Embryological manipulation to probe early evo-devo in the fish *Astyanax mexicanus*

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Abstract

The fish species *Astyanax mexicanus* with its sighted and blind eco-morphotypes has become an original model to challenge vertebrate developmental evolution. Recently, we demonstrated that phenotypic evolution can be impacted by early developmental events starting from the production of oocytes in the fish ovaries. *A. mexicanus* offers an amenable model to test the influence of maternal effect on cell fate decisions during early development, however, the mechanisms by which the information contained in the eggs is translated into specific developmental programs remain obscure due to the lack of specific tools in this emergent model. Here we describe methods for the generation of gastruloids from yolkless-blastoderm explants to test the influence of embryonic and extraembryonic properties on cell fate decisions, as well as the production of chimeric embryos obtained by intermorph cell transplantations to probe cell autonomous or non-autonomous processes. We show that *Astyanax* gastruloids recapitulate the main ontogenetic events observed in intact embryos, including the internalization of mesodermal progenitors. In addition, intermorph cell grafts resulted in proper integration of exogenous cells into the embryonic tissues, with lineages becoming more restricted from mid-blastula to gastrula. The implementation of these approaches in *A. mexicanus* will bring new information about the cascade of events from the maternal pre-patterning of the early embryo to the evolution of brain regionalization.

Introduction

Emergent model organisms offer novel possibilities to unravel specific questions related to developmental and cell biology. However, some limitations, inherent to each animal system, often render difficult the implementation of novel methodologies.

Astyanax mexicanus species has thrived as an emergent model organism for evolutionary developmental biology studies. Its success in this field is due to the existence of two extremely different eco-morphotypes within the same species. *A. mexicanus* comprises river-dwelling fish populations, “surface fish”, and several populations adapted to the life in caves in complete and permanent darkness, “cavefish”. During cave adaptation, striking morpho-functional modifications occurred. Compared to the surface fish, the cave-adapted morphs have completely lost their eyes and pigmentation. In addition, some constructive traits have also emerged such as larger olfactory organs and more numerous facial neuromasts and taste buds, which probably contribute to a sensory compensation to the loss of the visual system (Bibliowicz et al., 2013; Blin et al., 2018; Hinaux et al., 2016; Varatharasan et al., 2009; Yoshizawa et al., 2010). Most of the morphological differences observed in the nervous system of *A. mexicanus* morphotypes have an early embryonic origin (Hinaux et al., 2016; Pottin et al., 2011; Rétaux et al., 2016; Yamamoto & Jeffery, 2000). In fact, recent evidence has shown that maternal determinants, present in the oocyte before fertilization and before developmental programs are initiated, have an important contribution to later phenotypes (Ma et al., 2020, Ma et al., 2018; Torres-Paz et al., 2019). Indeed, any differential composition of maternal determinants in the eggs is susceptible to lead to changes in early developmental events, such

as activation of the zygotic genome, embryonic patterning and establishment of signaling centers, thus affecting later ontogenetic processes.

In fish, the extraembryonic yolk cell (of maternal origin) is an important source of inductive signals that pattern the overlying blastoderm, the embryo proper. Asymmetric segregation of maternal determinants leads to the induction of the embryonic organizer in the prospective dorsal side of the blastoderm. This symmetry breaking event will lead to localized production of different morphogens, creating gradients of signaling activity within the developing embryo. The integration of these signals by embryonic cells gives them positional information and instructs them to follow a particular developmental program (A. F. Schier & Talbot, 2005). Thus, changes in the information contained in the oocytes, represented by maternally inherited RNAs and proteins, will affect the subsequent sequence of developmental events. *A. mexicanus* offers a unique model to test the maternal influence in precocious and later development. However, methods to assess the effect of signaling centers (embryonic and extraembryonic) and the potential of cells to respond to these signals have not been developed yet in this model.

