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Dorsal and Ventral Positional Cues Required for the Onset of Planarian Regeneration May Reside in Differentiated Cells

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We previously showed by grafting experiments that the dorsoventral (DV) interaction evokes morphogenetic events similar to those that occur in regeneration. However, it is not yet understood whether the stem cells themselves or differentiated cells have the ability to induce regeneration. Here we demonstrated by a combination of X-ray irradiation and grafting experiments that the dorsal and ventral positional cues inducing morphogenetic events are retained in X-ray-irradiated tissues, suggesting that the differentiated cells may be responsible for the positional cues. We grafted a small piece of irradiated worm, in which the stem cells were certainly eliminated, to an intact one in DV-reversed orientation. We observed that projections were developed from the host–donor boundary, as in the previous experiments. Whole-mount *in situ* hybridization with several markers demonstrated that the projections had a newly established DV axis and also had anterior or posterior characteristics. Furthermore, chimeric analysis with a strain-specific marker showed that the projections consisted of nonirradiated cells and that *IFb*-expressing cells, which normally belonged to the ventral tissue, could be generated even from the stem cells located on the dorsal side. Taken together, the findings suggest that the stem cells may simply differentiate depending on the surroundings and that differentiated cells may present positional cues that induce morphogenesis. © 2001 Academic Press

Key Words: **regeneration; dorsoventral interaction; planarian; positional cues; X-ray irradiation; stem cells.**

INTRODUCTION

Adult planarian have a strong ability to regenerate from even a tiny fragment. Several studies suggested that dorsoventral (DV) interaction evoked by wound closure is a trigger for the regeneration (Chandebois, 1979; Kato *et al.*, 1999). However, it is not well understood what types of cells are responsible for providing environmental cues for the regeneration. The cells of adult planarians are divided into two major populations, stem cells, referred to as "neoblasts," and differentiated cells. In this study, we aimed to investigate whether the ability to induce the regeneration resides in the neoblasts or the differentiated cells.

The neoblasts have been identified as a particular cell type based on morphological studies (Pederson, 1959; Morita, 1967; Hori, 1992) and are believed to have totipotency (Wolff and Dubois, 1998; Baguñà *et al.*, 1989). The neoblasts are only mitotic cells and distributed throughout most of the body (Shibata *et al.*, 1999; Newmark and Sanchez Alvarado, 2000). X-ray irradiation causes elimination of the neoblasts and loss of regenerative ability (Lange, 1968). There have been conflicting results about whether positional cues are remain in the irradiated tissue. Brønsted (1969) concluded from data on irradiation and grafting experiments that ". . . polarity is not retained in irradiated tissue." However, Saló and Baguñà (1985) suggested that anteroposterior positional cues are retained in the irradiated

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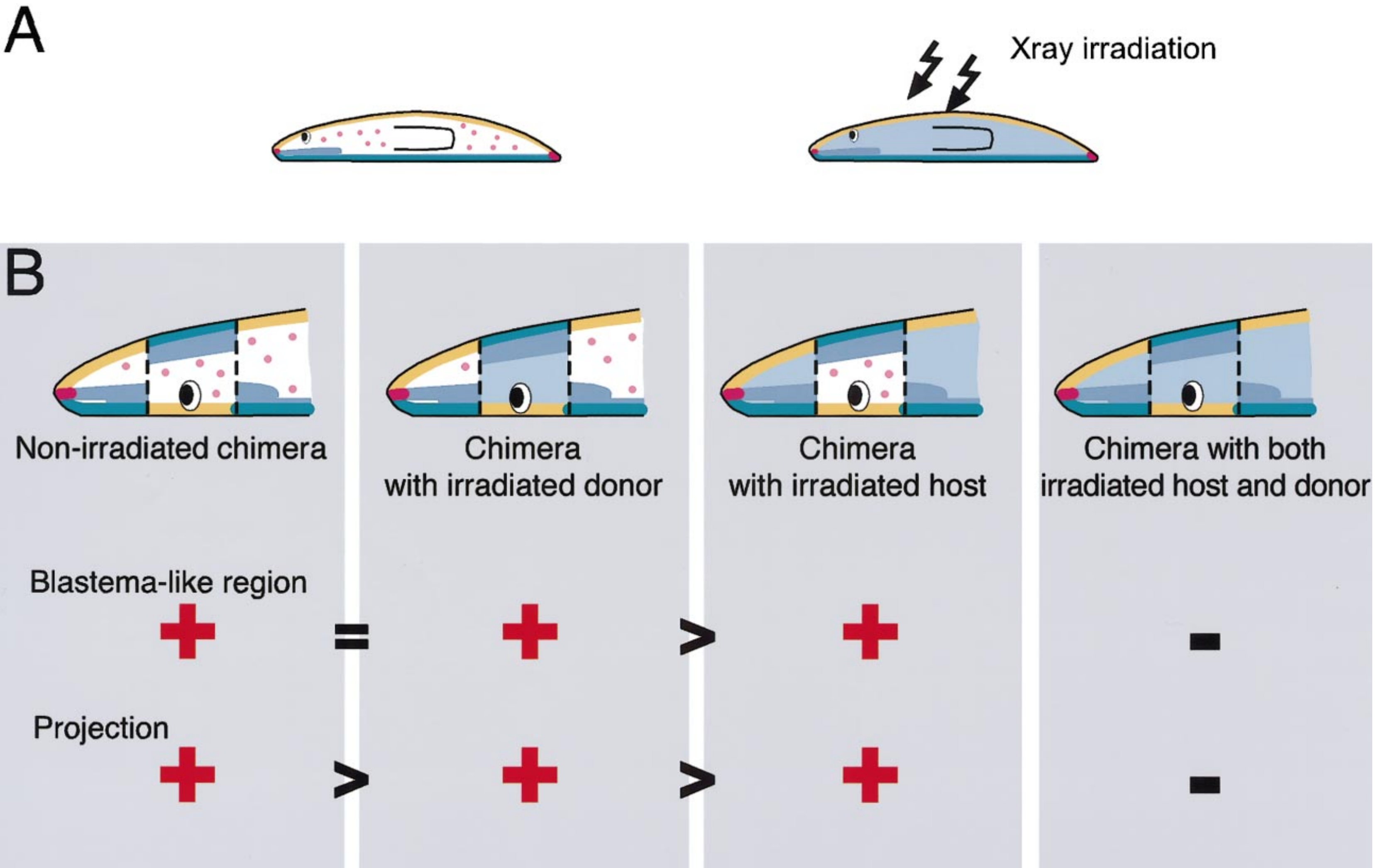

FIG. 1. Schematic representation of grafting experiments with X-ray irradiation. (A) The scheme represents sagittal sections of the whole body of an intact (drawing on the left side) and a X-ray-irradiated planarian (drawing on the right side). (B) Experimental design and summary of the results are represented. The scheme represents sagittal sections of the anterior region. The piece sucked out from an irradiated worm using a Pasteur pipette was rotated 180° to reverse the DV orientation and grafted to an equivalent position of a nonirradiated worm (a chimera with irradiated donor) and vice versa (a chimera with irradiated host). A nonirradiated chimera (a chimera with both intact host and donor) and a chimera with both irradiated host and donor are also represent in the scheme. A red plus sign indicates the formation of the blastema-like region or the projection. A black minus sign indicates failure of the formation of the blastema-like region or the projection. A sign of equality or inequality indicates the size of the blastema-like region or the projection. Yellow, dorsal side; green, ventral side; dark blue, brain and ventral nerve cord; purple, DV boundary; light blue, irradiated tissue; pink dot, neoblast.

