Embryonic expression of endothelins and their receptors in lamprey and frog reveals stem vertebrate origins of complex Endothelin signaling

Tyler Square1, David Jandzik1,2,†, Maria Cattell1,‡, Andrew Hansen1 & Daniel Meulemans Medeiros1

Neural crest cells (NCCs) are highly patterned embryonic cells that migrate along stereotyped routes to give rise to a diverse array of adult tissues and cell types. Modern NCCs are thought to have evolved from migratory neural precursors with limited developmental potential and patterning. How this occurred is poorly understood. Endothelin signaling regulates several aspects of NCC development, including their migration, differentiation, and patterning. In jawed vertebrates, Endothelin signaling involves multiple functionally distinct ligands (Edns) and receptors (Ednrs) expressed in various NCC subpopulations. To test the potential role of endothelin signaling diversification in the evolution of modern, highly patterned NCC, we analyzed the expression of the complete set of endothelin ligands and receptors in the jawless vertebrate, the sea lamprey (Petromyzon marinus). To better understand ancestral features of gnathostome edn and ednr expression, we also analyzed all known Endothelin signaling components in the African clawed frog (Xenopus laevis). We found that the sea lamprey has a gnathostome-like complement of edn and ednr duplicates, and these genes are expressed in patterns highly reminiscent of their gnathostome counterparts. Our results suggest that the duplication and specialization of vertebrate Endothelin signaling coincided with the appearance of highly patterned and multipotent NCCs in stem vertebrates.

Neural crest cells (NCCs) are multipotent migratory embryonic cells that give rise to an array of adult tissues and cell types, including parts of the heart, peripheral ganglia, pigment cells, and much of the head skeleton1. Because NCCs are unique to vertebrates, and form a variety of vertebrate-specific derivatives, the origin and evolution of NCCs is considered a major facilitator of vertebrate diversification and success2–4.

The NCCs of all living vertebrates are divided into distinct subpopulations with unique migration routes and developmental fates5,6. Modern NCCs presumably evolved step-wise from a more homogenous population of migratory neural tube cells with limited developmental potential7,8. When and how NCCs acquired their multipotency, patterning, and stereotyped migration routes, is unclear.

In modern jawed vertebrates, Endothelins are key regulators of NCC differentiation, migration, and patterning9–19. Gnathostomes possess multiple (2–6) endothelin receptors (ednrs)20 as well as multiple (3–6) endothelin ligands (edns)21. Phylogenetic analysis places the ligands into four paralogy groups21: edn1, edn2, edn3, and edn4, while the receptors form three groups20: ednra, ednr1b1, and ednr2b2. Importantly, most modern vertebrates possess subsets of these (discussed below).

1Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309, USA. 2Department of Zoology, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, 84215, Slovakia. †Present address: Department of Zoology, Charles University, 128 44 Prague, Czech Republic. ‡Present address: Department of Pediatrics, University of Colorado Denver, Anschutz Medical Campus, Aurora, Colorado 80045, USA. Correspondence and requests for materials should be addressed to T.S. (email: square@colorado.edu) or D.M.M. (email: daniel.medeiros@colorado.edu)
Endothelin ligands are translated as ~200 aa (amino acid) polypeptides (Preproendothelins) that are then processed into short, and invariably 21 aa signaling ligands. This occurs as Preproendothelins obtain disulfide linkages between cysteine residues 1 and 15, and 3 and 11 (present on all known Edn ligands), and lose their amino and carboxyl termini via cleavage by a furin endopeptidase, making an intermediate product known as “Big Endothelin”. Big Endothelins are subsequently trimmed on the carboxyl end again by an Endothelin converting enzyme (ECE)22-24, leaving the 21 aa polypeptide. In jawed vertebrates there are two main groups of ECEs, ECE-1 and –2, which have unique biochemical properties25 as well as unique developmental roles in mouse26.

Despite these differences, both ECE-1 and ECE-2 have a similar hierarchy of Big Endothelin cleavage rates in vitro (Edn1 \( \gg \) Edn2 \( > \) Edn3)27 (Edn4 has not been assayed in this context). Once fully processed and secreted by the cell, mature Edn ligands interact with the various Ednrs to drive cell fate decisions and provide positional information to NCCs16-18. In cell culture assays, each Ednr appears to have a unique set of binding affinities for the different mature Edn ligands27-29, though the early developmental relevance of these differences remains unknown.

The first role described for Endothelin signaling was in vasconstriction and circulatory development30-33. Subsequently, Endothelin pathways were found to perform a variety of functions in NCC including cell fate determination, migratory pathfinding, and patterning of cranial NCC. Specifically, the Edn1/Ednra pathway works to pattern skeletogenic cranial NCC into distinct skeletal progenitor populations along the dorso-ventral axis of the pharynx, thereby positioning the jaw joint, and also has a function in cardiac NCC development34,35,36. In non-skeletogenic NCCs, the Edn3/Ednrb pathway is necessary for pigment cell specification and migration10,13,18, as well as enteric neuron development16,19. edn2, though present in all major vertebrate lineages, does not have known expression in early development to our knowledge. edn4 is retained only in some lineages of ray-finned fishes21, and its expression and function in early development is unknown. Interestingly, ednrb2 has been lost in both zebrafish and therian mammals30, despite this receptor’s importance in NCC-derived pigment cell development in other vertebrates13,18,37.