Here, we describe the implementation in *A. mexicanus* of methods used in well-established fish models to tests aspects of cell/tissue specification during early embryogenesis. First, we have adapted a recent method of embryonic explant culture developed in zebrafish and known as "pescoids" to grow the blastoderm after removal of the extraembryonic yolk cell (Fulton et al., 2020; Schauer et al., 2020). Under these conditions of altered embryonic geometry and physical constraints, the explants are able to recapitulate the main processes observed in intact embryos such as symmetry breaking, germ layer specification and elongation. In addition to previous reports on fish gastruloids we found clear indications of mesoderm internalization. These

gastruloids will allow comparative analyses of gene expression in *Astyanax* morphs in the absence of yolk-derived signaling. Second, we have set up the conditions to efficiently achieve inter-morph cell transplantations at matching stages during early embryogenesis. Cell grafting have been widely performed in zebrafish embryos to test cell autonomy and potential during development, as well as to dissect lineage and timing aspects during cell specification. Grafts have also been performed between distinct species such as zebrafish and medaka to study developmental heterochronies (Fuhrmann et al., 2020). In *A. mexicanus,* inter-morphs cell transplantation will allow asking similar questions in a micro-evolutionary context. Further, the implementation of these methodologies to generate gastruloids and chimeric embryos in *A. mexicanus* will help to explore the effect of embryonic and extraembryonic signals in cell decisions during early development.

Materials and methods

Fish and embryo collection

Our *A. mexicanus* colonies were obtained in 2004 from the Jeffery laboratory at the University of Maryland, College Park, United States. The surface fish stock derives from rivers in Texas, United States and the cavefish from the Pachón cave in San Luis Potosi, Mexico. Fish were since then maintained on a 12:12 hr light:dark cycle at a temperature of 22°C for cavefish and 26°C for surface fish. Reproductions were induced every other week by changes in water temperature: for cavefish temperature was increased to 26°C, and for surface fish temperature was decreased to 22°C during 3 days followed by an increase to 26°C (Elipot et al., 2014). Fish

from both morphotypes spawn regularly the first and second days following the increase in temperature. Here, embryos were obtained exclusively by *in vitro* fertilization in order to ascertain synchronous early development. Embryo dechorionation was performed by enzymatic treatment with Pronase 1mg/mL (Sigma) and embryos were maintained in Embryo Medium (EM) at 24°C. Animals were treated according to the French and European regulations for handling of animals in research. SR's authorization for use of *Astyanax mexicanus* in research is 91-116. This work did not necessitate a protocol authorization number from the Paris Centre-Sud Ethic Committee. The animal facility of the Institute received authorization 91272105 from the Veterinary Services of Essonne, France, in 2015.

Generation of chimeric embryos by cell transplantations

Donor embryos were injected at the 1-cell stage with 3-5nL of 1% Dextran-FITC 10,000 MW (Molecular Probes) and 0.05% Phenol Red (to see the solution) using a FemtoJet (Eppendorf). Glass pipettes for microinjection and cell transplantation were prepared on a Narishige PN-30 puller using borosilicate glass capillary (GC100F15 Harvard Apparatus LTD and B120-69-10 WPI, respectively). Microinjection pipettes were sealed at the tip and broken for opening at the moment of the injection using forceps. Cell transplantations pipettes were prepared in advance, the tip was broken at the desired internal diameter (15-30 µm) and polished using a Micropipettte grinder (Narishige EG-44) at an angle of 35° in order to create a smooth needle-shaped tip. Our cell transplantation system consisted of a holder for the glass pipette (WPI) connected to a 1mL syringe by a Teflon tubing (Narishige). Under a fluorescent macroscope (Giger et al., 2016), labelled donor cells were aspirated into the tip of the glass pipette filled with EM, and 3-12 cells expelled into the host embryo with gentle pressure. Host embryos were

let to develop in EM until fixation. In this study, isotopic and isochronic intermorphs grafts were performed (Surface animal pole cells to Cave animal pole).

Generation of gastruloids

A. mexicanus gastruloids were produced following a recent description in zebrafish (Fulton et al., 2020; **Figure 1A**). Briefly, at the 512-1K cell stage the yolk was carefully removed from embryos using eyebrows knives (**Supplemental video 1**). Blastoderm explants were cultured until the corresponding 10 hours post-fertilization (hpf) at 24°C in L15 medium (Gibco) supplemented with 3% Fetal Bovine Serum (FBS, Biosera).

