pattern of the anterior-posterior (AP) axis in chordates is regulated by the spatiotemporal expression of a common set of genes in early development, although extreme variation in adult morphology can be observed. Several gene families are involved in orchestrating this patterning process. Among them are *Hox* genes and retinoic acid (RA) which play a well-characterized causal role in AP patterning and the organization of the chordate bauplan. However, knowledge of the tissue-specific deployment of *Hox* genes and RA in the course of evolution remains elusive. Great potential for a deeper understanding of this question is held by the intermediate mesoderm (IM) due to its unique mode of development.

All vertebrate kidneys are derived from the IM which differentiates sequentially from anterior to posterior into several types of kidney tissues at specific positions along the AP axis. The pronephros, the first to develop, is essential for the subsequent formation of the two other kidneys, the mesonephros and metanephros. Early kidney genes such as the transcription factors *Lim1* and *Pax2* are positioned in amniotes only in IM located posterior to the 6th somite axial level, which is also the border between the head and the trunk. Previous studies in our lab revealed that *Hoxb4* and RA play critical roles in regulating the expression of kidney genes along the AP axis by conferring competence to IM cells to respond to kidney inductive signals (TGF-β family). These signals emanate from surrounding tissues along the entire axis, including anterior non-kidney generating IM1;2;3. We proposed a model in which competence of IM cells to respond to TGF-β signaling and express kidney genes is driven by RA and mediated by *Hoxb4.* However, the interaction between these two factors and the mechanisms through which they regulate kidney gene induction are unknown.

Several studies in the last two decades have discovered homologues of *Lim1* and *Pax2* in amphioxus, lamprey and catshark that reveal expression in nephric structures. In this project we take a comparative evolutionary developmental approach using these three model organisms to elucidate the origin of the nephric system and its molecular regulation in vertebrates. In catshark, *Scyliorhinus canicula*, a chondrichthyans, the initiation and patterning of the IM and the pronephros are mostly unknown. Due to the phylogenetic position of chondrichthyans as the sister group to all other gnathostomes (jawed vertebrates), understanding the molecular mechanisms that govern the formation of the IM and pronephros promises to provide valuable information regarding the evolution of nephric system development in all jawed vertebrates. The lamprey, *Lampetra fluviatilis*, is representative of the cyclostomes (jawless fish), a sister group to all other vertebrates. It exhibits unique features such as lack of jaws and paired appendages and serves as a crucial taxon for reconstructing the evolutionary events leading to the elaborated nephric system in vertebrates4. The amphioxus, *Branchiostoma lanceolatum,* is a member of a group of non-vertebrate chordates, cephalochordates. In our comparative analysis, it serves as an outgroup to elucidate the evolutionary origin of the vertebrate kidney. Several interesting and unexpected preliminary results point to evolutionary transitions in the molecular control of the nephric system that correlate with the development of the vertebrate body and the origin of the head and mesodermal tissues (Esc2019). Discovering these basal pathways for kidney regulation will deepen our understanding of the evolution of vertebrate kidney development and elucidate molecular mechanisms that regulate the amniote nephric system.

**D. Detailed description of the proposed research**

**D1. The origin of the intermediate mesoderm**

**D1a. Positional and developmental relationships of lamprey and catshark nephric and somitic markers**

 From our preliminary results (Figs. 1,2) it is clear that nephric genes in catshark are expressed in a specific ventrolateral territory within the epithelial somite. From the double immunostaining of Pax2 and Pax3, and *Pax1* WMIHS, is clearly observed that Pax2 and Pax3 share a sharp expression border, whereas *Pax1* occupies a domain that is ventromedial (Fig.2 D-G, arrows). However, defining the precise domain expression of all three genes within one section is imperative for understanding nephric gene territories within the organization of the somitic domain65. This includes the timing of rearrangement of budding in the IM. To achieve this goal we will perform triple immunostaining, if antibody cross reactivity will permit this. If not, we propose double immunostaining of Pax2 and Pax3 following WMISH for *Pax1*. If this method is unsuccessful, we will perform alternate *Pax1* in situ hybridization (ISH) and double immunostaining of Pax2 and Pax3 in sequential sections.