When the various roles for endothelin signaling in vertebrate NCC development evolved is unknown. ednrs appear to be unique to chordates, and are not found in the genomes of protostomes or non-chordate deuterostomes20; no tunicate genome appears to contain any edn- or edr-like genes20,21. Amphioxus (Branchioptera floridaelae) also lacks any Edn ligands20,21, though it possesses a single ednrtl-like gene that is not expressed during early embryogenesis (unpublished results, ref. 38). The Japanese lamprey, Lethenteron japonicum, a jawless vertebrate, possesses at least one ednr and six putative edn ligands39, suggesting that at least a simple form of Endothelin signaling (i.e. a single receptor with its peculiar ligand affinities) arose before the common ancestor of jawed and jawless vertebrates.

After reviewing available genomic assemblies and multiple transcript assemblies from adult and larval tissues, here we identify and characterize the expression of what is likely the complete set of ednrs and edn ligands in the sea lamprey, Petromyzon marinus. We find dynamic embryonic and larval expression of two ednrs which are likely orthologous to gnathostome ednra and ednrb. We also identify six P. marinus edn ligand genes, four of which are expressed in temporally-spatially restricted patterns in embryos and early larvae, with a fifth transcribed diffusely throughout the animal. We then use comparisons with Xenopus laevis ednra and ednrb expression to assign tentative functional overlap between some lamprey and gnathostome Endothelin receptors and ligands. Our results suggest that at least one edn histidine occurred prior to the divergence of cyclostomes and gnathostomes, and that sophisticated NCC patterning by duplicated and sub- or neofunctionalized ednrs and edns likely evolved in stem vertebrates. We posit that the deployment of Ednra signaling in NCC, followed by duplication and divergence of Edn signaling components, paved the way for fine-tuned control over NCC fate determination, migration, and patterning in modern vertebrates.

Methods
Gene cloning and sequence acquisition. Polymerase chain reactions (PCRs) were performed according to standard protocols using the primers listed in Table S1. Fragments corresponding to P. marinus ednA, ednC, ednE, ednra, and ednrb were amplified from a 5’ RACE library made from combined cDNA. These genes were named based on their deduced orthology to L. japonicum sequences (see below) except ednrb, which has not been identified in L. japonicum to our knowledge. Notably, part of the ednrb sequence fragment we cloned via RACE had previously been identified20 in the 2007 P. marinus genomic assembly on contig56749, though only ~350 nt of this sequence is represented there. Unfortunately, this contig is only ~4.9 kilobases long, and does not contain any other predicted coding sequences that could be used to determine synteny. This sequence is absent from the 2010 P. marinus genomic assembly. Using the nucleotide sequences published for the Japanese lamprey20 as a reference, fragments corresponding to the putative P. marinus ednB, ednD, and ednF transcripts were amplified from genomic DNA or cDNA by designing primers against sequenced transcripts20. In the case of ednD, our P. marinus gene fragment appears to be completely situated within the 3’ UTR. Similarly, seven P. marinus metalloprotease transcript fragments (with sequence similarity to gnathostome ECEs) were identified in transcriptome assemblies derived from adult40 and combined adult and larval tissues (unpublished data). All sequences have been deposited to GenBank (see Table S2 for accession numbers).

As is typical within the tetraploid X. laevis genome, we found two duplicates of each edn and ednr gene, except in the case of ednrb2, for which there appears to be three copies (a: NM_001086238.1; b: NM_001085878.1; c: KU680750). edn1-b, edn3-a, ednra-a, ednrb1-b, ednrb2-a, and ednrb2-b were previously described and/or sequenced11,18 (see accession numbers in Table S2). The riboprobe template for X. laevis ednra-b was a gift from the Mayo Lab; the rest of these transcripts were subcloned from gDNA or cDNA using available sequence information. Using X. laevis genomic assemblies 7.1-8.0 (www.xenbase.org), we identified and cloned fragments of ednr1-a, ednr2-a, edn2-b, edn3-b, ednrb1-a, ednrb1-b, and ednrb2-c out of genomic DNA or cDNA. These novel edn and ednr
sequence fragments have been deposited to GenBank (see Table S2 for accession numbers). ClustalW alignments with translated sequence fragments, and subsequent ML trees confirmed the identity of these Xenopus sequences.

**In situ hybridizations, cryosectioning, and imaging.** Using riboprobes generated from the amplified gene fragments described above, in situ hybridization (ISH) was performed as previously described\(^41,42\). Developmental staging for lamprey followed Tahara, 1988, while *X. laevis* staging followed Nieuwkoop and Faber, 1994. For simplicity, we use “st. 33,” “st. 35,” and “st. 37” to describe the *X. laevis* developmental windows usually referred to as “st. 33/34,” “st. 35/36,” and “st. 37/38,” respectively. Interestingly, riboprobes designed against the different *X. laevis*-specific gene duplicates of ednra (ednra-a and ednra-b) and edn2 (edn2-a and edn2-b) displayed differential staining despite high sequence similarity and regional overlap of the probe binding sites, as previously noted for other *X. laevis*-specific gene duplicates\(^42\) (see below). Cryosectioning and imaging was performed as previously described\(^42,43\).