Histology and imaging

Colorimetric ISH was performed as previously described (Pottin et al., 2011). Digoxigenin-labeled riboprobe was prepared using PCR products as templates. *no-tail* (*ntl*) cDNA was obtained from our ESTs library (accession number ARA0ABA99YL22). Procedure for revelation of FITC-labeled donor cells combined with fluorescent *ISH* was adapted from our *ISH* protocol (Alié et al., 2018). Following the hybridization with the Digoxigenin-labeled probe, embryos were incubated for 1 hour in blocking solution (Tris 0.1M pH 7.5, NaCl 150mM, Tween 20 0.1% and 5% blocking reagent Roche) and then with POD-conjugated anti-FITC antibody (11426346910; Roche, 1/400) diluted in blocking solution overnight. Embryos were washed in PBS/Tween 0.1% (PBST) 10 times for 10 minutes and incubated for 30 min at room temperature with TAMRA-tyramide 1/1000. Peroxidase activity was activated by H2O2 (0.003%, Sigma) for 1 hr and samples were washed again 10 times for 10 minutes in PBST. Revelation of the dig-labeled probe was performed using an anti-Digoxigenin antibody coupled to POD (11207733910; Roche,

1/400) and revealed using a FITC-tyramide (1/400). After several washes on PBST embryos were stained with DAPI at a final concentration of 1 mg/ml, overnight at 4˚C, washed in PBS before dissection and mounting (Vectashield, Vector Laboratories). Embryos stained by colorimetric ISH were imaged on a Nikon AZ100 multizoom macroscope. Confocal acquisitions were done on a Leica-SP8 confocal microscope using the Leica Application Suite software. Images were processed on ImageJ.

Results and discussion

Generation of Astyanax gastruloids

Recent advances in the field of gastruloids have highlighted the robustness of animal development and the key steps taking place during this process. In zebrafish pescoids (yolk-less blastoderm explants) the main aspects of early development observed in intact embryos are recapitulated. Symmetry breaking, axis elongation and neural specification occur despite the absence of extraembryonic signaling (Fulton et al., 2020; Schauer et al., 2020). In fish, these events are controlled maternally (Marlow, 2020; Solnica-Krezel, 2020) and *A. mexicanus* with its two morphotypes has become an excellent model to challenge the role of maternal determinants in embryogenesis (Ma et al., 2018, 2020; Torres-Paz et al., 2019).

Pescoids derived from embryos of both *Astyanax* surface and cavefish embryos (surfoids and caveoids, respectively) developed similarly to those described in zebrafish. After removal of the vitellus at the 256-2K cell stage (**Figure 1B**), blastoderm explants sealed the wound and became rounded during the first hour post culture (hpc, **Figure 1C**). Then, during the next 3-4 hours in

culture a cavity was formed (**Figure 1C**), which may correspond to a "blastocoel" (Schauer et al., 2020). After 5 hpc, the first signs of axis elongation were observed, similarly in terms of timing in surfoids and caveoids (not shown). Cultures were stopped after 7 hpc, the time corresponding to the tailbud stage in intact embryos, and gastruloids were fixed for further processing and imaging. The extent of elongation at the end of the culture was variable between pescoids and differed similarly in the two morphs: gastruloid elongation was observed in 79% of surfoids and 84% of caveoids. The shape of the elongated gastruloids was asymmetrical and pear-shaped, with a narrow tip at one end and a larger rounded form at the opposite extremity. We studied the expression of the mesodermal marker *ntl* in *Astyanax* pescoids and found a pattern reminiscent of the developing notochord observed in intact fish embryos (**Figure 1F, G**). Microscopic observations suggested that cells expressing *ntl* had been internalized, an aspect of fish gastruloids that may have been overlooked. We compared the expression of *ntl* in confocal acquisitions after fluorescent ISH in intact embryos at tailbud stage and in pescoids at equivalent stages (**Figure 2**). In confocal reconstructed sections of pescoids and control embryos, we observed similar organization of *ntl* expressing cells, always underneath an overlying layer of superficial cells (**Figure 2**). Thus, these data confirm a conserved internalization process of axial mesoderm in explants despite the absence of vitellus. These observations highlight the robustness of cellular processes during vertebrate gastrulation.

