Deliverable 5.5
Identification of biomarkers and in vitro GSC amplification

Version 1

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Contents

1. Objective

The two main objectives of the deliverable were to identify genes specifically expressed in spermatogonial stem cells (SSC) and to develop reliable cell culture procedures allowing the *in vitro* amplification of these SSC.

2. Background

2.1. Germ stem cell definition and functional properties

Spermatogonial stem cells (SSCs) are the primitive germ cell populations of the germ cell lineage and they are required for the continuous or seasonal production of gametes within the testes throughout the life of males. Transplantation studies of testicular cells harvested from adult rainbow trout have demonstrated that SSC are present in adult testes and they remain bipotent since SSC can colonize the gonads of the recipients and generate functional sperm or oocytes depending on the sex of the recipient embryos (Takeuchi *et al.* 2003; Okutsu *et al.* 2006a). Similarly, it is demonstrated that ovogonial stem cells (OSC) from adult female ovaries share the same stemness properties (Yoshizaki *et al.* 2010). Therefore, SSC and OSC should be considered as a unique adult germ stem cell population. The cellular dynamics and the underlying molecular mechanisms that ensure the establishment of their stock in the testes of adult males remain poorly understood. Transplantation studies in rainbow trout and other species have suggested that very few SSC could reside within the testes of adult males and even fewer in female ovaries. Indeed, despite the transplantation of thousands of spermatogonial cells, only 1 to 3 foci of colonization and clonal expansion are observed in the gonads of recipient embryos. We have observed that this capacity for colonization and clonal expansion decreases with the age of the donor animal between 2 and 12 months of age (Bellaiche *et al.* 2014). In the latter study, we demonstrated in adult rainbow trout that spermatogonial stem cells expressed *nanos2* at higher levels than other germ cell populations. However, we demonstrated that the morphology and transcriptome of the germ stem cells change during ontogenesis in trout testes suggesting that successive populations with different stemness properties likely emerge during ontogenesis (Maouche *et al.* 2018). **There is a great need to identify new gene markers to unravel the cellular dynamics of juvenile and adult germ stem cell populations. This knowledge could also be used to improve the SSC enrichment in testicular cell suspensions used in cell grafting.**

The cryopreservation of SSC is now well mastered (Okutsu *et al.* 2006b; Aliakbari *et al.* 2016). SSC grafting is the only truly reliable and operational method for the long term conservation and regeneration of valuable genetic resources (nuclear and mitochondrial DNA) with potential application for the aquaculture sector. Technologies based on germ stem cell grafting can also be used in the field of conservation biology for vulnerable or endangered wild fish species (biodiversity conservation). **As mentioned above, the limited number of SSC is a limitation for the dissemination and implementation of the germ stem cells grafting. This could be circumvented by amplifying the germ stem cells using *in vitro* cell culture systems.**

2.2. Germ stem cell *in vitro* cultures

Although it is well admitted that spermatogonial stem cells are slow dividing cells, it has been demonstrated that germ stem cells can be maintained and amplified in vitro for several days using different culture systems and appropriate medium conditions. In trout, mixed testicular cells were successfully cultured for two weeks in plastic wells coated with fibronectin to identify factors stimulating cell proliferation (Loir 1999a, b). More recently, a Sertoli cell feeder layer was developed to culture trout spermatogonial cells (Iwasaki-Takahashi *et al.* 2020). Such a system expanded the number of ASGs by 37.8-fold over a 28-day culture period. The successful grafting of these cultured cells into triploid sterile recipient embryos resulted in the production of functional spermatozoa and eggs (Iwasaki-Takahashi *et al.* 2020). In zebrafish and rainbow trout, organotypic cultures for 7 to 10 days have been successfully developed to unravel paracrine and endocrine regulatory mechanisms (Sakai 2006; Sambroni *et al.* 2012, 2013; Nóbrega *et al.* 2015). *In vitro* sperm production was reported from zebrafish spermatogonial stem cells collected from testicular hyperplasia (Kawasaki *et al.* 2015). In 2009, Hans Clevers and colleagues have developed intestinal organoids from adult stem cells derived from the crypt-villus structure (Sato *et al.* 2009). Interestingly, such organoids can be cryopreserved and amplified *in vitro*. In mammals, attempts to produce testicular organoids were reported in pig, human (Sakib *et al.* 2019a, b; Vermeulen *et al.* 2019) and mouse (Richer *et al.* 2024). In fish, no testicular organoid had been reported to date.

3. Methodology

The identification of new gene markers of gem stem cells populations was carried out using candidate approach (see milestone 27) and single cell RNA sequencing (scRNA-seq) as previously described (Tang *et al.* 2009). This latter technique allows one to determine the mRNA expression levels in each cell and to classify all cells according to the similarity of their transcriptome profiles. In other terms, the scRNA-seq technique allows one to analyse the cell heterogeneity in complex tissues in order to identify all cell types including rare cell populations, the dynamics of cell lineages, and molecular interactions between cells.

Different commercial extracellular matrix and culture media were tested in 3D testicular culture systems to maintain survival and proliferation of spermatogonial stem cells.

