Development of a Robust Gel-Free 3D Culture System for Generating Spheroids from Axolotl Blastema Cells

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Abstract

Regenerative biology is a pivotal field aimed at understanding and harnessing the ability of organisms to restore damaged tissues and organs. The axolotl (Ambystoma mexicanum) is a key model organism renowned for its exceptional regenerative capabilities, particularly in limb regeneration. However, current *in vitro* research is hampered by the lack of well-defined axolotl blastema cell lines and unreliable primary culture protocols. To address these challenges, we present a novel, robust, gel-free 3D culture system for axolotl blastema cells. This system overcomes limitations of 2D culture methods by enabling the formation of spheroid structures that closely mimic the *in vivo* environment. Using this protocol, we observed that spheroids derived from axolotl blastema tissues retained key regenerative markers, including PRRX1, and exhibited stable expression of crucial blastema markers through extended culture periods. Our approach facilitates the study of cellular and molecular mechanisms underlying limb regeneration in axolotls and provides a valuable tool for drug testing and regenerative research. The development of this 3D culture system represents a significant advancement in regenerative biology, offering a more consistent and reliable model for exploring the regenerative potential of axolotl cells.

Key Words: Axolotl, Blastema cells, 3D cell culture, Limb regeneration, Spheroid culture

INTRODUCTION

Regeneration, the process of restoring the function of organs and tissues after damage, is a critical area of research in regenerative biology [1, 2]. A key model organism in this field is the axolotl, known for its extraordinary regenerative abilities, particularly in limb regeneration [3-20]. However, despite extensive *in vivo* studies on blastema, there is currently no established cell line that retains the essential characteristics of blastema cells for *in vitro* research. The only known axolotl cell line, AL-1, lacks detailed documentation regarding its origin, characterization, and acquisition methods, which limits its utility in regenerative studies [21]. Additionally, existing protocols for primary blastema cultures are plagued by low reproducibility and significant limitations. Kumar et al. (2010) reported that these cultured cells were unsuitable for passaging and could not proliferate. Consequently, a well-defined and consistently usable regenerative cell

group for axolotl studies is absent. This underscores the need for a 3D blastema culture system to better mimic the *in vivo* environment, enabling various drug trials, elucidation of molecular pathways, and targeted studies. In contrast, significant progress has been made in blastema cell culture development in other model organisms like Xenopus laevis, where *in vitro* cultures have been pivotal for exploring limb regeneration, offering insights into the regenerative capacities and limitations in amphibians [22, 23]. However, despite the advancements with Xenopus, similar cell line cultures for axolotl blastema cells are notably lacking. The absence of a reliable and reproducible *in vitro* system for studying axolotl blastema cells has impeded progress in understanding the full regenerative potential of this model organism since it has higher regenerative capacity then Xenopus [24].

In this study, we present the first detailed, robust, and highly applicable gel-free 3D axolotl blastema culture protocol. This system overcomes the limitations of previous methods, allowing the formation of spheroid structures from blastema cells, thus providing a new platform for *in vitro* studies of axolotl limb regeneration. Our culture system maintains cell-cell interactions and the cellular microenvironment, paving the way for a deeper understanding of the regenerative processes.

MATERIALS &METHODS

Ethics Statement

The experimental work was conducted in accordance with procedures approved Istanbul Medipol University (IMU) Medical Research Center. The Institutional Ethics Committee of the IMU approved the following experimental procedures in this study with authorization number E-38828770-772.02-1111.

Animal Handling and Collection of Blastema

Axolotls (Ambystoma mexicanum) measuring 15–20 cm from snout to tail tip were housed on a 12-hour light/dark cycle and fed every two days. Axolotls were placed into the 0.03% benzocaine solution for 7-10 minutes to anesthetize. Then, animals were put onto a tissue paper to eliminate the excess benzocaine solution. Both forelimbs were cut in the middle of elbow and digits using Surgical Design No. 10 Carbon Scalpel Blade. Excess skin was pushed backward and trimmed the protruding bone with micro scissors to make the cut area flat. Then the animals were kept in tap water for 16 days following their maintenance conditions. At day 16, amputated animals were anesthetized in 0.03% benzocaine solution for 7-10 minutes then, they were placed onto a bunch of tissue paper one by one and remove the excess benzocaine solution. The amputated limbs were hold proximal to the elbow with a curved pattern forceps and blastemas were cut with 11 cm iris scissors. Harvested blastemas were placed into 35 mm sterile petri dish with 1 ml povidone iodine solution for up to 3-4 minutes.

