Supplementary Information for:

Genetically tractable embryonic cell lines from sea urchins

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Supplementary Methods

Transfection of cultured sea urchin embryonic cells

Transfection of cultured sea urchin cells was attempted using mRNA encoding red fluorescence protein (RFP) produced from the plasmid (NPM1-RFP-pCS2+) (a gift from Clifford Brangwynne at Princeton University)[73], a plasmid encoding cyan fluorescent protein (CFP) mCerulean behind the promoter of the *L.variegatus* polyubquitin-C gene (LvPolyUb::H2B-CFP) (a gift from Amro Hamdoun at Scripps Institution of Oceanography, UCSD)[74], and dextran tetramethylrhodamine (3000MW, ThermoFisher Scientific) as reporters. To generate the RFP mRNA, the NPM1-RFP-pCS2+ plasmid was linearized using NotI and gel-purified using a QIAquick Gel Extraction Kit (Qiagen, 28704). Linearized plasmids were column-cleaned under RNAse-free conditions using a Zymo DNA clean and concentrator kit (Zymo, D4034). Linearized plasmids were used as a DNA template for in vitro transcription using the HiScribe SP6 Kit (New England Biolabs, E2070S) and capped with a 3´-O-Me-m7G(5')ppp(5')G RNA cap structure analog (New England Biolabs, S1411S) following manufacturer's instructions (New England Biolabs, RNA Synthesis Protocol, E2070). mRNA was column purified using the Zymo RNA clean and concentrator kit (Zymo, R1013) and quantified using a Qubit 4 Fluorometer and broad range RNA kit (Invitrogen, Q10211). mRNA was aliquoted and stored at -80°C until use.

Lipid-mediated transfections were conducted with NPM1-RFP mRNA using Lipofectamine™ MessengerMAX™ (Invitrogen, LMRNA001) according to the manufacturer's recommendations. Trials were conducted to increase the amount of mRNA added, to test different complex formation medias (basic urchin media vs. OptiMEM), the number of cells per

transfection (200,000 vs. 400,000), the presence/absence of serum in the recovery medium, and the time plated cells were in media without serum as summarized in Table S8.

Electroporation was performed with the BioRad Gene Pulser Xcell Electroporation System in Gene Pulser 0.2 cm gap cuvettes with $2x10^5$–$5x10^5$ cells using the RFP mRNA, the LvPolyUb::H2B-CFP plasmid, and dextran tetramethylrhodamine, (3000MW). Multiple media types were tested including basic urchin media (BUM) and a mannitol-based electroporation buffer ('T+K' buffer) used previously for the electroporation of sea urchin sperm and eggs[75,76]. A broad range of voltages, length of voltage (ms), intervals between pulses and number of pulses were tested (Table S8). Nucleofection was performed using the Lonza 4D-Nucleofector X Unit Transfection System (Lonza Biosciences) using RFP mRNA, the LvPolyUb::H2B-CFP plasmid, and dextran tetramethylrhodamine, (3000MW). A total of $2x10^5$–$2.5x10^5$ cells were used per condition using different electroporation media (BUM, T+K, and PBS). Cell viability was assessed after electroporation by trypan blue exclusion or by evaluating the amount of adherent cells. The electroporation and nucleofection conditions tested are summarized in Table S8.

Attempts were made to transfect the *L. variegatus* cells with the LvPolyUb::H2B-CFP plasmid using linear PEI (Polyethylenimine; Polysciences, Inc. cat #23966). Briefly, 400,000 cells were seeded in UM with 10% FBS in 96 well tissue culture plates 24 hours prior to PEI transfection to achieve ~70% confluence. Cells were boosted by the addition of 200 µL fresh UM with 10% FBS on the day of transfection. Plasmids and PEI were diluted in BUM or OptiMEM. Diluted PEI concentrations were varied to test various mass ratios of PEI:DNA (from 1:1 to 5:1). Diluted plasmids were added to diluted PEI dropwise while gently vortexing. DNA-PEI mixes were incubated at room temperature for 15 min before adding dropwise to plated cells.

Following transfections, cells were imaged at 24, 48, 72, and 96 hours post-transfection on an Olympus IX83 inverted microscope using the Olympus cellSens Dimension software.

Cryopreservation of cultured sea urchin cells

A variety of cryopreservation protocols were tested for our sea urchin embryonic cultures (slow freezing, vitrification, different cryoprotective agents), detailed in Table S5. Cells were counted using a hemocytometer with trypan blue exclusion to determine viability. Aliquots of 2–10 million cells (along with any spheroids and embryoid bodies in the culture) were centrifuged at 800 x g for 5 minutes. The cell pellets were gently resuspended in 1 mL of the appropriate freezing media (see Table S5) and placed in a 2 mL cryovial in a Mr. Frosty™ freezing container at -80℃ to cool at a controlled rate (~1℃/min). After 24 hours, the cells were transferred to the vapor phase of a liquid nitrogen freezer (Taylor Wharton 10K K Series Cryogenic System) for at least 24 hours before reviving. To revive cells, the cryovial was quickly warmed in a 25℃ water bath until just thawed (< 1 minute) and 1 mL of room temperature urchin media with 15% FBS was added to the cryovial before transferring into 10 mL of urchin media with 15% FBS to dilute the cryoprotectants. The sample was then centrifuged at 800 x g for 5 minutes. The cell pellets were gently resuspended in 5 mL urchin media with 15% FBS and transferred to a T25 tissue culture flask maintained at room temperature. Cells were counted and viability assessed on a hemocytometer using trypan blue exclusion after thawing and once per week thereafter, and the media was changed two times per week until the experiment was terminated.

