Primordial Blasts, a population of blood borne stem cells responsible for whole body regeneration in a basal chordate

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Abstract:

The invertebrate chordate *Botrylloides leachii* regenerates entire new bodies from small fragments of blood vessels in a process called whole body regeneration (WBR). During the early stages of WBR, proliferation occurs only in small, blood borne cells expressing *integrin-alpha-6, pou3* and *vasa*. Proliferating blood cells can be ablated using Mitomycin C (MMC), and MMC treatment blocks regeneration. However, regeneration could be rescued by injection of blood borne cells from healthy individuals, and using limiting dilution approaches we found that injection of a single Integrin-alpha-6 (IA6)-positive cell is capable of rescuing whole body regeneration. The contribution of IA6+ cells to WBR was confirmed via lineage tracing: following transplantation of EdU-labeled IA6+ cells into MMC treated recipients, EdU label is detected in regenerating tissues. Our data suggest that *ia6+pou3+* cells are proliferating blood borne stem cells that are responsible for whole body regeneration, and we named these cells Primordial Blasts. Primordial Blasts express *notch* and *frizzled* receptors, and inhibitors of either Notch or canonical Wnt signaling block regeneration and proliferation of Primordial Blasts.

Introduction:

The ability to regenerate missing structures is widespread among the metazoan phyla. Some invertebrate species in the groups of Cnidarians, Planarians and Asteriods can regenerate whole bodies from small fragments, while some vertebrates such as amphibians and fish can regenerate distal structures and some organs. In contrast, some groups such as insects, birds and mammals have nearly lost the ability to regenerate [1-3]. Just like their general regeneration capacities, the cellular sources for regeneration are highly variable among regenerating species. In the Cnidarian *Hydractinia* and the Planarian *Schmidtea*, pluripotent stem cells are present in the adult and are responsible for regeneration, while in amphibians and zebrafish, lineage-committed progenitors arise from de-differentiation [1, 4]. In many cases, proliferation of undifferentiated cells (whatever their source) will lead to the formation of a blastema in the early stages of regeneration [1].

In many chordates, regeneration is limited to wound healing. Some species, such as amphibians, can regenerate missing limbs and the spinal cord, while some fish can regenerate fins and part of the heart. In contrast, invertebrate chordates of the genus *Botrylloides* can regenerate whole bodies from very small fragments of vasculature (Figure 1) [5, 6]. We are studying this process, called whole body regeneration (WBR) in *Botrylloides leachii,* a colonial ascidian of the family Styelidae. Ascidians are the basal chordates, and begin life as a tadpole larvae with a typical chordate body plan. Following a free-swimming stage, the larvae settle and metamorphose into a sessile invertebrate adult, called an oozooid. In colonial species, metamorphosis is followed by a lifelong asexual reproduction program during which entire new bodies regenerate, eventually creating a colony of genetically identical individuals, called zooids. Zooids are connected to each other by a large extracorporeal vasculature and the entire colony is embedded within a gelatinous tunic (Figure 1A). Each week each zooid gives rise to 1-4 new bodies, which are derived from a region of the zooid called the peribranchial epithelium. This process is called palleal budding, and leads to expansion of the colony.

B. leachii also undergoes a regeneration process following injury, called Whole Body Regeneration (WBR). When fragments of the extracorporeal vasculature are surgically separated from the colony, regeneration of a new zooid is triggered. During the first 24 hours after separation of the vessel fragment from the colony, the blood

vessels undergo regression and remodeling (stage 1, Figure 1B). Blood flow continues and is powered by contractions of the remaining ampullae [5]. During the next several days, the blood vessels continue to remodel and form a dense, contracted network (stage 2, Figure 1B). Within this dense, highly pigmented vascular tissue, an opaque mass of non-pigmented cells becomes apparent, progressing to form a hollow, blastula-like structure. The vascular epithelium wraps itself around this vesicle, leading to the formation of a distinctly visible double vesicle (stage 3 Figure 1B). Folding of this vesicle leads to development of the germ layers, and regeneration begins to resemble the developmental stages of palleal buds (stage 4 Figure 1B). Although multiple double vesicles can initially form, only one will continue to develop into a functional zooid [7]. The heart begins to beat before the development of other organs is complete (stage 5 Figure 1B). After about 10-14 days of regeneration, the fully mature body will begin filter feeding.

In this study we aimed to assess whether whole body regeneration (WBR) in *Botrylloides leachii* is driven by a population of preexisting somatic stem cells. In planarians, a population of proliferating somatic stem cells called neoblasts gives rise to the missing body parts during whole body regeneration [8]. A population of blood borne cells with an undifferentiated appearance termed Hemoblasts are present in all colonial ascidians and have been suggested to play roles in WBR [7]. In *Botrylloides violaceus,* a species closely related to *Botrylloides leachii,* 15-20 small hemoblasts that express piwi have been shown to aggregate under the epidermis of a blood vessel during early WBR [9]. In a previous study in *B. leachii*, Blanchoud et al showed an increase in the population of hemoblasts very early after injury during WBR in *B. leachii* [5]. During WBR in *B. violaceus*, circulating hemoblasts that express *piwi* appear near the early vesicle stage and occasionally within the epithelium of a vesicle [7]. These results had suggested that blood borne stem cells might play a role in WBR, but to date, it has never been directly tested whether such cells give rise to regenerating tissues.

In colonial ascidians, long lived mobile germline stem cells are present in the blood and give rise to gonads during asexual reproduction. The cells express genes associated with germ cells, such as *vasa*, *piwi* and *pumilio*. We have previously shown that in *Botryllus schlosseri*, a population of cells enriched for expression of these germline genes can be prospectively isolated based on expression of Integrin-alpha-6 [10].

Here, we show that during the very early stages of WBR in *B. leachii*, cell proliferation occurs only in blood borne cells that express Integrin-alpha-6 and the transcription factor pou3. We used IA6 as a marker to purify a population of blood borne cells that is enriched for expression of pou3 and germline multipotency genes such as vasa and piwi, and show that this population is required for regeneration and gives rise to regenerating tissues. We named these cells Primordial Blasts and show that their proliferation during WBR is regulated by Notch and Wnt signaling.

Results:

The majority of proliferating cells during early stages of WBR express Integrin-alpha-6, pou3 and vasa.