**Phylogenetic reconstruction.** To assign the orthology of the *P. marinus* edn ligand fragments described above to those previously published for *L. japonicum* edn genes, a ClustalW nucleotide alignment was constructed using only endothelin ligand DNA sequences derived from both lamprey species. The Maximum Likelihood (ML) method of phylogeny reconstruction was employed in MEGA6 thereafter (Fig. S1). The *P. marinus* genes were named corresponding to their orthology with their closest *L. japonicum* relative, for which all ortholog pairs show strong support in a bootstrap analysis.

In an attempt to address the identity of the lamprey Endothelin signaling components amongst all vertebrate Endothelin ligands (save EdnB and EdnD), Endothelin receptors, and Endothelin converting enzymes, ClustalW protein alignments were built using inferred aa sequences derived from transcript or genomic data, and thereafter used to build ML trees (Figs S2, S3 and S4). For the Edn ligands, an ML tree was first built by using aa sequences derived from a variety of gnathostomes (specifically excluding those that were missing the conserved region containing and surrounding the functional ligand), testing many parameters for the aa alignment and ML tree preliminarily. Given the heterogeneity in sequence conservation across Edn aa sequences, these trees were sometimes structured very differently from each other when different parameters were employed, as reflected by the range of bootstrap values and overall tree topology in Fig. S2. We thus selected parameters that would maximize our tree to reflect the accepted relationships of (1) vertebrate taxa, and (2) the syneteny analysis on edn genes performed previously\(^21\) which supported edn1/3 and edn2/4 clades. We then added *P. marinus* aa sequences to the dataset, realigned, and rebuilt the tree, bootstrapping it 100 times using these gnathostome-optimized parameters (*P. marinus* EdnB and -D, and *L. japonicum* EdnB, -D, -E, and -F were discounted from this analysis due to their incomplete nature). An Endothelin receptor ML tree was similarly constructed, using amphioxus Ednr-like as an outgroup (Fig. S3). To address the identity of putative *P. marinus* ECEs and other related metallopeptidase\(^44,45\) genes, a phylogeny was built using the inferred translations from each of seven sea lamprey M13 family metallopeptidase transcripts, along with aa sequences for these genes from an assortment of deuterostomes, using three LTA4H sequences as an outgroup (an M1 family peptidase\(^46\); Fig. S4).

**Results**

**Cloning and sequence analysis of endothelin pathway genes.** In order to understand the relative timing of the appearance and diversification of Endothelin pathway gene groups, we gathered available sequences from a wide range of deuterostomes (mainly gnathostomes). No gene with moderate sequence similarity to an Edn has been identified in any invertebrate by our own, or previous analyses\(^20,21\), however ECE-like1 metalloproteases do appear to exist in amphioxus and uurchin (Fig. S4), and an amphioxus edn-like gene has been previously identified\(^38\). It is important to note that ECE-like1 (also known as X-converting enzyme, XCE) is a distinct group of proteins found throughout deuterostomes, and is closely related to, but separate from the clade of proteins containing gnathostome ECE-1 and ECE-2 (Fig. S4). Conversely, the single *Ednr*-like gene in amphioxus appears to be a true ortholog of vertebrate *Ednrs*\(^35\); there is no known clade of “edn-like” genes in vertebrates.

The ML trees generated here fail to strongly support strict orthology of lamprey and gnathostome Edn ligands and ECE peptidases (Figs S2 and S4). Interestingly, Sea lamprey EdnA clusters with the tetrapod Edn1 group with moderate bootstrap support (71%), however in this analysis, the ray-finned fish Edn1 sequences were excluded from this group. Conversely, *P. marinus* Ednra and Ednrb cluster separately with their putative gnathostome orthologs, with moderate bootstrap support (59% and 58% respectively; see Fig. S3). Thus while a parsimonious view would suggest that these two lamprey receptors do belong to these two main groups of Ednrs, it remains possible that one or both of these lamprey Ednrs are actually duplicates that were lost in gnathostomes, which would make them a unique Edn subtype. Sea lamprey appear to possess five ECE genes, which we have named ECE-A, -B, -C, -D, and -E. Notably, ECE-A and -B cluster with the gnathostome ECE-1/2 clade with high bootstrap support (see Fig. S4), whereas ECE-C, -D, and -E have low support, and thus these latter three independent duplicates might not actually code for enzymes capable of processing Edns. We have named them as such simply because they show the strongest sequence similarity to ECEs. All accession numbers corresponding to these sequenced transcripts can be found in Table S2.