The same approach will be done for lamprey embryos. We will clone lamprey somitic markers such as *Pax3*, *Pax1* and *MyoD* and perform similar triple expression experiments starting at stage 21, the earliest stage nephric genes are observed. Our preliminary results suggest that the dynamics of expression and the expression domain of the nephric genes is different from the those observed in catshark. Therefore, this part of the project will be done at several developmental stages and will serve as a reference for understanding the regulation of somitic territories and the initiation of the IM from an evolutionary perspective.

**D1b. The formation of the pronephros and the nephric duct in catshark and lamprey**

**D1b1. Catshark**

Following detailed analyses of the origin of the pronephros in somitic domains we aim at understanding the budding and architecture of the pronephros and nephric duct formation. Our preliminary frontal sections of catshark embryos expressing *Pax2* reveal valuable information about the emergence of the pronephros from somites 6-10 (Fig. 1H-K). Since the expression of nephric genes extends posteriorly in somitic domains of the mesonephros, analyses of additional stages will be done to investigate the contribution of more posterior somites to the nephric duct. The frontal sections are in agreement with results obtained in cross sections (Fig. 1A-G) and provide another physical dimension to understand the process. Despite these informative histological sections a clear 3D image of the cells involved in the process is imperative. For this purpose Z-series’ of fluorescent Pax2 immunostaining (Fig. 2) will be collected using a Nikon A1R laser scanning confocal microscope (LSCM) and three-dimensional reconstruction will be performed using Bitplane software (Imaris).

**D1b2. Lamprey**

Our preliminary results at stage 25 revealed expression of nephric genes in a ventral domain. But whether expression was within somitic mesoderm or forms a distinct one is not clear (Fig.3D-F). In these somites a tubule connected to the *Pax2/Lim1* expression domain was observed that revealed no expression of these genes (Fig.3E,F,). At the early stage 22 nephric genes were expressed in the lateral aspect of a domain that included progenitors displaying somitic properties (Fig. 3C). Our preliminary results already indicate there are significant differences between lamprey and catshark in pronephros formation that raise important questions. How is the expression domain at early stages extended ventrally to create the distinct region of *Pax2/Lim1*? How are the short tubules formed and connected to the nephric duct? To answer these questions, we will use two methodologies. First, we will conduct detailed analyses of early and late stages in different section orientations of embryos expressing *Lim1* or *Pax2* (frontal and sagittal). This should teach us the morphological aspects by which the pronephros is formed and the tubules and nephric duct are assembled. Second, depending on availability and specificity of antibodies against Pax2, Lim1 and whether selected genes identified from the catshark transcriptome exhibit specific expression in lamprey tubules and nephric duct, we propose to use LSCM to create fluorescence-based 3D images of developmental stages 21 to 25 in catshark utilizing Imaris Z-series analyses.

**D1b3. Understanding assembly of the nephric duct by DiI experiments in catshark**

 As discovered in our preliminary results, the ventrolateral domain of somite 6 and posteriorly develops laterally to form the nephric duct. However, in somite 8 (Fig. 2E), two Pax2 domains are observed, one that is lateral to the somite and resembles a tube-like structure and one that is within the epithelial somite. At least four somites (6-9) (Fig. 1H-J) seem to contribute to the nephric duct lateral to the somites raising several questions. Is the pronephric duct formed by cells originating from the 6th to the 9th or 10th somite and extending posteriorly or by cells originating in each somite (Fig. 1K) that join the formation of the pronephric duct in a segmented manner? Assuming a multiple somitic origin, how are the cells connected to assemble the duct structure? Alternatively, if the duct originates from one somitic source, how does it extend posteriorly while assembled into the duct? To answer these questions, we developed a new methodology rarely used before in catshark. Two fluorophores were injected into sequential somites in an alternate manner. Our preliminary results show that the injections were successful and revealed alternate staining in the ventrolateral somitic domains of somites 6 to 10 (see Fig.4 for somite 6, arrows). After 24h the two dyes started to rearrange along the pronephric duct as separate entities indicating different somitic origins (Fig. 4E,F). Elaboration of this experiment will extend the period of incubation up to 96 h and include injection to more posterior somites and all developmental stages from stage 19 to 24. Contiguous staining with one dye will indicate one somitic source of the pronephric duct. Alternately, dye staining with two fluorophores will provide evidence for multiple somitic sources. Two methodologies will be applied to further examine the injected embryos. First, selected embryos from a time lapse experiment will be sectioned in all orientations to confirm the somitic source of dyes. This will exclude the possibility that dyes are stuck in somites and possibly permit the tracking of cell movements and assembly of the nephric duct. Second, we will use LCSM as described in section D1b1. Because of technical restrictions, at the moment, live imaging methods cannot be practiced in this animal model.