To assess whether blood borne stem cells are involved in responding to injury, we analyzed the expression of stem cell markers in proliferating cells during the early time points after surgical separation of blood vessels from the colony.

In *Botryllus schlosseri*, we had previously identified a population of germline stem cells (GSCs) that expresses *integrin-alpha-6* and *vasa* [10]. While most GSCs are found in germline niches in the developing zooids, a subset of these cells is also found in the circulation. We hypothesize that these pluripotent GSCs may be the source of WBR. To assess whether *integrin-alpha*-6 positive stem cells proliferate during WBR in *B. leachii,* we analyzed expression of mitosis-specific *histone 3* and *integrin-alpha-6* by double fluorescent in situ hybridization (FISH) and found that 89% of all *integrin-alpha-6* + cells proliferate in blood vessels from a healthy colony, named stage 0, (Figure 1C). During stage 1, 24 hours after injury, 93% of *integrin-alpha-6* + cells are actively proliferating, and the overlap between s-phase specific *histone 3* and *integrin alpha 6* stays high during stage 2 (92% Figure 1C). Furthermore, integrin-alpha-6-positive cells make up the majority of proliferating cells during the early stages of WBR, as 95 and 82% of all proliferating cells are integrin-alpha-6-positive in stages 1 and 2 (Figure 1C). During stage 2, some *ia6*+ cells appear to form small aggregates (arrows), and we hypothesize that these locations may be early regeneration niches. When the formation of the double vesicle begins (stage 3) *integrin-alpha-6* + become increasingly quiescent, although 60% still proliferate, and cell proliferation occurs in the regenerating double vesicles (arrow, Figure 1C) and other ia6- cells.

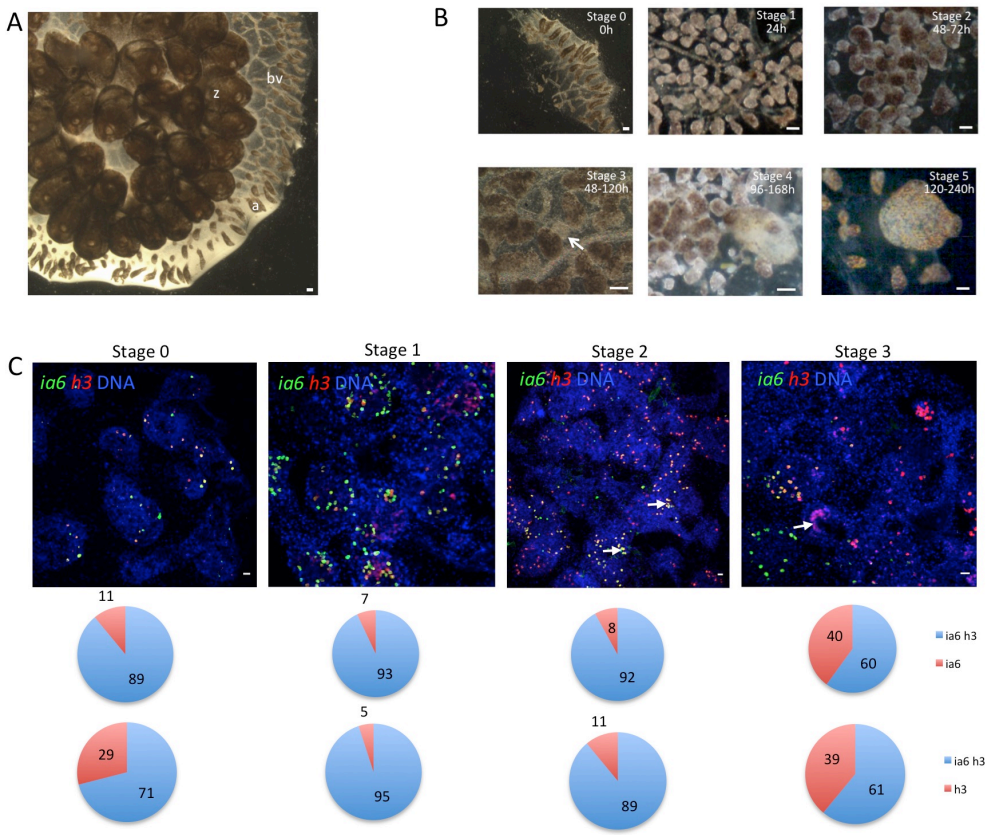


Figure 1: **Whole body regeneration is associated with proliferation of blood borne integrin-alpha-6+ cells.** A) *Botrylloides leachii*, whole colony brightfield image, dorsal. Zooids (z) are embedded in a common, transparent tunic. Blood vessels (bv) extend throughout the tunic and are shared by the zooids of the colony. At the periphery of the colony, blood vessels form terminal sacs termed "ampullae" (a). Scale bars are 200um. B) Stage of whole body regeneration, brightfield images. Blood vessels are surgically separated from the colony (stage 0). During the first 24h, the blood vessel begin to remodel (stage 2). After 48h, blood vessels become condensed and highly pigmented (stage 2). Regeneration begins when a double vesicle is formed, consisting of two layers of epithelium (stage 3). This double vesicle undergoes organogenesis (stage 4) and gives rise to a new, filter feeding body (stage 5). C: Fluorescent in situ hybridization (FISH) showing expression of integrin-alpha-6 (ia6, green) and histone 3 (h3, red) during stages 0, 1, 2 and 3 of WBR. Arrows in stage 2 indicate clusters of ia6+ cells. Arrows in stage 3 indicate developing double vesicles. DNA was stained with Hoechst (blue). Pie graphs show percentages of ia6/h3 double positive and ia6 single positive cells among all ia6+ cells (top), as well as ia6/h3 double positive and h3 single positive cells among all h3+ cells (bottom). Scale bars 20um.

