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Mesodermal cell interactions in the sea urchin embryo: properties of skeletogenic secondary mesenchyme cells

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SUMMARY

An interaction between the two principal populations of mesodermal cells in the sea urchin embryo, primary and secondary mesenchyme cells (PMCs and SMCs, respectively), regulates SMC fates and the process of skeletogenesis. In the undisturbed embryo, skeletal elements are produced exclusively by PMCs. Certain SMCs also have the ability to express a skeletogenic phenotype; however, signals transmitted by the PMCs direct these cells into alternative developmental pathways. In this study, a combination of fluorescent cell-labeling methods, embryo microsurgery and cell-specific molecular markers have been used to study the lineage, numbers, normal fate(s) and developmental potential of the skeletogenic SMCs. Previous fate-mapping studies have shown that SMCs are derived from the veg_2 layer of blastomeres of the 64-cell-stage embryo and from the small micromeres. By specifically labeling the small micromeres with 5-bromodeoxyuridine, we demonstrate that descendants of these cells do not participate in skeletogenesis in PMC-depleted larvae, even though they are the closest lineal relatives of PMCs. Skeletogenic SMCs are therefore derived exclusively from the veg_2 blastomeres. Because the SMCs are a heterogeneous population of cells, we have sought to gain information concerning the normal fate(s) of skeletogenic SMCs by determining whether specific cell types are reduced or absent in PMC(−) larvae. Of the four known SMC derivatives: pigment cells, blastocoelar (basal) cells, muscle cells and coelomic pouch cells, only pigment cells show a major reduction (>50%) in number following SMC skeletogenesis. We therefore propose that the PMC-derived signal regulates a developmental switch, directing SMCs to adopt a pigment cell phenotype instead of a default (skeletogenic) fate. Ablation of SMCs at the late gastrula stage does not result in the recruitment of any additional skeletogenic cells, demonstrating that, by this stage, the number of SMCs with skeletogenic potential is restricted to 60-70 cells. Previous studies showed that during their switch to a skeletogenic fate, SMCs alter their migratory behavior and cell surface properties. In this study, we demonstrate that during conversion, SMCs become insensitive to the PMC-derived signal, while at the same time they acquire PMC-specific signaling properties.

Key words: cell interactions, primary mesenchyme cells, secondary mesenchyme cells, cell fate, skeletogenesis, gastrulation, sea urchin embryo

INTRODUCTION

Cell interactions play an important role in the specification of cell fates in the early sea urchin embryo. An interaction between the two principal populations of mesodermal cells, primary and secondary mesenchyme cells (PMCs and SMCs, respectively), is critical in regulating SMC fates and the formation of the larval skeleton (reviewed by Ettensohn, 1992). Although fate-mapping studies have shown that during normal embryogenesis the skeleton is synthesized exclusively by PMCs, certain SMCs also have the potential to express a skeletogenic phenotype under appropriate experimental conditions (Fukushi, 1962; Langelan and Whiteley, 1985; Ettensohn and McClay, 1988). Elimination of the PMCs by microsurgical or fluorescence-based methods results in the conversion of 60-70 secondary mesenchyme cells to a skeletogenic fate (Ettensohn and McClay, 1988; Ettensohn, 1990a). During this change in cell fate, SMCs express PMC-specific molecular markers and acquire the ability to respond to migratory cues in the blastocoel normally recognized only by PMCs (Ettensohn and McClay, 1986, 1988). Signals transmitted by the PMCs therefore regulate both the migratory behavior and program of gene expression of SMCs.

Several important properties of the cells that switch fate in response to PMC ablation remain unelucidated. The SMCs are a diverse cell population and the normal fate of the skeletogenic SMCs has not been established. Time-lapse videorecordings of PMC(−) embryos show that some (but not all) SMCs that ingress late in gastrulation switch fate in such embryos (Ettensohn and McClay, 1988). Recent studies have re-emphasized the heterogeneous nature of the

