smedinx-11 is a planarian stem cell gap junction gene required for regeneration and homeostasis

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The largely unknown mechanisms that regulate adult stem cells probably involve signals from neighboring differentiated cells. Gap junction channels providing direct cell-cell communication via small molecules are a crucial component of morphogenesis and normal physiology. However, no specific gap junction protein has yet been functionally linked to adult/somatic stem cell behavior in vivo or to organ regeneration. We report the identification and characterization of smedinx-11—an innexin gap junction channel gene expressed in the adult stem cells (neoblasts) of the planarian Schmidtea mediterranea. smedinx-11 RNAi treatment inhibits regeneration and abrogates neoblast maintenance. Moreover, smedinx-11 expression is enriched in an irradiation-sensitive subpopulation (‘X2’) and is required for proper expression of other stem cell-specific markers. Analyses of the smedinx-11 downregulation phenotype revealed a striking anterior-posterior neoblast gradient. Our data demonstrate a novel role for gap junction proteins and suggest gap junction-mediated signaling as a new and tractable control point for adult, somatic stem cell regulation.

KEY WORDS: Planarian, Gap junctional communication, Stem cell, Regeneration

INTRODUCTION

The planarian flatworm exhibits a remarkable ability to regulate complex pattern formation, providing an exciting opportunity to understand well-conserved and widely relevant molecular controls of morphogenesis. Planarian stem cells (neoblasts) have the potential to differentiate into all of the different tissue types found in the adult flatworm, and worms show no evidence of senescence as neoblasts continually replace damaged or aging cells (Reddien and Sánchez Alvarado, 2004). Thus, neoblasts must effectively integrate the signals required for self-control, controlled proliferation, migration, differentiation and cell incorporation into target tissues during physiological turnover and maintenance of complex pattern (morphostasis). Furthermore, after injury, neoblasts proliferate and regenerate all missing tissues in a short period of time, restoring the original form and proportion of the organism (Reddien and Sánchez Alvarado, 2004). All of these properties highlight the interactions between adult stem cells and the context of their existing differentiated neighbors (Joseph and Morrison, 2005; Li and Xie, 2005; Scadden, 2006; Yamashita et al., 2005). Understanding the endogenous controls of stem cell behavior is of high priority for therapeutic applications in regenerative medicine and cancer biology, as well as for the fundamental understanding of highly regulative morphogenesis. However, the molecular mechanisms of the crosstalk between stem cells and their surrounding differentiated tissues are largely unknown.

Gap junctional (GJ) communication is increasingly revealed as an important regulatory modality functioning alongside traditional secreted signaling molecules. In metazoans, gap junction proteins are conserved membrane channels involved in direct cell-cell communication via small molecule signals during numerous physiological events (Levin, 2002; Nicholson, 2003; Phelan, 2005). Gap junction-permeable signals have been implicated in embryonic morphogenesis and neoplasm (Bruzzone et al., 1996; Levin, 2002; Mesnil et al., 2005). Importantly, GJ communication is not just a housekeeping event necessary for cell survival; several studies have revealed subtle alterations of embryonic patterning, but not generalized inhibition of cell proliferation or toxicity, following abrogation of GJ communication (GJC)-dependent signaling (reviewed in Levin, 2007). Invertebrate gap junctions are composed of proteins from the innexin family, whereas vertebrate gap junctions consist of the homologous pannexins as well as connexins. Innexins have been shown to be a crucial component for tissue polarity, morphogenesis, locomotion, electrical cell coupling, and germ-line cell maintenance and differentiation (Bauer et al., 2004; Gilboa et al., 2003; Nogi and Levin, 2005; Phelan, 2005; Tazuke et al., 2002); however, no specific gap junction protein has yet been functionally shown to regulate adult somatic stem cells in vivo. Moreover, no specific gap junction gene product has been implicated in the interplay of signals required during the regeneration of complex structures.

In recent work, we characterized the expression of a family of innexin genes in planaria (Nogi and Levin, 2005). Crucially, however, the specific innexins involved in patterning, and the molecular mechanisms by which innexin function could regulate cell growth, fate specification and regeneration, remain completely unknown. The molecular implication of a specific innexin gene in regulative morphogenesis and stem cell behavior, as well as the characterization of the consequences of its abrogation, are the key steps that must now be addressed. Thus, we investigated the potential roles of GJ-mediated signals in regeneration and homeostasis, using the planarian species Schmidtea mediterranea as a tractable model system in which to probe novel cellular mechanisms of communication between stem cells and their niche.

We identified 12 transcripts encoding innexin proteins (smedinx-1–12). Additional qualitative and quantitative expression studies suggested that neoblast-associated innexins are present and enriched in specific adult stem cell subpopulations. Functional analysis using RNA interference (RNAi) revealed a neoblast-
associated gene, smedinx-11, that is specifically required for tissue regeneration, homeostasis and neoblast maintenance. Reduction of smedinx-11 expression resulted in a phenotype that is different in several key properties from those obtained with known genetic and radiation-based treatments. Our data show that smedinx-11 is required for the normal expression of well-conserved key stem cell regulators such as PIWI and other proteins. Taken together, our data reveal a novel role for GJC proteins and characterize a new element functioning in the regulation of adult stem cells in their natural environment.