In mammals, Oct4 (Pou5F1) is a transcription factor that is required for pluripotency [4, 11]. Pou class 5 is a vertebrate specific group of pou genes that likely split from pou3 during vertebrate evolution. Therefore, invertebrate *pou3* is most similar to vertebrate

pou5 (Gold et al). Since vertebrate *pou5* genes play roles in pluripotency, it is likely that *pou5* inherited this function from an ancestral pou paralog, such as *pou3*. In the cnidarian Hydractinia echinata, *pou3* plays a role in stem cell pluripotency [12] and we hypothesized that *pou3* might play a similar role in *B. leachii* stem cells. We cloned *pou3* from *B. leachii* and the closely related colonial ascidian *Botryllus schlosseri* and constructed a phylogenetic tree that confirms the close relationship of pou3 to pou5 (Figure S1A). *Pou3* is expressed in germ cells in B. leachii colonies (Figure S1B) Analyzing expression of mitosis-specific *histone 3* and *pou3* by double FISH, we found that proliferating cells in the blood of *B. leachii* express *pou3* in uninjured steady state and during the early stages of regeneration (Figure 2A). 91% of all pou3-positive cells proliferate in steady state, and this percentage stays high during stage 1. During stage 1, *pou3*-positive cells make up the majority of proliferating cells (87%, Figure 2A). During stage2, the number of proliferating pou3+ cells decreases to 77% and some pou3+ cells form aggregates in putative early regeneration niches (arrow). By stage 3, 64% of pou3-positive cells become quiescent, and cell proliferation is predominant (71%) in *pou3*-negative regenerating double vesicles (arrows) and blood cells (Figure 2A). results show that *ia6+/pou3+* cells proliferate in steady state and make up the majority of proliferating cells during the early stages of WBR. Based on these findings, we hypothesize that IA6+ pou3+ proliferating cells are stem cells responsible for WBR.

We isolated Integrin-alpha-6-positive cells by flow cytometry (Figure S1C) [13] and performed gene expression analysis by qPCR. Germline pluripotency genes such as *vasa*, *piwi1* and *piwi2* and *pou3* are highly expressed in IA6+ cells (Figure 2B). Previously, only one piwi gene had been reported in *B. leachii [14]*, but we found that, like most animals, *B. leachii* has two *piwi* genes ([15, 16], supplemental file). Using Double FISH, we confirmed that 81% of either *ia6-* or *pou3*-positive cells are double positive, while 9 and 10% express only *pou3* or *ia6,* respectively (Figure 2C). (Figure 2C). The overlap between *vasa* and *pou3* is 87% (Figure 2C).

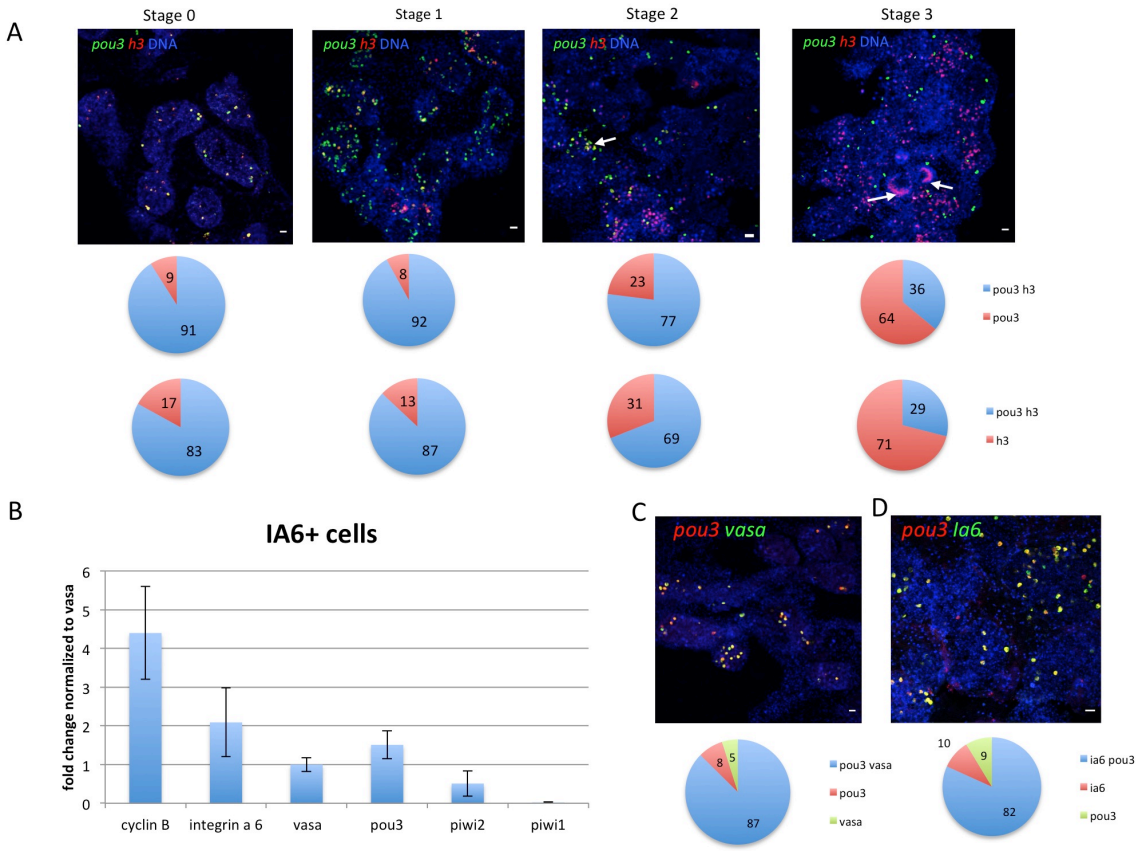
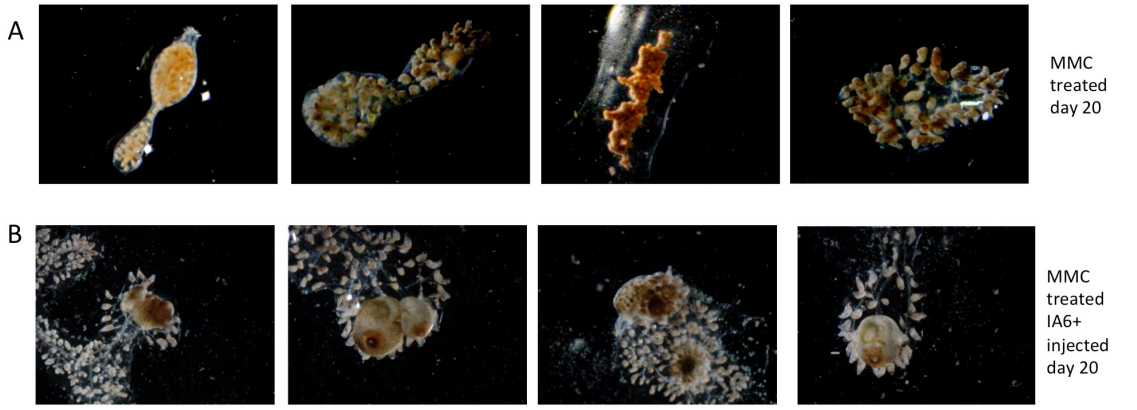