SMCs and point to the need to characterize more fully the subpopulation of skeletogenic SMCs. At least four populations of SMCs arise during embryogenesis. First, in those species that have been examined carefully, a population of prospective pigment-forming SMCs ingresses relatively early in gastrulation (Gustafson and Wolpert, 1967; Gibson and Burke, 1985). Later in gastrulation, larger numbers of cells are released from the tip of the archenteron. Some of these cells move into the blastocoel and adopt a fibroblast-like morphology; they have been referred to as basal cells (Cameron et al., 1991) or blastocoelar cells (Tamboline and Burke, 1992) although this population might include several different cell types. At the completion of gastrulation, two coelomic pouches appear as bilateral outpocketings of the foregut (Gustafson and Wolpert, 1963). Subsequently, 10-15 SMCs migrate out of each coelomic pouch and extend slender processes that surround the foregut. These cells express several distinctive cytoskeletal proteins and produce the circumesophageal musculature of the pluteus larva (Ishimoda-Takagi et al., 1984; Cox et al., 1986; Burke and Alvarez, 1988; Wessel et al., 1990).

It is well established that the SMCs are descendants of the veg_2 blastomeres formed at the 64-cell stage (Hörstadius, 1973). One qualification to this generalization is that some cells of the coelomic pouches are derived instead from the small micromeres, four small cells that arise from the unequal division of the micromeres at the fifth cleavage (Endo, 1966; Pehrson and Cohen, 1986; Tanaka and Dan, 1990; Cameron et al., 1991). The small micromeres undergo one additional round of division to produce eight cells that remain at the tip of the archenteron during gastrulation and later contribute to both the right and left coelomic pouches. Two considerations raise the possibility that descendants of the small micromeres might also contribute to the population of skeletogenic SMCs. (1) The small micromeres are the siblings of the large micromere daughter cells, the founder cells of the PMC lineage, and are therefore the closest lineal relatives of the PMCs. (2) The small micromere descendants are present at the tip of the archenteron during gastrulation and re-enter the cell cycle later in development (Pehrson and Cohen, 1986). Although during normal larval development, the small micromere descendants do not ingress into the blastocoel during gastrulation or exhibit a mesenchymal phenotype, it cannot be assumed that patterns of cell migration or division are the same in normal and PMC(−) embryos.

The number of SMCs with skeletogenic potential has not been determined. Removal of the entire complement of PMCs in *Lytechinus variegatus* results in the conversion of an average of 60-70 SMCs to a skeletogenic fate (Ettensohn and McClay, 1988). It is not known, however, whether this represents the limit of the embryo's regulative capacity in this regard, or whether elimination of the skeletogenic SMCs might result in the conversion of additional cells to this fate. As noted above, those SMCs that switch fate following PMC ablation exhibit a number of PMC-specific properties, including the expression of skeletogenic cell surface markers, migration to PMC-specific target sites and the synthesis of spicules. The PMCs have another distinctive characteristic - the ability to transmit a signal that regulates SMC fate. Although the mechanism of the signaling is unknown, it provides a different functional test of the extent to which converted SMCs acquire PMC-specific properties.

This study further examines the cellular basis of the PMC-SMC interaction, focusing on the properties of the responding cells. We have attempted to define more fully the lineage, numbers and normal developmental fate(s) of the skeletogenic SMCs. We have also further explored the regulative capacity of the embryo by asking whether there are additional populations of SMCs (or other cells) with skeletogenic potential. Finally, the effect of fate-switching on the properties of the SMCs has been investigated. We have asked whether converted SMCs remain responsive to the PMC-derived signal and whether they acquire PMC-specific signaling properties.

MATERIALS AND METHODS

Embryo culture

Adult *Lytechinus variegatus* were collected at the Duke University Marine Laboratory (Beaufort, NC) or were obtained from a commercial supplier (Susan Decker, Hollywood, FL). Animals were induced to shed gametes by intracoelomic injection of 0.5 M KCl. Eggs were washed twice with Instant Ocean (IO) (Aquarium Systems, Inc.), fertilized with a dilute suspension of sperm and cultured in IO in glass bowls. The developmental rate of the embryos was controlled by maintaining cultures at 18-24°C in temperature-controlled water baths.

Cell transplantations and fluorescent cell marking

Vital fluorescent labeling of cells with rhodamine B isothiocyanate (RITC) and microsurgical manipulations of embryos were carried out as previously described (Ettensohn and McClay, 1988; Ettensohn, 1990b).