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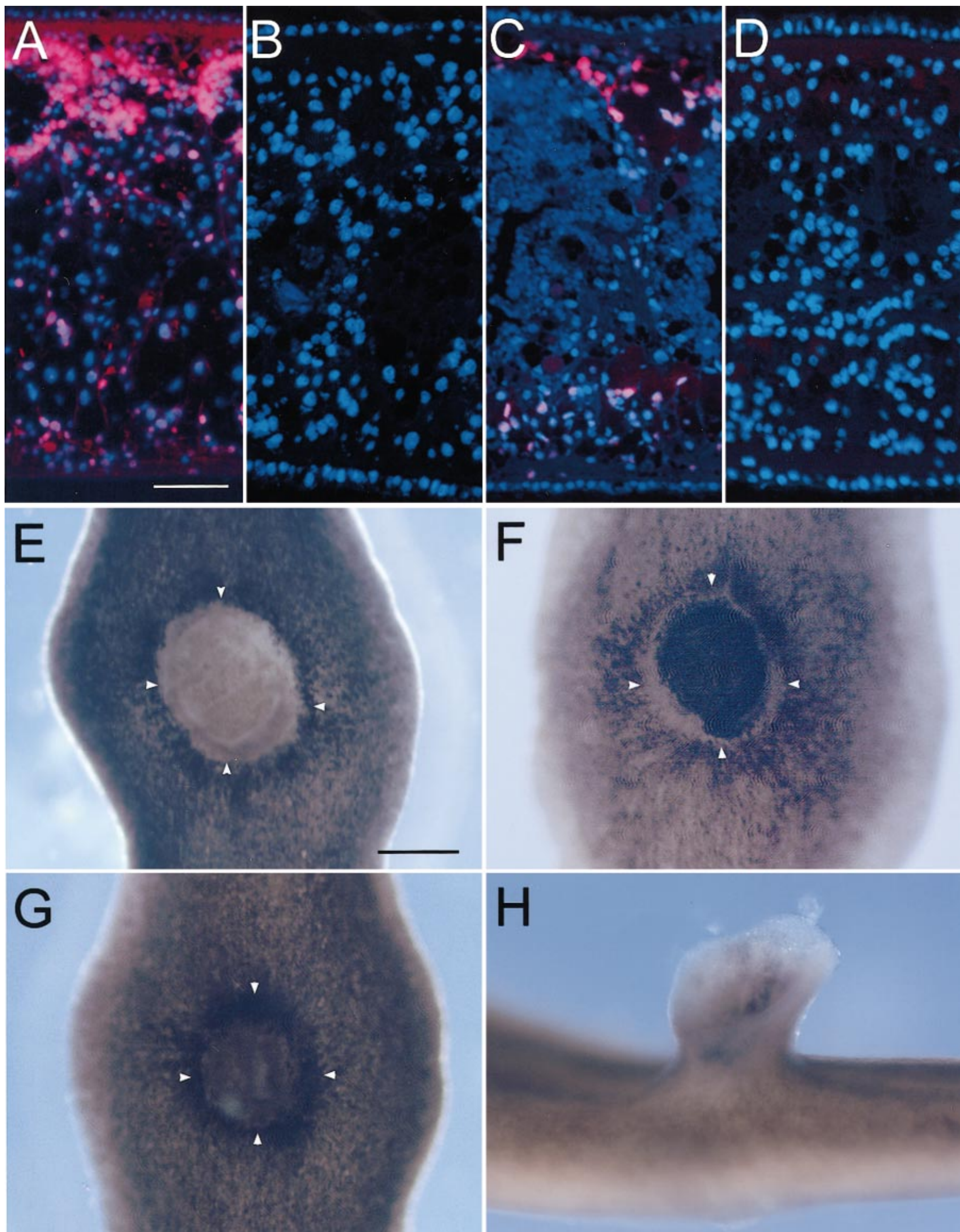

FIG. 2. (A–D) Sagittal sections stained with anti-PCNA antibody in nonirradiated and irradiated worms. Double staining of PCNA in red and the nuclei in blue (Hoechst 33342). Anterior is on the left and dorsal is on the top. Bar, 50 μm. (A) An intact SSP. (B) SSP. 3 days after irradiation with 65 R. PCNA-positive cells could not be observed. (C) An intact HI. (D) HI. 3 days after irradiation with 55R. PCNA-positive cells could not be observed. (E–G) Dorsal view. 4 days after grafting. Anterior on the top. Bar, 500 μm. (E) A nonirradiated chimera. A blastema-like region is formed between the host and the donor (arrowheads). (F) A chimera with an irradiated donor. A blastema-like region is formed between the host and donor (arrowheads). (G) A chimera with both irradiated host and donor. No blastema-like region is formed (arrowheads). (H) A projection on dorsal side of a chimera that had an irradiated donor developed for 30 days.

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tissue. When a nonirradiated piece and an irradiated piece from different anteroposterior levels were joined, intercalary regeneration occurred, and missing parts were restored. However, it was not verified before the graft whether the neoblasts of the irradiated tissue were certainly eliminated, because no molecular marker for the neoblasts was available at that time. Thus, it is still unclear whether positional cues, especially dorsal and ventral positional cues, reside in the neoblasts or differentiated cells.

Mammals also have multipotent stem cells in the adult. Recently, it was demonstrated that adult stem cells, such as neural stem cells and bone marrow stromal cells, can generate various types of cells depending on the surroundings (Woodbury *et al.,* 2000; Ferrari *et al.,* 1998; Pittenger *et al.,* 1999). For instance, neural stem cells could generate a variety of blood cell types when they were injected into vein (Bjornson *et al.,* 1999). It seems that differentiation of the adult stem cells is regulated by environmental cues, which may be given by differentiated cells. In contrast, a fertilized egg, a totipotent stem cell, forms an entire organism. Further, in most aspects of development, proliferating cells themselves may participate in positional cues, which regulate the differentiation of other proliferating cells.

In this study, to investigate whether the dorsal and ventral positional cues reside in the differentiated cells or the neoblasts, we eliminated the neoblasts by X-ray irradiation. By immunohistochemistry with antiproliferating cell nuclear antigen (PCNA) antibody (H. Orii *et al.,* unpublished data), we verified that the neoblasts were certainly eliminated at least by 3 days after irradiation. Subsequently, we examined whether the dorsal (ventral) tissue without the neoblasts could interact with intact ventral (dorsal) tissue to induce typical morphogenetic events.

MATERIALS AND METHODS

Animals

Two clonal strains of the planarian *Dugesia japonica* were used, namely, SSP and HI, which were established in our laboratory. SSP and HI originated from the Irima River, Gifu Prefecture, and Iwayadani Park, Hyogo Prefecture, respectively. The length of each strain is approximately 1.3 cm.