Figure 2: **Blood borne cells proliferating during WBR express pou3, ia6 and vasa.** A) FISH showing expression of pou3 (green) and histone 3 (h3, red) during stages 0, 1, 2 and 3 of WBR. DNA was stained with Hoechst (blue). Scale bars 20um. Pie graphs show percentages of pou3/h3 double positive and pou3 single positive cells among all pou3+ cells (top), as well as pou3/h3 double positive and h3 single positive cells among all h3+ cells (bottom). B) qPCR analysis showing expression of cyclin B, integrin-alpha-6, vasa, pou3, piwi2 and piwi1 in IA6+ cells isolated by flow cytometry. Data are expressed as averages of fold changes normalized to vasa. n=4. C) FISH showing co-expression of pou3 (red) and vasa (green) in stage 0. DNA was stained with Hoechst (blue). Pie graph shows percentages of pou3/vasa-double-positive cells as well as pou3 and vasa single positive cells. D) FISH showing co-expression of pou3 (red) and ia6 (green) in stage 0. DNA was stained with Hoechst (blue). Pie graph shows percentages of pou3/ia6-double-positive cells as well as pou3 and ia6 single positive cells. Scale bars 20um.

<u>IA6+pou3+ cells are required for regeneration:</u>

To functionally assess whether proliferating IA6+*pou3*+ cells are involved in whole body regeneration, we ablated proliferating cells using the drug Mitomycin C (MMC). Vascular tissue was soaked in MMC immediately after surgery and treated until stage 1, when

overlap between ia6 and the proliferation marker histone 3 is 93% (Figure 2). In MMC treated vessels at 5 days post treatment, no *pou3*+ or *histone3*+ cells remain (Figure S1D). Elimination of proliferating cells with Mitomycin C at stage 0-1 results in loss of regenerative capacity (Figure 3). Normally, whole body regeneration is complete after 11-14 days (Figure 1), but MMC treated samples do not reach stage 4 or stage 5 even after 20 days (Figure 3A), and most MMC treated blood vessels appear to be permanently arrested in stage 2 (Figure 3A). Regeneration can be rescued in MMC treated animals by injection of blood cells isolated from an untreated, healthy animal, demonstrating that WBR depends on blood borne cells (Figure 3B). To identify the population of cells responsible for this rescue, we injected different populations of cells isolated by flow cytometry. For example, 50 cells that are in G2/M phase of the cell cycle, isolated from the blood of a healthy animal, can rescue regeneration, whereas the same number of cells in G0 phase does not have this ability (Figure 3C). 50 Integrin-alpha-6 negative cells are not able to rescue WBR, and 57% rescue is achieved by injection of 1000 IA6- cells (Figure 3C), indicating that IA6- cells capable of rescuing WBR are very rare (about 1/2000). It is possible that a rare population of IA6- stem cells exists, as almost 20% of pou3+ cells are ia6-. (Figure 2c). In contrast, 90% rescue is achieved by injecting 50 IA6+ cells (Figure 3C). We found that injection of a single IA6+ cell isolated from the blood of a healthy animal can rescue whole body regeneration in 20% of MMC treated samples (Figure 3C). By performing Limiting dilution analysis using ELDA analysis software [17], we calculated the estimated frequency of IA6+ cells capable of rescuing WBR to be about 1 in 7.55 (upper estimate 4.63, lower estimate 12.3). These results show that IA6+pou3+vasa+ cells are required for WBR, and we named these cells “Primordial Blasts” in accordance with the primordial stem cell hypothesis [18] and because of their potential relationship with the previously described hemoblasts [19].



C

Injected cell population	none	IA6-1000	IA6-50	G2/M 50	G0 50	IA6+ 50	IA6+ 10	IA6+ 5	IA6+ 3	IA6+ 1	Whole blood
% WBR Stage 5	2%	57%	0%	62%	0%	90%	75%	73%	50%	20%	95%

Figure 3: **IA6+ cells are required for whole body regeneration.** A: Mitomyinc C treatment prevents regeneration. Vessel fragments were treated with MMC for 16h immediately after surgery. MMC was removed and the samples followed for 20 days. Brightfield images of vessel fragments 20 days post MMC treatment. B: Injection of IA6+ cells 24h after MMC treatment rescues WBR. Brightfield images of MMC treated vessel fragments 20 days post injection of IA6+ cells. C: Table showing WBR rates 20 days after injection of different cell populations.

IA6+ Primordial Blasts give rise to regenerating tissues in developing bodies:

Since IA6+ cells appear to be functionally required for WBR, we wanted to assess whether progeny from IA6+ cells gives rise to regenerating tissues. We used EdU to label IA6+ cells in steady state and subsequently track the presence of their progeny in regenerating tissues by transplanting them into regenerating vessels. The modified thymidine analogue EdU is incorporated into newly synthesized DNA and detected in fixed tissues using a fluorescent dye. We ensured that EdU could be detected in blood borne cells at 24h post injection and at stage 3 of WBR (Figure S2A). IA6+ cells were isolated from donor animals that had been injected with EdU the day before to allow EdU incorporation into the DNA of cycling cells. EdU-labeled IA6+ cells were injected into MMC treated donors as in the rescue experiments described above. When recipients reached stage 3 or stage 4 of WBR, the regenerating tissues were fixed and stained for EdU. EdU-positive cells were present in the inner cell mass of early double vesicles in stage 3 (green arrows, Figure 4A). At later stages, when tissues begin to differentiate

(stage 4), EdU-positive cells are found in differentiating tissue layers within the developing stage 4 regenerating bud. (Figure 4B, red arrows). In Botryllids, bodies and vessels are lined by a layer of ectodermal epithelial cells, and some of these cells appear to be labeled as well (Figure 4B, green arrows). These results show that IA6+ cells give rise to regenerating tissues in newly developing bodies.