Antibodies

Monoclonal antibody (mAb) 6a9b10 (Ettensohn and McClay, 1988) was used as a marker for the skeletogenic phenotype. This mAb recognizes the PMC-specific glycoprotein msp130 (Leaf et al., 1987; Farach et al., 1987; Parr et al., 1990) as well as several other related PMC-specific cell surface glycoproteins (Ettensohn and Fuhrman, unpublished observations). A polyclonal antiserum that recognizes a larval form of myosin heavy chain was provided by Dr G. M. Wessel, and was used as a marker for muscle cells (Wessel et al., 1990).

Immunofluorescent staining of experimental embryos

After microsurgical operations, embryos were released from microinjection chambers by gently removing the upper coverslip of a chamber and placing it in a glass dish filled with IO. Using a mouth pipette and working under a dissecting microscope, a stream of IO was directed into the chamber until the embryos were forced out into the dish. Embryos were then transferred by mouth pipette to glass depression slides and cultured in a humid chamber until the desired stage. For some experiments, embryos were collected and subjected to additional microsurgical manipulations. In such cases, the embryos were re-loaded into microinjection chambers by concentrating them in a minimal volume of water in a finely drawn mouth pipette, transferring them onto a fresh polylysine-coated coverslip and assembling a new chamber.

At the conclusion of each experiment, embryos were fixed as whole mounts in 100% methanol and stained by indirect immunofluorescence. Microsurgically manipulated embryos were fixed for

5 minutes in ice-cold 100% methanol in the depression slide, rinsed with IO (2×10 minutes), then incubated for 1-2 hours at room temperature in 50 μl of full-strength mAb 6a9b10 supernatant or a 1:100 dilution of anti-myosin heavy chain in IO on a square piece of Parafilm on the bottom of a 35 mm plastic dish placed in a humid chamber. After rinsing again in IO (2×10 minutes), the embryos were mouth-pipetted into 50 μl of a 1:50 dilution of FITC-conjugated goat anti-mouse IgA/IgG/IgM or goat anti-rabbit IgG (FITC-GAM and FITC-GAR, respectively) (Organon Teknica) on a fresh piece of parafilm and stained for 1-2 hours at room temperature. After rinsing again with IO, embryos were mouth-pipetted into a drop of 'FITC-Guard' anti-photobleaching mounting medium (Testog, Inc.) and gently mixed. For counts of converted SMCs, stained embryos were squashed beneath a no. $1\frac{1}{2}$ coverslip. For confocal microscopy (below), embryos were mounted on glass slides with Scotch double-sided tape spacers (Scotch no. 665 tape) to prevent compression and covered with no. 0 coverslips. All samples were sealed with fingernail polish and stored at −20°C. Whole mounts prepared in this way could be stored for many weeks with no detectable loss of fluorescence.

Labeling of small micromeres with 5-bromodeoxyuridine

Small micromeres were specifically labeled with 5-bromodeoxyuridine (BrdU) after the method of Tanaka and Dan (1990). This method takes advantage of the fact that, after their formation at the 32-cell stage, the small micromeres divide only once until late in larval development, while all other cells of the early embryo undergo several additional rounds of DNA replication and division. Stock solutions of BrdU (10 mM) and thymidine (50 mM) (Sigma) were prepared in distilled water and stored at 4°C. Stock solutions were diluted in IO just before use. 1-cell zygotes were incubated in 1 μM BrdU from 20-50 minutes postfertilization (23°C). At this temperature, embryos were beginning the first cleavage division at the end of the labeling period. They were washed with IO, chased for 10 minutes in 500 μM thymidine, and then washed with fresh IO and cultured to the desired stage. BrdU-labeled plutei were fixed in 70% ethanol for 30 minutes at −20°C. An equal volume of 4 N HCl/1% Triton X-100 was added and the embryos incubated for an additional 30 minutes at room temperature. After washing several times with 0.05% Tween 20 in phosphate-buffered saline (T-PBS), the embryos were stained by indirect immunofluorescence as described above, using a 1:20 dilution of an anti-BrdU monoclonal antibody (Becton Dickinson) in T-PBS followed by FITC-GAM.