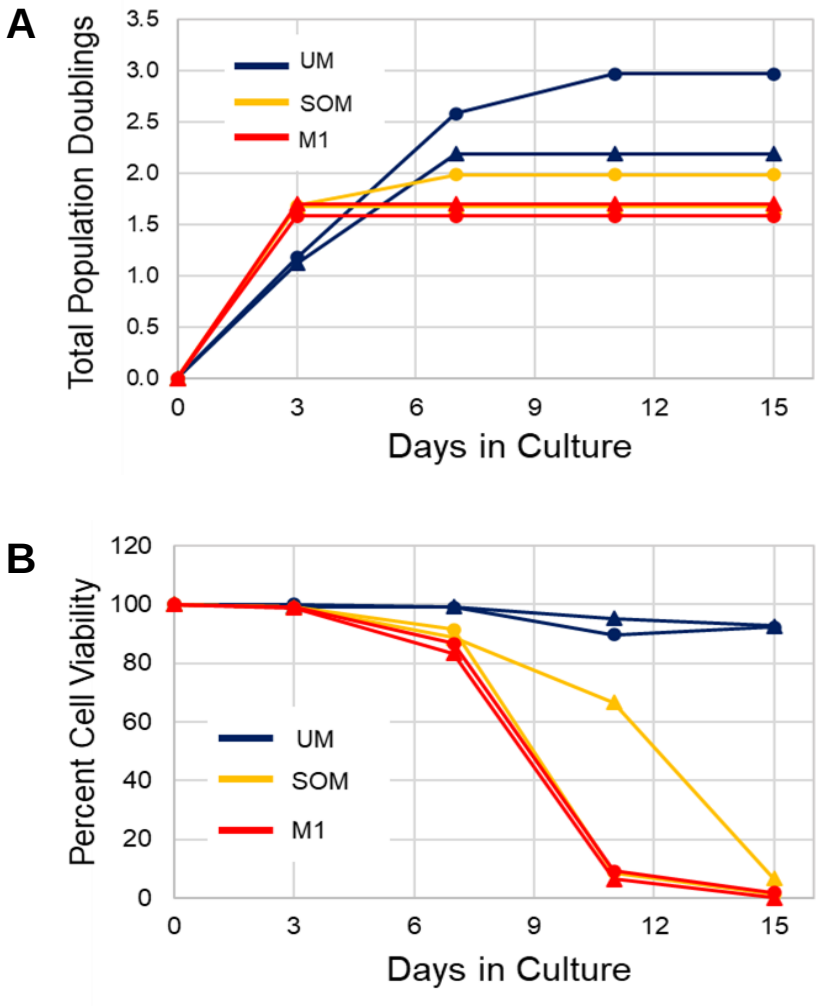
Vitrification, or fast freezing, was also tested using two different cryoprotectant solutions[77]. Vitrification solution A (VSA) contained 40% (v/v) Ethylene Glycol and 0.6 M sucrose while vitrification solution B (VSB) contained 20% (v/v) Ethylene Glycol, 0.6M sucrose and 16% (v/v) 1,2 Propanediol. Embryoid bodies and spheroids, single cells and adhered

colonies were collected and centrifuged for 2 minutes at 100 x g. First the cells were equilibrated in equilibration solution 1 (ES1; 10% ethylene glycol in basic urchin media) for 3.5 minutes. Cells were then centrifuged for 2 minutes at 100 x g, ES1 was removed and equilibration solution 2 (ES2; 25% ethylene glycol in basic urchin media) was added and incubated for 3.5 minutes. The cells were centrifuged for 2 minutes at 100 x g, ES2 was removed and 500 µL of VSA or VSB was added. The cells were equilibrated for 3.5 minutes in cryovials at room temperature before being placed directly in a liquid nitrogen freezer for a minimum of 24 hours. To revive cells, the cryovial was quickly warmed in a 25℃ water bath (<1 minute) before adding 1 mL of room temperature 1M sucrose. The cells were left to equilibrate for 5 minutes before sequentially diluting with sucrose to 0.7 M by 0.175 M with each step, equilibrating for 2.5 minutes after each addition. Then cells were spun for 2 minutes at 100 x g and resuspended in 5 mL of urchin media with 15% FBS and plated on a T25 flask. Cells were counted using a hemocytometer with trypan blue exclusion to determine growth and viability post-thaw.

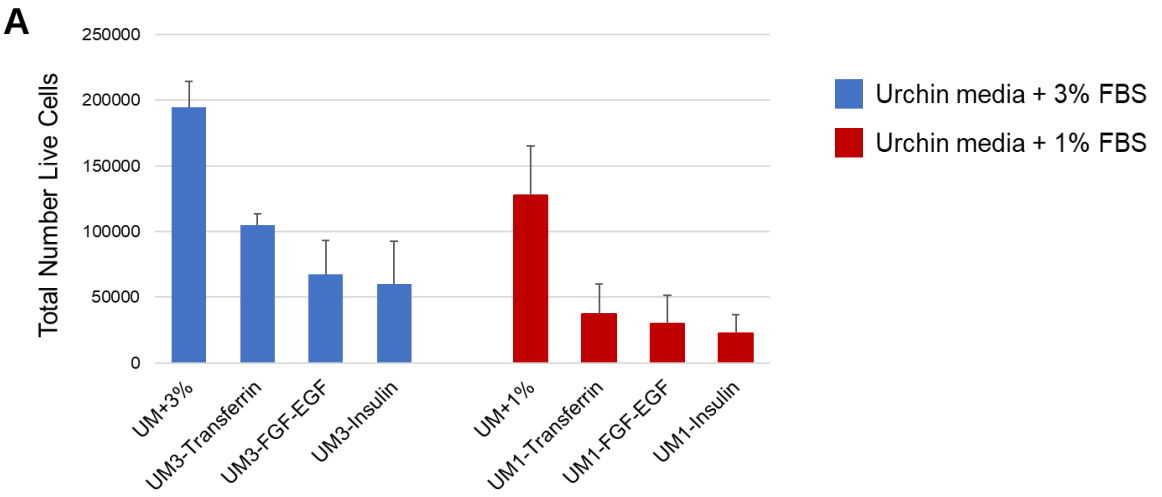
References for Supplementary Methods

73. Brangwynne, C. P., Mitchison, T. J. & Hyman, A. A. Active liquid-like behavior of nucleoli determines their size and shape in *Xenopus laevis* oocytes. *Proceedings of the National Academy of Sciences* **108**, 4334-4339, doi:doi:10.1073/pnas.1017150108 (2011).
74. Jackson, E. W. *et al.* Stable germline transgenesis using the Minos Tc1/mariner element in the sea urchin Lytechinus pictus. *Development* **151**, doi:10.1242/dev.202991 (2024).
75. Swezey, R. R. & Epel, D. Stable, resealable pores formed in sea urchin eggs by electric discharge (electroporation) permit substrate loading for assay of enzymes in vivo. *Cell Regulation* **1**, 65-74 (1989).
76. Takao, D. & Kamimura, S. Single-cell electroporation of fluorescent probes into sea urchin sperm cells and subsequent FRAP analysis. *Zoological science* **27**, 279-284 (2010)
77. Magalhães, R. *et al.* Vitrification Successfully Preserves Hepatocyte Spheroids. *Cell Transplantation* **17**, 813-828, doi:10.3727/096368908786516765 (2008).

Supplementary Fig. 1. Growth and viability of *L. variegatus* embryonic cells in three different media. (A) Total cumulative population doublings of the embryonic cells over the first 15 days in culture in three different media: Urchin Media with 3% FBS (UM), Supplemented Oyster Media (SOM), and Marine Media M199 (M1). (B) Percent viable cells in each media condition over the first 15 days of culture. Two replicate T25 flasks (represented by triangles and circles) were monitored for each media condition represented by the different colored lines.