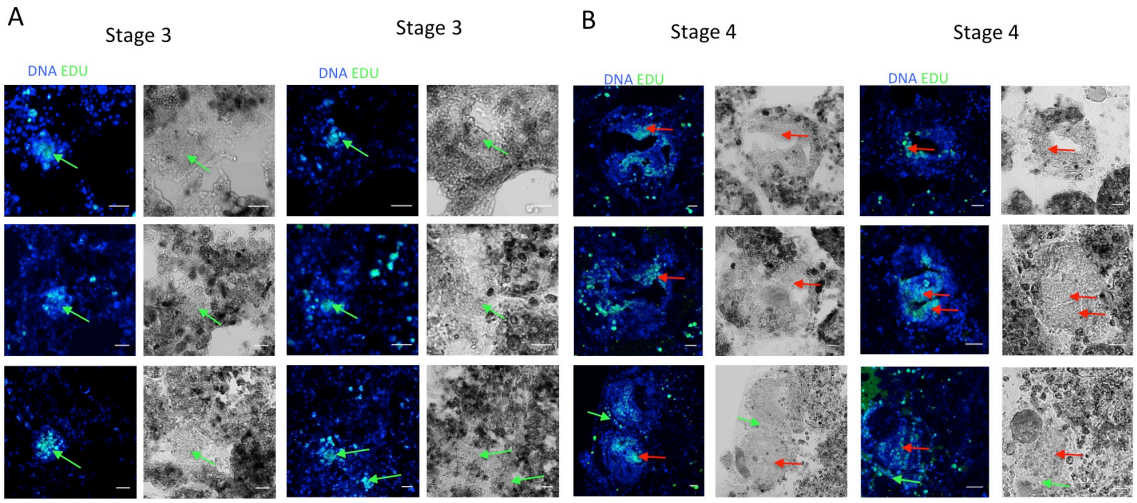


Figure 4: IA6+ cells give rise to regenerating tissues. A) IA6+ cells were isolated from Edu-treated animals and injected into MMC treated vessel fragments. Left panels: Overlay of nuclear staining (Hoechst, blue) and Edu (green) shows Edu-positive cells in the inner cell mass of the double vesicle at stage 3 (green arrows). Right panels are brightfield images of the same tissue. Scale bars 20um. B) Several samples from the same experiment as in A were followed to stage 4 (organogenesis). Edu-positive cells are present in several layers of differentiating tissue (red arrows) and occasionally in the outer epithelial layer (green arrows). Right panels: Overlay of nuclear staining (Hoechst, blue) and Edu (green). Right panels are brightfield images of the same tissue. Scale bars 20um.

<u>Inhibition of Notch or Wnt signaling blocks regeneration and proliferation of primordial Blasts:</u>

As expected, cycling cells (G2/M) isolated from the blood of healthy, unmanipulated animals (0h), express high levels of *integrin-alpha-6*, *cyclin b*, *pou3* and *myc* (Figure 5A). We also analyzed this cycling cell population for expression of components of the Notch and Wnt signaling pathways. These pathways are known to play roles in regulating stem cell activity, proliferation of blastema cells and cellular differentiation in other organisms [20-27] and are upregulated during WBR in *B. leachii* [6]. *Notch 1, notch 2* and the downstream gene *hes1*, as well as the Wnt pathway components *frizzled8, frizzled5,*

disheveled and *beta catenin* are highly expressed in G2/M cells, indicating active Notch and Wnt signaling in these cells (Figure 5B). 24h after injury, *notch2, frizzled 8* and *frizzled 5* are upregulated in G2/M cycling cells by qPCR. 48 after injury, *notch2* and *frizzled 8* are highly upregulated. *Notch1* is downregulated at 24 and 48h (Figure 5C). *Pou3* and *integrin alpha 6* are highly upregulated in G2/M cells at 24 and 48h (Figure 5C). *Piwi2* is highly upregulated in cycling G2/M cells at 48h together with *vasa* (Figure S2B). These results show that *pou3, ia6, piwi2* and *vasa* are highly expressed in cells that proliferate during the early stages of WBR. Double FISH for notch1 and histone 3 shows that notch1 positive cells proliferate during stage 0 and stage 1, but fewer of these cells are present in stage 2 (Figure 6A). However, the number of notch2 positive cells increases by stage 2 (Figure 6A).

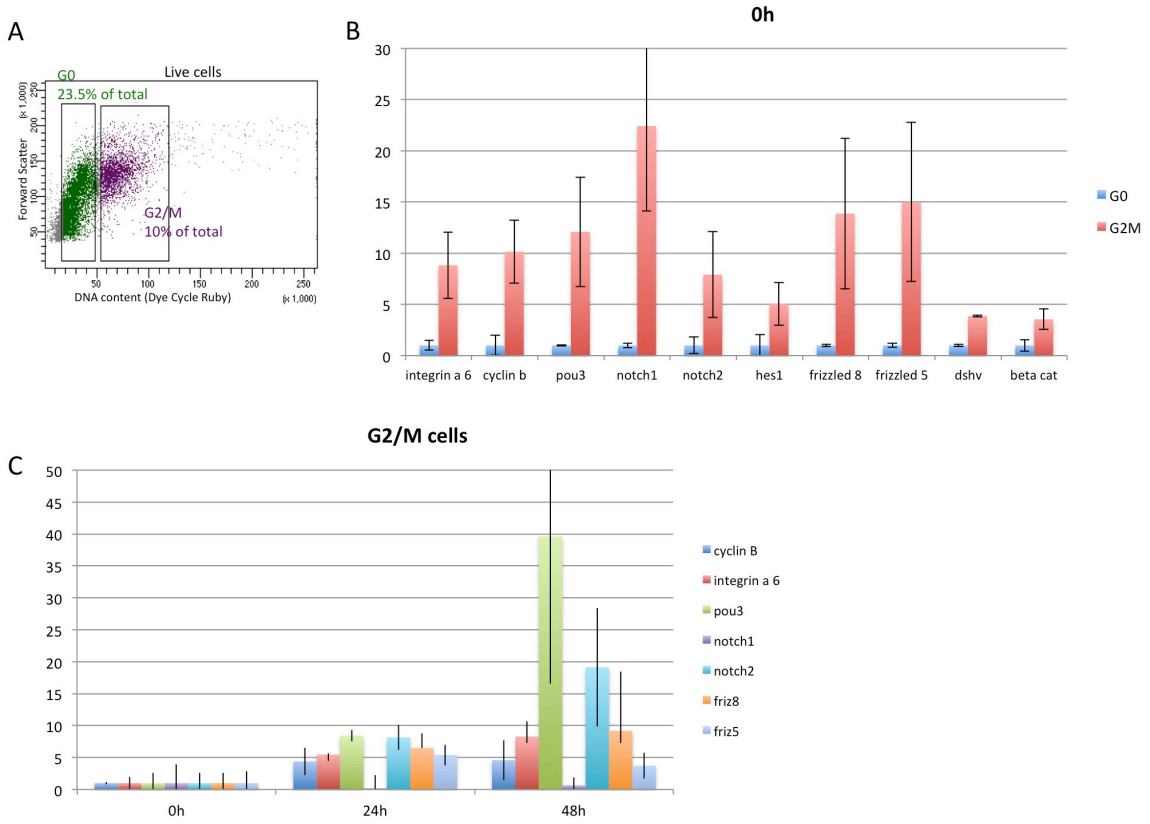
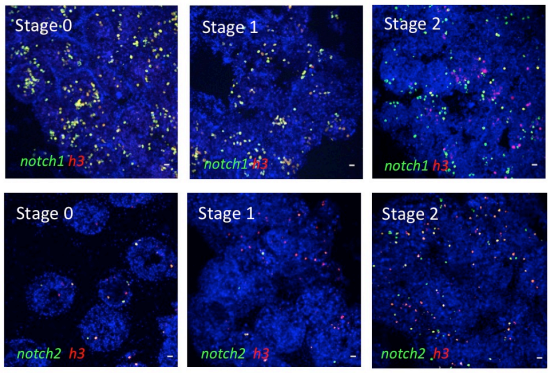