MATERIALS AND METHODS

Planarian culture
In all experiments, the clonal line CIW4 of S. mediterranea provided by A. Sánchez Alvarado (University of Utah, UT, USA) was used and maintained as previously described (Cebrià and Newmark, 2005).

Xenopus GJ assay
Full-length innexin gene sequences were obtained by RACE and subcloned into the pCS2+ expression vector. Full-length smedinx-11 was entered into Genbank under accession number DQ851133 (formerly AY067505). Ventral or dorsal blastomeres of four-cell Xenopus embryos were injected with in vitro-synthesized, full-length, capped mRNA encoding smedinx-11, Dugesia japonica innexin-11 (Djinx-11, the smedinx-11 homolog) or a truncated mutant of smedinx-11 (for additional details, see Fig. S2 in the supplementary material). Effects were assayed as described previously (Levin and Mercola, 1998). Injection of mRNA encoding smedinx-11 or Djinx-11 into ventral blastomeres, in which cells are normally gap-junctionally isolated (Levin and Mercola, 1998), specifically randomized embryonic laterality (35% and 26% heterotaxia, respectively, n=119, P<0.01 by χ2 test with Pearson correction); by contrast, injections into dorsal blastomeres (expressing the innexin in a zone that is already well-coupled by gap junctions) had no effect on embryonic asymmetry. Similarly, a non-functional smedinx-11 mutant did not randomize in any injection locale (P=0.3). Thus, smedinx-11 behaved in this assay identically to all of the connexin gap junction constructs tested previously (Levin and Mercola, 1998).

Quantitative real-time PCR (qRT-PCR)
The Xenopus isolation procedures and internal control used were as previously described (Reddien et al., 2005b). We used the iScript cDNA synthesis kit and IQ SYBR green supermix (BioRad); PCR reactions and analyses were performed using iCycler (BioRad).

RNAi
In vitro double-stranded (ds)RNA preparation (including the 65-1469 bp fragment) and injection procedure was as previously described (Reddien et al., 2005b). The injection schedule was: a total of three injections distributed over two consecutive days, one day of rest, followed by one more day of injections (one injection per day with three pulses of 32 nl of respective dsRNA). To rule out the possibility of local neoblast depletion, microinjections in some experiments targeted either pre- or post-pharyngeal areas. In most cases, dsRNA injections were targeted to the gastro-vascular system, to assure effective dsRNA incorporation as well as homogeneous distribution throughout the body. Some animals were amputated at 8 days after first injection and, when possible (for all S. mediterranea innexin genes other than smedinx-11), a second round of injections plus amputation was performed. The control group in RNAi experiments was injected with water. Some animals remained intact for more than 3 weeks, and then were fixed and processed for immunostaining or in situ hybridization (ISH).

In situ hybridization
ISHs were performed as previously described (Reddien et al., 2005b). Once the ISH signal for positive controls was reproducible, experiments were carried out with different S. mediterranea innexin riboprobe dilutions to determine the optimal ratio of positive-signal:background. The specificity of the ISH pattern was also confirmed by three sets of experiments performed in parallel (Fig. 2A): (i) γ-irradiation (6000 rad), which resulted in the selective removal of stem cells (neoblasts), significantly reduced the signal of two S. mediterranea innexins compared to the un-irradiated worms (see below; results were additionally confirmed by qRT-PCR); (ii) sense probe, which resulted in no detectable signal; and (iii) specific RNAi exposure, which reduced the signal of the target genes.

Flow cytometry experiments
Planarian dissociation and cellular labeling was performed as described (Reddien et al., 2005b) with only one filtration through a 35 µm Nitex filter. Propidium iodide was added (5 µg/ml) to eliminate dead cells. Labeled cell fractions were sorted using a MoFlo high-speed cell sorter and analyzed with Summit software (Dako). Combinations of Hoechst and calcine stains were used in standard protocols to separate cells on the basis of dye efflux and DNA content (Goodell et al., 1996).

Image collection and processing
Digital images were collected using a Nikon SMZ1500 microscope connected to a Cool Snap CF camera (Roper Scientific Photometrics), and with Openlab software (Improvision), a Leica TCS SP2 Spectral Confocal Imaging System and an Olympus BX61 motion-controlled compound microscope attached to a Hamamatsu Orca AG high-resolution, monochrome, cooled CCD camera with iPlabs analysis software.