**D1c. Regulation of somitic domains in catshark and lamprey**

Based on the precise triple expression patterns at different developmental stages we will perform gain and loss-of-function experiments of signaling molecules and transcription factors known to effect somite compartmentalization and nephric gene regulation2;19;65 in both species.These signaling molecules will include TGF-β family members such as Activin, Nodal-like morphogen, BMP4, BMP4 antagonist noggin, Wnts family members, and Shh.

In the first experiments, we will use pharmacological agents to block such signaling pathways. Evidence for the involvement of such signaling factors and validation of our method come from our preliminary results in which Wnt signaling was blocked (Fig. 2H,I). This significantly affected dorsal neural tube and somite formation with no clear effect on *Pax2* expression and budding of the nephric duct. These pharmacological experiments will be analyzed for expression of nephric genes and somitic markers such as *Pax3*, *Pax1* and *MyoD*. In these experiments many new genes discovered in the transcriptome (see section C2b) will be examined to elucidate upstream regulatory control of their expression. Further details in section….

 In the second experiments, we will electroporate gene constructs into particular somites of catshark to overexpress (gain-of-function) or inhibit (loss-of-function) particular pathways. This will be done at later stages (21-23) to achieve better survival for long incubation periods (4-6 days) following the injection/electroporation procedure. The implications of this experimental timing are bias towards post pronephros initiation events. Since development proceeds from anterior to posterior, analyses of more posterior somites at later stages may reveal results similar to anterior somites at earlier stages. We are aware this may result in examination of the mesonephros domain. However, as observed in our preliminary results (Fig.1K) posterior somites also express nephric genes in their lateral domain, resembling their expression in anterior somites of the pronephric domain. Such relatively late stage experiments may manifest themselves as important developmental understandings relevant to our questions regarding formation of the nephric field. We intend to overexpress constructs of the *Hox4* gene family in anterior somites. These genes are expressed in association with nephric genes (6 somite anterior border) and are affected in RA+/- experiments (Schmidt et al., 2021). We will also use the *cyp26A1* to interfere with the RA synthesis pathway and analyze the full gene repertoire discovered in our transcriptome analysis. The methodology of injection and electroporation of gene constructs in catshark embryos is currently under development. We anticipate that promising results obtained for our dye injections and the wide use of electroporation in chicks in my lab will soon provide positive results. Assuming that the electroporation methodology will encounter technical problems such as salinity of the medium resulting in inappropriate PH and electric conductivity, we will use the injection technique to locally introduce pharmacological agents (a systemic problem when using them in the medium) or directly block gene expression by morpholinos or siRNAs specifically targeting genes of interest. In such experiments scrambled morpholinos or siRNA controls will be used after analyses of antibodies (if available) against the investigated gene to assess the procedure. These experiments are expected to elucidate essential aspects of the regulation of nephric gene expression domains, initiation of the IM and establishment of the nephric duct.

**D1d. Budding from the somite in catshark: EMT and MET or evagination by proliferation?**

How is the IM established, and how is the nephric duct formed? Our preliminary results indicated that IM cells and the nephric duct originate from ventrolateral somitic domains (Figs. 1,2). Initial contradictory observations indicated the formation of a Pax2/Lim1 positive somitic domain by evagination (Fig. 1G), whereas a ball of cells emanating from the epithelial somite was observed in other sections (Fig.2D,E). To clarify the precise mechanism by which somitic cells reorganize to form the nephric duct, detailed expression patterns of markers for three potential cell processes (EMT, MET, evagination by cell proliferation) will be performed at several developmental stages. For EMT and MET we will examine the expression of the crucial genes *snail*, *E-cadherin, N-cadherin, Vimentin, Laminin* and *c-Met*66;67 by either *in situ* RNA localization or immunostaining. To understand evagination we will examine cell proliferation markers such as proliferating cell nuclear antigen (PCNA) and changes in extracellular matrix composition. Should clear indications be obtained regarding the process, gain and loss-of-function experiments (depending on available methodologies) will be performed to assess the role each gene plays in IM and nephric duct development. Moreover, identified genes will be evaluated in other gain and loss-of-functions experiments proposed in the previous section.