4. Results and Discussion

4.1. Identification of new spermatogonial stem cell markers in teleost fish

In order to identify different early spermatogonial cell populations and unravel cell-specific gene markers, we initiated a single cell RNA sequencing approach from sexually immature juvenile rainbow trout testis. Cells were isolated from 6- and 12-month old males testes showing high numbers of putative spermatogonial stem cells. Our first attempt in 2022 to produce single cell RNA sequencing libraries, failed due to a technical problem. A severe Lactococcosis epizooty occurred in 2023 at the INRAE experimental fish farm (PEIMA) and all animals had to be eradicated. As a result, we were not

able to repeat the experiments from rainbow trout testes due to the lack of animals. Consequently, we decided to carry out the single cell RNA sequencing from adult zebrafish testes.

In contrast to rainbow trout, the production of functional spermatozoa is not seasonal in zebrafish and spermatogenesis occurs constantly in adult testes. In consequence, spermatogonial stem cells remain a rare cell population in zebrafish adult testes compared to juvenile rainbow trout testes. In order to increase the proportion of spermatogonial stem cells, 4 month-old adult zebrafish were exposed to 10 nM 17β-estradiol for 3 weeks. Such hormonal treatment supresses meiosis and spermiogenesis due to a feed-back effect on Fsh and Lh gonadotrophins production and release into the blood stream. Zebrafish spermatogenesis is then allowed to progressively recover from the hormonal treatment to mimic spermatogenesis onset (Figure 1). Full spermatogenesis is recovered by four days after hormonal treatment arrest.

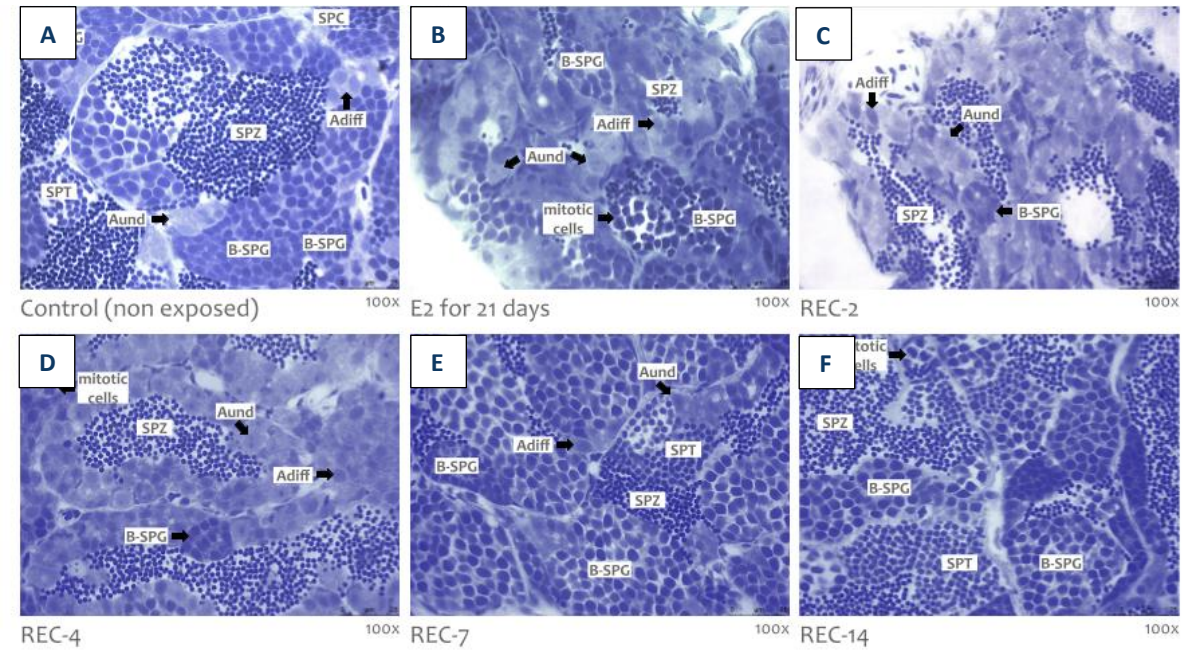

Figure 1 : Histological analyses of zebrafish testes after hormone treatment.

Testes were collected from non-treated zebrafish (A), 21 days-treated zebrafish using 10 nM 17β estradiol (B) or treated zebrafish euthanatized 2 (C), 4 (D), 7 (E) or 14 (F) days after the hormone treatment. All germ cell populations including differentiated and differentiated spermatogonia, spermatocytes, spermatids, and spermatozoa are present in the control testes (A). Note the absence of meiotic and spermiogenic cells, and the fewer numbers of spermatozoa after 21 days of estradiol treatment. Proliferation of B spermatogonia is observed 4 days after stopping hormone treatment (D). Meiosis is resumed by 7 days after hormone treatment (E). Aund: undifferentiated A spermatogonia; Adiff: differentiated A spermatogonia, B-SPG: B spermatogonia; Spt: spermatids; Spz: spermatoazoa