RESULTS

Twelve innexin clones (smedinx-1–12) were identified in the planarian S. mediterranea
Among planarian species, S. mediterranea is the most convenient model in which to perform molecular and genetic analyses (Newmark and Sánchez Alvarado, 2002; Saló, 2006; Sánchez Alvarado, 2006; Sánchez Alvarado and Kang, 2005). Twelve cDNAs coding for innexins were identified in two public S. mediterranea databases (Sánchez Alvarado et al., 2002; Zayas et al., 2005) and numbered smedinx-1 to smedinx-12 (Fig. 1A, and see Fig. S1 in the supplementary material). Phylogenetic analysis and in situ hybridization (ISH) revealed considerable similarity of sequence and expression pattern to Dugesia japonica innexins (inter-species comparison in Fig. S1 in the supplementary material). These transcripts were segregated into four categories of expression: excretory, digestive, mesenchymal and nervous tissue (Fig. 1A). Although innexin genes are now widely known as encoding gap junction proteins, we indirectly confirmed this function in a Xenopus assay (see Materials and methods and Fig. S2 in the supplementary material).

The expression of mesenchymal S. mediterranea innexin genes is widely distributed. To confirm that these patterns are correct and specific, and to conclusively establish expression of these genes, several rounds of optimization of the ISH protocol used were performed with negative (sense probe) and positive (other genes with expression patterns known to involve different tissues) (Newmark and Sánchez Alvarado, 2002; Sánchez Alvarado et al., 2002) controls (data not shown). Moreover, wild-type intact animals were sectioned (transversally and longitudinally along the antero-posterior axis) and ISH was performed on sections (Reddien et al., 2005b) to ensure that probe penetration differences did not bias the results (Fig. 1B and see Fig. S3 in the supplementary material). The results of whole-mount and section ISH under a variety of conditions consistently revealed expression in nervous, excretory and digestive systems, as well as in the mesenchyme—a tissue that contains a number of different cell types (Fig. 1 and see Fig. S3 in the supplementary material). Please see the legend to Fig. S1 in the supplementary material for a discussion of the comparison of innexin expression patterns among S. mediterranea and D. japonica.
Expression of *smedinx-11* and *smedinx-2* was associated with neoblasts

Because neoblasts are the only proliferative cells in adult planaria, γ-irradiation provides a convenient way to selectively kill them and thus identify neoblast-associated gene expression (Guo et al., 2006; Hayashi et al., 2006; Ogawa et al., 2002; Orii et al., 2005; Reddien et al., 2005b; Rossi et al., 2006; Salvetti et al., 2000; Salvetti et al., 2005; Shibata et al., 1999). ISH and quantitative real-time PCR (qRT-PCR) revealed that the expression of only two *S. mediterranea* innexin genes (*smedinx-11* and *smedinx-2*) was reduced by γ-irradiation (Fig. 2A,B and data not shown), suggesting a relationship between these *S. mediterranea* innexins and neoblasts.

Interestingly, the expression patterns of these two neoblast-associated *S. mediterranea* innexin genes revealed an additional domain not present in some neoblast markers (Guo et al., 2006; Hayashi et al., 2006; Ogawa et al., 2002; Orii et al., 2005; Reddien et al., 2005b; Rossi et al., 2006; Salvetti et al., 2000; Salvetti et al., 2005). Proliferative neoblast-associated gene expression is commonly observed throughout the mesenchyme, but not in tissue in front of the photoreceptors or in the pharynx (Guo et al., 2006; Ogawa et al., 2002; Orii et al., 2005; Reddien et al., 2005b; Rossi et al., 2006; Salvetti et al., 2000; Salvetti et al., 2005). However, in addition to the *smedinx-11* signal in mesenchymal cells, irradiation-sensitive expression of *smedinx-11* was also present in areas known to be postmitotic (i.e. cells anterior to the photoreceptors, surrounding cephalic ganglia, and in the pharynx) (Figs 1, 2 and see Fig. S3A,B in the supplementary material). Such irradiation-sensitive gene expression in areas in front of the photoreceptors and pharynx (see Fig. S3A,B in the supplementary material) has also been described for another stem cell-associated transcript, *vasa*-related (Shibata et al., 1999). Moreover, although the expression of both *smedinx-11* and *smedinx-2* was reduced after irradiation, a small irradiation-insensitive component remained (Fig. 2B).

*smedinx-11* gene expression is enriched in the postmitotic X2 subpopulation

Recent work using fluorescent activated cell sorting (FACS) identified two irradiation-sensitive subpopulations (X1 and X2) with neoblast-like morphology (Hayashi et al., 2006; Reddien et al., 2005b). The X1 subpopulation in *S. mediterranea* consists mainly of dividing neoblasts expressing the piwi genes *smedwi-1* and *smedwi-2*, and *smedcyclinB*; however, no marker or functional characterization are yet available for the X2 population (which consists of both irradiation-sensitive and -insensitive cells). Gene expression experiments using FACS-isolated cells demonstrated that *smedinx-11* is mainly expressed in the X1 and X2 compartments, and its expression levels are specifically enriched in X2 cells (Fig. 2C).