Studies in zebrafish have shown that asymmetric translocation of maternal determinants leading to the dorsal determination occur as early as the 16-32 cell stage (Jesuthasan & Strähle, 1997). The activation of the zygotic genome, a process dependent on the maternal transcriptomic machinery, starts around the 64 cell stage (Chan et al., 2019), whereas clear

zygotic transcription is observed from the 512 cell stage. Concurrently, at this stage, called the mid-blastula transition, embryonic cell cycles become asynchronous and the extra-embryonic yolk syncytial layer (YSL) is formed at the interphase between the yolk and the blastoderm (Kimmel et al., 1995). Similarly to zebrafish, in *Astyanax* the mid-blastula transition takes place around the 512-1K cell stage (Hinaux et al., 2011; **Supplemental video 2**). Here, the explant experiments were performed between the 256-2K cell stage, i.e., the end of the maternal-to-zygotic transition, thus it must be taken into account that maternally-derived pre-patterning already exists in the cultured blastoderms. Consequently, in order gain insights into the temporal sequence of maternal patterning events, the generation of earlier explants must be considered. The comparative analysis of earlier explants in the two *Astyanax* morphs will allow dissecting precisely the timing and the impact of the maternal contributions to developmental evolution. Recently, it was shown in zebrafish that the extraembryonic YSL layer does not form properly in yolkless-explants and that correctly developing pescoids can be obtained even from very precocious 64 cell stage embryos (Schauer et al., 2020). This indicates that the gastruloids are able to develop into embryo-like structures in the absence YSL-derived signals (Chen & Kimelman, 2000; Rodaway et al., 1999). Conversely, animal caps explants are able to develop into structures similar to pescoids only if Nodal and downstream planar cell polarity signaling pathway are active (Williams & Solnica-Krezel, 2020), indicating that Nodal activity in pescoids must come from marginal cells. Nodal signaling is necessary for the induction of endomesodermal fates at the blastoderm margin (Schier et al., 1997; Vopalensky et al., 2018). Visual inspection of the mesodermal marker *ntl* expression in our pescoids at the different states of axial elongation clearly shows that the point where *ntl*-expressing cells are internalized

corresponded to the marginal zone, where the wound closed (not shown). Thus, the wounded margin in the blastoderm explants would be topologically and functionally equivalent to the blastopore in intact embryos, being both the source of Nodal signaling and the point where endomesoderm is internalized. Given the previously described differences in organizer formation and mesoderm internalization between the two *Astyanax* morphs embryos (Torres-Paz et al 2019), pescoids will prove powerful tools to study the origin and outcomes of these processes.