Testicular cells were dissociated for 1 hour at 28°C in L15 containing 0.075% trypsin-1 mM EDTA and 0.2% collagenase. Testicular cells were filtered through 70 and 40-µm nylon meshes, centrifuged and resuspended in L15, 10% FBS to be concentrated and counted. Libraries were

constructed from 16500 cells using the Chromium Next GEM Single Cell 3' Kit (v3.1) according to manufacturer's instructions (10X genomics, California, USA). Raw sequencing data from single cell RNA sequencing libraries have been processed using the Cell Ranger software (v8.0, 10X Genomics) to obtain the gene count matrices, using the zebrafish genome and gene annotation from Ensembl as reference datasets (Ensembl release 112, May 2024). The R package Seurat (v5; Hao et al., 2023) was used i) for data filtering (retention of genes detected in at least 5 cells; retention of cells that express at least 200 and no more than 5,000 genes; retention of cells in which the mitochondrial genes represent no more than 10% of the transcripts), ii) data normalization; iii) cell clustering (based on the Principal Components Analysis [PCA] and determination of the k nearest neighbours; Figure 2); iv) obtention of differentially expressed genes (DEGs) and differentially enriched regulatory or signalling pathways among the cell clusters, and v) discovery of specific cluster markers (Figure 3).

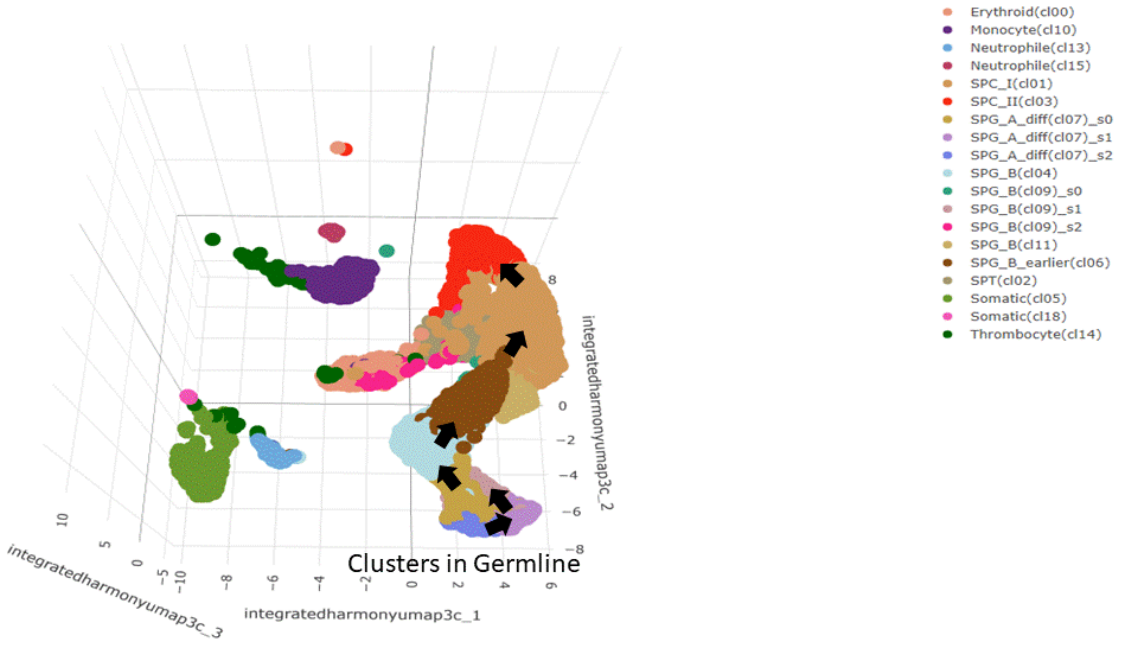

Figure 2: Visualization of testicular cell clusters using 3D UMAP (Uniform Manifold Approximation and Projection). Testicular cells exhibiting similar expression profiles of thousands of genes were grouped in clusters. Cell clusters were annotated according to known cell-specific genes. Note that clusters 3, 6, 2 and 1 regrouped A spermatogonia, B spermatogonia, Spermatocytes, and spermatids, respectively. This clusters organization illustrate the continuum of the germline differentiation (black arrows).