**Expression of *P. marinus* endothelin signaling components.** We assayed the expression of *edn* and *ednr* transcripts at stages 15, 17, and 21–28 via in situ hybridizations. This developmental series extends from mid-neurulation until the initial differentiation of the head skeleton. The expression of *L. japonicum* *ednA*, *ednC*, *ednE*, and *ednra* was previously described at mid-pharyngula stages\(^39\). While the expression patterns of these genes in these two lamprey species are generally similar, our analysis revealed additional expression domains not apparent in *L. japonicum*. These are detailed below.
Expression of ednA-F. ednA expression was first detected in ectoderm surrounding the forming mouth (stomodeum) weakly at st. 22, and more strongly at st. 22.5 (Fig. 1A). This expression domain was also found in *L. japonicum* 39. This ectoderm is fated to eventually cover the nasohypophyseal plate, contributing to the epithelium of the nostril 47. At st. 23.5, expression around the mouth appeared in a more distinct "comma" shape, and expression in the anteriormost dorsomedial pharyngeal arches (PAs) was first detected (Fig. 1B). Through st. 25.5, ednA expression in the pharynx expanded to the posteriormost PAs, while transcription became reduced around the mouth and in the anteriormost PAs (Fig. 1C). Sectioning at this stage revealed expression in both the center of the posteriormost seven PAs (in the mesodermal 'core', Fig. 1C') as well as the ectoderm overlying these PAs. At st. 26.5, expression was reduced to a small patch of mesoderm within each of these PAs (Fig. 1D). This expression persisted until st. 27.5, also reappearing in the region of the dorsal PA1 (not shown).

ednB expression was detected weakly in the upper lip, ear, heart, and somites at st. 25.5, and later in the brain of st. 28 larvae (Fig. S5A,B). ednC expression was first strongly detected at st. 21.5 along the flank in the ectoderm, and weakly in a medial spot within the anterior brain and in the ectoderm overlying lateral portions of the head (Fig. 1E). By st. 23, the ectodermal flank expression largely faded, while the brain expression expanded, and the expression flanking the head shifted more ventrally around the stomodeum to occupy a similar region as ednA transcripts (compare Fig. 1B' to F'). Deeper mesenchymal expression in the posterior future PAs was also first detected at st. 23 underneath the faded flank expression (arrows in Fig. 1F). At st. 25.5, expression around the mouth shifted to mesenchyme of the lower lip, but still remained in tissues on the lateral sides of the mouth (Fig. 1G); only this lower lip expression was previously characterized in *L. japonicum* 39. Sectioning revealed that all expression at st. 25.5 was mesenchymal (Fig. 1G'). At stages 26.5 (Fig. 1H) and 27.5 (not shown), the posterior PA expression was progressively diminished, and the more anterior expression around the mouth and in PA1 became progressively
refined. 
ednD
expression was not detected at any embryonic or larval stage assayed here. 
ednE
transcripts were first visualized weakly at st. 22 in head ectoderm (not shown), and more robustly in the st. 23 head ectoderm and along the boundary between the anterior yolk and the somites (Fig. 1I). Up to st. 25.5, this superficial PA expression in the head proceeded in an anterior to posterior fashion, and was only present in the most posterior PA by st. 28 (Fig. 1J-L; st. 28 not shown). At st. 25.5 expression began in deeper mesenchymal tissues within the PAs (Fig. 1K), and in a horizontal stripe dorsal to the PAs, presaging pigment deposition (Fig. 1K); this latter expression domain was previously described in L. japonicum. 
ednF
dexpression was detected at st. 25.5 diffusely throughout the entire head, but by st. 28 was mainly expressed in the brain (Fig. S5C–F).
the eye from st. 35–37, and within the eye. Interestingly, our future pronephros from st. 30–37 (Fig. S6G), subsequently in dorsal pharyngeal mesenchyme surrounding each region out of embryonic cDNA. Thereafter, riboprobes were designed mainly against a portion of the 3'UTR of each specimen is indicated in the bottom right corner of a panel. (A–D) edn1-b expression in X. laevis marks ectoderm around the nasal placodes, and ventral non-NCC mesenchyme and epithelia in the pharyngeal arches. (E–H) edn3-a expression in X. laevis marks ectoderm where future ednr2b-positive cells will migrate. All scale bars represent 100μm, save those in E and G which represent 500μm.