Supplementary Fig. 2. *L. variegatus* embryonic cells grown in Urchin Media without selected additives. (A) Dissociated cells were seeded into the wells of 24-well tissue culture plates (150K/well), media changed every 3-4 days, and counted 14 days after seeding. The cells were seeded into Urchin Media containing 3% FBS (blue bars) or 1% FBS (red bars) with the indicated additives omitted from the media: transferrin, FGF and EGF, insulin. Values are the average of four counts with error bars representing standard error of the mean. (B-E) EMBOSS Needle pairwise sequence alignments for the commercially available recombinant human additives versus the predicted protein sequences from *L. variegatus*: FGF (B), EGF (C), insulin (D), transferrin (E).



B **FGF Similarity 48.3%**

```
Hs_rFGF  AAGSITTLPALPEDGGSGAFPPG-----HFKDPKRLYCKNGGFFLRIHPD
                          |...|     :.|.|.:|:|:. .|.|.:|.|
Lv_FGF   ------------------ASSRGSPLIYNSKQPTQLFCRT-NFRLAVHED

Hs_rFGF  GRVDGVREKSDPHIKLQLQAEERGVVSIKGVCANRYLAMKEDGRLLASKC
         |.::|.|:..|.:..|.:|::.|.:|||||:.:..|:.:.::|.|.::.
Lv_FGF   GTINGTRDNMDVYSSLYIQSQRRSIVSIKGLKSQLYVCVDDNGNLYGNRR

Hs_rFGF  VTDECFFFERLESNNYNTY---------RSRKYTSWYVALKRTGQYKLGS
         |:..|:|.|:||.|.:|||         :.|::.:.::::.:.|:.::..
Lv_FGF   VSRNCYFQEKLEPNFFNTYAYKMPDSTNKRRRHRTLFLSINKYGESRIAK

Hs_rFGF  -KTGPGQKAILFLPMSAKS---
          :|   ||...|:|:...:
Lv_FGF   VRT---QKKAQFIPLVPPTELL
```

C **EGF Similarity 55.2%**

```
Hs_rEGF  NSDSE---CPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDL
         .||:.   |...:||||||.|||.::..:||.:|:|:.||.|.|||||.|
Lv_EGF   TSDAHIEPCEPFYDGYCLHGGVCKWLTGIDKPSCDCISGYRGARCQYRSL

Hs_rEGF  ----------KWWELR-
                   .:||:.
Lv_EGF   ATEGEERLFGDYWEVFI
```

D **Insulin A Similarity 71.4%**

```
Hs_InsulinA    GIVEQCCTSICSLYQLENYCN
               .||.:||.:.||:..||:|||
Lv_IGF1        LIVTECCHNRCSVSHLESYCN
```

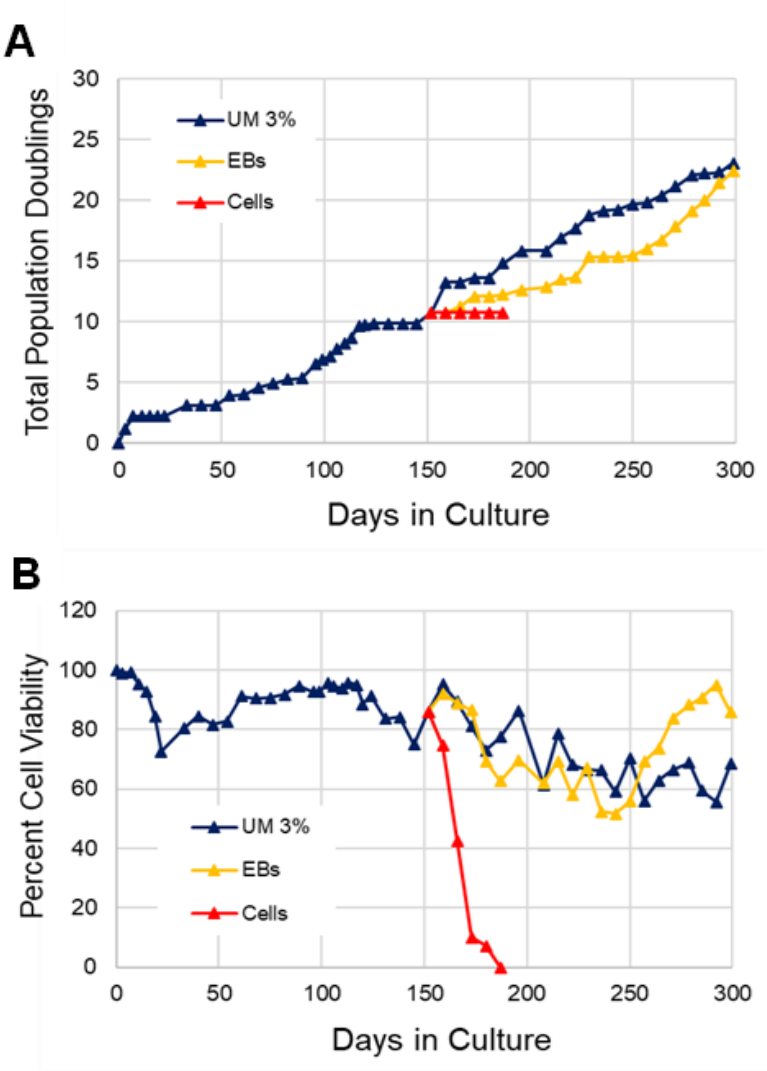
Insulin B Similarity 46.7%

```
Hs_InsulinB    FVNQHLCGSHLVEALYLVCGERGFFYTPKT
                 ...|||..||.|:..||..||::..|..
Lv_IGF1        --FPLLCGQELVNAVAFVCDGRGYYGQPSK
```