X-Ray Irradiation

Intact planarians were exposed to X rays after 1 to 3 days of starvation. Irradiation was carried out using a Softex B-4 X-ray source operating at 18 kV, 5mA. Worms were placed on ice to anesthetize them and then on a dish with the dorsal side up at about 4 cm under the X-ray source. Subsequently, SSP and HI were irradiated directly with a total of 65 R and 55 R, respectively. Under these conditions, irradiated worms could not regenerate after amputation and died within 20 days (Y. Saito *et al.,* unpublished data).

Microsurgery

SSP-HI chimeras were made by microsurgery. A small piece of the head or tail region of SSP or HI was sucked out using a Pasteur pipette and grafted into the equivalent region of HI or SSP in the dorsoventrally reversed orientation (Fig. 1). In most cases, irradiated HI was used as the host (chimeras with irradiated host) or donor (chimeras with irradiated donor) 3 days after irradiation. We refer to both of these types of chimeras as "irradiated chimeras." We also made chimeras with both nonirradiated host and donor (nonirradiated chimeras) and chimeras with both irradiated host and donor. The method of microsurgery was described previously in detail (Kato *et al.,* 1999).

Immunohistochemistry

Modified relaxant solution was used for fixation (Kato *et al.,* 1999). Paraffin sections of 4 μm thickness were prepared. After the sections were dewaxed and rehydrated, they were blocked in PBS containing 0.1% Triton X-100 and 10% goat serum for 30 min. The rabbit anti-planarian PCNA polyclonal antibody (H. Orii *et al.,* unpublished data) was used at 1:400 dilution and the signal was detected using Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories).

In Situ Hybridization

Fixation and preparation of sections were performed using methods similar to those used to prepare sections for immunohistochemistry. *In situ* hybridization was performed as described previously (Kato *et al.,* 1999). Double fluorescent *in situ* hybridization was performed using a TSA-indirect kit (NEN Life Science Products). Biotin-labeled probes were detected by staining with Texas red, and then sections were treated with the modified relaxant solution at room temperature overnight. Subsequently, DIG-labeled probe was detected by staining with FITC. Cell nuclei were stained with Hoechst 33342 (Sigma). Whole-mount *in situ* hybridization was performed as described previously (Umesono *et al.,* 1997; Agata *et al.,* 1998). The probes used in this study were *DjotxB* (planarian *Otd/otx* homologue; Umesono *et al.,* 1999), *Plox5-Dj* (planarian *Hox/HOM-C* gene; Orii *et al.,* 1995, 1999), *IFb* (intermediate filament gene; A. Tazaki *et al.,* unpublished), *PN5* (mucous component gene; Y. Umesono *et al.,* unpublished), and *PH20* (planarian retrotransposon; K. Agata *et al.,* unpublished).

Staining of Epidermis

To stain the nuclei of the epidermis, living planarians were soaked in 10 μg/ml Hoechst 33342 (Sigma) solution for 1 h. Under these conditions, only the epidermis was strongly stained, and the staining was maintained for at least 7 days. The worms stained with Hoechst 33342 were used for microsurgery as described above. After 3, 4, and 7 days, the worms were quickly frozen with propanol chilled by dry ice and then fixed with Carnoy's solution for 1 h. Fixed samples were embedded in paraffin and serially sectioned at 4 μm. In these processes, water was excluded, because the dye was dispersed in water. The sections were observed without dewaxing. After the sections were observed and photographed, they were dewaxed and rehydrated. Subsequently, double fluorescent *in situ* hybridization analysis of *IFb* and *PH20* was performed as described above.

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RESULTS

Grafting a Piece of Irradiated Planarian to a Nonirradiated One in DV Reversed Orientation Induced a Cup-Shaped Projection

We made chimeras between two clonal strains of *D. japonica,* SSP and HI, in order to distinguish between cells of the host and donor. To eliminate neoblasts, which were the only mitotic cells, planarians were X-ray irradiated with 65 R for SSP and 55 R for HI, respectively. The distribution of PCNA protein, which is expressed specifically in proliferating cells, was analyzed in the irradiated specimens by immunohistochemistry. The number of PCNA-positive cells decreased 1 day after irradiation, and most of the remaining PCNA-positive cells were not stained with Hoechst 33342, indicating degradation of the nucleus. Two days after irradiation, most of the PCNA-positive cells disappeared, and 3 days after irradiation, PCNA-positive cells could not be observed (Figs. 2A–2D). We also confirmed by an electron microscopic study that neoblasts were not observed under the conditions in which the PCNA-positive cells disappeared (data not shown). Thus, the worms at 3 days after irradiation must be composed solely of differentiated cells, and we used such worms for grafting experiments.

As shown in Fig. 1B, we designed graft experiments and defined the terms indicating the state of the experimental design (See also "Microsurgery" under Materials and Methods). When we grafted a small piece of an irradiated donor to a nonirradiated host in DV reversed orientation (chimeras with irradiated donor, Fig. 1B, $n = 102$), a blastema-like white region was formed on the boundary of the host and donor (Fig. 2F). This region increased in size and formed a cup-shaped projection on both the dorsal and the ventral sides of the host (Fig. 2H). We could not observe any morphological differences in the formation of the blastema-like region between the chimeras with an irradiated donor and nonirradiated chimeras (Figs. 2E and 2F). However, the projections in the chimeras with an irradiated donor were smaller than those in the nonirradiated chimeras (data not shown). In the chimeras with an irradiated host ($n = 58$), the blastema-like region appeared narrower and the projections were smaller than those of the chimeras with an irradiated donor (data not shown). Further, chimeras with both irradiated host and donor ($n = 9$) never formed such blastema-like regions (Fig. 2G) or cup-shaped projections. A summary of the experimental results is presented in Fig. 1B. These results clearly indicated that proliferating cells were indispensable for forming a cup-shaped projection, but not necessarily to induce the formation of a cup-shaped projection.

The Projections Were Formed as Results of Morphogenetic Event

We previously demonstrated that the projections induced by DV interaction resulted from morphogenetic events (Kato *et al.,* 1999). To investigate whether the cup-shaped projections induced by the interaction of nonirradiated dorsal (ventral) and irradiated ventral (dorsal) region resulted from morphogenetic events, we performed whole-mount *in situ* hybridization with several markers. We examined the expression of *IFb* (intermediate filament gene), which is a specific marker of the DV boundary. Ectopic expression of *IFb* was observed in the irradiated chimeras near the host–donor boundary (Fig. 3A). In contrast, ectopic expression of *IFb* could not be observed in the chimeras with both irradiated host and donor (Fig. 3B). These results suggested that the DV interaction did not lead to migration of *IFb*-expressing cells from elsewhere, but rather led to differentiation of such cells. Further, double staining for *IFb* and another DV boundary marker, PN5, clearly showed that an ectopic DV boundary was established in the projections of irradiated chimeras, the same as in nonirradiated chimeras (Figs. 3C and 3D). These results demonstrated that interaction of irradiated dorsal (ventral) and nonirradiated ventral (dorsal) regions established the ectopic DV axis.