Blastema Cell Culturing and Spheroid Formation

Blastema tissues were taken out with sterile fine tip dissecting forceps and dipped into 20% bleach solution in a 35 mm sterile petri dish for 4-5 seconds for sterilization. Then, they were placed into

35 mm sterile petri dish with 2 ml of dissection medium composed of DMEM-F12 (Multicell, 319-075-CL), 0.1 % BSA (Capricorn, BSA-1U), %2 Antibiotic-Antimycotic (Thermo Fisher, 15240062), 10% ddH_2O. Pipette tips were coated with coating medium containing 0.1% BSA in 1x PBS in 2 ml Eppendorf tube by taking the solution in and ejecting out pipette wisely to prevent blastema tissues stick on the surface of tips. Note that every tip that will be used must be coated with coating medium pipette wisely right before use. Therefore, coating medium must be kept in an Eppendorf tube through the whole culture process. The blastema tissues were transferred to 2 ml Eppendorf tube with 500 µl of fresh dissection medium and triturated with 1000 µl coated pipette tips for 2-3 minutes. Next, the tissues were triturated with 200 µl coated pipette tips for 1 minute. Note that blastema covering skin tissue will remain intact as tissue fragments. Then, the samples were centrifuged at 200 x g for 5 minutes. Supernatants were discarded and 1 ml of culture medium containing DMEM-F12 (Multicell, 319-075-CL), 0.1 % BSA (Capricorn, BSA-1U), %2 Antibiotic-Antimycotic (Thermo Fisher, 15240062), 10% ddH_2O, %1 ITS (Thermo Fisher, 41400045) +%1 non-essential amino acid (Thermo Fisher, 11140050) +10% ddh2O + 0.2 µM L-ascorbic acid (Sigma, A0278) was added to each tube. Cells were mixed with the culture medium using coated tips and divided into two 35 mm sterile petri dishes as 500 µl sample for each. Note that for every 2 blastema, one 35 mm petri dish should be used to obtain efficient amount of sphere structures. For a greater number of cultures, multiple blastema can be pooled and divided into petri dishes later. Each petri dishes were completed with 1 ml culture medium right after and left for incubate in 2% CO_2 at 20ºC. The sphere structures will start to appear next day as indicated in **Figure 1**. On day second, 500 µl of culture medium was added to the culture and every two days, half of the medium was change with freshly prepared culture medium. It is observed that this way cells can be maintained up to 1 month.

Immunofluorescent Staining

The spheroid structures obtained from the cultures were collected into Eppendorf tubes, the medium was removed, and the spheroids were fixed with 4% paraformaldehyde for 3 days. After fixation, the samples were incubated in a blocking solution containing 5% goat serum, 1% Triton-X, and 1x PBS for 1.5 hours. The homemade primary PRRX1 antibody (a kind gift from TANAKA Lab) was diluted in the blocking solution, and the samples were incubated with the primary antibody 3 days at +4°C. After this incubation, the samples were washed three times for 30 minutes each with PBS solution. Goat anti-Rabbit IgG (H+L) secondary antibody (Thermo Scientific, 35552) were then diluted at a 1/750 ratio in the blocking solution, and the samples were incubated at +4°C for 24 hours. Subsequently, the samples were incubated with DAPI (4', 6-Diamidino-2-Phenylindole) for 2 hours at room temperature and washed again three times for 30 minutes each with PBS. Finally, the samples were transferred to glass bottom 35 mm petri dishes for visualization using a confocal microscope (Zeiss LSM 800 with Airyscan). As a control group, only secondary antibody were applied.