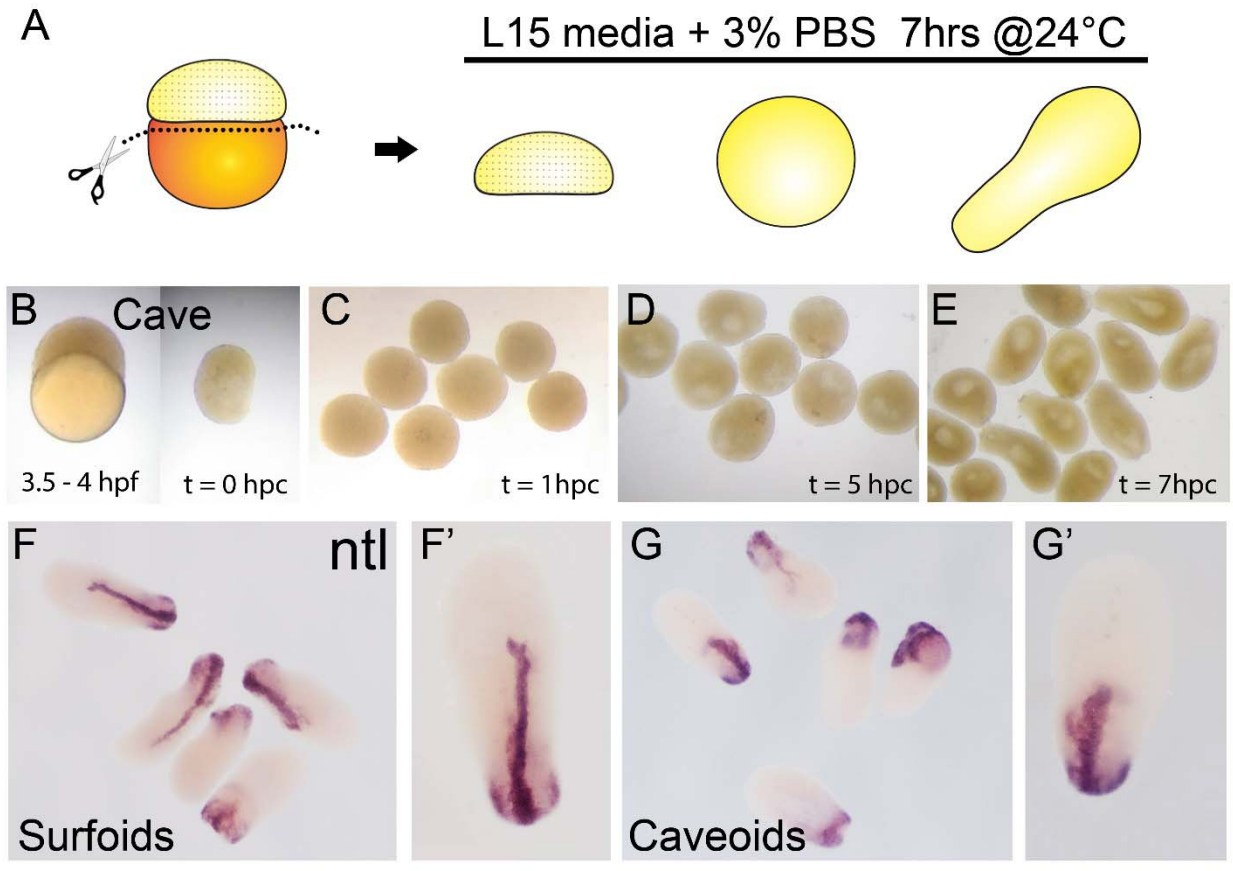

Figure 1.- Generation of pescoids in *A.* mexicanus. Procedure for the generation of *Astyanax* pescoids (**A**). Intact cavefish embryo at 1K-cell stage (**B**, left) and after yolk removal (**B**, right). Development of caveoids after 1, 5 and 7hpc (**C**, **D** and **E**, respectively). Expression of *ntl* in surfoids (**F**, **F'**) and caveoids (**G**, **G'**) after 7hpc.