Together, *smedinx-11* quantitative and qualitative expression analyses in FACS-isolated cells also revealed that this gene is expressed at low levels in most (~60%) proliferative neoblasts (X1), whereas, in X2 cells, in which the total *smedinx-11* levels are higher, the number of cells expressing this gene only reach approximately 24% (Fig. 2C,D). Because there is more total...
expression of smed-11 in X2 cells than in X1 cells, but fewer X2 cells express detectable smed-11 mRNA, we suggest that smed-11 is expressed at high levels in a subset of X2 cells.

The expression of *S. mediterranea* piwi genes is strongly concentrated in proliferative neoblasts (~90%) (Reddien et al., 2005b), suggesting that the expression in many X1 cells must overlap for *S. mediterranea* piwi genes and smed-11. To investigate whether smed-11 expression in X2 cells might be related to other neoblast markers, we stained FACS-isolated cells with a recently characterized SMEDWI-1 antibody that recognizes postmitotic irradiation-sensitive cells (Guo et al., 2006). In both X1 and X2 cell groups, over 90% were positive for SMEDWI-1 protein (see Fig. S4 in the supplementary material), suggesting that smed-11 expression in X2 neoblasts overlaps with SMEDWI-1-positive cells. Together, these quantitative and spatial expression data suggest that smed-11 represents a novel marker for irradiation-sensitive cells that provides a molecular entry-point into understanding the differences among stem cell subpopulations (see also the legend for Fig. S1 in the supplementary material).

**smed-11 is required for tissue regeneration and for proper neoblast function**

To investigate whether *S. mediterranea* innexins proteins regulate neoblast biology, RNAi experiments were performed (Sánchez Alvarado and Newmark, 1999) for each *S. mediterranea* innexin clone, and the effects on regenerating fragments and intact animals were evaluated (Reddien et al., 2005a). Although five sequences encoding *S. mediterranea* innexin (smed-5 to smed-9) were previously included in an RNAi screen, no significant phenotypes associated with regeneration or homeostasis were described (Reddien et al., 2005a). We used RNAi to screen all *S. mediterranea* innexin genes and found that only two out of 12 (smed-10 and...
smedinx-11) produced a detectable phenotype (100% penetrance) in both regenerating and intact worms. smedinx-10 is expressed in the excretory system and its abrogation produced the disruption of body homeostasis, revealed by a conspicuous swelling followed by subsequent lysis of the animals within 10 days (data not shown). The swelling phenotype, together with the spatial expression of smedinx-10, might suggest that loss of innexin function results in a failure in the excretory system to maintain efficient osmoregulation.

By contrast, smedinx-11 RNAi treatment (referred to as smedinx-11(RNAi)) in regenerating worms strongly inhibited blastema formation (Fig. 3A, the ‘early’ phenotype – inability to regenerate) with no toxicity or other visible abnormalities and normal wound healing. In intact planarians, smedinx-11(RNAi) did not produce any external abnormality until approximately 14 days after RNAi exposure (the ‘late’ phenotype, Fig. 3B and Fig. 7A-D). In both regenerating and intact worms, visible signs of the late phenotype began with tissue bending, restricted to the pre-pharyngeal area, which progressed and extended through the borders of the body with the exception of the anterior end (Fig. 3B, white arrows). Curling around the ventral surface and the inability to regenerate have been associated with impaired function of neoblasts following γ-irradiation or RNAi for several genes, including smedwi-2 (Guo et al., 2006; Reddien et al., 2005b; Reddien et al., 2005b). Although regression of the tissue in front of the photoreceptors (head regression) was not observed even after 1 month following the first injection of smedinx-11 dsRNA, all other phenotypes of irradiated worms (Reddien et al., 2005b) were recapitulated. These results suggest that smedinx-11 is required for proper neoblast function.

**smedinx-11 function is required for the proper expression of other neoblast markers**

To link innexin gene function with known molecular pathways and to further characterize the smedinx-11(RNAi) phenotype, we studied whether smedinx-11 is required for the proper expression of neoblast markers (Reddien et al., 2005b). qRT-PCR analyses showed that, at 7 days after smedinx-11(RNAi), smedwi-1 expression was reduced, whereas smedwi-2 expression was slightly increased; but, by 1 week later, the expression of both S. mediterranea piwi genes was severely reduced (Fig. 4A). We characterized the spatial expression of the S. mediterranea piwi genes, smedcyclinB and smedbruno-like (smedbruli) in smedinx-11(RNAi) worms. Expression of these neoblast markers disappeared gradually (Fig. 4B,C), consistent with the quantitative assay data (Fig. 4A). By contrast, markers of tissues other than neoblasts (S. mediterranea innexins expressed in excretory, nervous and digestive tissue) did not disappear in smedinx-11(RNAi) worms at 14 days after RNAi (Fig. 4C). Interestingly, smedbruli expression, which is observed in neoblasts and differentiated tissues (Guo et al., 2006), was strongly reduced in the neoblast compartment but remained in differentiated CNS cells (Fig. 4C). Although it is not known whether the regulation of other neoblast genes by smedinx-11 is cell-autonomous, taken together, these data firmly establish smedinx-11 as an endogenous regulator of neoblast biology (it is required for the proper expression of key stem cell genes in the piwi, cyclin B and bruno families).