**Expression of P. marinus ednra and ednr.** P. marinus ednra was first detected in a small paired patches of anterior mesoderm at st. 21 (Fig. S5G,H), and more robustly at st. 22 (Fig. 2A). At this stage, expression also began at the base of the pharynx where the heart will eventually develop (arrowhead in Fig. 2A). These expression domains were maintained until st. 27. ISH revealed ednra expression in late migratory and post-migratory skeletogenic NCCs in the head beginning at st. 24, first staining the ventralmost NCCs (future mucocartilage), as well as the upper and lower lips, and the more anterior PAs (Fig. 2B). PA expression thereafter proceeded to initialize in an anterior to posterior wave (Fig. 2B–D). By st. 26.5, this NCC expression was found throughout all PAs (Fig. 2D,D'). At st. 26.5 ednra was also detected in nascent lateral plate mesoderm (arrows in Fig. 2D), similar to expression of lamprey Lbx-A48. ednra transcription was maintained throughout st. 29, strongly marking the future head skeleton as cartilage began to differentiate (Fig. 2E). Most of these expression domains have also been observed in the Japanese lamprey (Fig. 2F). By st. 22, these ednrb-positive cells in the head were migrating ventrally towards their destinations in the pharynx (Fig. 2G), while the majority of those in the trunk were still poised at the dorsal neural tube (arrows in Fig. 2G). This migratory NCC expression continued through st. 24, becoming progressively extended ventrally and more diffuse as more NCCs had begun their migration (st. 23 shown in Fig. 2H). ednrb transcripts at st. 25.5 were detected around the mouth and in pre-skeletal NCCs in the PAs (Fig. 2I,1'), as well as future pigment cells (arrowhead in I) and peripheral nervous system components scattered along the lateral sides of the head, anterior yolk, and somites, including those that resemble dorsal root ganglia (arrows in Fig. 2I). At st. 26.5 expression in the pharynx was largely lost, though the expression in pigment cells was still apparent (not shown). Bleached larvae revealed this expression persists in melanophores until at least st. 28, being found in cells on the dorsal ridge and flank of the animals (st. 27 shown in Fig. 2J).

**Expression of X. laevis endothelin signaling components.** We performed ISH for all edn and ednr genes spanning st. 17 to st. 40, (mid-neurulation to the onset of head skeleton differentiation). Except in the cases of edn2 and ednra, all expressed X. laevis-specific gene duplicates analyzed here were discovered in completely overlapping domains at all stages assayed. We failed to clone the X. laevis duplicate ednrb-2-b.

**Expression of X. laevis edn1-3.** X. laevis edn1 was first detected at st. 19 in two patches of ectoderm just above the future stomodeum, overlying the developing telencephalon (Fig. 3A; see Fig. S6A–C for st. 28 sections). This expression persisted until st. 33, eventually becoming restricted to the ectoderm surrounding each nasal placode. At st. 26 we first observed edn1 transcription within the ventral PAs, beginning in the region of the ventral hyoid stream. At later stages, expression of edn1 spread posteriorly, then anteriorly to the rest of the ventral PA endoderm, mesoderm, and ectoderm, but not NCC-derived mesenchyme (Figs 3B–D and S6D–F). By st. 37 edn1 was detected ventrally in each PA (Fig. 3D). To assay the expression of edn2-a, first a probe corresponding to 387 nt of coding sequence was amplified from cDNA (“cds probe”). This probe produced signal in many different tissues in the head, though this was found to be largely background staining in the brain cavities and in the ear, and possibly also in the notochord (Fig. S6H,1). Sectioning revealed that mesenchymal staining within the eye and surrounding the eye at st. 35 and 37 was within cells. We were unable to clone a fragment of the identified edn2-b coding region out of embryonic cDNA. Thereafter, riboprobes were designed mainly against a portion of the 3'UTR of each edn2-a (645 nt) and edn2-b (684 nt), also including the last ~70 bases of coding sequence (“3' UTR probe”). These were amplified from genomic DNA. Using these UTR probes, edn2-a, but not edn2-b was detected within the future pronephros from st. 30–37 (Fig. S6G), subsequently in dorsal pharyngeal mesenchyme surrounding the eye from st. 35–37, and within the eye. Interestingly, our edn2-a 5' UTR and cds probes produced different staining patterns, despite having been designed to detect the same transcript. This may reflect differences in splice
variants of the edn2-a gene. edn2-b transcripts were never detected. edn3 expression was first seen at st. 23 surrounding in the nasal placodes, along the flank, and in the anterior trunk of the neural tube (Fig. 3E). Expression along the flank expanded and was maintained in lateral plate mesoderm surrounding the pronephros through st. 37, and ceased thereafter (Figs 3E–H and S6K). ISH signal around the dorsal neural tube proceeded in an anterior to posterior fashion, and was no longer detected by st. 37. In the head, expression in the nasal placode expanded and was joined by other expression in mesenchyme overlying the border between PAs 1 and 2 and around the eye (Fig. 3G,H).

Expression of X. laevis endothelin receptors. As previously described, ednra-a but not ednra-b expression was detected along the neural plate border during mid-neurulation (Fig. 4A). Thereafter, transcripts from both ednra-a (not shown) and ednra-b (Fig. 4B) genes were detected in completely overlapping regions. Expression was found in the otic placodes, uniformly throughout migratory cranial neural crest at st. 23, and along the flank in migratory trunk NCCs. These trunk NCCs could later be seen positioned between the boundaries of the somites, and aggregating along the future lateral line (Fig. 4C). By st. 31, expression had begun in the heart region, which continued throughout the entire series assayed here (Fig. 4C–E; st. 38–40 not shown). At stages 33–37, transcripts in the PA NCCs were apparently depleted ventrally compared to dorsal mesenchyme (Fig. 4D,E). Transcripts were also detected strongly in the medial fin at stage 40 (Fig. 4F). ednra transcripts were also detected in nephrostomes at stages 30–40 (Fig. 4D–F; arrows in Fig. S6R,S). ednrb1 expression was observed only in the medial PAs from st. 31–40, and in the eye from st. 35–40 (Figs 4H–J and S6L–N; st. 38–40 not shown). ednrb2-a and ednrb2-c were