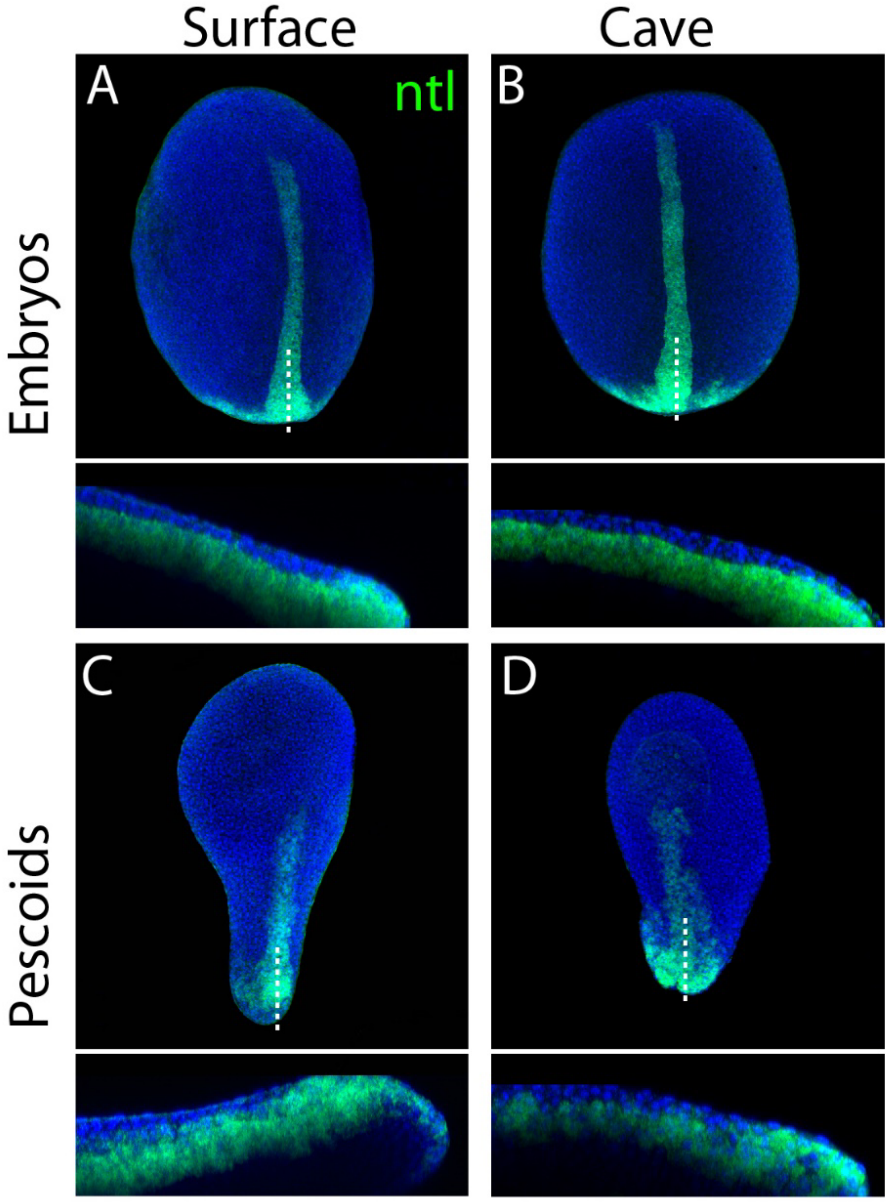

Figure 2.- Internalization of mesoderm in *Astyanax* pescoids. Expression of *ntl* in intact surface and cavefish embryos at 10 hpf (**A** and **B**, respectively), and in surfoid and caveoid after 7hpc (**C** and **D**, respectively). Embryos are oriented in dorsal views, anterior to the top. Bottom images are confocal reconstructions at the indicated level (dotted lines), anterior to the left and dorsal to the top.

Generation of chimeric embryos by cell grafting

Cell transplantation methodologies have been widely used in zebrafish to test cell-autonomy processes during embryogenesis in different experimental contexts (Cavodeassi et al., 2013; Fauny et al., 2009; Giger et al., 2016). *A. mexicanus* with its two eco-morphotypes offers a great opportunity to test autonomy during cell fate specification through intermorph cell transplantations. However, as a model it has not succeeded yet for this research purpose, mainly due to technical challenges that must be circumvented. A major challenge is the simultaneous collection of embryos of both morphotypes at equivalent developmental stage. *A. mexicanus* reproduce only in the dark (Simon et al., 2019), thus in order to obtain early developing embryos to work with during the day, the circadian cycle of fish was inverted in a special fish room dedicated to reproduction. In addition, to obtain early embryos developing synchronously, *in vitro* fertilizations must be performed using ready-to-spawn females, hence mating behavior must be monitored during the days following the induction of reproductions (see methods). Inductions of surface and cavefish were then coordinated in order to find spawning females of the two morphs at the same time.