**D2. Discovering the nephric regulatory gene network**

**D2a. RNA-seq**

We propose to discover novel genes, gene combinations and molecular pathways in early IM cell specification and PMF formation using catshark. According to our preliminary results (C2) this model organism reveals an unexpected IM origin from a specific ventrolateral domain within the epithelial somite. RNA-Seq analyses comparing two regions (anterior vs. posterior) along the AP axis at four developmental stages revealed 522 differentially expressed genes. Among these were 43 GO enriched genes with significant profiles that may advance our understanding of the establishment of the nephric system. Indeed, validation of some of these genes revealed expression patterns strongly associated to pronephros formation (Fig. 6). Validation analyses of all 43 genes will be done by WMISH. Genes expressed in somitic domains, especially in the ventro-lateral domain, budding nephric duct or nephric mesenchyme, will be investigated further. Previously validated genes (Fig. 6) plus the 43 new ones whose expression fulfills the criteria mentioned previously will be cloned in chicks and lamprey. Their expression patterns by WMISH will then be examined. Based on careful literature searches and genomic databases such as String and Enrichr,we will select genes for further analyses and experiments. For example, the complete ORF of chick *Uncx,* which presented an unknown expression pattern in the pronephros will be cloned into an expression vector and overexpressed in chick IM2 at early stages following analyses of nephric and somitic markers. In catshark and lamprey this gene and several other selected ones will be analyzed following pharmacological experiments.

**D2b. Tomo-seq**

To discover genes related to pronephros formation in a forthright manner, we will apply RNA tomography (tomo-seq) methodology to spatially resolve genome-wide expression. This method will complement and advance the RNA-Seq results from the previous section. We will apply Tomo-Seq to produce a genome-wide 3D atlas of gene transcription in the embryonic domain that extends from somite level 5 to somite 11-12 in stage 19 catshark embryos. At this stage from somite 6 (mature) to 12 all developmental events related to pronephros formation occur. Tomo-Seq has been successfully applied in the Mazan lab to catshark head explants in stage 17 embryos. The protocol used will therefore be adapted from Mayeur et al. (2021). A 3D model of the catshark embryo will be constructed by imaging of stage 19 samples by LSCM. A 3D binary mask was built using Fiji plugin to ImageJ to obtain a binary image of the tissue. This image was oriented transversally usingn the Interactive Stack Rotation plugin and resized to match sectioning planes and section numbers along all three planes. For generation of transcriptome data, embryos at stage 19 were cryosectioned in three dimensions (sagittal, frontal, transverse) and total RNA was extracted from each frozen section. Following Illumina RNA-Seq library construction, sequencing will be conducted on the DNB-seq platform (BGI). Reads will be mapped to the reference database of predicted gene models from the recently released catshark draft genome. From this data we will calculate the quantification, normalization and expression along each of the three axes. We will then extract genes that exhibit regionalized expression patterns essentially described in Mayeur et al. (2021). The final genome-wide 3D transcription profile will be analyzed to detect genes and pathways related to IM budding and nephric duct formation.

**D2c. Analysis of selected genes**

**D3b2. Gain and loss-of-function experiments for selected genes**

WMISH validation will be applied to selected genes that exhibit expression patterns that correlate (overlap or complement) with various developmental events (as described in Preliminary Results) and participate in IM and pronephric duct formation. These genes will be subjected for further experimental procedures. We will use a range of methodologies in catshark and chick embryos (according to availability in the animal model) to investigate the role of each gene in IM specification and pronephros formation and the relationships between the genes and morphological changes. Our methodologies will including siRNA/morpholino, electroporation of expression constructs and chemical inhibition of signal transduction pathways .

All live animal experiments will take place in the lab of Sylvie Mazan in France, where the animals are kept. All cloning and analyses as well as chick experiments will be done at the University of Haifa.

**Resubmission letter for ISF 1364/20**

**General**

The new proposal differs from the previous one in several aspects.

1. The abstract of the previous proposal was enhanced significantly based on our newest results. In addition, the project now focuses mainly on rationale which was probably not clear enough.

2. Aim and its related experiments in section of the previous proposal were completed and an article describing the findings and their implications was submitted for publication (see also a paragraph in the Scientific Background section of the current proposal).

3. Due to concerns of reviewers (mainly reviewer No. 4) regarding the scRNA-Seq proposed in the previous proposal, we replaced this method with more suitable Tomo-Seq RNA tomography of the investigated tissues which should reveal new genes *in situ* that participate in our processes of interest. This methodology has several advantages that are described in the Detailed Research Plan section.