The inability to regenerate within the first 7 days after smedinx-11(RNAi) demonstrates that the smedinx-11 phenotype actually begins well before external signs are visible (the early phenotype). Within the first week after smedinx-11(RNAi), animals failed to form a regeneration blastema despite a slight increase in smedwi-2 expression and the presence of mitotic activity. Thus, the early obligate role of smedinx-11 in regeneration does not function via smedwi-2 but is required to form a blastema upon amputation. By 14 days after smedinx-11(RNAi), there was a strong reduction in the expression of S. mediterranea piwi genes. By contrast, 8 days after smedwi-2(RNAi) exposure [when smedwi-2(RNAi) animals are unable to regenerate], smedinx-11 expression was not affected (see Fig. S5 in the supplementary material). This epistatic analysis strongly suggests that smedinx-11 function is required for the expression of the neoblast genes smedwi-1 and smedwi-2 during the late phenotype. It is possible that a significant component of the reduction in the expression of neoblast markers might result from a physical loss of neoblasts after smedinx-11(RNAi). However, the simple possibility that the loss of marker expression is due entirely to the disappearance of expressing cells is refuted by the upregulation of smedwi-2 following smedinx-11(RNAi); instead, this indicates the control of gene expression by SMEDINX-11 function. Taken together, our results suggest that SMEDINX-11 activity is
required for the normal expression of S. mediterranea piwi genes in the animal and has additional consequences that are not dependent upon smedwi-2.

**smedinx-11 is required for the maintenance of the neoblast pool**

FACS analysis of *smedinx-11(RNAi)* worms demonstrated that the decline in neoblast markers was accompanied by a reduction of neoblast subpopulations (≥10 days after RNAi) (Fig. 4D). At 10 days after *smedinx-11(RNAi)*, the number of dividing neoblasts was reduced, whereas the X2 subpopulation was not affected in this way (Fig. 4E). However, as the phenotype progressed, the X2 irradiation-sensitive subpopulation became strongly reduced (comparable to the irradiated group), a phenomenon observed only after γ-irradiation and in the late phases (≥18 days) of *smedwi-2 RNAi* treatment (Reddien et al., 2005b) (Fig. 5). The time-dependent way in which neoblast subpopulations disappear in *smedinx-11(RNAi)* animals suggests that *smedinx-11* is crucial in order to maintain a pool of proliferative stem cells and to support continuity of its postmitotic progeny. Thus, these data reveal functional, molecular differences between X1 and X2 neoblast subpopulations. Based on these expression and functional studies, we propose that *smedinx-11* is required for stem cell maintenance and that both neoblast subpopulations require *smedinx-11* in order to participate in body homeostasis and regeneration.

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**Fig. 4. smedinx-11 is required for neoblast maintenance.** (A) Differences in expression of neoblast markers, analyzed by qRT-PCR using total RNA extracted at 7 and 14 days after *smedinx-11(RNAi)* injection. Downregulation of *smedwi*-1 is observed within the first week after *smedinx-11(RNAi)*, whereas a slight increase in *smedwi*-2 expression was noted during this time. At 1 week later, the expression for both *S. mediterranea* piwi genes was severely reduced. qRT-PCR experiments were the result of triplicate experiments; values represent the difference between control and *smedinx-11(RNAi)*; error bars represent s.d. Gene expressions are relative to the ubiquitously expressed clone H.55.12e (Reddien et al., 2005b). (B) Expression changes in neoblast X1 markers at different days after *smedinx-11* dsRNA injection. Representative whole-mount in situ hybridization (ISH) results using the *smedwi*-1 probe are shown. Control (left-most) gives a regular signal distributed throughout the mesenchyme (Reddien et al., 2005b). Notice that changes in *smedwi*-1 expression are revealed by a gradual disappearance of the signal in a time-dependent manner. Within the first 2 weeks after *smedinx-11* dsRNA exposure, the *smedwi*-1 signal is dramatically reduced and, as the phenotype progresses, no signal is detected (>14 days) in the whole organism. In all cases, anterior end is up. At least n=7 worms were included at each time-point. (C) Spatial expression for different markers detected in undifferentiated tissues after *smedinx-11(RNAi)*. Differentiated (excretory, *smedinx*-10; CNS, *smedinx*-3; and digestive, *smedinx*-9) and undifferentiated (*smedwi*-1, *smedcyclinB* and *smedbruli*) tissue probes were assayed in control animals (upper row) and 14 days after *smedinx-11(RNAi)* (bottom row). Although expression for differentiated tissue markers remains similar to their control counterparts (5/5 each), the signal for neoblast markers was strongly reduced in *smedinx-11(RNAi)* worms (5/5 each). Interestingly, the component of *smedbruli* expression that is observed in differentiated tissue (i.e. CNS) remained, whereas its neoblast-related expression was reduced. (D) FACS profiles highlighting X1 subpopulations (insets from each profile) from dissociated planarians; control (top), 7 days post-irradiation (6000 rad; middle) and 10 days after *smedinx-11(RNAi)* (bottom). Notice that the population of dividing neoblasts was sharply reduced after irradiation and *smedinx-11(RNAi)*. (E) Percentage of cells from different FACS-isolated cell populations from control, irradiated and *smedinx-11(RNAi)*, 10 and 14 days after first injection (see Fig. 5 for corresponding FACS profiles showing all subpopulations). Interestingly, the number of X2 cells 10 days after *smedinx-11(RNAi)* is comparable to the control animals. However, as the phenotype progressed, the X2 cell numbers were reduced to levels comparable to the irradiated group. In all FACS experiments, n=20 animals were dissociated for each condition. Scale bars: 0.2 mm.
**DEVELOPMENT**