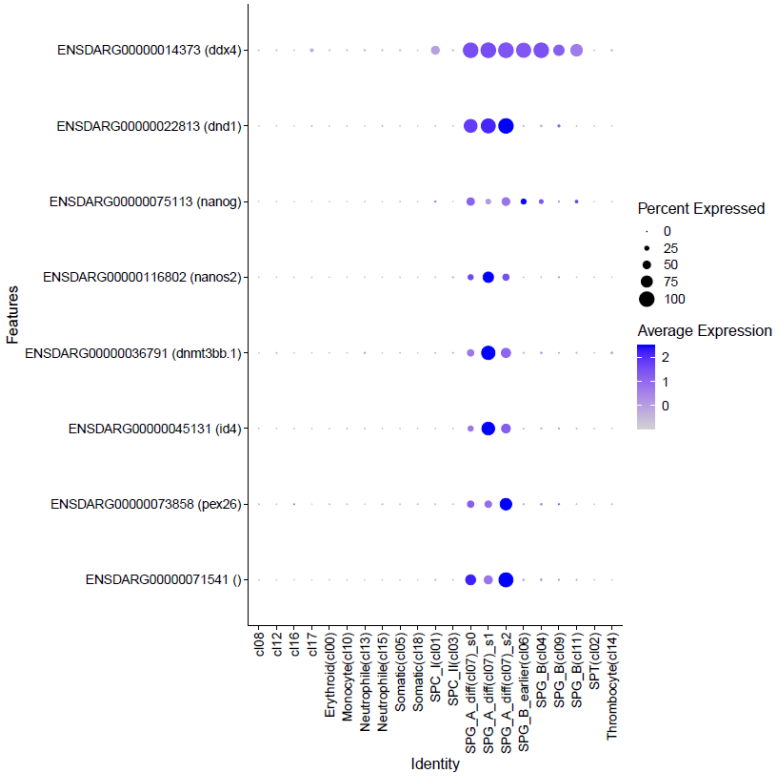

Figure 3: Dot plot analysis of gene expression of selected genes mainly expressed in spermatogonial cell clusters. Our analysis showed that undifferentiated A spermatogonia segregate in three main cell clusters. Cells segregating in the first cluster named s0 express high levels of *vasa* and *dnd1* transcripts but rather low levels of *nanog*, *nanos2*, *Id4*, *dnmt3bb.1*, and *shiza 10.4* transcripts. The second cell cluster named s1 is characterized by high expression levels of the *vasa*, *dnd1*, *nanos2*, *id4* and *dnmt3bb.1* transcripts. Finally, the third cell cluster named s2 shows higher expression levels of the *pex26* and *shiza 10.4* genes. The size of the circles represents the percentage of cells that express the gene of interest and the intensity of the colour illustrates the relative gene expression levels.

Taken together, our data suggest that undifferentiated A spermatogonia could segregate in three different clusters. The first cluster s0 regrouped cells exhibiting high levels of *vasa* and *dnd1* transcripts but rather low levels of *nanog*, *nanos2*, *Id4*, *dnmt3bb.1*, and *shiza 10.4* transcripts. The second cell cluster s1 was characterized by high expression levels of the *vasa*, *dnd1*, *nanos2*, *id4* and *dnmt3bb.1* transcripts. Finally, the third cell cluster s2 shows higher expression levels of the pex26 and shiza 10.4 genes. Our previous studies have demonstrated that *nanos2* transcripts were expressed at higher levels in paired spermatogonia (Bellaiche *et al.* 2014) suggesting that cells of cluster s1 would correspond to spermatogonial doublets. Cells regrouped in s0 or s2 clusters could originate or derive from paired spermatogonial doublets (cells of cluster s1).

We really need to better understand the relationship between cells included in s0 to S2 clusters before investigating cell specific genes. We are currently using the Monocle package (Trapnell *et al.* 2014) for the analysis of the single-cell transcriptomes dynamics along the spermatogenesis. This package will allow us to trace the cells' trajectories based on RNA velocity and to reorder the cell cluster occurrence to generate a "pseudotiming" analysis. Additionally, because paracrine interactions among the testicular cell types are critical for the process of spermatogenesis, we will explore the cell-cell communication networks in our dataset using the R toolkit CellChat (Jin *et al.* 2021).

4.2. *In vitro* culture of GSC

We have decided to explore innovative approaches based on testicular organoids production or organ cultures to achieve a reliable *in vitro* amplification of undifferentiated spermatogonia.

4.2.1. In vitro organ cultures

In the first experiments, we compared the L15 and DMEM/F12 basal media to culture zebrafish adult testes. Testes cultured in L15 media were incubated at 28°C under air with the HEPES supplement at 10 mM to maintain the pH at 7.4. Testes cultured in DMEM/F12 medium were cultured at 28°C under 5% CO2 to maintain pH at 7.4. Each experimental group was containing 6 replicates of organ cultures. After 9 days of culture, histological analyses were carried to investigate the survival and proliferation of the testicular cells including undifferentiated A spermatogonia. Our first investigations showed that the DMEM/F12 medium was poorly maintaining the undifferentiated A spermatogonia and topological structures of the testes compared to L15 basal medium.

In a second series of experiments, we compared the L15 medium supplemented with 10 mM HEPES and the DMEM/F12 medium supplemented with 10 mM HEPES. As mentioned above, testes were cultured for 9 days at 28°C under air or 5% CO2 depending on the L15 and DMEM/F12 used, respectively. We observed the maintenance of undifferentiated A spermatogonia in the testes cultured in the DMEM/F12 medium containing 10 mM HEPES (Figure 3). In addition, we observed spermatogonial doublets with cell morphologies similar to that observed in the testicular tissue before culture. We noted spermatogonial doublets with either large cells and poorly condensed chromatin or doublets with smaller cells with condensed chromatin. In the testes cultured in basal L15 medium, we hardly observed such spermatogonial doublets. This suggests that our new culture conditions could favour the renewal of undifferentiated A spermatogonia.

In a third series of experiments, we sought to determine whether androgen supplementation could affect the maintenance of undifferentiated A spermatogonial or the differentiation process of these cells. Unfortunately, bacterial contaminations of DMEM/F12 medium containing 10 mM HEPES occurred in our culture the last two days of culture and we could not conclude on the androgen function.