Quantitative PCR

Total RNA was extracted from spheroid and blastema samples using TRIGent reagent (equivalent to TRIZol, K5161) following the manufacturer's protocol. Subsequently, equal amounts of RNA were reverse transcribed into cDNA using the Advanced cDNA Synthesis Kit (WISENT

MULTICELL, 801-100) as per the manufacturer's instructions. Quantitative PCR (qPCR) analysis was then conducted using gene-specific primers for *Msx1* (Fwd:TCGGTCAAGTCGGAGAACT, Rev:GGGCTCAGATGTCTGGGC), *Prrx1* (Fwd:AACGAGTACCTATCCTGGG, Rev:GCACAAGGAGGAGAATAAGT), *CTGF* (Fwd:CACTAGCTGTGTACAGACAG, Rev:GAGCTCGTTGTCATTGGT), *Sox2* (Fwd:ATGTACAGCATGATGGAGACC, Rev:CTTCTCGGCCTCGGAGAG), *Oct4* (Fwd:GACAGCGGAGACGAGGAAGC, Rev:AGCTCCTGCAGGTTCTCGTTG), *and Nanog* (Fwd:CAGCAGCAGTCCAGTCCTAC, Rev:CCAGGTTTTCACCTGCTTAC), employing the SensiFAST™ SYBR® No-ROX Kit (Bioline-BIO-98005). *GAPDH* was used as a reference gene for normalization, and relative mRNA expression levels were quantified using the delta delta Ct ($2^{-\Delta\Delta Ct}$) method.

RESULTS

Formation of 3D Sphere Structures From Axolotl Blastema Tissues

Cells isolated from day 16 blastema tissue of axolotls were cultured without enzymatic dissociation, resulting in the formation of tissue fragments within the culture. Notably, by the following day, these tissue fragments began to form spherical structures, hereafter referred to as blastemal spheroids **(Figure 1A)**. Over the course of three days, these spheroids progressively got separated from the surrounding tissue fragments and became individual entities **(Figure 1B)**. It was observed that the size of the spheroids increased proportionally with the size of the originating micro-tissue. Furthermore, cells located outside of the tissue fragments were also capable of forming spheroids, though these were significantly smaller in size compared to those derived from tissue fragments **(Figure 1C)**. By using the protocol, spheroids can be obtained from blastema tissue.