Figure 4. Expression summary of ednr s in X. laevis. Left-lateral views in all non-prime panels save (A,B), which are dorsal (A) an anterior (B) views. All prime panels show anterior/oral views of the correspondingly lettered panel save K’, which is a dorsal view. Developmental stage for each specimen is indicated in the bottom right corner of a panel. (A–G) ednra expression in X. laevis. (A) ednra-a uniquely marks pre-migratory NCCs. (B–G) ednra-b expression in migratory and post-migratory NCC derivatives, nephrostomes, the heart, and fin mesenchyme. (H–J) ednrb1-a expression in X. laevis marks the eye and medial pharyngeal arch NCCs. (K–M) ednrb2-c expression in X. laevis marks migratory pigment cells, nerves, and the forebrain. All scale bars represent 100 μm, save those in (D,G,K’,L,M) which represent 500 μm.
Table 1. A summary of ednr subfunctionalization in vertebrates. Note: Genes are separated by commas to indicate a temporal change, while a + sign indicates they are coexpressed. For X. laevis, ‘ednra’ indicates that both the ‘-a’ and ‘-b’ copies are coexpressed. All zebrafish duplicates are listed by their specific name. Mouse ednrb is referred to here as ednrb1 since it is an ednrb1 gene (see Fig. S3). DRGs, dorsal root ganglia; NCCs, neural crest cells.

| Migratory, pre-skeletal NCCs | Post-migratory, Pre-skeletal NCCs | Migratory trunk NCCs (non-melanophores) | Post-migratory DRGs | Heart (post-migratory NCCs?) | Migratory and post-migratory melanophores |
|-----------------------------|----------------------------------|---------------------------------------|---------------------|----------------------------|-----------------------------------------|
| Sea lamprey ednrb           | ednrb, ednra                      | ednrb                                 | ednrb               | ednra                      | ednrb                                   |
| Zebrafish ednra1^30^        | ednra1 + ednra2^28^               | ednra1^36^                           | ednra1^26,23^       | ednra1^30^                 | ednra1^26,23^                           |
| Xenopus laevis ednra         | ednra + ednra1 (minor subset)     | ednra                                 | ednra2               | ednra                      | ednra2                                  |
| Chick/quail ednrb1^24^      | ednrb1^24^                       | ednrb1^34^                           | ednrb1^26,23^ + ednrb2^37^ | ednra1^34,37^           | ednra1^24,37^                           |
| Mouse ednra1^27,24^         | ednra1^27,24^                    | ednra1^27,24^ + ednrb1^26,37^        | ednra1^26,37^       | ednra1^24,37^             | ednra1^26,37^                           |

Both detected in pre-migratory and migratory nascent pigment cells, cranial ganglia, the forebrain, and peripheral nervous system derivatives of the trunk (Fig. 4K–M and S6O–Q).

Discussion

Duplication and specialization of Endothelin signaling pathways occurred in stem vertebrates.

To better understand the ancestral roles of the endothelin pathway in vertebrate development, we performed an exhaustive search of lamprey genomic and transcriptome sequences for edn and ednrb homologs. We then determined their expression throughout early development. To facilitate comparisons with gnathostomes, we also characterized and analyzed the embryonic and larval expression of all edns and ednrs in X. laevis. To our knowledge, this is the first report detailing the embryonic and larval expression of all edns and ednrs in a single gnathostome.

Phylogenetic analysis lends weak support to a scenario wherein a single ancestral ednrb gene was duplicated in stem vertebrates, with ednrb later duplicated in stem gnathostomes (Fig. S3). Our data showing similar expression of lamprey and gnathostome ednra and ednrb paralogs supports this, with ednras marking the heart and post-migratory skeletal NCCs, and ednrb1s marking migrating pigment cells, cranial glia, and other peripheral nervous system precursors. Overall, duplication and functional specialization of ednrs is similar to that of other regulators of NCC development, including Sox9, Tgf2, Dlx1, and Id1,2,52, which all have multiple paralogs expressed in NCC. Furthermore, like Sox9, Tgf2, Dlx1, and FoxD5 there is evidence that duplication of ednrs was accompanied by biochemical subfunctionalization and/or neofunctionalization. In mouse, Ednra binds most strongly to Edn1 and Edn2, while mouse Ednrb (a type B1 receptor) binds all Edns with similar affinity 27; in X. laevis Ednrb2 preferentially binds Edn328. Furthermore, rescue experiments have shown that mouse Ednrb cannot rescue the function of Ednra in maxillary skeletogenic cranial NCC, due to the inability of Ednrb to signal through Gq/11 proteins29. Whether these differences reflect the loss of Ednrb functionality or a gain of Ednra functionality awaits testing of amphioxus Ednr, the only invertebrate Ednrb known, and the only appropriate outgroup.