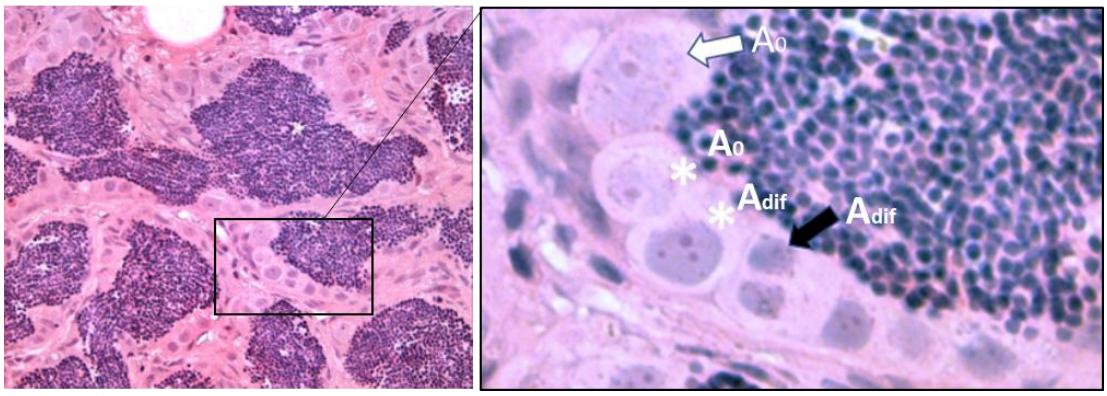

Figure 3: Morphology of the undifferentiated A spermatogonia in organ cultures.

Histological section of zebrafish adult testes cultured for 9 days in DMEM/F12 supplemented with 10 nM HEPES. Note the presence of undifferentiated A0 spermatogonia either in isolated or in doublets. The large isolated A0 spermatogonia (white arrow) could be germ stem cells. In some doublets (see asterisks), one of the two cells showed fragmented nucleoli and

darked nuclei indicating higher condensation levels of the chromatin. Other doublets are formed with two identical smaller cells with fragmented nuclei and darker nuclei. The absence of meiotic cells is due to the absence of androgen in the culture medium. Only spermatozoa are maintained in the organ during the time of culture. A0: putative germ stem cells; Adiff: spermatogonial doublets with progenitor cells.

4.2.2. Production of testicular organoids from zebrafish

In order to demonstrate that testicular cells of the germinal niche were capable to reaggregate after testicular cell dissociation, we first collected testicular cells from two independent zebrafish lines, one expressing the green fluorescent protein (GFP) in Sertoli cells and the other expressing the red fluorescent protein (DsRed) in spermatogonia. Testicular cells from the two zebrafish lines were mixed and embedded in Matrigel and DMEM/F12 medium. We observed few cell aggregates with green and red fluorescent proteins demonstrating that Sertoli and spermatogonial cells can reaggregate (Figure 4).

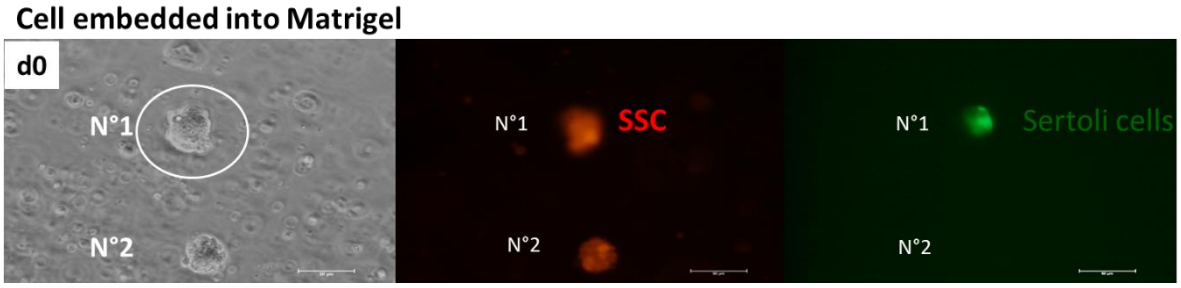

Figure 4: Reaggregation of the testicular cells following tissue dissociation and embedding in Matrigel.

Testicular cells were collected from two independent zebrafish lines, one expressing the green fluorescent protein (GFP) in the Sertoli cells and the other line expressing the red fluorescent protein (DsRed) in spermatogonia. Testicular cells from the two zebrafish lines were mixed and embedded in Matrigel and incubated at 28°C, DMEM/F12 medium under 5% CO2. Note that the upper cell aggregate (left panel) includes red (medium panel) and green (right panel) fluorescent cells demonstrating the reaggregation of the Sertoli and spermatogonial cells.

We tested the influence of commercial extracellular matrix on the morphology and growth of the cell aggregates (Figure 5). The number of cell aggregates obtained in Matrigel was similar to the number of cell aggregates in the Vitrogel. However, the periphery of the cell aggregates imbedded in the Vitrogel showed loose cells in contrast to cell aggregates imbedded in Matrigel suggesting the better properties of the Matrigel. The morphology of the cell aggregates changed over the culture time but the growth was very limited to 50 -75 µm after 19 days of culture (Figure 6).