Analysis of SMC derivatives in PMC(–) embryos

Counts were made of the numbers of pigment cells, coelomic pouch cells, and blastocoelar cells in control and PMC(−) larvae. Pigment cells were counted by compressing live, late pluteus larvae (60 hours postfertilization) and viewing them with differential interference contrast (DIC) optics. The dark-red pigment cells could be clearly distinguished from the transparent cells that constitute the remainder of the embryo. Blastocoelar cells were counted by optically sectioning live plutei (60 hours postfertilization) using DIC optics and directly counting the numbers of blastocoelar cells. The fibroblast-like morphology of these cells (Tamboline and Burke, 1992) can be distinguished from that of the PMCs, which have spherical cell bodies that are evenly spaced along the spicule rods. Counts of coelomic pouch cells were made from three-dimensional reconstructions (below) of 40-hour larvae that had been stained with propidium iodide, a nuclear stain (Cameron et al., 1991). Larvae were fixed in ethanol and treated with HCl/Triton as described above for BrdU labeling, washed three times with T-PBS and stained for 30-60 minutes in 0.2-0.5 mg/ml propidium iodide in IO. Propidium iodide was prepared as a stock solution in IO (1 mg/ml) and stored at 4°C. Muscle cells were counted by optically sectioning embryos that had been stained by indirect immunofluorescence with anti-myosin heavy chain and FITC-GAR (below).

Confocal laser scanning microscopy

To count the numbers of muscle cells and coelomic pouch cells in larvae accurately, fluorescently stained embryos were examined by confocal laser scanning microscopy. Whole mounts that had been mounted with tape spacers to avoid compression (see above) were examined with a Bio-Rad MRC-600 confocal laser scanning microscope, equipped with an 15 mW krypton-argon laser. For counts of coelomic pouch cells, 35-57 images (256×256 pixels) of the foregut region of each specimen were collected with a z-spacing of 0.6 μm, using a 60× (NA 1.3) planapochromat oil immersion lens. Each image was collected with the pinhole aperture at the mimimum setting in order to optimize z-axis resolution and was Kalman filtered. Z-series were reconstructed using BDSRender software (Biological Detection Systems, Inc), which generates rotating, three-dimensional renderings of optical data sets as either gray scale images or red/green stereopairs. By examining such three-dimensional reconstructions, it was possible to clearly distinguish the coelomic pouches from nearby cells and to make accurate counts of nuclei. Muscle cells were examined by optically sectioning immunostained embryos in a similar manner, but because fewer cells were stained, it was possible to count the cells directly by focussing up and down through each specimen, rather than storing z-series and generating three-dimensional reconstructions.

RESULTS

Lineage of the skeletogenic SMCs

Because the small micromeres are the sister cells of the four large micromeres that establish the PMC lineage, we examined the possibility that these cells might contribute to the skeletogenic secondary mesenchyme. Pulse-labeling of *L. variegatus* zygotes with 5-bromodeoxyuridine after the method of Tanaka et al. (1990) resulted in specific labeling of the small micromere descendants at the pluteus stage (Fig. 1A). As reported by Endo (1966) and Pehrson and Cohen (1986) in other species, the four small micromeres undergo one round of cell division during gastrulation, giving rise to a cluster of eight cells at the tip of the archenteron. The left coelomic pouch is usually larger than the right and the distribution of small micromere descendants in the two pouches is often unequal (Gustafson and Wolpert, 1963; Pehrson and Cohen, 1986). Fig. 1B shows the small micromere derivatives in a PMC(−) larvae fixed 36 hours after PMC removal (24 hours after the conversion of SMCs to a skeletogenic fate). In 48 such embryos examined, no reduction in the number of small micromere derivatives was apparent compared to control embryos. Some variability in the numbers of small micromere descendants was observed in both control and PMC(−) larvae, although the most common number was eight in both cases. Since BrdU unambiguously labels the small micromere daughter cells and would be diluted were these cells to initiate additional rounds of cell division, these observations demonstrate that the small micromeres do not contribute to the population of skeletogenic SMCs. It can therefore be con-