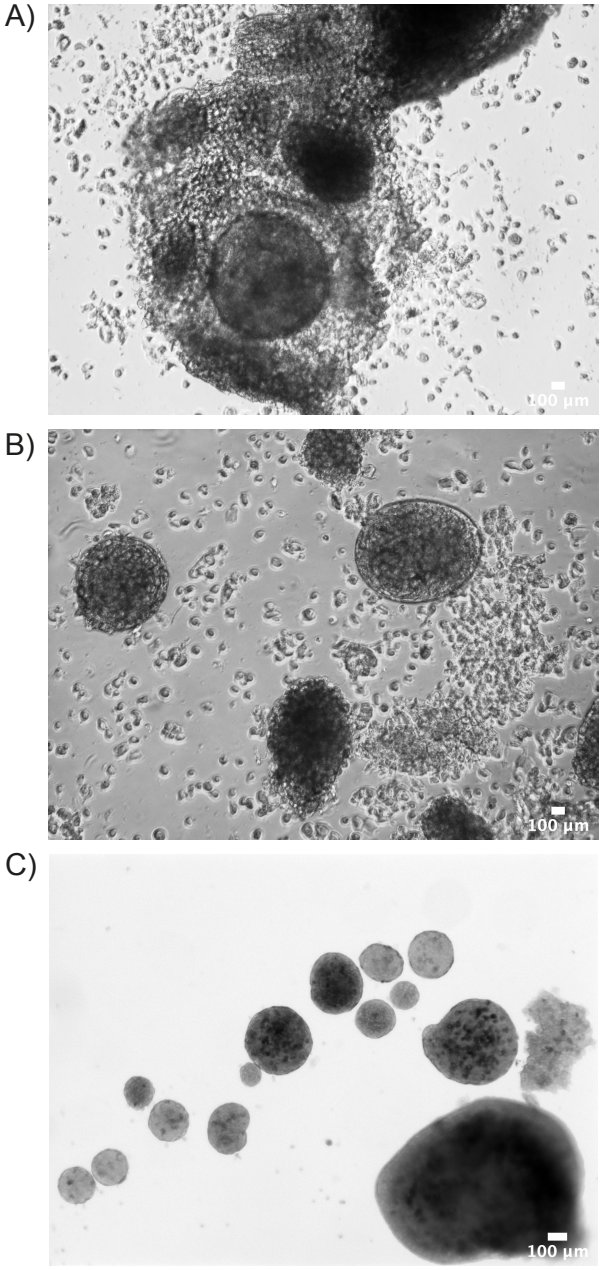

Figure 1: Axolotl blastemal spheroids in culture. Cells were isolated from day 16 blastema of 12 cm animal. A) Spheroids after 24 h in culture; B) Spheroids after 72 h in culture. C) Different sized blastemal spheroids obtained after 10 days in culture. Scale bar, 100 µm.

Blastemal Spheroids Retain Expression of Key Blastema Markers

To assess whether the blastemal spheroids maintain the expression of characteristic blastema markers over time, spheroids were harvested after 3 days (short-term culture) and 10 days (long-term culture). Immunofluorescent staining using a custom PRRX1 antibody (kindly provided by Dr. Elly Tanaka) was used to detect protein expression within the spheroids. PRRX1 is a transcription factor crucial for controlling gene expression during tissue regeneration. In axolotls,

it impacts the formation and differentiation of cells necessary for limb and tissue regrowth. Its role in epithelial-mesenchymal transition (EMT) is essential for the regenerative abilities of axolotl tissues [25-27] . The results demonstrated that PRRX1 was expressed by both short-term and long-term cultured spheroids **(Figure 2)**.

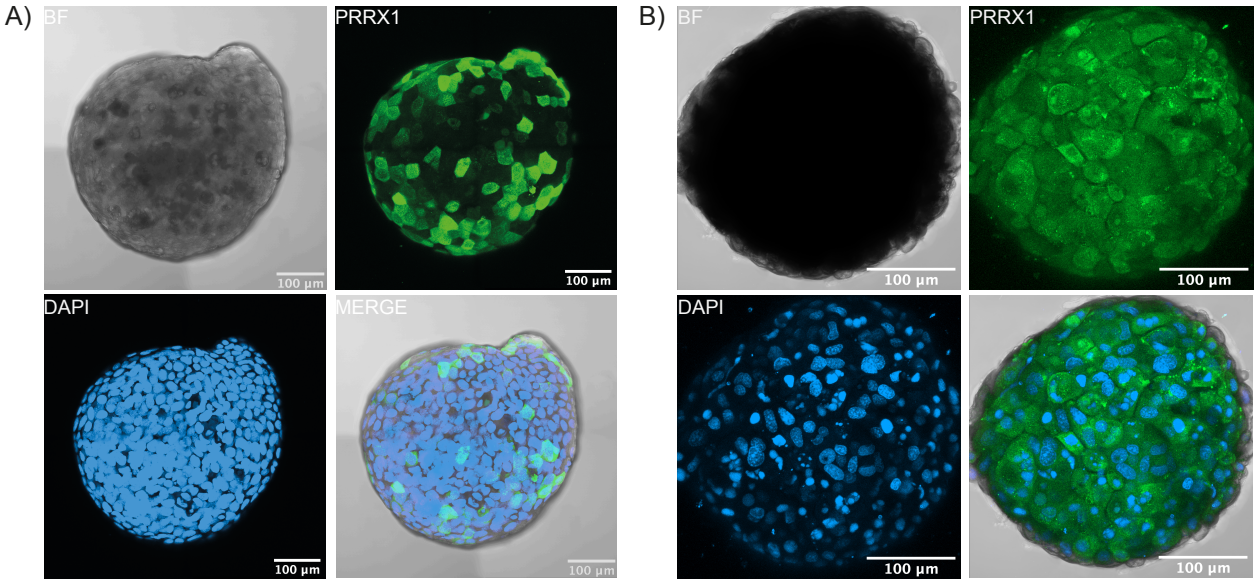

Figure 2: PRRX1 Expression in Axolotl Blastema Cells. Antibody staining of spheroids collected from day 3 and 10 of culture. (A) Expression of PRRX1 in axolotl blastema cells after 72 hours in culture. (B) Expression of PRRX1 after 10 days in culture. BF: Brightfield. Scale bar: 100 µm.