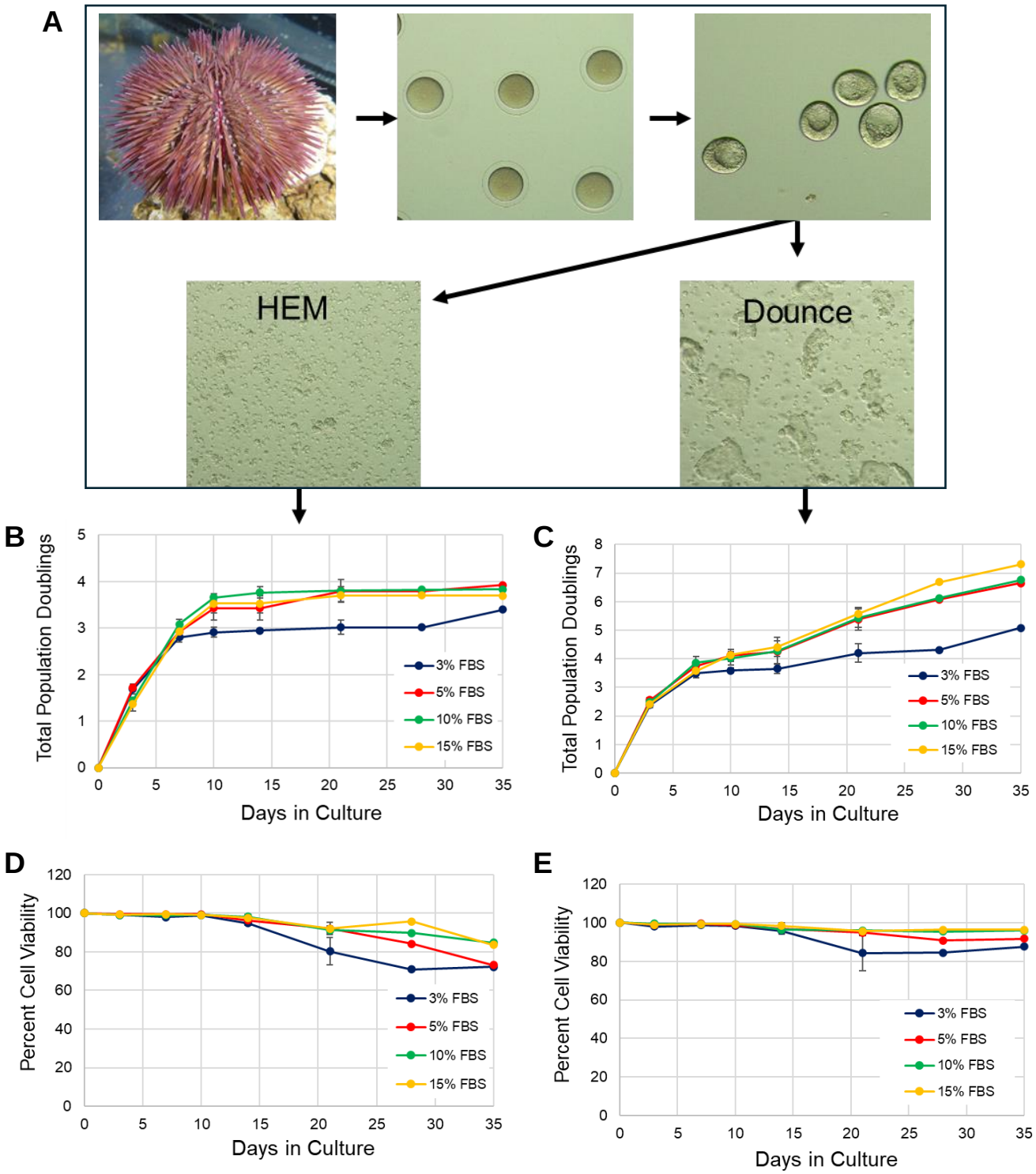
E **Transferrin Similarity 51.6%**

```
Hs_Trans  MRLAVGALLVCAVLGLCLAVPDKT-----VRWCAVSEHEATKCQSFRDHM
              :.|:::|..|.|.:.|...|     :|||.:|..|:.||.:.|...
Lv_Trans  ----MNAMMMCIFLCLLMTVSLTTAATTQMRWCTISNEESQKCVAMRTAF

Hs_Trans  KSVIPSDGPSVACVKKASYLDCIRAIAANEADAVTLDAGLVYDAYLAPN-
          .|  .|..|.|.||.:...||::.:...:|:.:|||.|   |.:||..
Lv_Trans  SS--NSLSPEVVCVAGSDIPDCMQKVQDEQANLITLDGG---DIHLAGKE

Hs_Trans  -NLKPVVAEFYGSKEDPQTFYYAVAVVKKDS-GFQMNQLRGKKSCHTGLG
           :|.||:.|.|.     |..||.:||||..| ..:::.|.||.|||||:.
Lv_Trans  FDLVPVIQEVYA-----QDTYYGIAVVKATSTDLRLSNLEGKNSCHTGVR

Hs_Trans  RSAGWNIPIGLLYCDLPEPRKPLEK------------AVANFFSGSCAP
          |:||||||:|.|          ||:             ||:.||:.||||
Lv_Trans  RTAGWNIPVGFL----------LEQGYMTPTDCGDDITAVSQFFNQSCAP

Hs_Trans  CA-------DGTDFPQLCQLC--PGCGCSTLNQYFGYSGAFKCLKDGAGD
          .|       .|.:...||.:|  |.|...:...|..|:|||:||.:|.||
Lv_Trans  GAYSERNDPYGQNPQNLCGICTDPRCPPDSTELYQSYAGAFRCLAEGDGD

Hs_Trans  VAFVKHSTIFENL-ANKAD---RD----QYELLCLDNTRKPVDEYKDCHL
          |||:|.:|:.||. .|..|   ||    .:.:||.|.|:..||:.::|:|
Lv_Trans  VAFIKPATVIENTDGNGQDTWNRDLSSADFRILCPDGTQSTVDQAENCNL

Hs_Trans  AQVPSHTVVA--RSMGGKEDLIWELLNQAQEHFGKDKSKE-FQLFSSP--
          |:.|:|.:|.  |:...:.....::|.||.:.||.|.::. |::|.|.
Lv_Trans  AKSPAHAIVTSPRTTDAQIQAFQDVLRQAVDLFGDDNNQNGFKMFDSTDF

Hs_Trans  HGKDLLFKDSAHGFLKVPPRMDAKMYLGYEYVTAIRNLREGTCPEAPTDE
          :|.||||||||.....:...:.:||| .|...|..|:  .|||.
Lv_Trans  NGHDLLFKDSAQNLANLESGLTYRTYLG-TYADTIDGLK--MCPEG----

Hs_Trans  CKPVKWCALSHHERLKCDEWSVNSVG-----KIECVSAETTEDCIAKIMN
            .::||..|..|..||.:.|....|     :|.|....:...|:::::.
Lv_Trans  --SLRWCTTSAVETQKCRDMSAAFKGEGWSPQISCYEEMSKGHCVDRVIA

Hs_Trans  GEADAMSLDGGFVYIAGKCGLVPVLAENYNKSDNCEDTPEAGYFAIAVVK
          |:||.::||||.:::.||. .:.|::.|:|:...   :.|:|.|||:||.:
Lv_Trans  GDADVVTLDGGDLFEAGN-DVAPIVGESYSSGG---ENPDASYFAVAVAR

Hs_Trans  KSASDLTWDNLKGKKSCHTAVGRTAGWNIPMGLLYNKINHCRFD------
          | .:....|.|:.|||||.:|:|:|||:|:|.|.:. .|...|
Lv_Trans  K-GTQFGMDELQDRKSCHTGIGKTSGWNVPVGHLIDN-GHISVDDNCDIP

Hs_Trans  ----EFFSEG-CAPGSKKD---------SSLCKLCMGSGLNLCEPNNKEG
              ||||.| ||||:|.|         :|||.||:|:|.|.|..|.|.
Lv_Trans  KAVGEFFSAGSCAPGAKTDKYDPEGTNPNSLCSLCIGTGNNFCVRNANEP

Hs_Trans  YYGYTGAFRCLVEK-GDVAFVKHQTVPQNTGGKNPDPWAKNLNEKDYELL
          ||.|.||||||.|: ||||||||.|||.||.|:..:.|::|||...||||
Lv_Trans  YYDYAGAFRCLAEQAGDVAFVKHTTVPDNTDGQGTEDWSRNLNSSHYELL
```