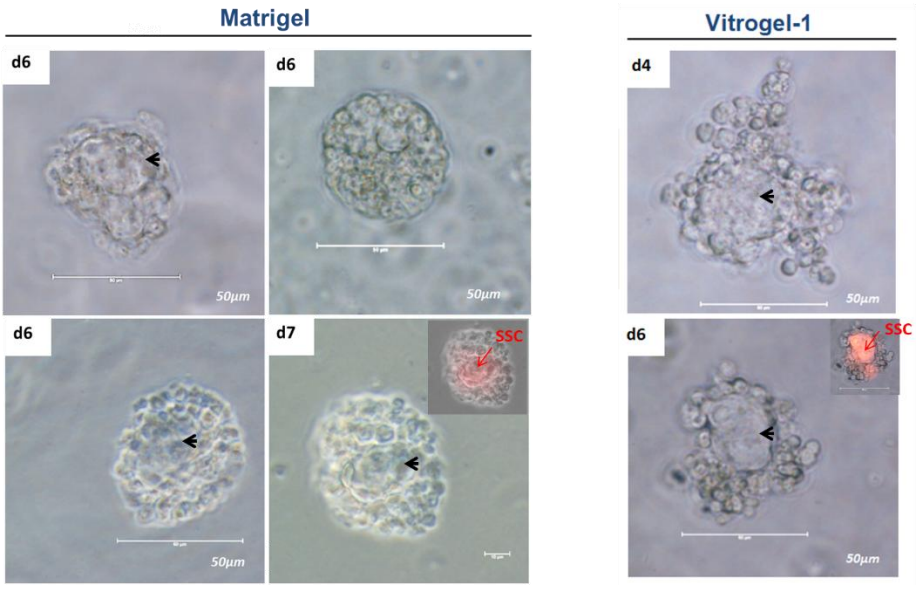

Figure 5: Testicular cell aggregates following tissue dissociation and embedding in different extracellular matrix.

Testicular cells were collected from adult zebrafish testes and dissociated using different 0.2% collagenase and 0.2% dispase in L15 medium. Cells were imbedded in the commercial extracellular matrix (Matrigel or Vitrogel). The formation and culture of cell aggregates in L15 medium were followed for 7 days. Note the different morphology of the cell aggregates after 6 or 7 days of culture, the limitation of the size to 50 µm, and the presence of fluorescent spermatogonial germ cells in the aggregates. The cell aggregates in Matrigel appear homogeneous with regular periphery in contrast to cell aggregates cultured in Vitrogel.

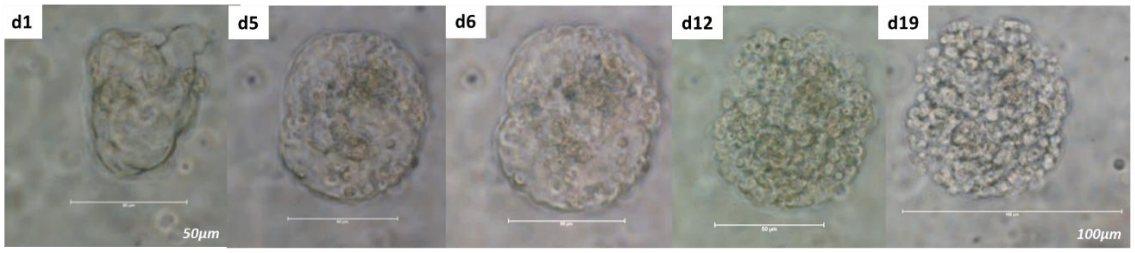

Figure 6: Evolution of testicular cell aggregates imbedded in Matrigel extracellular matrix.

Testicular cells were collected from adult zebrafish testes and dissociated using different 0.2% collagenase and 0.2% dispase in L15 medium. Cells were imbedded in the commercial Matrigel extracellular matrix. The formation and culture of cell aggregates in L15 medium were followed from the first day of culture (d1) up to 19 days of culture (d19). Note the low and limited growth of the cell aggregates and the apparent increase in the number of small cells at the surface of the cell aggregates.

4.2.3. Production of testicular organoids from Sturgeon

The cell reaggregation was tested from testicular cells dissociated from 2 years- old immature sturgeon. The dissociated and reaggregated cells were incubated in microwell plates. Cell aggregates were observed although their growth was limited to 50 µm. The histological analysis of the testicular cell aggregates is similar to spheroid structures (Figure 7). Spheroids are considered as primitive organoids.