PRRX1 proteins show the presence of connective tissue precursors, however to confirm the blastema properties of the spheroids, transcription of multiple blastema marker genes need to be tested. For this purpose, quantitative reverse transcription PCR (qRT-PCR) analysis was conducted to compare the mRNA levels of key blastema markers, *CTGF, MSX2,* and *PRRX1*, between the spheroids cultured for 3 days and the original blastema tissue. The analysis revealed that the spheroids express comparable amount of these markers to original blastemas **(Figure 3A)**. Moreover, the expression levels of pluripotency markers, *OCT4, SOX2*, and *NANOG*, were also similar in the spheroids and blastemas after 3 days in culture **(Figure 3B)**. We conclude that the blastemal spheroids maintain their blastemal properties assessed via expression of blastema markers.

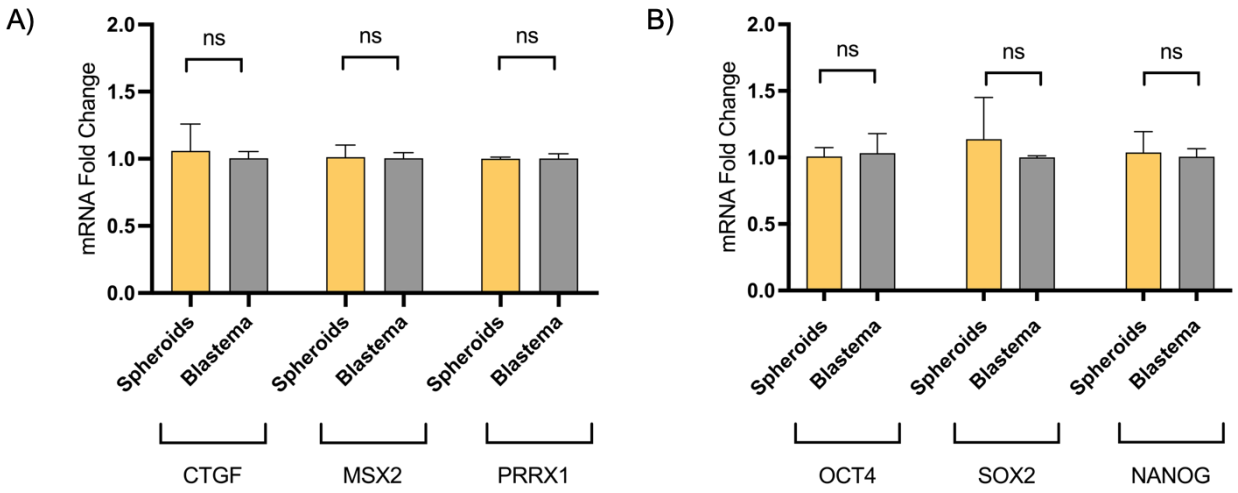

Figure 3: qRT-PCR Analysis of Spheroids and Blastema Tissue. (A) Relative mRNA expression levels of blastema markers in spheroids and blastema tissue. (B) Relative mRNA expression levels of pluripotency markers in spheroids and blastema tissue. Blastema tissues were collected on day 16 for both cell culture and direct analysis. No significant differences were observed between spheroids and blastema tissue ($p > 0.05$, Student's t-test). Error bars represent the mean ± SEM.

DISCUSSION

This study marks a significant advancement in axolotl limb regenerative biology by establishing the first protocol for spheroid production from axolotl blastema cells. By elucidating the culture conditions required for the formation and maintenance of blastemal spheroids, we have provided a crucial tool for investigating the underlying mechanisms of tissue regeneration. The successful generation of these spheroids represents a foundational step in creating *in vitro* models that can potentially replicate the complex regenerative processes observed *in vivo*. As such, this protocol not only contributes to our understanding of spheroid formation dynamics but also opens new avenues for research into the cellular and molecular pathways that drive regeneration in axolotls.