Figure 5: **Gene expression in cycling blood borne cells in steady state and during WBR.** A) Isolation of cycling G2/M cells by flow cytometry. Cells were stained with the live DNA stain Dye Cycle Ruby. Linear analysis of fluorescence intensity plotted against forward scatter (cell size) shows a clear separation of G2/M cells with 2n DNA content and G0/G1 cells with 1n DNA content. G2/M cells comprise 10% of live cells. B: B: qPCR analysis showing expression of, integrin-alpha-6, cyclin b, pou3, notch1, notch2,

hes1, frizzled 8, frizzled 5, disheveled (dshv) and beta catenin in G2/M cells in steady state (0h). Data are expressed as averages of fold changes normalized to G0 cells, n=3.
To assess whether Notch or Wnt signaling are required for WBR, vessel fragments were allowed to regenerate in the presence of inhibitors of either Notch or Wnt signaling. Inhibition of either Notch or canonical Wnt signaling blocked regeneration in a dose dependent manner (Figure 6B). In either 2uM of the Notch inhibitor DAPT or 1uM of the canonical Wnt signaling inhibitor IWR, the vessel tissue underwent remodeling up to stage 2 in both drugs, but never reached stage 3, and appeared otherwise healthy and alive. Double FISH for *histone 3* and *pou3* at 72h of treatment showed that only very few proliferating *pou3*-positive cells are present in drug treated vessels compared to controls (Figure 6C). These results show that WBR depends on both Wnt and Notch signaling, and that both signaling pathways control proliferation of pou3+ Primordial Blasts after injury.

A



B

Condition	% stage 4 day 10
Control (no drug)	90%
Notch inhibitor 2uM	0%
Notch inhibitor 0.2uM	55%
Wnt inhibitor 1uM	0%
Wnt inhibitor 0.1uM	12.5%

C

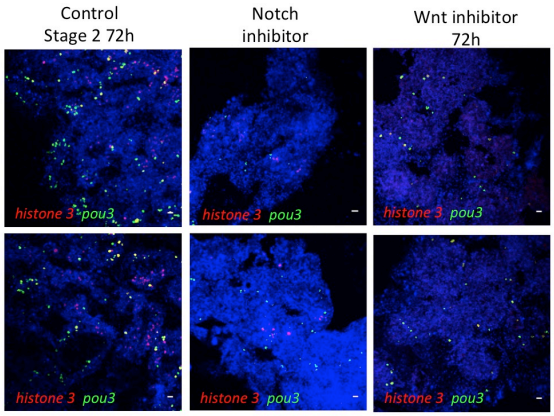


Figure 6: **Inhibition of Notch or Wnt signaling prevents WBR and blocks proliferation of pou3+ cells.** A: top panel: FISH for notch1 (green) and h3 (red) during steady state (stage 0) and during early stages of WBR. Bottom panel: FISH for notch2 (green) and h3 (red) during steady state (stage 0) and during early stages of WBR. B: table showing the percentage of vessel fragments reaching stage 4 at day 10 post injury

during treatment with different doses of inhibitors of canonical wnt signaling or Notch signaling. Controls received vehicle only. C) FISH for pou3 (green) and h3 (red) showing a reduction in proliferating pou3+ cells 72h after surgery when treated with an inhibitor of either notch or wnt signaling.

Discussion:

Here we provide functional evidence that blood borne stem cell are responsible for whole body regeneration and give rise to regenerating tissues in an invertebrate chordate. Based on the evidence presented here, *pou3/ia6*-positive cells appear to be a pool of constantly dividing pluripotent stem cells present in the blood of *Botrylloides leachii*. We are naming these cells Primordial Blasts for the following reasons: Germ plasm components such as *vasa, nanos* and *piwi* are expressed in stem cells with somatic potential in some animals and have therefore been defined as germline multipotency (GMP) genes [28]. Primordial stem cells are defined as germ plasm containing cells with both somatic and germline potential that arise during embryogenesis. In some species, primordial stem cells can be present in the adult animal and are often involved in asexual reproduction and regeneration [18, 29]. Examples include small micromeres from sea urchins, neoblasts, i-cells in cnidarians and archeocytes in sponges and the 4d lineage in annelids. We hypothesize that *pou3/ia6*+ cells share similar features and are a population of primordial stem cells present in the blood of *B. leachii*. The common stem cell gene repertoire of ancestral metazoan primordial stem cells comprises *cyclin B1* and *vasa* [29]. Both of these genes are expressed in *ia6*+ *pou3*+ cells. Expression of *vasa* and *piwi* in *pou3/ia6*+ cells from *B. leachii* suggests that the germline multipotency program is operating in Primordial Blasts. In tunicates, the germline is generally segregated by preformation early in embryonic development. In colonial ascidians, *vasa*-positive germline stem cells are blood borne and persist throughout adult life and give rise to gonads during repeated rounds of asexual reproduction. We hypothesize that in some colonial ascidians, the *vasa*+ stem cell population retains somatic potential that is realized during whole body regeneration. Transplantation of individual IA6+ cells into MMC treated recipient vessels showed that 20% of single IA6+ cells could restore regenerative capacity, with an estimated stem cell frequency of about 1 in 7.5, indicating that some IA6 cells may be pluripotent and able to give rise to all 3 somatic germ layers. Future studies will test whether Primordial Blasts are pluripotent at the single cell level and whether they are

able to give rise to both somatic and germline tissues. Like neoblasts [30], *Ia6+pou3+* cells might be highly heterogeneous, with only a subfraction being toti- or pluripotent. Single cell sequencing will reveal the degree of heterogeneity and the number of sub-populations within the IA6+ population in the future.