DjotxB (a planarian *otx* homologue) is usually expressed specifically in the brain, while *Plox5-Dj* (a planarian *Hox/HOM-C* gene homologue) is expressed specifically in the tail region. In the projections formed in the anterior region, expression of *DjotxB* was clearly observed, but that of *Plox5-Dj* was not (Figs. 3E and 3G). In contrast, the projections formed in the posterior region expressed *Plox5-Dj,* but not *DjotxB* (Fig. 3F,H). These results demonstrated that the projections had anterior or posterior characteristics according to their position along the AP axis. Taken together, these findings indicate that the cup-shaped projection was not a mere "projection," but was formed as a result of morphogenetic events, and also indicate that tissues without neoblasts were sufficient to cause the DV interaction.

The DV Boundary Was Not Always Identical to the Host–Donor Boundary in the Irradiated Chimeras

Whole-mount *in situ* hybridization showed that the ectopic *IFb*-expressing region appeared broad and meandering in the irradiated chimeras (Figs. 3A, 4B, and 4C), while the region appeared to maintain a uniform width in the nonirradiated chimeras (Fig. 4A). Furthermore, the chimeras with an irradiated host showed clearly that some of *IFb*-expressing cells were off to the side from the major expressing region (Fig. 4C, arrow).

We investigated the contributions of the host and donor cells to the projections. Expression of *PH20* is useful for distinguishing between the two strains SSP and HI (Kato *et al.,* 1999), because *PH20* is expressed more strongly in SSP than in HI. Double staining of *IFb* and *PH20* in the nonirradiated chimeras after 7 days of grafting showed that the host and donor cells were present in the projections roughly equally and abutted each other at the top of the projections (Fig. 4D). Furthermore, this result indicated that

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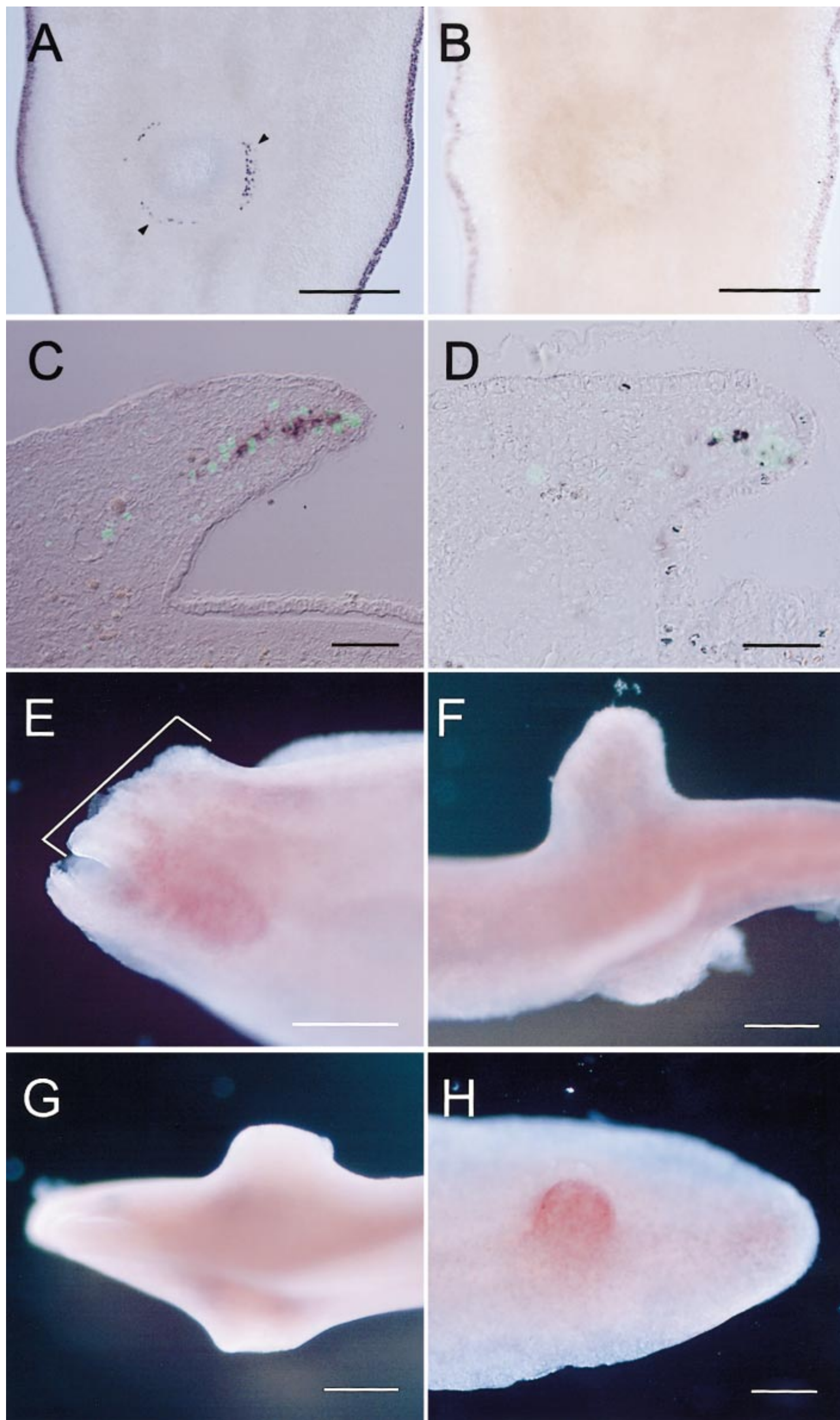

FIG. 3. (A, B) Whole-mount *in situ* hybridization with probe for *IFb*. Bar, 500 μm. (A) Ventral view of a chimera with an irradiated donor, developed for 6 days. Ectopic expression of *IFb* is observed (arrowheads). (B) Dorsal view of a chimera with both irradiated host and donor, developed for 10 days. Ectopic *IFb* expression is not observed. (C, D) *IFb* is stained in green and *PN5* is stained in dark purple by BCIP and NBT, respectively. Anterior is on the left. Dorsal is on the top. Bar, 50 μm. (C) A projection formed on the dorsal side of a nonirradiated graft 7 days after grafting. Ectopic expression of two DV boundary markers, *PN5* and *IFb*, is observed in the projection. (D) A projection formed on the dorsal side of a graft with an irradiated donor, developed for 7 days. Ectopic expression of *PN5* and *IFb* is also observed in this projection. (E–H) Projections formed in chimeras with irradiated donors, developed for 20 days. Bar, 500 μm. (E) Ventral view. A projection developed in the anterior region expressed *DjotxB*. A bracket indicates the projection. (F) Lateral view. A projection developed in the posterior region did not express *DjotxB*. (G) Lateral view. A projection developed in the anterior region did not express *Plox5-DJ*. (H) Dorsal view. A projection developed in the posterior region expressed *Plox5-DJ*.