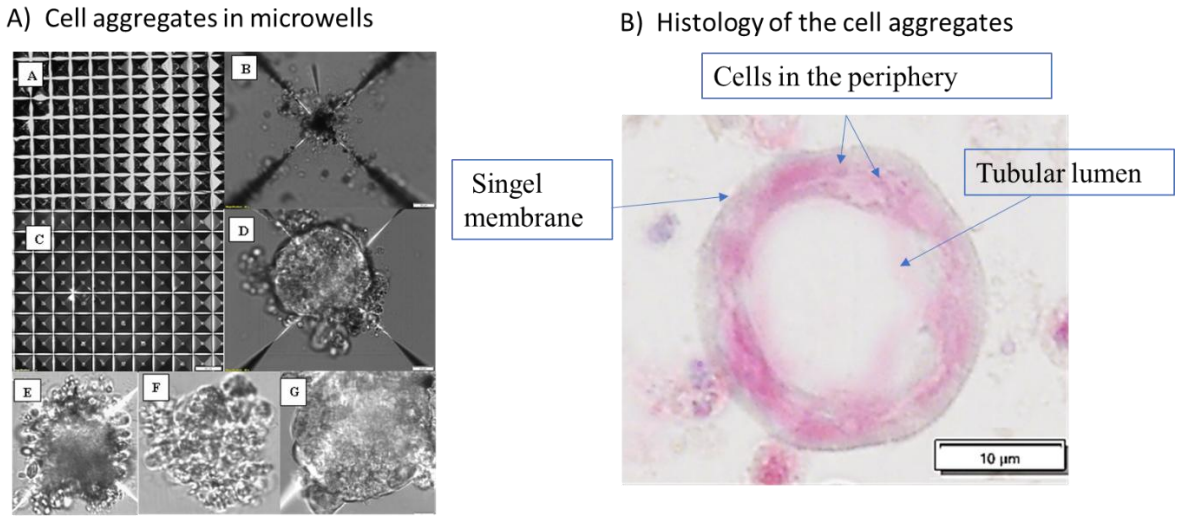

Figure 7: Cell aggregates of sturgeon testicular cells cultured in microwells.

A) Testicular cells were collected from immature sturgeons and dissociated using 0.2% collagenase and 0.2% dispase in L15 medium. Cells were cultured in microwell plates containing L15 medium and under air. The formation and the morphology of the cell aggregates were followed from the first day of culture (B left panel) up to 10 days of culture (D, left panel). Note the morphological changes of the cell aggregate during d1 (E, lelft panel), d5 (F, left panel) and d10 (G, left panel). B) histological analysis of the cell aggregates showing a structure similar to spheroid.

4.2.4. Analysis of the rainbow trout testicular matrix

In order to improve the limited growth of the testicular cell aggregates, we have characterized the components of the rainbow trout extracellular matrix in the testes using a large scale proteomic approach. Three experimental conditions were compared in order to enrich the protein extracts with matrisome proteins: snap freeze testes, frozen testes washed in PBS (partial decellularization) and decellularized testes using a 0.1% SDS solution. Six replicates were considered in each groups. The quality of the decellularization process was determined using the scanning electron microscopy (SEM). The quality of the extracellular matrix and collagen fibrils were studied using transmission electron microscopy (TEM). We have identified 15672 testicular proteins and 544 proteins were annotated as matrisome proteins according to the matrisome database. The testicular extracellular matrix contained 25% of collagen entities and only 3% of laminins. In contrast, it is well known that commercial Matrigel contain 60% of laminins and only less than 1% of collagens. We also identified matrisome proteins and matrisome interacting proteins mainly expressed in male or both sexual gonads. We also identify proteins involved in the softness and tensil index of extracellular matrix. These physical parameters are important for organoid formation and growth. In consequence, we believe that a supplementation of the Matrigel with different collagen or testicular proteins of decellularized Rainbow trout testes may be an interesting approach to improve the limited growth of the cell aggregates.

5. Conclusion

5.1. Search for GSC biomarkers

A candidate gene approach described in milestone 27 allowed us to identify a transcript encoding for a membrane bound protein and expressed at higher levels in trout undifferentiated A spermatogonia. The production of antibodies is costly, time consuming, and involves high levels of risk of failure. We decided to await single cell RNA sequencing experiment before starting the production of antibodies to make sure to have selected the best candidate GSC cell biomarker.

The single cell RNA sequencing allowed us to discriminate three different populations of undifferentiated A spermatogonia although we need to further established their dynamics. Genes specifically expressed in s0, s1 or s2 clusters (dnmt3bb.1, Id4, pex26…) will be used in double fluorescence *in situ* transcript hybridizations (RNAscope technology) to identify whether genes are accumulated in single spermatogonia or 2 to 8 cells cysts.

To make sure we are not missing a very rare and initial population of GSC from which s0 to s2 cell clusters could originate, we are currently repeating experiments from testicular cells of 17β-estradiol treated zebrafish. This will allow us to increase the number of spermatogonial cells to be clustered.

Although we need to further determine the cell entities regrouped in clusters s0 to s2, we have established a first list of genes that are differentially expressed in s0, s1 and s3 clusters. RNAscope experiments will be carried out to validate the cell-specific or differential expression patterns of transcript encoding for putative cell surface GSC biomarkers.

5.2. GSC *in vitro* cultures

We made some progress towards developing organoids. Primitive testicular organoids known as spheroids were obtained from zebrafish and sturgeon but their growth remained limited to 50 µm. This suggests that culture conditions need to be improved (composition of the culture medium, composition of extracellular matrix, …).

The proteomic characterization of the 15672 testicular proteins highlights the complexity of endogenous extracellular matrix and major differences compared to the commercial Matrigel matrix.

The scRNA-seq described above in section 4.1 will allow us to identify membrane bound receptors and their cognate ligands expressed at the surface of GSC and in Sertoli cells, respectively. The knowledge gain could highlight paracrine signalling pathways that could be active in GSC to maintain cell survival and GSC renewal. The addition of activators and inhibitors of such signalling pathways could be used to improve the culture media.

6. References

Aliakbari F, Yazdekhasti H, Abbasi M, Hajian MM & Baazm M 2016 Advances in cryopreservation of spermatogonial stem cells and restoration of male fertility. *Microsc.Res.Tech.* **79** 122–129.