In this work surface fish embryos were labeled with dextran-FITC at the one-cell stage (donors), and cells were grafted homo-chronically into unlabeled cavefish embryos (hosts) at two developmental time points, the mid-blastula transition (512-2K cell stage) and the onset of gastrulation (30-50% epiboly) (**Figure 3A**). As a source of donor cells we choose the embryonic animal pole, in order to compare to fate maps studies performed in zebrafish and showing that ectodermal precursors arise from this field, whereas endomesodermal precursors derive from more marginal cells (A. F. Schier & Talbot, 2005; Woo & Fraser, 1997). After transplantation of fluorescently labeled cells (**Figure 3B**), embryonic development was observed to occur normally during the following hours post grafting (hpg), with labeled cells integrated in the embryo (**Figure 3C, D**). After fixation and methanol storage of chimeric embryos, FITC fluorescence in labelled cells was completely lost (not shown), rendering necessary a revelation through immunohistochemistry. Fluorescent revelation of labeled cells with FITC-coupled tyramide was avoided because an extensive bleed through of fluorescence to channels at lower wavelengths was observed (not shown). Instead, revelation with TAMRA-coupled tyramide, whose fluorescent excitation/emission occurs at higher wavelengths (557nm and 583nm, respectively) than FITC (495nm and 521 nm, respectively), did not produce bleed through to lower wavelengths channels (**Figure 3G', G" and H', H"**).

We compared the organization and repartition of grafted cells in host embryos at the two developmental stages, blastula *versus* gastrula, and observed clear differences between the two conditions. Clones transplanted at blastula stage were distributed extensively throughout the embryos and in a more disorganized manner compared to those transplanted at gastrula stages which in some cases showed clear sings of symmetry (compare **Figure 3E** and **F**). Using the

positional information and the expression of *ntl* as reference, we observed that grafts at blastula stages were able to produce both ectodermal (n = 15/15 embryos, superficial cells in **Figure 3G, G', G"**) and endomesodermal (n = 6/15 embryos, *ntl* positive cell in **Figure 3H, H', H"**) derivatives in chimeric embryos. On the other hand, grafts performed at gastrula stages gave rise to only ectodermal cells (n = 24/24 embryos, **Figure 3F** and not shown). Confocal reconstructions suggested a correct integration of transferred cells in the developing host tissues (**Figure 3E', E"** and **F', F"**), and supported an expected progressive lineage restriction from mid-blastula to early gastrulation stages. Similar results were also observed in reciprocal experiments, *i.e.* transplants of cavefish donor cells into a surface fish host (not shown).

Intermorph grafting will shed light on the cell autonomy and the effect of the embryonic signaling environment on previously described heterochronies, heterotopies and differences of gene expression levels of during development of *Astyanax* morphs (Hinaux et al., 2016; Pottin et al., 2011; Torres-Paz et al., 2019; Yamamoto et al., 2004). The combination of these grafting methods with the use of transgenic reporter lines such as the cavefish and surface fish *Zic1::GFP* lines labelling eyefield precursors as we have recently produced (Devos et al., 2019), will allow the detailed investigation of intrinsic and extrinsic factors implicated in eye specification and degeneration.