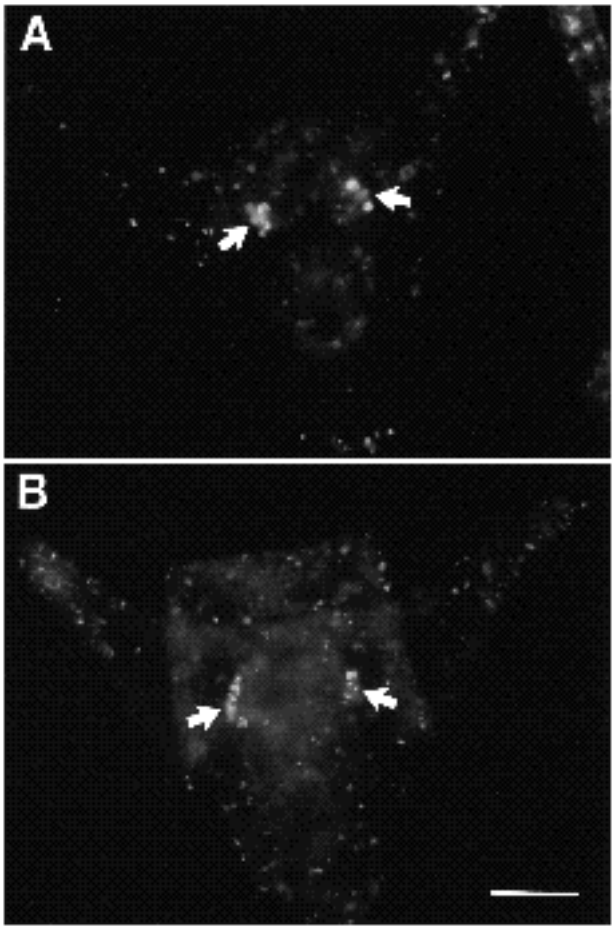

Fig. 1. The small micromeres do not contribute to the population of skeletogenic SMCs. Nuclei of small micromeres were specifically labeled by briefly incubating 1-cell zygotes in 5-bromodeoxyuridine after Tanaka and Dan (1990). (A) Control larva, 36 hours postfertilization. (B) PMC(−) embryo, 48 hours postfertilization (36 hours after PMC removal). In both cases, the typical bilateral arrangement of small micromere descendants is visible adjacent to the foregut (arrows). Scale bar, 50 μm.

cluded that the skeletogenic SMCs are derived exclusively from the veg_2 layer of blastomeres of the 64-cell-stage embryo, which gives rise to most of the secondary mesenchyme (Hörstadius, 1973).

Analysis of SMC derivatives in PMC(–) embryos

To gain information concerning the normal fate(s) of the skeletogenic SMCs, PMC(−) larvae were analyzed to determine whether specific SMC derivatives were reduced or absent (Figs 2-4). By examining living embryos using differential interference contrast microscopy, cell counts were made of two major derivatives of these cells; pigment cells and blastocoelar cells. To compare numbers of myogenic cells in control and PMC(−) embryos, larvae that had been stained with a polyclonal antiserum that recognizes larval myosin heavy chain (Wessel et al., 1990) were optically sectioned using confocal microscopy. Finally, the numbers of cells in the coelomic pouches at the pluteus larva stage were determined by propidium iodide staining in combination with confocal fluorescence microscopy and three-dimensional image reconstruction (see Methods).

For pigment and blastocoelar cell determinations, control and PMC(−) larvae were scored at advanced larval stages (60 hours postfertilization) to insure that cell differentiation was complete. For muscle cell determinations, it was not possible to compare control and PMC(−) embryos of the same absolute age in hours postfertilization, as we observed a reproducible delay in the appearance and morphogenesis of these immunopositive cells in PMC(−) embryos relative to controls, a difference that we have not explored further. Control and PMC(−) embryos were therefore scored at the time of release of myogenic cells from the coelomic epithelium, before the formation of an extensive array of circumesophageal strands made it difficult to distinguish individual cells (Fig. 3B). In control embryos, this stage of myogenesis was reached at 26-28 hours postfertilization, while PMC(−) embryos reached an equivalent developmental stage at 32-34 hours. In addition, examination of large numbers of control (n>100) and experimental (n=54) embryos at later larval stages revealed no apparent differ-