**smedinx-11(RNAi) reverses the endogenous anterior-posterior neoblast gradient**

Strikingly, after *smedinx-11(RNAi)*, the mitotic activity of neoblasts disappeared in anterior-posterior (AP) progression (Fig. 6A). We quantified three regions (pre-pharyngeal, pharyngeal and post-pharyngeal, Fig. 6B) (Reddien and Sánchez Alvarado, 2004) and counted mitotic activity in each region (Reddien et al., 2005a; Reddien et al., 2005b). In wild-type animals, we detected an intrinsic AP gradient of mitotic activity (more dividing cells in anterior than in posterior areas, Fig. 6C), consistent with previous reports on the distribution of neoblasts and mitotic activity (Baguñà, 1976; Brandstätter, 1969). However, we noted a sharp reduction in mitotic activity in pre-pharyngeal and pharyngeal, but not in post-pharyngeal, regions within the first week after *smedinx-11(RNAi)*. Eventually, as the phenotype progressed, the reduction in mitotic activity also reached the post-pharyngeal region, ending with the depletion of mitotic cells in the whole animal after 14 days following *smedinx-11(RNAi)*. A similar microinjection schedule targeting only pre- or post-pharyngeal areas was performed to test whether the depletion of mitotic activity in the anterior areas was due to a local neoblast response to repair the wounds caused by the three successive rounds of microinjections. In both cases (microinjections in the pre- or post-pharyngeal area, data not shown), mitotic activity disappeared in the same AP spatiotemporal fashion, suggesting that this is a specific response to the functional abrogation of SMEDINX-11 and not because of localized effects at the injection site. These data also revealed that, even though the anterior region normally possesses more mitotic cells than the posterior region in the worm, this is also the area with the highest propensity to lose mitotic activity after the loss of SMEDINX-11 function. Remarkably, this pattern of mitotic activity disappearance has not been reported for any type of γ-irradiation treatment or RNAi of genes required for stem cell maintenance, and is unique for the reduction of SMEDINX-11.

**DISCUSSION**

Our functional data indicate that *smedinx-11* is a neoblast-associated gene that regulates somatic adult stem cells during self-renewal, regeneration and homeostasis (Fig. 7A-C). The *smedinx-11(RNAi)* phenotype differs in important ways from the loss of regenerative ability following knockdown of other previously characterized transcripts (Guo et al., 2006; Reddien et al., 2005b; Salvetti et al., 2005). First, in known genes required for homeostasis, animals usually die <1 month after first exposure to RNAi. By contrast, *smedinx-11* inhibition did not lead to head regression; fragments of pre-existing tissue survived without brains or other head organs for over 1 month (verified by synaptotagmin expression, data not shown). This long survival will enable the characterization of morphallaxis and the transitions among stem cell states. The way in which irradiation-sensitive cells disappear after *smedinx-11(RNAi)* is unique to this phenotype (i.e. first proliferative cells, followed by the X2 population). Moreover, although *smedwi-2* is already known to be required for regeneration, abrogation of this gene seems not to affect stem cell maintenance—a stark contrast to *smedinx-11*. Lastly, the reversion of the AP gradient of proliferative cells that we observed after *smedinx-11(RNAi)* has not been described for any previous RNAi phenotype or γ-irradiation treatments.