The term ”Blasts” was chosen because *pou3/ia6+* appear to be constantly dividing in healthy animals and because they are part of the undifferentiated “hemoblast” population that has been described historically in colonial ascidians [31] [32] and has been implicated in whole body regeneration [7, 9]. WBR occurs in some but not all species of colonial ascidians [33], and appears to be closely related to a process of asexual reproduction, where new bodies spontaneously develop from the vasculature (vascular budding) under homeostatic conditions. Hemoblasts seem to play crucial roles in both processes. During vascular budding in *Botryllus primigenus*, observations by Oka and Watanabe strongly suggest that cells circulating in the blood stream form the inner part of the double vesicle [34]. Here, we have shown that IA6+ cells give rise to the inner cell mass of the double vesicle as well as early germ layers during WBR in *B. leachii*, and have thus conclusively proven for the first time that a population hemoblasts gives rise to regenerating tissues.

A previous study by Rinkevich et al. suggested that piwi-positive cells are involved in regeneration in B. leachii. That study had several notable differences versus our findings. First,piwi expression was not seen in blood vessels of steady state colonies and was only detected after 2 days post injury. In addition, Piwi expression was detected only in cells attached to the vascular epithelium, but not in circulating cells. Following intitiation of WBR, Piwi-protein expression was detected in regenerating vessel fragments, and siRNA-mediated knockdown of piwi suggested a role of piwi+ cells in WBR. [14], the nature of piwi+ cells was not explored in detail, and it is not clear which piwi-homolog was detected. In contrast, we show that mRNA for two *piwi* homologs is expressed in blood borne *pou3/ia6+* Primordial Stem Cells in steady state as well as in cycling cells at 24 and 48h of regeneration (Supplemental Figure 8B). We show that *Piwi2* is highly upregulated in cycling G2/M cells at 48h (Supplemental Figure 8B) together with *pou3, ia6* and *vasa*, supporting the idea that the germline multipotency program is operating in *pou3+* Primordial Blasts. We show that *pou3/ia6+* cells are

responsible for WBR and present conclusive evidence that they directly contribute to regenerating tissues during WBR.

We show that proliferation of Primordial Blasts is required for WBR, and that it is controlled by both Notch- and Wnt- signaling pathways. Both of these pathways have been shown to play roles in regulating regeneration and stem cell proliferation in many regenerating species [2]. Wnt/b-catenin signaling is a highly conserved signal transduction pathway with well defined roles in embryonic development. Many studies have uncovered the importance of Wnt signaling in regeneration in various species, from Cnidarians to planaria to vertebrates [1, 2]. Wnt signaling is involved in regulating regeneration polarity and the formation of organizing centers, but it is also required for blastema formation [25] and stem cell maintenance [24]. Notch regulates blastema cell proliferation during zebrafish fin regeneration, and it mediates cell fate decisions such as proliferation and differentiation in many stem cell types [22]. Notch also regulates blastema formation during distal regeneration in the ascidian Ciona [21]. In a large-scale gene expression study, Zondag et al. showed that Wnt and Notch signaling components are upregulated during early stages of regeneration in *B. leachii [6]*. Here we show that inhibition of either canonical Wnt or Notch signaling completely abrogates WBR. Significantly fewer *pou3*+ cells are found in vessels fragments treated with inhibitors of Wnt or Notch signaling, indicating that these pathways are required for proliferation and possibly maintenance of pou3+ cells.

Future studies will investigate which Notch and Wnt ligands are expressed by the regeneration niche, and how and when these signaling pathways act on the stem cells to regulate proliferation and differentiation. We will assess which pathways induce cell fate decisions such as self-renewal vs differentiation in primordial blasts, and the roles of pou3 and vasa in maintaining pluripotency and self-renewal.

Methods:

Animals

Botrylloides leachii colonies used in this study were collected in Santa Barbara, CA and allowed to attach to glass slides. Colonies were maintained in continuously flowing raw seawater and cleaned with soft brushes every 14 days.

Whole body regeneration

Under a stereo dissection microscope, blood vessel fragments were surgically separated from the rest of the colony using razor blades. Regenerating fragments were kept attached to slides in flowing filtered seawater at 19-23°C.

Integrin-alpha 6 and cell cycle flow cytometry

Genetically identical, stage matched samples were pooled, and blood was isolated by cutting blood vessels with a razor blade and gentle squeezing with the smooth side of a syringe plunger. Blood was diluted with filtered seawater and passed through 70 µm and 40 µm cell strainers. Anti-Human/Mouse-CD49f–eFluor450 (Ebioscience, San Diego,CA, USA, cloneGoH3) was added at a dilution of 1/50 and incubated on ice for 30 min and washed with sorting buffer. For cell cycle sorting, Vybrant Dye Cycle Ruby Stain (Thermo Fisher Scientific, Waltham, MA, USA) was added at a dilution of 1/100. Fluorescence activated cell sorting (FACS) was performed using a FACSAria (BD Biosciences, San Jose, CA, USA) cell sorter. A live cell gate was selected based on forward/side scatter properties, and samples were gated IA6 (CD49f)-positive or –negative based on isotype control staining (RatIgG2A-isotype-control eFluor450, Ebioscience, San Diego,CA, USA). Analysis was performed using FACSDiva software (BD Biosciences, San Jose, CA, USA). Cells were sorted using a 70 µm nozzle and collected into filtered seawater.