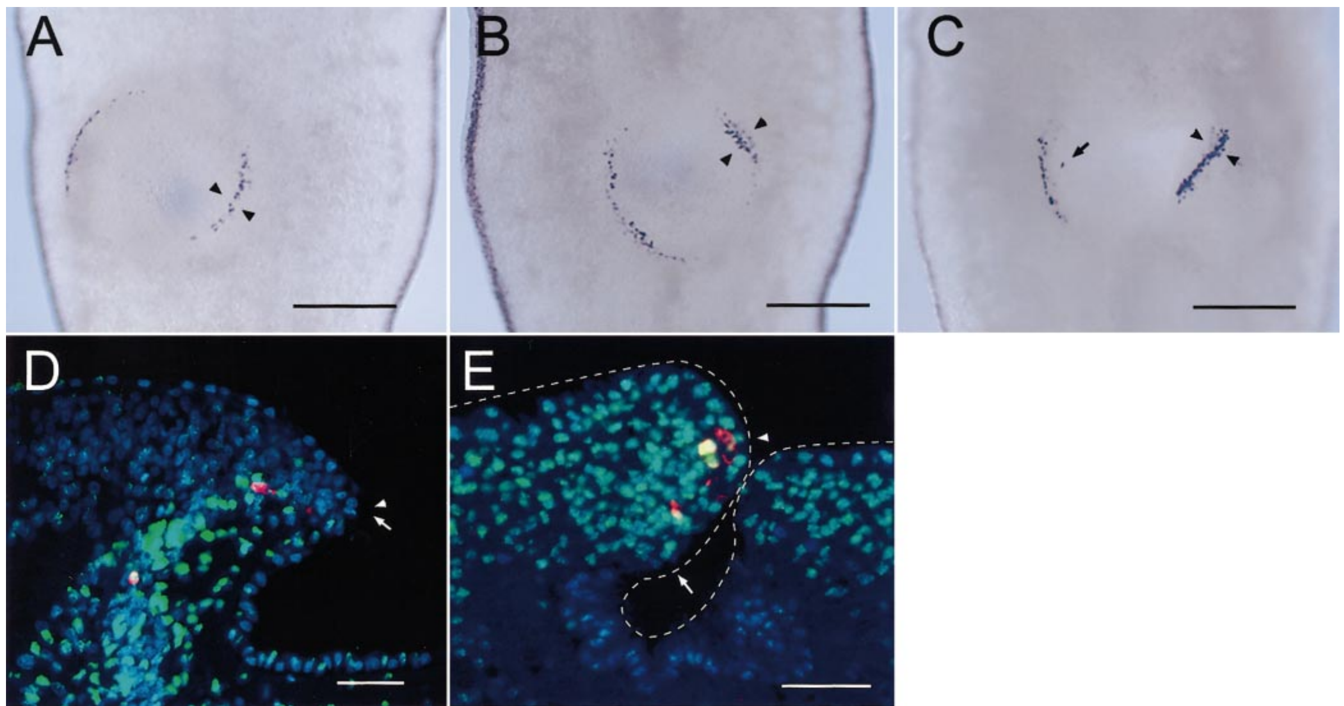

FIG. 4. (A–C) Whole-mount *in situ* hybridization with *IFb* probe after 7 days of grafting. Bar, 500 μm. (A) Dorsal view of a nonirradiated chimera. The ectopic *IFb*-expressing region is straight (arrowheads). (B) Ventral view of a chimera with an irradiated donor. The region between the arrowheads is meandering and broad. (C) Dorsal view of a chimera with an irradiated host, developed for 10 days. The *IFb*-expressing region is broad and meandering (arrowheads). Some *IFb*-expressing cells are off to the side of the major expressing region (arrow). (D, E) *PH20* is stained in green, *IFb* is stained in red, and double-positive cells appear in yellow. Anterior is on the left. Dorsal is on the top. Bar, 50 μm. (D) A projection formed in the dorsal side of a nonirradiated chimera developed for 7 days. The DV boundary (arrowhead) is identical to the host–donor boundary (arrow). (E) A projection formed in the dorsal side of chimera with an irradiated donor, developed for 7 days. *PH20*-expressing cells are nonirradiated host cells. The DV boundary (arrowhead) is not identical to the host–donor boundary (arrow). The dashed line indicated the outline of the projections.

the host–donor boundary was identical to the DV boundary (Fig. 4D, arrow and arrowhead). In the chimeras with an irradiated donor, irradiated cells still remained and the host-donor boundary could be observed clearly (Fig. 4E). In contrast to the nonirradiated chimeras, the projections mainly consisted of cells of the nonirradiated host. Interestingly, *IFb*-expressing cells were not found on the host–donor boundary but instead were found near the top of the projections in most cases (Fig. 4E, arrow and arrowhead), although the host–donor boundary was the region where dorsal and ventral tissues abutted each other at first.

Although *IFb* is a DV boundary marker, *IFb*-positive cells display ventral characteristics. As shown in Fig. 4D, *IFb*-positive cells seemed to be derived from ventral tissue (donor cells) in nonirradiated chimeras. In contrast, in the irradiated chimeras, *IFb*-expressing cells were derived from the host cells even on the dorsal side of the host (Fig. 4E). Since the DV boundary was already established, it is reasonable that dorsal and ventral characteristics around the ectopic DV boundary were also established. It seemed that, on the dorsal side of the host, the outer side of the ectopic DV boundary had dorsal characteristics, like the host, and the region between the DV boundary (Fig. 4E, arrowhead) and the host–donor boundary (Fig. 4E, arrow) had ventral characteristics, like the donor. These results implied that newly differentiated cells with ventral characteristics around the projections of the host dorsal side were generated from the dorsal cells of the host.

Differentiation of the Neoblasts May Be Dependent on the Surroundings

To confirm whether neoblasts of the dorsal (ventral) side can differentiate into ventral (dorsal) cells, we examined early expression of *IFb* during the projection formation. In the irradiated chimeras as well as nonirradiated chimeras, *IFb*-expressing cells were detected starting at 3 days after grafting by whole-mount *in situ* hybridization. While the *IFb*-expressing region appeared straight in nonirradiated chimeras (Fig. 5A), the region already appeared broad and meandering in the irradiated chimeras (Fig. 5B). The relation between the position of *IFb*-expressing cells and the

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TABLE 1
Position of the *IFb*-Expressing Cells

		On the host–donor boundary	Distant from the host–donor boundary	In both positions
Irradiated chimeras	R3	25% (1/4)	25% (1/4)	50% (2/4)
	R4	0% (0/7)	14% (1/7)	86% (6/7)
	R7	0% (0/6)	0% (0/6)	100% (6/6)
Nonirradiated chimeras	R3	80% (4/5)	0% (0/5)	20% (1/5)
	R7	100% (6/6)	0% (0/6)	0% (0/6)

Note. Each value indicates the number of chimeras with *IFb*-expressing cells in a given location/number of chimeras. Percentages are calculated from these data. R3, R4, and R7 indicate days after grafting.

host–donor boundary was investigated by chimeric analysis. After 3 days of grafting, in the chimeras with an irradiated donor, 29% (4/14) of specimens had *IFb*-expressing cells. Among the chimeras with *IFb*-expressing cells, 25% of chimeras had *IFb*-expressing cells only on the host–donor boundary, 25% of chimeras had such cells only at a distance from the host–donor boundary, and the remaining 50% of chimeras had such cells in both positions (Figs. 5D–5F; Table 1). On the other hand, most *IFb*-expressing cells were found only on the host–donor boundary in the nonirradiated chimeras (Fig. 5C, Table 1). Since the host and the donor cells appeared not to mix after 3 days of grafting, it was unlikely that neoblasts of the host ventral side had migrated to the host–donor boundary of the host dorsal side and differentiated into ventral cells there. *IFb*-expressing cells may differentiate just at that position. These results suggest that neoblasts differentiated into ventral or dorsal cells regardless of their original positions. The fact that *IFb*-expressing cells were observed at a distance from the host–donor boundary dose not rule out the possibility that direct cell-to-cell or cell-to-extracellular matrix contact was involved in the morphogenetic events. However, the result rather suggests that some secreted factors may be involved in the morphogenetic events. Thus, the results indicate that differentiated cells are at least responsible for the DV positional cues.