4. Although potentially significant results were obtained with *Hox* construct xenoplastic experiments (aim…and research plan section…in the previous proposal) this Aim was omitted from the new proposal. These promising results will serve as part of another project designed to answer questions regarding the *Hox* regulatory network.

5. RNA-Seq which **was not** included in the previous proposal was successfully completed, and these results are described partially in the Preliminary Results section. With the genes emerging from this screen we plan to discover significant parts of the gene network controlling pronephros and nephric duct formation.

6. Based on the latest analyses of new genes, the new proposal is focused on the cyclostome-to-gnathostome transition with some reference to cephalochordates. This reduces the role of amphioxus and leaves it for another project specified in this model.

In the last year several parts of our previous proposal were successful and many new results were obtained. While validating our hypotheses and concepts, many parts remain to be accomplished and several new avenues are now open for further investigation.

**Specific answers:**

**Reviewer No. 1**

"A final aim of the proposal is to perform single cell RNA transcriptomic using the catshark model,... This technique is very promising. Yet, it presents several challenges

from cell-dissociation protocol, definition of zones, depth and quality scRNA-seq, analysis of data. For these reasons it would be advisable to advance its application in the schedule."

The scRNA-Seq methodology proposed in the previous proposal raised multiple concerns from several reviewers. In this proposal, we switched to new RNA screen that is straightforward and more appropriate to our system and questions. This new method, 3D tomography RNA-Seq, was already in use in the lab of our collaborator in France (Sylvie Mazan), and an article describing its results in the head region was just accepted for publication (Mayeur et al., 2021). We believe that this method will reveal new genetic components *in situ*.

"The experimental approaches to be used for loss of function experiments (i.e. morpholino or

CRISPR-Cas9 system) in lamprey and catshark are not detailed."

Indeed, the methodologies for loss-of-function experiments are still under development in these two model systems. The injection technology in catshark was improved, but the main caveat is the survival of embryos. In the last year we made significant advances and improved the method such that we are able to keep embryos in development for another 4-5 days.

**Reviewer No. 2**

"The application is highly ambitious, proposing to examine gene expression across several organisms, examine the effects of Hox and RA on patterning, followed by other GOF and LOF experiments, cell dynamics and single cell-seq analyses."

I agree with the reviewer comment. Indeed, this is a very challenging project in many aspects. The chosen model organisms lack molecular data on pronephros formation (besides some gene expression patterns without functional data) and mainly lack methodologies for such experiments. Despite this we were able to generate important findings that are now under submission for publication. Furthermore, our preliminary data (especially the transcriptome analyses and validation, Figs. 5,6) provides further evidence supporting our ability to successfully proceed with this project and to significantly extend our data regarding the genes involved in early development of the nephric system. We are indeed behind in terms of specific, well controlled gain and loss-of-function experiments in these models organisms. However, our progress with local injection into biological materials at specific developmental stages indicates that this problem may soon be solved.

"It may be that a reduced scope is better suited at these relatively early stages of the investigation."

In the new proposal we reduce the use of the amphioxus only to expression patterns of newly discovered genes for clues regarding their involvement in regulation of the nephric system/Hatschek's nephridium in early chordates. The new proposal is focused on the cyclostome-to-gnathostome transition which appears to be crucial for the development of the nephric system in vertebrates.

**Reviewer No. 3**

"In section D1c, plasmids incorporating "full hox genes" with a linked reporter will be introduced into embryos and their effect on ectopic expression of nephric genes will be assessed. Does this indicate that hox genes will be under the control of their own promoters? If so, would there be reason to expect expression outside of their own domains? More detail on the exact design of transgenes would clarify the proposal."

This is an important question that we clarify in the new proposal. Essentially, all gene constructs we prepare are under a constitutive promoter (usually CMV) and following IRES we enter GFP or RFP as reporter gene. Electroporation of any such construct to a desired location will eventually cause ectopic or overexpression of the gene.

"The weakness of the proposal is the descriptive nature of many of the proposed experiments using known markers of kidney development on different organisms. While descriptive, it can be argued that the experiments are necessary to provide a foundation for the novel manipulations using ectopic Hox gene expression and alteration of signaling pathways. Also, much of the initial descriptive experiments have been done (preliminary data) and it is anticipated that the project could quickly progress to prioritizing mechanistic studies of kidney gene regulatory networks."