One major consequence of the *smedinx-11(RNAi)* phenotype is the inability to regenerate (despite the presence of cell proliferation and the lack of early toxicity), unlike in bipolar heads previously described in *D. japonica* using heptanol (Nogi and Levin, 2005). This probably reflects the different dynamics of GJ closure caused by the two techniques: external heptanol exposure targets different gap junctions non-selectively, changing the open-state probability in cells accessible to the drug; by contrast, RNAi abrogates *smedinx-11*-dependent GJC. The RNAi is specific for one innexin transcript and thus allows us to separately characterize GJ signals involved in AP polarity versus stem cell regulation. Combinatorial analysis of individual innexins will be necessary to understand these distinct roles and to determine whether the lack of phenotype from RNAi of the other innexin family members might indicate that the roles of individual innexins can be compensated for by other innexin family members (as is evident in the connexin literature on vertebrate gap junctions in patterning).

Because *smedinx-11* and *smedwi-2* expression was irradiation-sensitive (Fig. 2A,B), these postmitotic *S. mediterranea* innexin expression domains are likely to reveal committed progeny of neoblasts, in addition to cells expressing the known *smedwi-1* and *smedwi-2* markers (dividing neoblasts) (Reddien et al., 2005b). The *smedinx-11* gene expression pattern in X1 and X2 cells opens an important opportunity to dissect the transition between proliferative and postmitotic neoblasts. Moreover, expression studies for *smedinx-11* revealed its irradiation-sensitive expression in postmitotic areas (Fig. 2 and see Fig. S3 in the supplementary material), its presence in X1 with enrichment in X2 cells (Fig. 2C,D) and its overlapping expression with other neoblast markers (see Fig. S4 in the supplementary material). Together with the way in which the X1 fraction disappears before the X2 cells after *smedinx-11(RNAi)* (Figs 4, 5), these data are suggestive of a progression between X1 and X2 cells. Therefore, one likely possibility is that X2 represent the postmitotic progeny of the proliferative X1 compartment [consistent with our observation that both *smedinx-11* and SMEDWI-1 are observed in irradiation-sensitive postmitotic areas (Guo et al., 2006), and that, whereas SMEDWI-1 protein is expressed in both X1 and X2, its mRNA is expressed mostly in X1 cells]. Future work will further elucidate the complexity and composition of the X2 cells and the possible roles of SMEDINX-11-mediated signaling as a component of the transition of stem cells between sub-population states (Fig. 7D).

Analysis of *smedinx-11(RNAi)* also revealed unique effects on the endogenous neoblast AP gradient (Fig. 6). Because we detected no obvious AP gradient in *smedinx-11* expression, the...
higher number of neoblasts in the pre-pharyngeal area might be due to the anterior regions of planarians possessing a higher density of complex organs and systems that are subject to greater cellular turnover compared with posterior regions. The neoblasts anterior to the pharynx support more-varied tissues and/or tissues with higher cellular demand. Thus, after smedinx-11(RNAi) (which limits the available neoblast pool), the anterior regions might deplete the neoblasts more rapidly than posterior tissues, consistent with the requirement of neoblast maintenance for smedinx-11. Another possibility is that the propensity of neoblasts to respond to gap junction-mediated signal differs in a graded manner along the AP axis. This AP progression confirms the existence of AP gradients that were hypothesized to exist by classical workers in the regeneration field (Brondsted, 1969), provides a molecular entry-point into the known but still not understood relationship between position along the AP axis and proliferation rates of neoplastic and normal cells in mammals (Auerbach and Auerbach, 1982), and suggests that a primary component of physiological gradients are the neoblasts themselves. These data additionally demonstrate that the control of neoblast proliferation by gap junction signals functionally interacts with the morphogenetic polarity of the animal; future work must address the mechanisms integrating neoblast behavior with large-scale axial patterning.

Fig. 6. smedinx-11(RNAi) gradually reverses the endogenous AP mitotic gradient. (A) Confocal projections of whole-mount immunostained animals using an antibody against the phosphorylated form of Histone-3 (H3P) (green dots are positive signal). Control (left); smedinx-11(RNAi) worms at different times after dsRNA exposure (7, 11 and 14 days). H3P signal is disappearing in a gradient, anterior to posterior, in a time-dependent manner. Each time-point consisted of n=7 worms. (B) Schematic of worm regions quantified in C. C H3P signal quantification from different anatomical areas – pre-pharyngeal (PP), pharyngeal (P) and post-pharyngeal (T) – from control and smedinx-11(RNAi) animals. The H3P signal was quantified (Reddien et al., 2005b) at different time points after dsRNA exposure. H3P signal as detected in whole-mount immunostaining is disappearing gradually and inhomogeneously throughout the worm. In controls (7/7 worms at each time-point), H3P signal from the PP area always contained the highest numbers compared with other regions. By contrast, in smedinx-11(RNAi) worms, the H3P signal from the PP region always contained the smallest number (7/7 worms at each time-point). Mitotic activity completely disappeared by >14 days post-smedinx-11(RNAi). Values are average, and error bars represent s.d.; groups were compared using the Student’s t-test (**P<0.001, *P<0.02, *P<0.08, and ND for no difference). RNAi in planarians (either by feeding or microinjections) results in uniform distribution throughout the body once the dsRNA molecules are incorporated into the worm (Reddien et al., 2005a; Reddien et al., 2005b). Thus, specific gene expression disappears spatially uniformly after RNAi, in a time-dependent manner. Interestingly, the inhibition by smedinx-11 of both neoblast marker expression (S. mediterranea piwi genes, smedcyclinB and smedbrul) and of mitotic activity was characterized by a progressive AP disappearance of the signal patterned along the AP axis (i.e. first depletion of pre-pharyngeal areas followed by most-posterior ones) as the phenotype progressed. This response was consistently observed in neoblasts but not in markers of differentiated tissues – in animals subjected to RNAi for a given gene followed by ISH (Fig. 2A and Fig. 4B,C). In addition, similar results in AP neoblast depletion were obtained as a consequence of microinjections targeting pre- or post-pharyngeal areas. Thus, the activity gradients we describe are specific to smedinx-11 and not a general feature observed in any other RNAi-induced phenotype or irradiation treatment described in the literature. Scale bar: 0.1 mm in A.
proper targeting of neural crest cells (Xu et al., 2006) – a cell population heavily involved in embryonic morphogenesis of diverse body regions.