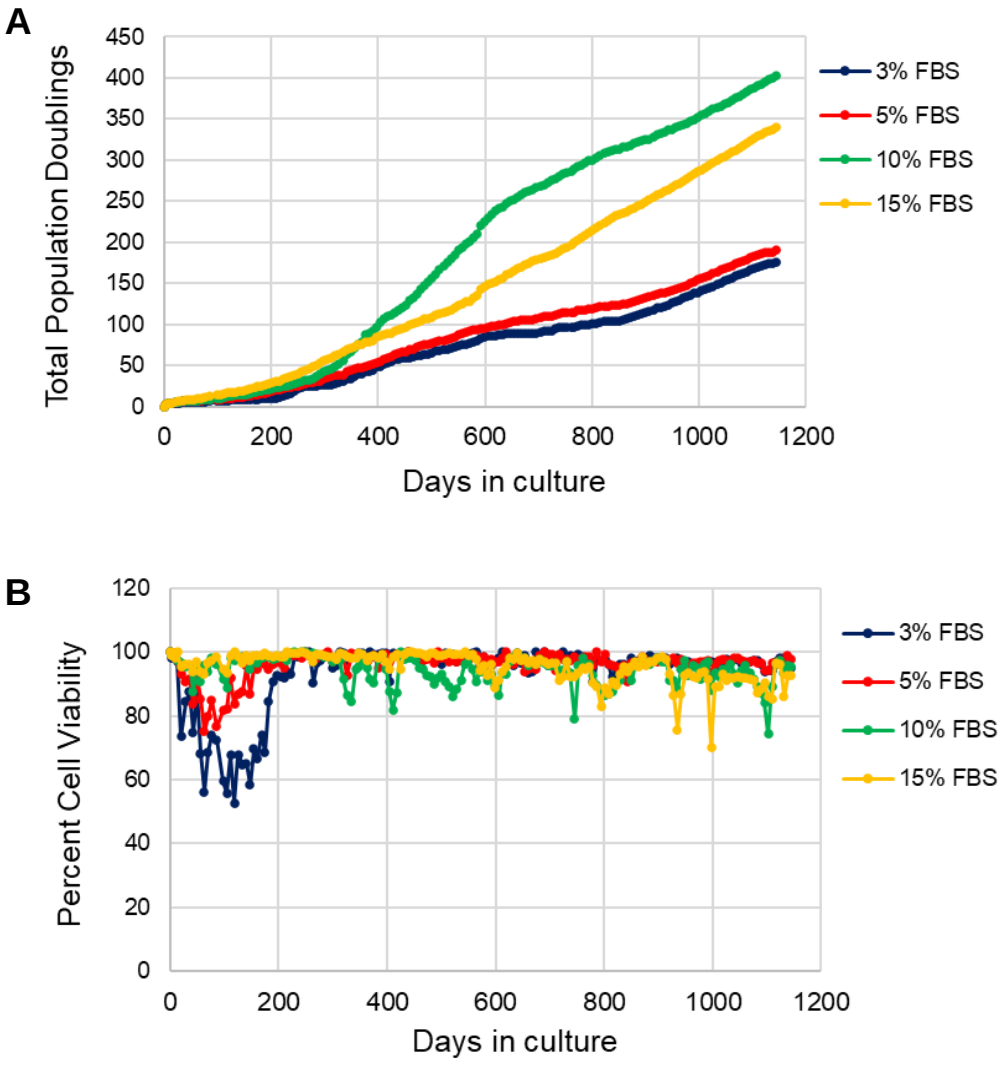
Supplementary Fig. 3. Growth and viability of size fractionated *L. variegatus* embryonic cell cultures. (A) Total cumulative population doublings over time for complete cell cultures containing a mixture of suspension and adhered cells and embryoid bodies (UM 3%, blue line). Cultures seeded with suspension cells that passed through a 40 µm strainer (Cells) are shown by the red line, and embryoid bodies larger than 40 µm (EBs) are shown by the yellow line. Population doublings were calculated by counting suspension cells. (B) Percent cell viability of the complete and size fractionated cultures determined by trypan blue exclusion. All cultures were in Urchin Media with 3% FBS.