Although the host–donor boundary was still distinguishable 3–7 days after grafting, the irradiated cells were finally replaced by the intact cells. As shown in Fig. 5G, after 30 days of grafting, in the chimeras with nonirradiated HI as the host and irradiated SSP as the donor, *PH20* was not expressed, but ectopic *IFb* was still expressed in the projections. This result clearly indicated that the DV polarity of the projection was still maintained, while irradiated donor cells were replaced by nonirradiated host cells. The neoblasts might migrate from the intact host to the irradiated donor side and differentiate into the appropriate cells depending on the positional cues of the donors. Taken together, the findings show that differentiated cells have positional cues which regulate the differentiation of the neoblasts, and the neoblasts might differentiate depending on the surroundings.

Old Dorsal Epidermis Covered the Ventral Mesenchyme in the Irradiated Chimeras

In planarians, the epidermis does not have proliferative ability. When worms are cut, the cells of the epidermis stretch and cover the wound and are gradually replaced by newly differentiated epidermal cells (Morita and Best, 1974; Hori, 1979). Dorsal and ventral epidermis have different characteristics. The ventral epidermis has numerous cilia and microvilli. The dorsal epidermis is thicker than the ventral epidermis and has less cilia (Morita and Best, 1974; unpublished observations). Thus, in the irradiated chimeras, it is possible that the epidermis between the DV boundary and the host–donor boundary kept the characteristics of the host, while the mesenchyme of this region may already have the same characteristics as the donor. Unfortunately, we did not have markers available for dorsal or ventral epidermis. Instead, we investigated the process in which the old epidermis was replaced by newly differentiated cells. To distinguish old epidermis and newly differentiated epidermis, only old epidermis was stained with Hoechst 33342 before grafting. Because the epidermis is formed by differentiation from the neoblasts in the mesenchyme, newly differentiated epidermis can be identified by negative staining of the dye.

After 3 days of grafting, most of the blastema-like region, which was recognized as an unpigmented region, was covered with old epidermis (Fig. 6A); however, some regions were covered with the newly differentiated epidermis in the same specimen. After 4 days, most of the old epidermis around the host–donor boundary was replaced by new epidermis (Fig. 6C), while in the chimeras with both irradiated host and donor, the formation of the blastema-like region and replacement of the epidermis did not occur (Fig. 6D). In a few cases of chimeras with an irradiated donor, as shown in Fig. 6B, we found that *IFb*-expressing cells were positioned at some distance from the host–donor boundary and were covered with old epidermis (Figs. 6A and 6B). Although the region between the DV boundary and the host–donor boundary was narrow, the mesenchyme of this region may already had ventral characteristics, since *IFb*-expressing cells themselves usually belong to the ventral

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tissue. This result suggests that the dorsal epidermis covered the ventral mesenchyme. Thus, the polarity of the epidermis may not alter that of the mesenchyme.

DISCUSSION

Differentiated Cells Are Responsible for the Dorsal and Ventral Positional Cues in Planarians

Appendages of insects and adult urodeles have strong regenerative ability. There have been many studies showing that ectopic AP or DV interaction induces formation of supernumerary legs (Iten and Bryant, 1975; Bryant and Iten, 1976; French, 1976; Campbell and Tomlinson, 1995). Each case of the supernumerary leg formation can be well explained by the "boundary model" (Meinhardt, 1983). The boundaries of different cell populations act as organizing centers for establishment of the ectopic proximodistal (PD) axis. This model is almost completely consistent with the molecular mechanism of PD axis formation in early development of *Drosophila* legs and vertebrate limbs (Martin, 1995; Campbell and Tomlinson, 1995). We previously reported that ectopic DV interaction induces morphogenetic events in planarians and suggested that DV interaction has an important role in the onset of regeneration (Kato *et al.,* 1999). The boundary model can well explain this phenomenon.

Experiments using X-ray irradiation to examine formation of the supernumerary legs have been performed in urodeles, the imaginal discs of *Drosophila,* and the wing buds of chickens. These experiments gave contradictory results. Most studies showed that the irradiated tissues still had positional cues which could stimulate the formation of the supernumerary legs (Maden, 1979; Holder *et al.,* 1979; Smith *et al.,* 1978; Adler and Bryant, 1977). However, Carlson (1974) could not observe the formation of distinct supernumerary legs in adult axolotl. This contradiction may have been caused by different conditions of irradiation. The authors of these studies did not verify elimination of proliferating cells before grafting. Furthermore, the source of cells for morphogenesis seems to be different among different animals. In adult urodeles, dedifferentiated cells might participate in regeneration (Gardiner *et al.,* 1999) and also in formation of the supernumerary legs. In contrast, in chickens and *Drosophila,* proliferating embryonic cells may be involved in the formation of supernumerary legs. Given these facts, the interpretation is controversial. Although each case of the formation of supernumerary legs can be well explained by the boundary model, the roles of the proliferating cells and differentiating cells in those animals cannot be compared with each other. Here we have shown that in planarians the differentiated cells are at least responsible for the dorsal and ventral positional cues, and interaction with those cells could cause the neoblasts to form new structures. These results suggested that the dorsal and ventral positional cues that function in the onset of regeneration reside in the differentiated cells.

Saló and Baguñà (1985) joined two fragments from different AP levels in the planarian. One was irradiated tissue and the other was nonirradiated tissue. Subsequently, they observed intercalary regeneration. They suggested that AP positional cues resided in differentiated cells; however, they did not confirm whether the neoblasts were certainly eliminated. Indeed, the neoblasts were not eliminated immediately after irradiation in our experiments. If the grafting was done under such conditions, the remaining neoblasts might have affected the results. We previously showed that *Plox5-Dj* (a planarian *Hox/HOM-C* gene) was expressed only in the posterior region of intact planarians and regenerating body pieces. These results suggested that *Plox5-Dj* is involved in AP patterning in planarians (Orii *et al.,* 1999). Three days after irradiation, *Plox5-Dj* was still expressed as in intact worms, suggesting that *Plox5-Dj* is expressed at least in differentiated cells (Orii *et al.,* 1999). Although the relationship between positional cues and the hox gene in planarians is obscure, this result supports the idea that AP positional cues reside in the differentiated cells. Since we verified the depletion of the neoblasts in our experiments, our results clearly indicated that tissues without neoblasts are responsible for the DV positional cues. Analysis at 3 days after grafting indicated that the position of the *IFb*-expressing cells was not always identical to the host–donor boundary. This result suggests that some secreted factors were involved in the morphogenetic events, but does not eliminate the possibility that direct cell-to-cell or extracellular matrix-to-cell contact was involved. At least, differentiated cells were involved in the morphogenetic events in the irradiated chimeras. Thus, DV positional cues may reside in differentiated cells.