It is unknown if lamprey Ednra and Ednrb have diverged with regard to ligand binding affinities or signal transduction outputs. However, their divergent expression patterns support the hypothesis that distinct Ednra and Ednrb signaling pathways evolved in stem vertebrates. Because of the unique roles different Ednrs play in NCC migration and fate determination, it is tempting to speculate that duplication and divergence of vertebrate ednrs facilitated the evolution of the highly patterned and multipotent NCC of modern jawed and jawless vertebrates.

Given that Ednra cleaving enzymes are responsible for the final processing of endothelin ligands, their appearance was a critical evolutionary step in the advent of the first vertebrate-type Endothelin pathway. In an attempt to understand when the first ECE appeared, we analyzed M13 metallopeptidases from a variety of deuterostomes. Given the available genomic and transcriptomic resources, we were unable to identify coding sequences most closely resembling ECE-1/2 in any invertebrate. However we did find coding sequences resembling the closely related ECE-like1 and Nepriysin in amphioxus (Branchiostoma floridae) and sea urchin (Strongylocentrotus purpuratus), and PHEX in sea urchin only. It seems probable then that the gene duplications giving rise this suite of genes (ECE-like1, Nep, and PHEX) predate the divergence of echinoderms and chordates. Given that recombinant in vitro experiments have shown that ECE-like1 is unable to cleave Big Endothelin 156, and Nepriysin degrades Big Endothelin 1 in a manner that yields no detectable Edn1 ligand57, it seems plausible that the invertebrate genes identified here do not have a role in any type of rudimentary Endothelin ligand processing. Thus, in combination with the apparent absence of any edn-like gene outside of vertebrates22, there is no evidence that true Endothelin ligands or Endothelin converting enzymes exist outside of vertebrates based on deposited deuterostome sequences.

Despite the overall sequence similarity of amphioxus Edn-like to vertebrate Ednrs, this invertebrate Ednrb exhibits many divergent features, including the absence of a highly conserved lysine residue within the 2nd transmembrane domain known to be important for ligand binding20,28,58. While it is possible that invertebrate chordate ednr or ECEs may have been overlooked during genome sequencing or annotation, the available data strongly suggest that early vertebrates greatly expanded on the elaborate biochemistry underlying these pathways. If no modern invertebrate ECE or Edn ligand truly exists, this would suggest that the Endothelin signaling
Figure 5. Lamprey ednA expression is reminiscent of gnathostome edn1 expression, despite low support for strict orthology. Oral/anterior views in (A–C,E,F,H). Left lateral views in (C,D,G,I). Developmental stage for each specimen is indicated in the bottom right corner of a panel. (A–D) X. laevis expression of edn1-b from stage 19 to 33. Dotted outline in A surrounds the cement gland. Arrows in B indicate expression in ectoderm which will eventually contribute to the nostrils (E–I) P. marinus expression of ednA from stage 22.5 to 26.5. Arrows in F indicate expression in ectoderm which will eventually move rostrally to cover the nasohypophyseal plate, contributing to the future medial nostril. In a similar sequence, both genes are expressed in bilateral patches above the future mouth, surrounding the nasal placodes/nasohypophyseal plate, in pharyngeal arch mesoderm and ectoderm, and just below the stomodeum. All scale bars represent 100 μm. cg, cement gland; nhp, nasohypophyseal plate; np, nasal placode; st, stomodeum.

Differences in ednr expression reveals flexibility in the timing and extent of ednr transcription in NCC. While P. marinus and gnathostomes express their ednra and ednrb paralogs similarly, there are some clear differences (see Table 1). For one, lamprey ednra is restricted to NCCs destined for the head, while zebrafish60, X. laevis, chicken41, and possibly mouse17 all express ednra in trunk NCCs. Furthermore, while zebrafish60, Xenopus, and mouse17–24 express ednra in some migrating NCCs, lamprey and chicken60 do not express ednra in NCCs until they have reached their destinations. Whether these differences reflect an expansion of the ancestral ednra expression pattern in gnathostomes, or a restriction of ednra to post-migratory cranial NCC in lamprey and chicken is unclear.

P. marinus expresses ednrb more broadly in NCCs than any gnathostome, with strong expression apparent in the peripheral nervous system, pigment cells, and all skeletogenic NCCs during and briefly after migration. By contrast, Zebrafish62,63 and mouse18,37 restrict ednrb-type expression to pigment cells and peripheral nervous system derivatives, though early zebrafish ednrb expression has not been well described, leaving open the possibility of more widespread ednrb expression in this species. X. laevis expresses ednrb-type receptors in pigment cells, peripheral nervous system derivatives, and a minor subset of post-migratory skeletogenic NCCs. As in lamprey, avian ednrb115,64 appears to broadly mark early migratory cranial NCCs, although it is unclear if this expression persists after those cells reach the pharynx. Based on these comparisons, we speculate that lamprey-like ednrb expression in all, or most, migrating NCC represents the ancestral vertebrate state, with the loss of some expression domains occurring after duplication of ednrb in the gnathostome lineage.