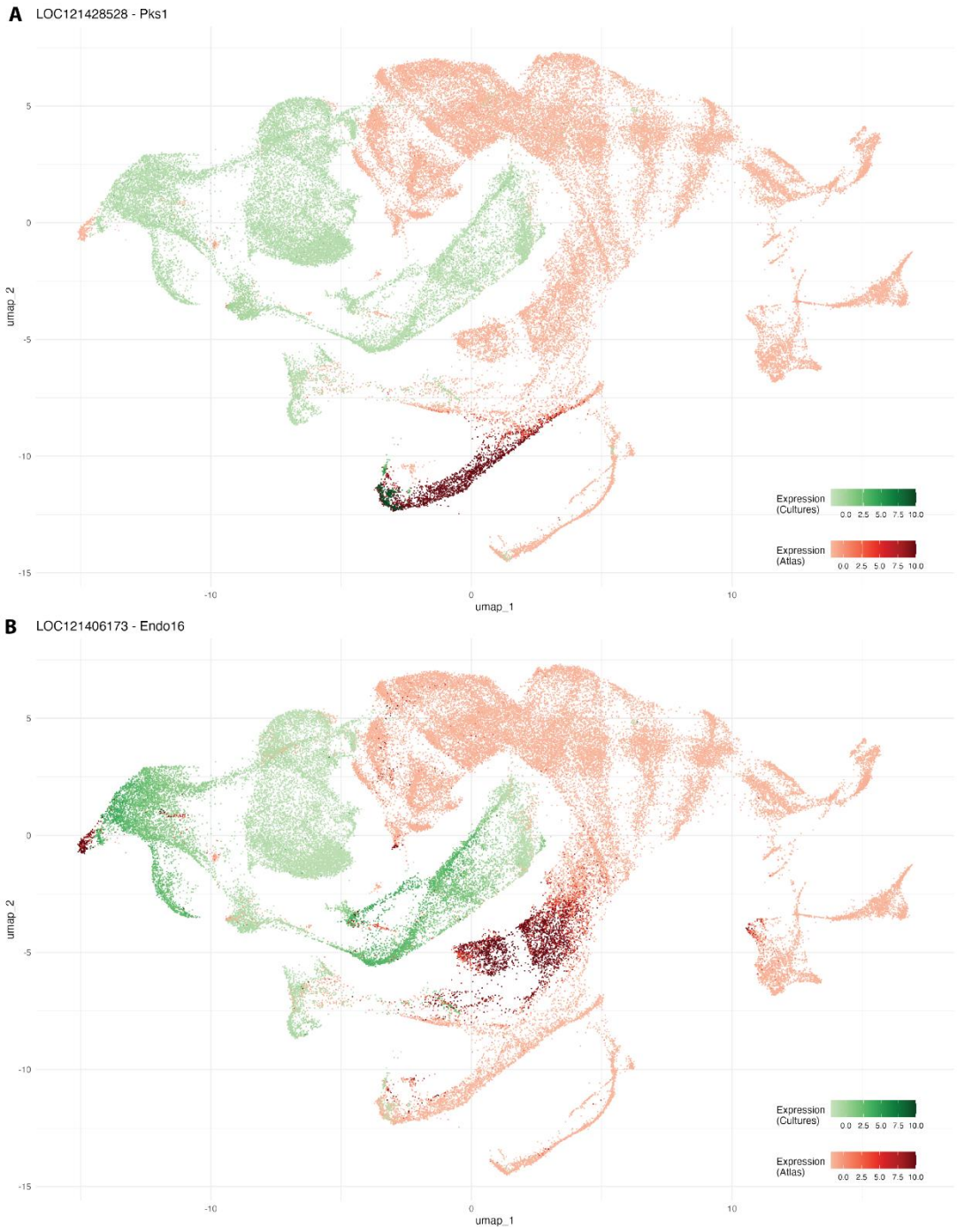
Supplementary Fig. 4. Growth and viability of *L. variegatus* embryonic cell cultures generated by mechanical and chemical dissociation. (A) Late-stage blastulae from *L. variegatus* dissociated with HEM or Dounce homogenization. Total cumulative population doublings for cells dissociated with HEM (B) or Dounce (C) over 35 days in culture in Urchin Media (UM) with different concentrations of FBS (3%, 5%, 10%, 15%). Population doublings were calculated by counting suspension cells and shown as an average of n=3 flasks with error bars representing SEM. Percent viability of cells dissociated with HEM (D) or Dounce (E) (n=3, SEM).



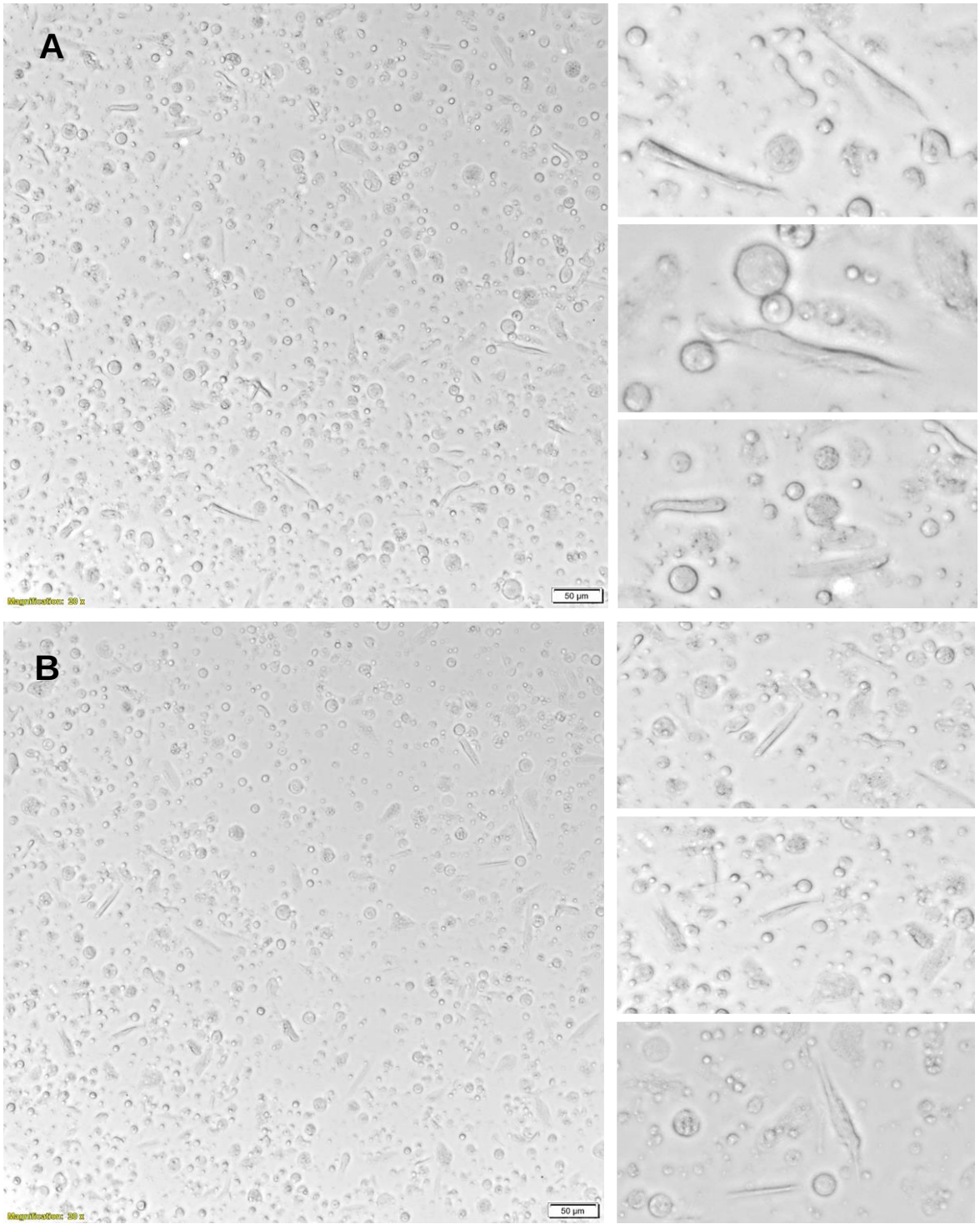
Supplementary Fig. 5. Growth and viability of *L. variegatus* embryonic cells for more than three years in culture. (A) Cumulative population doublings of *L. variegatus* embryonic cells in Urchin Media with different concentrations of FBS (3%, 5%, 10%, 15%). (B) Percent viable cells in each media condition