The Neoblasts May Differentiate Regardless of Their Original Position

Multipotent stem cells are found in various tissues in adult mice and humans. Recently, it has been indicated that some adult stem cells have the capacity to differentiate into various types of cells without restriction by the original embryonic germ layers *in vivo* and *in vitro.* Bone marrow stromal cells can generate myogenic cells *in vivo* (Ferrari *et al.,* 1998) and neurons *in vitro* (Woodbury *et al.,* 2000). Adult neural stem cells can generate several types of blood cells when they are injected into the vein (Bjornson *et al.,* 1999). They noted that the adult neural stem cells likely needed extra time to acquire another fate. Ultimately, adult stem cells may differentiate into appropriate cells in response to the surroundings.

In the present study, in the irradiated chimeras, *IFb*-expressing cells, which normally belong to the ventral tissue, were differentiated from the cells of the dorsal side. Although we cannot rule out the possibility that the neoblasts also have dorsal and ventral positional cues, the neoblasts may simply differentiate in response to the surroundings.

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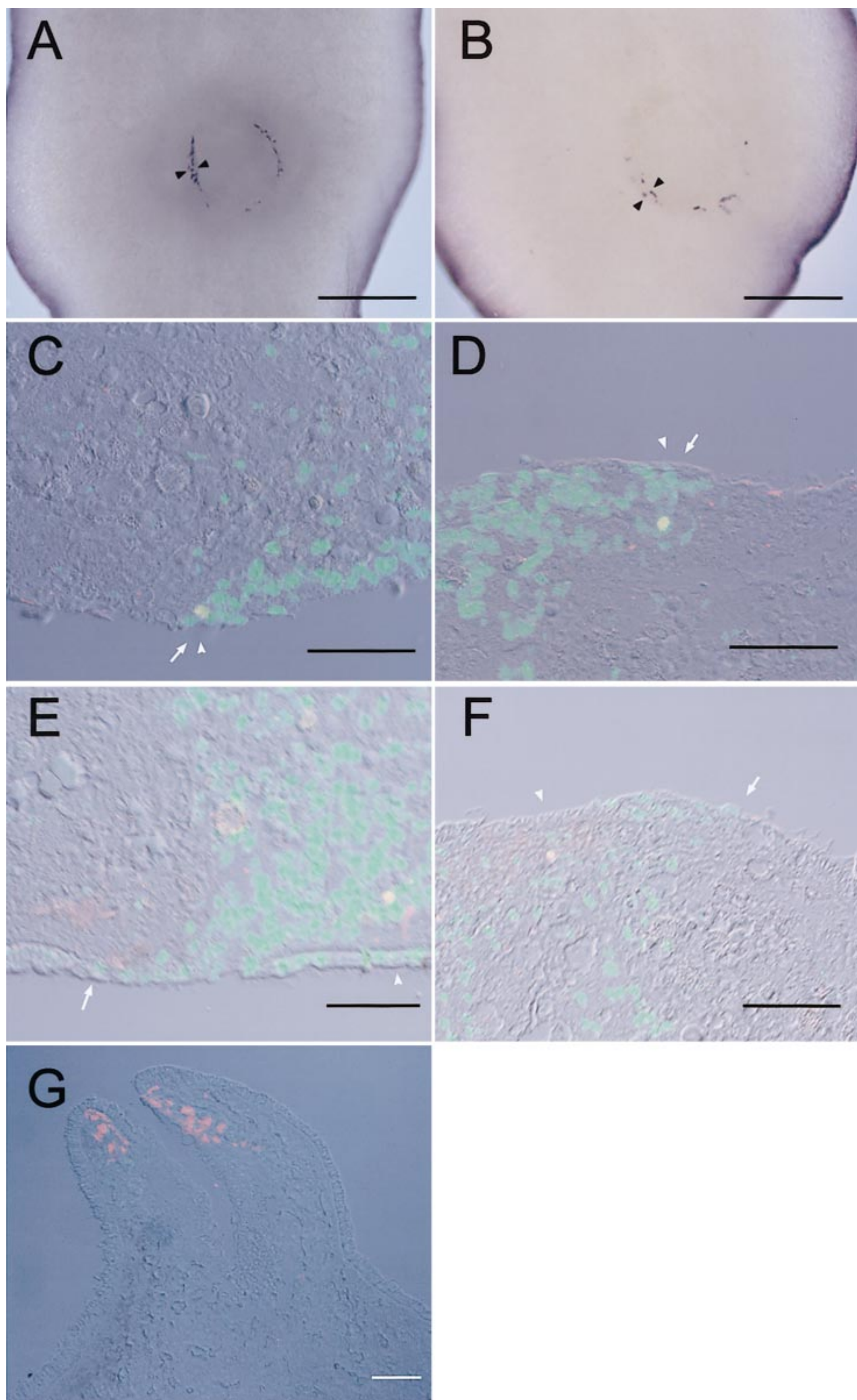

FIG. 5. (A, B) Whole-mount *in situ* hybridization with *IFb* probe after 3 days of grafting. Dorsal view. Bar, 500 μm. (A) The ectopic *IFb*-expressing region is straight in the nonirradiated chimera (arrowheads). (B) The ectopic *IFb*-expressing region is meandering and broad in the chimera with an irradiated donor (arrowheads). (C–F) Sagittal sections with *PH20* stained in green, *IFb* stained in red, and double-positive cells appearing in yellow. Bar, 50 μm. (C) Ventral side of a nonirradiated chimera developed for 3 days. Ectopic expression of *IFb* (arrowhead) can be observed on the host–donor boundary (arrow). (D) Dorsal side of a chimera with irradiated donor, developed for 3 days. *PH20*-expressing cells are nonirradiated host cells. Ectopic expression of *IFb* (arrowhead) is seen on the host–donor boundary (arrow). (E, F) Chimeras with irradiated donor, developed for 3 days. *PH20*-expressing cells are nonirradiated host cells. Ectopic expression of *IFb* (arrowhead) is not seen on the host–donor boundary (arrow). (E) Ventral side. (F) Dorsal side. (G) A projection formed on the dorsal side of a chimera with an irradiated donor, developed for 30 days. *PH20* (irradiated cells) is no longer observed, while *IFb* is still expressed.