Bellaiche J, Lareyre JJ, Cauty C, Yano A, Allemand I & Le Gac F 2014 Spermatogonial stem cell quest: nanos2, marker of a subpopulation of undifferentiated A spermatogonia in trout testis. *Biol.Reprod.* **90** 79.

Iwasaki-Takahashi Y, Shikina S, Watanabe M, Banba A, Yagisawa M, Takahashi K, Fujihara R, Okabe T, Valdez Jr DM, Yamauchi A & Yoshizaki G 2020 Production of functional eggs and sperm from in vitro-expanded type A spermatogonia in rainbow trout. *Communications Biology* **3** 308.

Jin S, Guerrero-Juarez CF, Zhang L, Chang I, Ramos R, Kuan C-H, Myung P, Plikus MV & Nie Q 2021 Inference and analysis of cell-cell communication using CellChat. *Nature Communications* **12** 1088.

Kawasaki T, Siegfried KR & Sakai N 2015 Differentiation of zebrafish spermatogonial stem cells to functional sperm in culture. *Development* dev.129643.

Loir M 1999a Spermatogonia of rainbow trout: I. Morphological characterization, mitotic activity, and survival in primary cultures of testicular cells. *Mol.Reprod.Dev.* **53** 422–433.

Loir M 1999b Spermatogonia of rainbow trout: III. In vitro study of the proliferative response to extracellular ATP and adenosine. *Mol.Reprod.Dev.* **53** 443–450.

Maouche A, Curran E, Goupil A-S, Sambroni E, Bellaiche J, Le Gac F & Lareyre J-J 2018 New insights into the evolution, hormonal regulation, and spatiotemporal expression profiles of genes involved in the Gfra1/Gdnf and Kit/Kitlg regulatory pathways in rainbow trout testis. *Fish Physiology and Biochemistry* **44** 1599–1616.

Nóbrega RH, Morais RDVDS, Crespo D, De Waal PP, De França LR, Schulz RW & Bogerd J 2015 Fsh Stimulates Spermatogonial Proliferation and Differentiation in Zebrafish via Igf3. *Endocrinology* **156** 3804–3817.

Okutsu T, Suzuki K, Takeuchi Y, Takeuchi T & Yoshizaki G 2006a Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. *Proc.Natl.Acad.Sci.U.S.A.* **103** 2725–2729.

Okutsu T, Yano A, Nagasawa K, Shikina S, Kobayashi T, Takeuchi Y & Yoshizaki G 2006b Manipulation of fish germ cell: visualization, cryopreservation and transplantation. *J.Reprod.Dev.* **52** 685–693.

Richer G, Goyvaerts C, Marchandise L, Vanhaecke T, Goossens E & Baert Y 2024 Spermatogenesis in mouse testicular organoids with testis-specific architecture, improved germ cell survival and testosterone production. *Biofabrication* **16** 045024.

Sakai N 2006 In vitro male germ cell cultures of zebrafish. *Methods* **39** 239–245.

Sakib S, Voigt A, Goldsmith T & Dobrinski I 2019a Three-dimensional testicular organoids as novel in vitro models of testicular biology and toxicology. *Environmental Epigenetics* **5** dvz011.

Sakib S, Uchida A, Valenzuela-Leon P, Yu Y, Valli-Pulaski H, Orwig K, Ungrin M & Dobrinski I 2019b Formation of organotypic testicular organoids in microwell culture†. *Biology of Reproduction* **100** 1648–1660.

Sambroni E, Rolland AD, Lareyre JJ & Le GF 2012 FSH and LH have common and distinct effects on gene expression in rainbow trout testis. *J.Mol.Endocrinol.* **50** 1–18.

Sambroni E, Lareyre JJ & Le GF 2013 Fsh controls gene expression in fish both independently of and through steroid mediation. *PLoS.One.* **8** e76684.

Sato T, Vries RG, Snippert HJ, Van De Wetering M, Barker N, Stange DE, Van Es JH, Abo A, Kujala P, Peters PJ & Clevers H 2009 Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459** 262–265.

Takeuchi Y, Yoshizaki G & Takeuchi T 2003 Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. *Biology of Reproduction* **69** 1142–1149.

Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, Wang X, Bodeau J, Tuch BB, Siddiqui A, Lao K & Surani MA 2009 mRNA-Seq whole-transcriptome analysis of a single cell. *Nature Methods* **6** 377–382.

Trapnell C, Cacchiarelli D, Grimsby J, Pokharel P, Li S, Morse M, Lennon NJ, Livak KJ, Mikkelsen TS & Rinn JL 2014 The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nature Biotechnology* **32** 381–386.

Vermeulen M, Del Vento F, Kanbar M, Pyr Dit Ruys S, Vertommen D, Poels J & Wyns C 2019 Generation of Organized Porcine Testicular Organoids in Solubilized Hydrogels from Decellularized Extracellular Matrix. *International Journal of Molecular Sciences* **20** 5476.

Yoshizaki G, Ichikawa M, Hayashi M, Iwasaki Y, Miwa M, Shikina S & Okutsu T 2010 Sexual plasticity of ovarian germ cells in rainbow trout. *Development.* **137** 1227–1230.

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Work Package	N°	5	Title	New biological models for aquaculture research using innovative biotechnologies
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Work Participants	IMR, MATE, IFREMER, JU, NTNU, ULPGC, WU, DTU CCMAR			

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