ednrb duplicates in X. laevis highlight divergent subfunctionalization in different gnathostome lineages. Recent phylogenetic analysis suggests that ednrb was duplicated in stem gnathostomes, giving rise to ednrb1 and ednrb220. Most modern gnathostomes retain both duplicates, with the exception of zebrafish and therian mammals, which have both lost ednrb220. In X. laevis, ednrb2 is expressed in pigment cells, while ednrb1 is only expressed in the eye, and a small population of post-migratory NCCs in the intermediate domain of the PAs, reminiscent of dixk42. In quail and chicken embryos, ednrb2 is also expressed in pigment cells. However, unlike X. laevis, both avian ednrb receptors are expressed in dorsal root ganglia and Schwann cells, though at different times64,65. Interestingly, both mouse and zebrafish appear to have compensated for the loss of ednrb2 by expressing ednrb1 in NCC-derived pigment cells16,37,62,63, an expression domain not seen in any other gnathostome examined to date. The differential ligand binding properties of ednrb127 and ednrb228 open up the possibility that these divergent expression patterns may reflect lineage specific differences in the migration patterns and/or fate of ednrb-expressing NCC, in particular, pigment cells.

Endothelin receptor expression in pre-migratory neural crest supports an ancestral function in NCC specification. Available expression data shows that only X. laevis and P. marinus transcribe an edn in pre-migratory NCC in a pattern reminiscent of neural crest specifiers such as Sox9/SoxE2 and FoxD3/FoxD-A11,66.
Work in *X. laevis* suggests a role for early Ednra signaling in the maintenance of NCC identity and regulation of neural crest specifiers.11 In *P. marinus*, ednrb rather than ednra is expressed broadly in premigratory NCC, however it is unknown if it performs a similar function. Similar expression domains support shared function of some lamprey and gnathostome Endothelin ligands. Despite poor sequence similarity, gene expression patterns lend support to shared functions of some lamprey and gnathostome Endothelin ligands. Specifically, gnathostome edn1 and lamprey ednA have similar expression patterns around the future mouth, pharynx, and in the presumptive nasal epithelia.47 (Fig. 5; arrows in Fig. 5B,F), while gnathostome edn3 and lamprey ednE have broadly similar expression in domains populated by ednrb-expressing melanophores (Fig. 6). While these similarities may be due to the direct orthology of these genes, it could also be due to similar regulatory subfunctionalization of non-orthologous Edns produced by duplications before or after the gnathostome/agnathan split. Such convergent subfunctionalization could have been facilitated by the organization of the ancestral Edn cis-regulatory landscape.

Phylogenetic analysis suggests a fourth Endothelin ligand gene, edn4, was lost in lobe-finned fish; its embryonic expression pattern has not been described. It is possible one of the other lamprey edns is an edn4 ortholog. Embryonic edn2 expression is only known in *X. laevis* (this work), and does not strongly resemble any of the lamprey edns alone (save the dorsal pharyngeal expression found in most lamprey edns). Further analyses of edn2 and edn4 may resolve these apparent discrepancies.

Differences in edn1/ednA expression correlate with differences in gnathostome and agnathan head skeleton patterning. While edn1 and ednA have broadly similar expression patterns, there are some clear differences. In gnathostomes, edn1 is expressed in the ectoderm, mesoderm, and endoderm of the...
ventralmost pharynx, where it acts through Ednra to drive expression of the ventral specifier genes hand1 and dlx5/6. In lamprey, ednA transcripts are restricted to the pharyngeal mesoderm and ectoderm in the dorsal-intermediate domain of the pharynx. Unlike gnathostomes, this expression does not abut hand-expressing NCC in ventral pharynx4 (Fig. 1). The significance of this positional difference in lamprey ednA and gnathostome edn1 is unclear, but we speculate that it may relate to differences in the dorso-ventral patterning of the pharyngeal skeleton between these groups, namely the pronounced asymmetry in gnathostome PAs as compared to lamprey.

Conclusions
To our knowledge, lamprey has the most edn ligands expressed during early development of any vertebrate. This could be the result of cyclostome- or lamprey-specific duplications, and/or the loss of edn ligand genes in gnathostomes. Regardless, the presence of multiple edns and ednrns in P marinus strongly suggests that sophisticated Endothelin signaling in NCCs predates the divergence of modern jawed and jawless vertebrates. Given the essential involvement of these pathways in NCC guidance and differentiation, we posit that Endothelin receptor duplication and sub- and/or neofunctionalization played a role in the evolution of the multipotent and highly patterned NCC of modern vertebrates. New methods for high-efficiency mutagenesis67 in lamprey should allow us to better deduce the ancestral roles of Edn signaling in vertebrates and test this hypothesis.

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**Author Contributions**
D.M.M. conceived the project. D.M.M. and T.S. designed the study. T.S., D.J., M.C., D.M.M. and A.H. cloned gene fragments. T.S., D.J., M.C. and A.H. performed in situ hybridizations. D.J. and T.S. performed cryosectioning. T.S., M.C. and D.J. took images. T.S. and D.M.M. wrote the manuscript. T.S. assembled the figures. All authors provided input on the final paper.

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