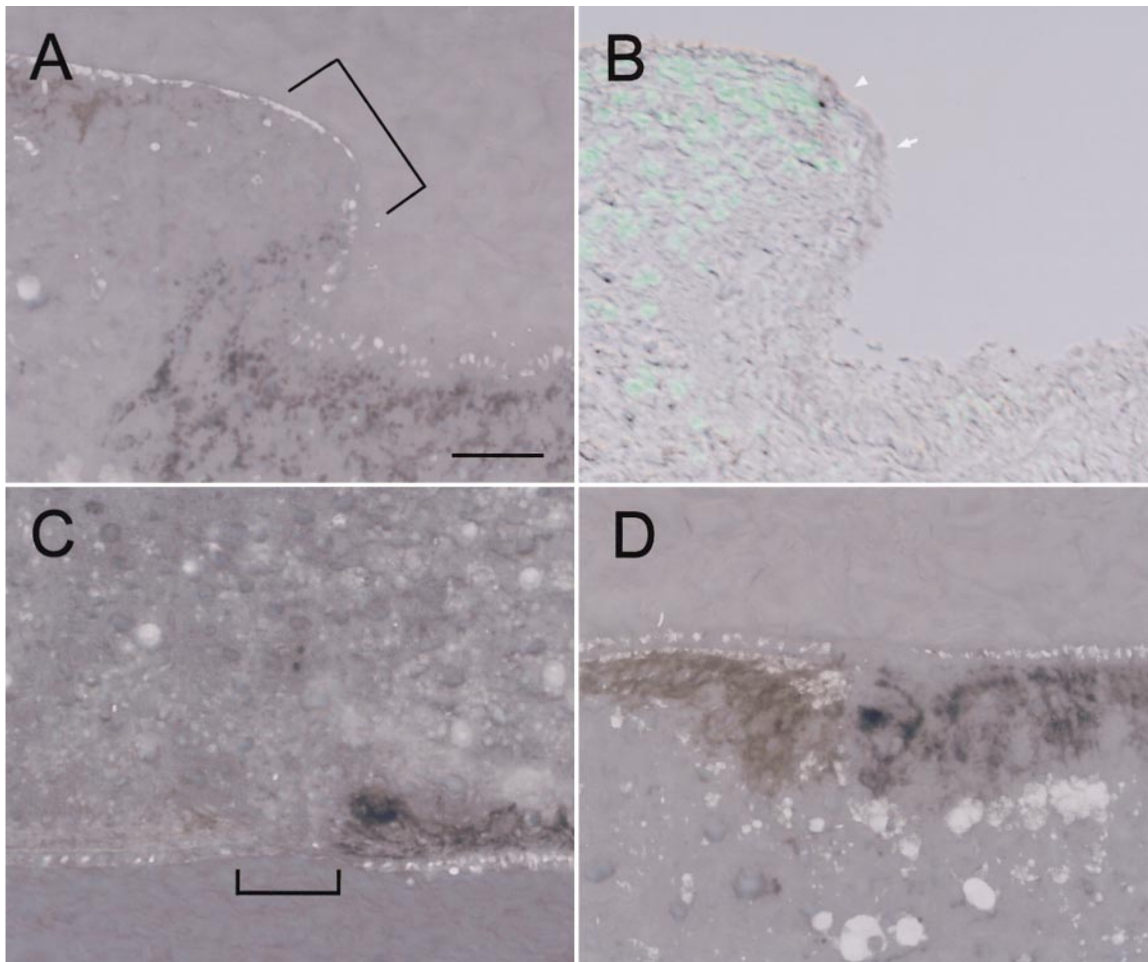

FIG. 6. Anterior is on the left and dorsal is on the top. (A) A projection on the dorsal side of a chimera with an irradiated donor, developed for 3 days. Tissue on the right side is from the irradiated donor. Staining by Hoechst 33342 can be observed in white. The bracket indicates a blastema-like region, which can be identified as an unpigmented region. Epidermis stained with Hoechst 33342 covers the blastema-like region. (B) A section of near that of (A). Double staining of *PH20* in green and *IFb* in dark purple by BCIP and NBT, respectively. *PH20*-expressing cells are nonirradiated host cells. Ectopic expression of *IFb* (arrowhead) is somewhat distant from the host–donor boundary (arrow). (C) Ventral side of a chimera with an irradiated donor, developed for 4 days. New epidermis (the Hoechst-negative cells) cover the blastema-like region. The bracket indicates the blastema-like region. (D) Dorsal side of a chimera with both irradiated host and donor, developed for 4 days. Staining by Hoechst 33342 can be observed in white. No blastema-like region (unpigmented region) is formed. Stretched epidermis covers the host–donor boundary, and nuclei cannot be observed. Bar, 50 μm.

Establishment of DV Axis

In the irradiated chimeras, cells constituting the blastema-like region were derived only from the nonirradiated tissue. Some of *IFb*-expressing cells were positioned at a distance from the host–donor boundary from the time they first appeared. Therefore, the DV boundary was not always identical to the host–donor boundary, and the DV axis was established mainly in the blastema-like region. Taken together, these facts indicate that it is possible that secreted factors are involved in the establishment of the DV axis. To get direct evidence supporting this idea, we try to interrupt the DV (the host and donor) interaction by insertion of a pored polycarbonate filter or aluminum foil. Unfortunately, because of experimental difficulties, we were unable to ascertain whether secreted factor(s) are involved or not.

Whole-mount *in situ* hybridization with a probe for *IFb* showed a broad and meandering ectopic DV axis in the irradiated chimeras. One explanation is the number of cells responsible for positional cues. We used worms for grafts 3 days after irradiation. Thus, the cells responsible for dorsal and ventral positional cues were not newly generated for at least 3 days. It is conceivable that the decrease of those cells in irradiated tissue led to an imbalance of the dorsal and

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ventral positional cues and resulted in the broad and meandering ectopic DV boundary in irradiated chimeras.

The Role of the Epidermis in Morphogenesis and DV Patterning

During regeneration of urodeles' limbs, the epidermis migrates rapidly and covers the wound. The wound epidermis itself proliferates, which is necessary for blastema formation (Géraudie and Ferretti, 1998). X-ray irradiation inhibits limb regeneration but allows the irradiated epidermis to migrate over the wound (Bulter, 1933). When nonirradiated muscle was transplanted to the irradiated limb, no regeneration occurred. In contrast, when nonirradiated skin was transplanted to the irradiated limb, regeneration occurred (Lheureux, 1983). These results suggested that the intact epidermis was required to promote regeneration. Recent work using *Xenopus* larva appendages clearly showed that regeneration ability is dependent on the expression of *fgf10* by mesenchymal cells (Yokoyama *et al.,* 2000). These results suggested that the epidermis is not sufficient, and the epidermis–mesenchyme interaction is also important for promoting the regeneration.

The DV polarity of the ectoderm is strongly concerned with that of the mesoderm during mouse and chicken limb development. It has been suggested that the patterning mechanisms of regeneration and development are the same in urodeles (Muneoka and Bryant, 1982). A recent study of limb regeneration of *Xenopus* larvae showed that the epidermis controls DV patterning of the regenerating blastema and suggested that epidermis–mesenchyme interaction is required to accomplish regeneration (Matsuda *et al.,* 2000).

In planarians, we showed that the epidermis was not renewed when ectopic expression of *IFb* was already detected. At the blastema-like region, the old host epidermis covered newly generated mesenchyme, which had the same polarity as the donor. Thus it seems that new epidermis is not necessary for the formation of the blastema-like region and that the polarity of the epidermis is not related to the DV patterning of the mesenchyme in planarians.

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