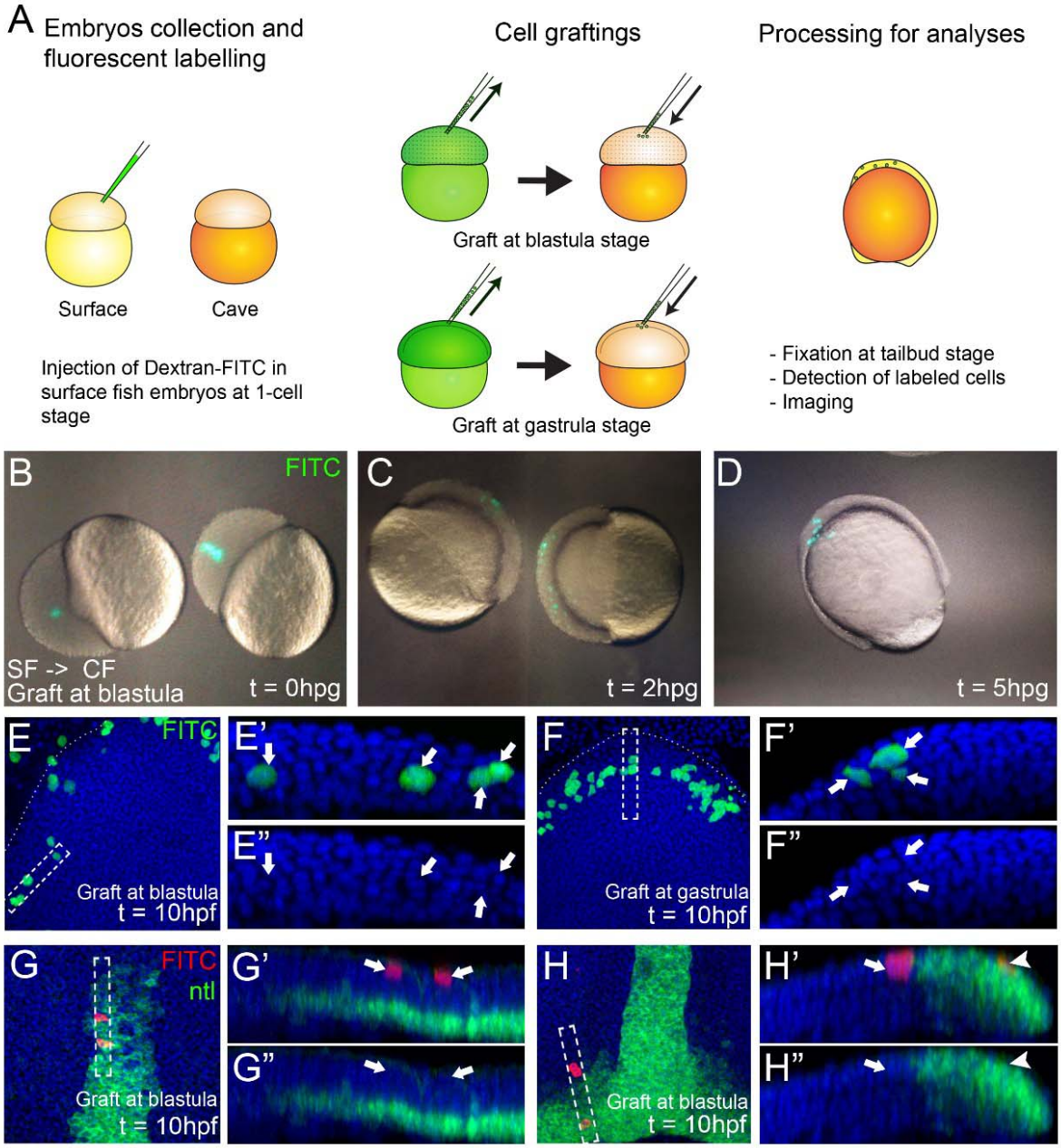

Figure 3.- Generation of chimeric embryos in *A. mexicanus* by inter-morph cell transplantation. Procedure for cell grafting between surface and cavefish embryos (**A**). Live host cavefish embryos that have been transplanted with FITC-labeled surface cells (green) at the blastula stage, photographed immediately after the grafting (0hpg, **B**), or after 2hpg (**C**) and 5hpg (**D**). Confocal images of dissected cavefish embryos at 10hpf at the level of the anterior neural plate (indicated in dotted curved lines). Labeling in green corresponds to surface fish cells

grafted at blastula (**E**) and gastrula (**F**) stages. Respective reconstructed sections were obtained at the levels indicated in rectangles (**E'**, **E"** and **F'**, **F"**). Confocal images of dissected cavefish embryos at 10hpf stained for *ntl* (green), at the level of the anterior notochord (**G**) and the tail bud (**H**). Labeling in red corresponds to surface fish cells grafted at blastula stage. Arrows in **G'** and **G"** indicate superficial ectodermal cells overlying the notochord anterior tip (green). Arrowheads in H' and H" indicate a cell in the mesodermal domain expressing *ntl*. Images in E, F G and H are dorsal views, anterior to the top. Corresponding reconstructions are oriented with anterior to the right and dorsal on top.

Conclusions

Implementation and optimization of new methods in emergent model systems is fundamental for tackling novel scientific questions. Here we describe the methodology and potential applications of cellular techniques to generate yolk-free gastruloids and chimeric embryos in *Astyanax mexicanus*. These methods will allow the characterization of developmental states during cell lineages differentiation in embryogenesis. In addition, these techniques will push forward genomic and cellular approaches to understand the key steps during eye development and degeneration in cavefish.

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