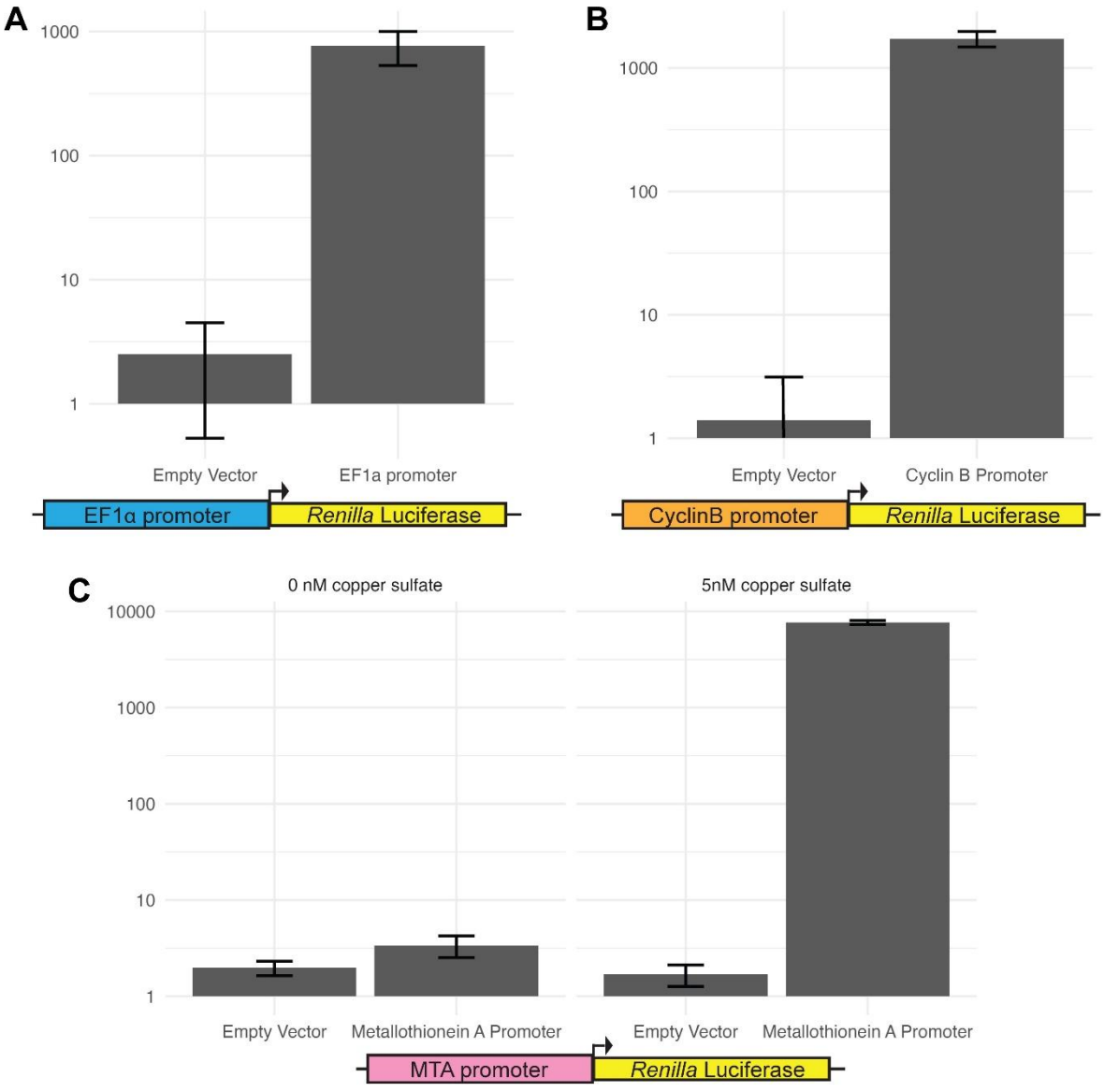
Supplementary Fig. 6. (A) Normalized Pks1 expression for cultured cells (green) and atlas cells (red) in integrated UMAP space. A max cutoff value of 10 and normalization to the maximum is imposed for visualization. Pks1 marks pigment cells. (B) Normalized Endo 16 expression for cultured cells (green) and atlas cells (red) in integrated UMAP space. A max cutoff value of 10 and normalization to the maximum is imposed for visualization. Endo16 is a marker of midgut epithelium and precursor vegetal plate cells.



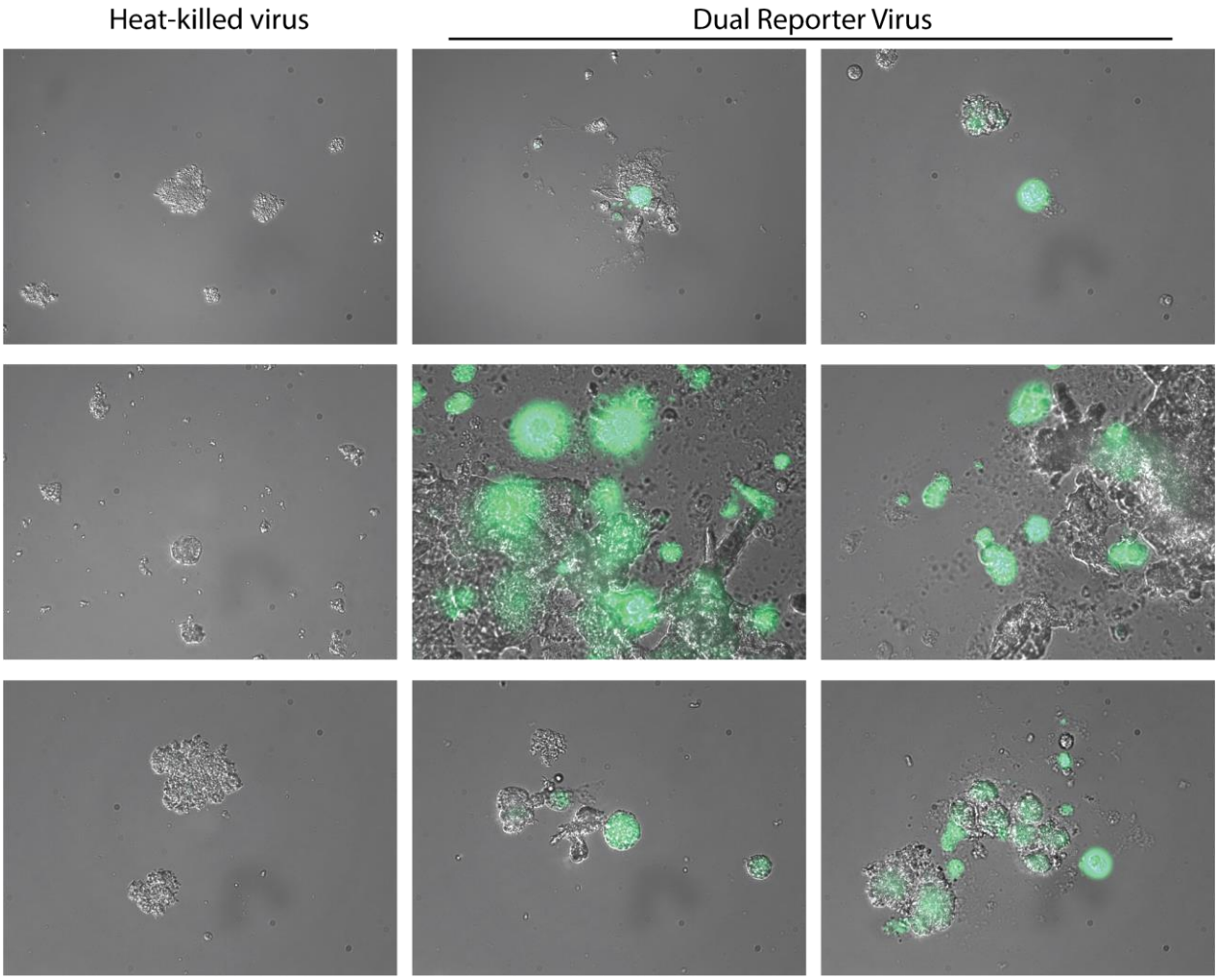
Supplementary Fig. 7. *L. variegatus* embryonic cells produced spicules demonstrating active biomineralization in culture. Panel A and B: Two images of cell cultures in UM + 5% FBS on Day 425 with notable spicules. Enlarged sections of the image are shown to the right of each image. Scale bar is 50 µm.