Our results in a regenerating system confirm in vitro studies in mammalian cells that suggest that gap junction expression is required to maintain proliferative state in neural progenitor cells and that the expression of gap junction-specific genes (e.g. connexin43) decreases as neuronal differentiation takes place (Cheng et al., 2004; Leung et al., 2002; Rozental et al., 1998). Although our data implicate gap junctions in the control of somatic, adult stem cells in a morphogenetic context for the first time, a number of recent studies have indicated that GJC is crucial for germ cell function in Drosophila (Gilboa et al., 2003; Tazuke et al., 2002), suggesting that GJC might provide an ancient and powerful mechanism for mediating the complex interplay of regulatory signals among differentiated and stem cells in multicellular systems (Sánchez Alvarado and Kang, 2005). Further molecular analyses of GJ roles in planaria and vertebrate systems will provide crucial opportunities to understand the evolution of morphogenetic controls as well as identifying novel therapeutic targets for manipulating stem cell behaviors in biomedical settings.

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Fig. 7. Summary schematic of smedinx-11 function in intact and regenerating planarians. (A) In intact animals, smedinx-11 is required to maintain proliferative neoblasts. Representative phenotype progression after smedinx-11(RNAi) shows that smedinx-11 is required for the maintenance of neoblasts with the capability to proliferate (red dots). Our data suggest that intact animals possess an anterior-posterior endogenous gradient of mitotic neoblasts that is gradually reversed as the neoblasts lose their proliferative capabilities after smedinx-11(RNAi). For simplicity, smedinx-11 postmitotic expression is not represented. (B) smedinx-11 is specifically required for blastema formation. Animals amputated 1 week after smedinx-11(RNAi) failed to regenerate even though proliferative neoblasts were present, suggesting a loss of normal neoblast behavior even prior to their disappearance. Although mitotic neoblasts disappear by about 2 weeks after smedinx-11(RNAi), the RNAi-treated animals survived longer than 1 month in both intact and trunk fragments, but were unable to mount a regeneration response, demonstrating the distinct requirement for smedinx-11 in blastema formation. (C) Summary of the key features of different cell populations (X1, X2 and XIns) obtained in flow cytometry experiments. Asterisk denotes the fact that approximately 10-12% of X2 cells can be positive for smedwi-1 and smedwi-2 gene expression while none will be positive for smedcyclinB (Reddien et al., 2005b). (D) Schematic model of the dynamics of different cells (X1, X2 and XIns) obtained in flow cytometry experiments. Proliferative neoblasts (X1) are shown in red, X2 cells in green and blue, and differentiated cells (XIns) in gray. The graded bar represents the level of smedinx-11 expression; the percentages of smedinx-11-positive cells are indicated. Neoblast-related cells are included in X1 and X2 subpopulations that express smedinx-11. The proposal that X2 cells represent the progeny of the proliferative neoblasts is based on: (i) irradiation-sensitive smedinx-11 expression is observed in both proliferative and postmitotic areas; (ii) the time-dependent manner in which neoblasts disappear after smedinx-11(RNAi) (i.e. first X1 cells disappear followed by X2); (iii) smedinx-11 gene expression is restricted to proliferative cells, but its protein signal is broadly observed in both proliferative and postmitotic irradiation-sensitive cells, consistent with the switching off of the normal smedwi-1 expression by cells as they exit X1 but retaining the protein in postmitotic stages prior to protein turnover; and (iv) smedinx-11 expression overlaps with that of smedwi-1 at both the gene and protein level. Therefore, it is possible that X2 cells might represent a transitional stage between X1 and XIns cells, which is consistent with results obtained in vitro for gap junction genes using mammalian neuronal progenitor cells.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/17/3121/DC1

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