Spheroid Formation Dynamics in Axolotl Blastema Cultures

We observed that axolotl blastema cells cultured on commercially treated surfaces initiate spheroid formation within tissue fragments, becoming distinct entities after 2-3 days. The progressive detachment and enlargement of these spheroids highlight a critical aspect of their formation, where the size of the originating micro-tissue plays a significant role. Moreover, the formation of smaller spheroids by individual cells underscores the heterogeneity within the culture, suggesting that spheroid size may correlate with distinct biological functions possibly forming different part and tissues of the limb structure. This variability in spheroid size warrants further investigation, as it may impact the regenerative potential and differentiation pathways of these structures.

During the course of method development, we tried using serum, however we found that addition of serum to the culture medium decreased both the number and size of spheroids, indicating that

serum components may negatively impact spheroid integrity or promote differentiation pathways that inhibit spheroid formation. To address this issue, we established completely serum-free culture conditions to optimize spheroid formation. This adaptation not only aligns with practices with the only known axolotl cell line, AL-1 but also provides a defined medium avoiding uncharacterized factors that could influence the regenerative potential of the cells [21]. In these serum-free conditions, ITS (insulin, transferrin, and selenium) is used as a supplement to support essential cellular functions and intracellular transport demands that would typically be met by serum. Additionally, we incorporated non-essential amino acids into the culture medium. The rationale for this supplementation lies in its ability to reduce metabolic stress on the cells by providing critical amino acids that are not synthesized at sufficient levels during culture. This helps to prevent the accumulation of toxic by-products, which can arise from the de novo synthesis of these amino acids by the cells. As a result, the inclusion of non-essential amino acids enhances cell viability and supports the consistent formation of healthy spheroids [28].

Given the elevated levels of reactive oxygen species (ROS) during axolotl regeneration, we incorporated antioxidants into the culture medium to counteract oxidative stress. The inclusion of L-ascorbic acid (L-aa) proved beneficial, as it significantly improved cell survival and functionality, indicating that managing oxidative stress is vital for maintaining healthy spheroid cultures. These findings suggest that antioxidant supplementation should be a standard practice in future regenerative studies involving axolotl cells.

Expression of Pluripotency and Blastema Markers

Our qRT-PCR and immunofluorescence analyses confirmed that the blastemal spheroids retained the expression of key blastema markers (Prrx1, Msx2, CTGF) across different culture durations. The sustained expression of PRRX1 proteins even after 10 days in culture demonstrates the stability and robustness of these spheroids as models for studying regeneration. The preservation of these markers over extended periods suggests that the spheroids maintain their regenerative capacity and cellular identity, making them valuable tools for dissecting the molecular pathways involved in tissue regeneration.

Technical Considerations for Spheroid Culture

A significant technical challenge identified was the tendency of spheroids to attach to the culture surface, which could compromise their integrity and lead to uncontrolled spreading. To mitigate this issue, we recommend the use of an incubator with shaker during culture, which would maintain the spheroids in suspension and preserve their structural integrity.

Conclusion

In conclusion, the establishment of optimized *in vitro* culture conditions for axolotl blastema cells has enabled the reliable formation of spheroids that retain key regenerative markers. The development of serum-free conditions, alongside antioxidant supplementation, has proven essential for supporting spheroid formation and maintenance. These spheroids represent a promising model for studying the cellular and molecular mechanisms underlying tissue regeneration in axolotls. Notably, the potential differentiation of blastema spheroids into distinct

cell or tissue types can be investigated through long-term incubation or by supplementing the culture with specific growth factors. Such approaches could shed light on the pathways involved in tissue specialization and even morphogenesis during regeneration. However, while this protocol is a significant step forward, further optimization is necessary to refine the conditions and maximize the consistency and scalability of spheroid production. Additionally, more in-depth studies are required to fully understand the functional capacities of these spheroids and their potential applications in regenerative medicine. The insights gained from this study lay a strong foundation for future research aimed at unlocking the regenerative potential inherent in these unique cellular structures.

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