Quantitative RT PCR

Sorted cells were pelleted at 700g for 10min, and RNA was extracted using the Nucleospin RNA XS kit (Macherey Nagel, Bethlehem, PA, USA), which included a DNAse treatment step. RNA was reverse transcribed into cDNA using random primers (Life Technologies, Carlsbad, CA, USA) and Superscript IV Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA). Quantitative RT-PCR (Q-PCR) was performed using a LightCycler 480 II (Roche Life Science, Penzberg, Germany) and LightCycler DNA Master SYBR Green I detection (Roche, Penzberg, Germany) according to the manufacturers instructions. The thermocycling profile was 5 min at 95, followed by 40

cycles of 95 °C for 10 sec, 60 °C for 10 sec. The specificity of each primer pair was determined by BLAST analysis (to human, *Ciona* and *Botryllus* genomes), by melting curve analysis and gel electrophoresis of the PCR product. Primer sequences are listed in Supplemental table 1. Relative gene expression analysis was performed using the $2^{-\Delta\Delta CT}$ Method. The CT of the target gene was normalized to the CT of the reference gene *actin* : $\Delta C_T = C_{T\,(target)} - C_{T\,(actin)}$. To calculate the normalized expression ratio, the ΔC_T of the test sample (IA6-positive cells) was first normalized to the ΔC_T of the calibrator sample (IA6-negative cells): $\Delta\Delta C_T = \Delta C_{T(IA6\text{-}positive)} - \Delta C_{T(IA6\text{-}negative)}$. Second, the expression ratio was calculated: $2^{-\Delta\Delta CT}$= Normalized expression ratio. The result obtained is the fold increase (or decrease) of the target gene in the test samples relative to IA6-negative cells. Each qPCR was performed at least three times on cells from independent sorting experiments gene was analyzed in duplicate in each run. The ΔC_T between the target gene and *actin* was first calculated for each replicate and then averaged across replicates. The average ΔC_T for each target gene was then used to calculate the $\Delta\Delta C_T$ as described above. Data are expressed as averages of the normalized expression ratio (fold change). Standard deviations were calculated for each average normalized expression ratio (n=6). Statistical analysis was performed using Student's T-test.

Mitomycin C treatment and rescue

Vessel fragments were cut and soaked in 60µM Mitomycin C (Tocris, Bristol, UK) in filtered seawater for 24 hours. MMC was removed and fragments were returned to flowing seawater for 24 hours before being micro-injected with cells (different numbers of IA6+, IA6-, G0 or G2M, as indicated) isolated from the blood of normal, healthy colonies. Rescue efficiency (number of fragments with completed WBR at stage 5) was scored after 21 days.

Fluorescent *in situ* hybridization on cryosections

Vessel fragments at different stages of regeneration were fixed overnight in 4% paraformaldehyde in PBS at room temperature. Samples were washed in PBS and soaked in 15% and 30% sucrose for 30 minutes each before embedding in OCT medium. 20µm sections were cut using a Leica cryostat. Fluorescent *in situ* hybridization was adapted from [35] for cryosections. Briefly, *B. leachii* homologs of genes of interest were identified by tblastn searches of the *B. leachii* EST database (https://www.aniseed.cnrs.fr/aniseed/default/blast_search) using human or Ciona (when

available) protein sequences. Primer pairs were designed to amplify a 500-800 bp fragment of each transcript (Primer sequences in Supplemental table 1). PCR was performed with Advantage cDNA Polymerase (Clontech, Mountainview, CA, USA, 639105) and products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, A1360). *In vitro* transcription was performed with SP6 or T7 RNA polymerase (Roche, Penzberg, Germany 10810274001, 10881767001) using either digoxigenin, fluorescein or dinitrophenol labeling. Cryosections were air dried and fixed with 4% PFA for 10 minutes and washed with PBS/1%Triton-X-100. Probes were diluted in hybridization buffer and hybridized at 65 C for 30 minutes. Probes were removed and slides washed with 2xSSC/1%Triton and 0.2xSSC/1%Triton for 15 minutes each at 65C. HRP-conjugated anti-digoxigenin antibody (Roche 1/1000), HRP-conjugated anti-fluorescein antibody (Roche 1/500) or unconjugated anti-DNP antibody (Vector labs 1/100) followed by anti-rabbit-HRP (Abcam, Cambridge, UK, 1/200) were used to detect labeled probes. Fluorophore deposition was performed by incubating slides for 20 minutes at RT in Tyramides (Fluorescein-Tyramide, Alexa-555 Tyramide or Alexa-594-Tyramide, all from Thermo Fisher) diluted 1/500 in PBS with 0.001% hydrogen peroxide. Slides were washed twice with PBS and Nuclei were stained with Hoechst 33342 (Life Technologies). Imaging of labeled samples was performed using an Olympus FLV1000S Spectral Laser Scanning Confocal.

Tracking of EdU labeled cells

For every 10 zooids, 2µl of 1mM EdU (Thermo Fisher) dissolved in filtered seawater was injected into the blood stream of healthy colonies. After 24 hours, Integrin-alpha-6-positive cells were isolated from EdU-injected colonies by flow cytometry and injected into recipient vessel fragments 24 hours after Mitomycin treatment (see above). At stage 3 (n=10) and stage 4 (n=10) of regeneration, injected samples were fixed and cryosections were prepared as described above. Sections were fixed with 4% PFA for 30 minutes and treated with proteinase K for 7 minutes. Sections were post-fixed with 4%PFA for 20 minutes and blocked with PBS/1% Triton and 3% BSA for 1h. The clickit-reaction cocktail was prepared according to the manufacturer's instructions and the reaction was stopped after 1h at room temperature. Nuclei were stained with Hoechst 33342. Regenerating samples injected with EdU were used as positive control, and uninjected regenerating samples were used as negative controls. Imaging of labeled

samples was performed using an Olympus FLV1000S Spectral Laser Scanning Confocal.

Small Molecule Inhibitor Treatment

Vessel fragments were cut and placed in the bottom of 24 well plates. 1ml of filtered seawater containing the Notch-signaling inhibitor DAPT (Tocris, 0.2-2μM) or the Wnt-signaling inhibitor endo-IWR1 (Tocris, 0.1-1μM) was added and replaced every other day. The number of colonies that reached stage 4 were counted at 14 days post injury.

Competing interests:

The authors state no competing financial interests.

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