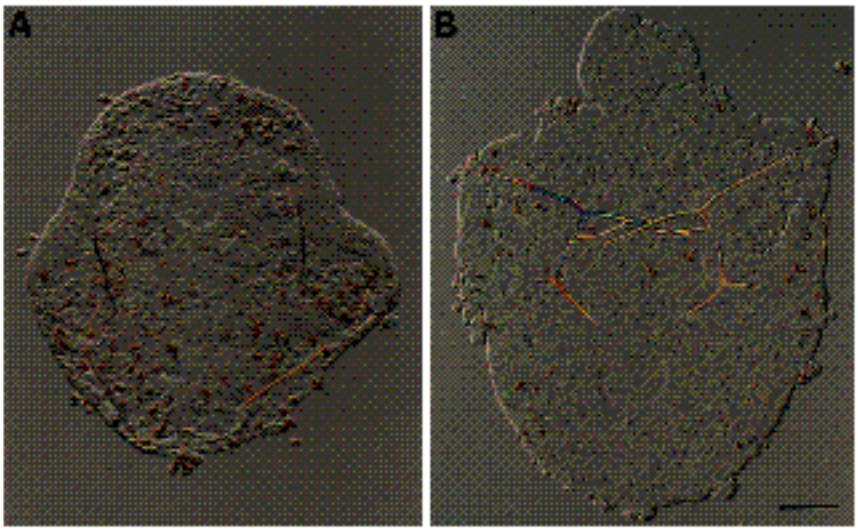

Fig. 2. SMC conversion leads to a decrease in the number of pigment cells. Squash preparations of an unfixed control larva (A) and a PMC(−) larva (B), showing a reduction in the number of dark red pigment cells following PMC removal. Counts of pigment cells made from such specimens are shown in Fig. 4. Scale bar, 50 μm.