Indeed, in the last year or two some of our work entered the stage of functional experiments. This is the nature of the submitted paper and also the beginning of other pharmacological experiments that are different than the RA+/- experiments (the submitted paper).

**Reviewer No. 4**

The reviewer raised important concerns which helped to focus the current proposal and to better explain the rational.

Answers to specific comments:

"important recent literature on the evolution of mesoderm among chordates (e.g. Prummel et al 2019; Aldea et al 2019) is absent form the proposal."

Indeed, these are important articles that were cited and incorporated into the new proposal.

"The approach proposed in the project is a classic comparative evolutionary developmental biology approach and as such, it is not particularly innovative. Also, the project will not lead to new methodologies or tools that could be exploited by other researchers."

First, "comparative evolutionary developmental biology approach" is indeed not innovative but why is this important if the project is fruitful and leads to discover of new genes, pathways and a basic understanding of the system? Second, this comparative evolutionary developmental approach is not in common use for molecular biology and especially xenoplastic experiments. The late are actually extremely rare. Innovation of methods is not the objective of the proposal. An old methodology, when suitable to answer well defined scientific questions, can reveal new knowledge and advance a field. This is the case for our two proposals. A manuscript that presents a completely new understanding of evolutionary transitions and trajectories was just submitted for publication. Promising new results came from the previous proposal leading to the current one. Furthermore, in the second part of the previous proposal scRNA-Seq we proposed to reveal the progenitors, new genes and molecular pathways involved in budding and nephric duct formation. For several reasons, we changed the RNA screen from scRNA-Seq to 3D tomography RNA-Seq in the new proposal which is more adequate. The methodology of Tomo-Sseq is absolutely the cutting-edge in the field. In the previous and the current proposals new methodologies are proposed based on promising results from local injection of materials into catshark embryos. The positional injection of pharmacological agents, morpholinos or siRNAs will significantly change the use of the catshark model in developmental experiments. Moreover, we propose to electroporate gene constructs into catshark embryos and to advance this method as well. Taken together, our scientific interest in the catshark and lamprey models represent their key positions in the vertebrate tree. Our results have already revealed significant new insights into the understanding of the development and evolution of the nephric system and provided advanced methodologies in the catshark model at a minimum.

"it is not clear what specific gaps in the literature the author wants to fill;"

I wonder about this concern since this reviewer well understands the goals of the proposal and earlier in his review wrote: "There is not so much information on the molecular mechanisms driving nephrogenesis outside vertebrates; therefore, a broader study covering additional taxa with important phylogenetic positions is necessary. The proposed project aims to fill the gap of knowledge by exploring chondrichthyes (catshark) as an outgroup to Euteleostomes, lampreys as an outgroup to gnathostomes and amphioxus, as an outgroup to vertebrates.

The choice of the experimental model systems is appropriate for addressing evo-devo

questions and possibly suitable for the dissection of the conserved and not conserved

regulatory mechanisms defining kidney development in chordates.

These are the gaps we aim to fill. This was very clear in the previous proposal, and I guess very clear in the current one too.

"- the methodology proposed is not particularly innovative, pointing rather to a low risk, low gain project"

I completely disagree with this statement. RNA-Seq methodologies, if done in the appropriate way (choice of tissues, timing or comparable conditions +/- factors of interest) and professionally analyzed can potentially be a "high gain project". Our preliminary results in this current proposal are extremely fruitful and demonstrate exactly this point (Preliminary Results Fig. 5, Fig.6).

"- the amphioxus system could be better exploited and the author does not really go deep in the state of the art and possible interesting questions that could be addressed using this system."

The amphioxus model is a great early chordate organism and proved to be excellent in revealing and explaining vertebrate traits. To be more precise and effective in the new proposal, this model will serve mainly descriptive purposes to examine expression patterns of newly chosen genes that emerged from the RNA screens for the purpose of getting a chordate perspective. Unfortunately, no experiments will be done to explore the role and function of these genes in this model. This will allow the project to focus on the cyclostome Gnathostomata transition and to gain a deeper understanding of pronephros and IM formation in early vertebrates and amniotes (chick embryos).

**Reviewer No. 5**

This reviewer, unfortunately, criticized the proposal in a very shallow way without specific arguments. In such a case I cannot seriously answer his comments.