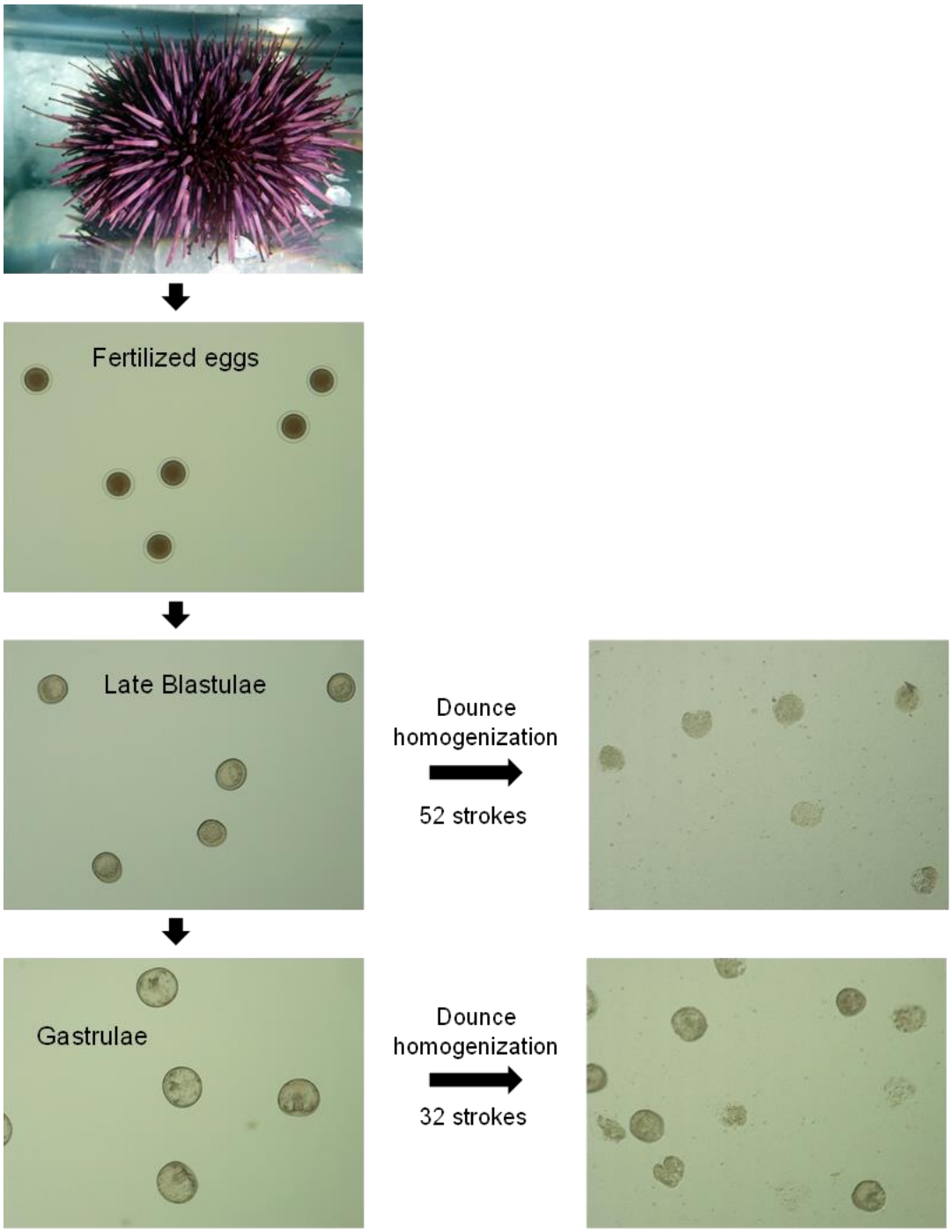
Supplementary Fig. 8. *L. variegatus* promoters drive robust transgene expression. 270 bases of sequence upstream of the putative transcriptional start site of the EF1α (A) Cyclin B (B) and Metallothionein A (C) genes was cloned upstream of luciferase and used to assess promoter function. (C) The Metallothionein A promoter drove robust luminescence upon induction with 5 nM $CuSO_4$. Error bars represent 95% confidence intervals for mean relative luminescence.



Supplementary Fig. 9. Additional fluorescence micrographs of *L. variegatus* embryonic cell cultures transduced with reporter lentivirus. Cells were imaged 2 days following transduction with either heat-killed or live dual reporter lentivirus, showing bright fluorescence in cells transduced with live virus.



Supplementary Fig. 10. Derivation of *S. purpuratus* embryonic cell cultures. Late-stage blastulae and early gastrulae from *S. purpuratus* were mechanically dissociated by Dounce homogenization and used to seed embryonic cell cultures.



Supplementary Figure 11. Temperature effect on growth and viability of *S. purpuratus* embryonic cells. (A) Cumulative population doublings of *S. purpuratus* embryonic cells in Urchin Media with different concentrations of FBS (5%, 10%, 15%). At 51 Days in culture replicate flasks of cells maintained in UM+10% FBS and 15% FBS were placed at room temp (22°C) (triangles) while all other cultures were maintained at 17°C (circles). (B) Percent viable cells in each media condition. Each line is an average of n=2 replicate flasks for each condition.