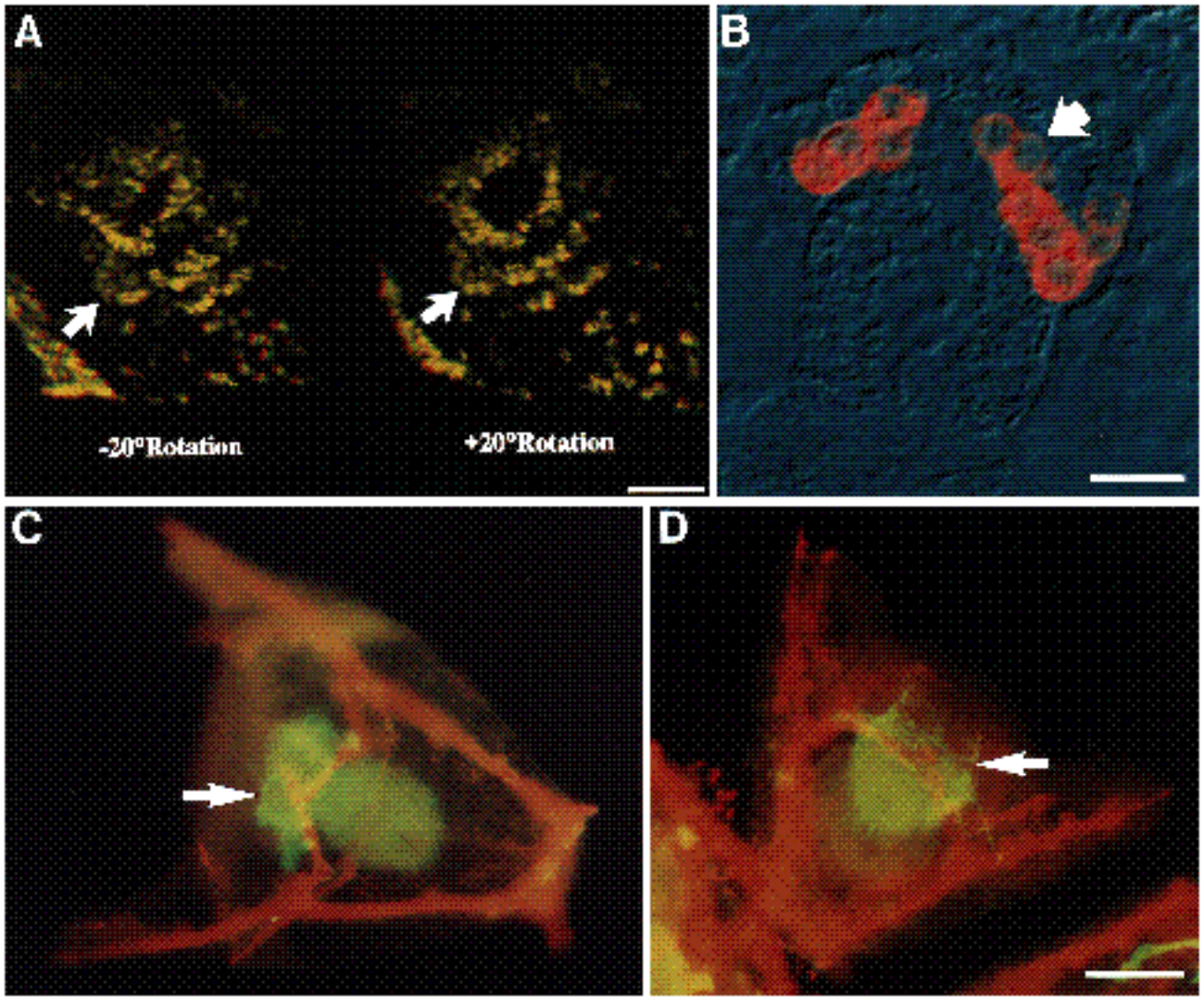

Fig. 3. Analysis of coelomic pouch cells and muscle cells in control and PMC(−) larvae. (A) Coelomic pouch cells in a PMC(−) larva. The embryo was fixed, stained with propidium iodide and optically sectioned with a BioRad MRC-600 confocal laser scanning microscope (see Methods). A three-dimensional reconstruction was generated with BDSRender software. The views shown here are two of the twenty different frames in a movie of this specimen. The views are displayed as red-green stereopairs (red-green glasses are required for viewing). In this orientation, the nuclei in one coelomic pouch (arrow) are clearly labeled and can easily be distinguished when the specimen is viewed in different orientations. Counts of coelomic pouch cells made from such reconstructions are shown in Fig. 4. Scale bar, 30 μm. (B) Muscle cells in a PMC(−) larva. An overlay of transmitted light (blue) and confocal fluorescent (red) images of a 34-hours larva stained with an anti-myosin antibody (see Methods). Myogenic cells are clearly visible at the base of each coelomic pouch and can be counted accurately due to the lack of staining within the cell nuclei (arrow). Scale bar, 20 μm. (C,D) Control (C, 36 hours postfertilization) and PMC-depleted (D, 48 hours postfertilization) larvae stained with anti-myosin heavy chain (green) and monoclonal antibody 6a9b10, a marker for skeletogenic cells (red). There is no apparent effect on the extent or organization of the muscular strands that surround the esophagus (arrows) following SMC conversion. Fainter staining of endodermal epithelial cells, especially those comprising the sphincters separating gut segments, is also apparent in both control and PMC(−) larvae (see Wessel et al., 1990). Scale bar, 50 μm.

ences in the organization or abundance of the circumesophageal musculature (Fig. 3C,D). Coelomic pouch cells were examined in 40-hour larvae, after the release of myogenic cells from the coelomic epithelium. Because of the relatively small size of the pouches and their tendency to flatten along the wall of the foregut (Gustafson and Wolpert, 1963), it was difficult to distinguish propidium iodide-stained nuclei in the pouches from those in surrounding tissues. This problem was circumvented by generating rotating, three-dimensional reconstructions of propidium iodide-stained larvae and making nuclear counts from such reconstructions (Fig. 3A).

The results of these cell counts show that the numbers of coelomic pouch cells, blastocoelar cells and muscle cells are similar in control and PMC(−) larvae (Fig. 4). The difference in the mean number of blastocoelar cells, although small, was statistically significant by a *t*-test ($P<0.001$), while differences in numbers of coelomic pouch cells and

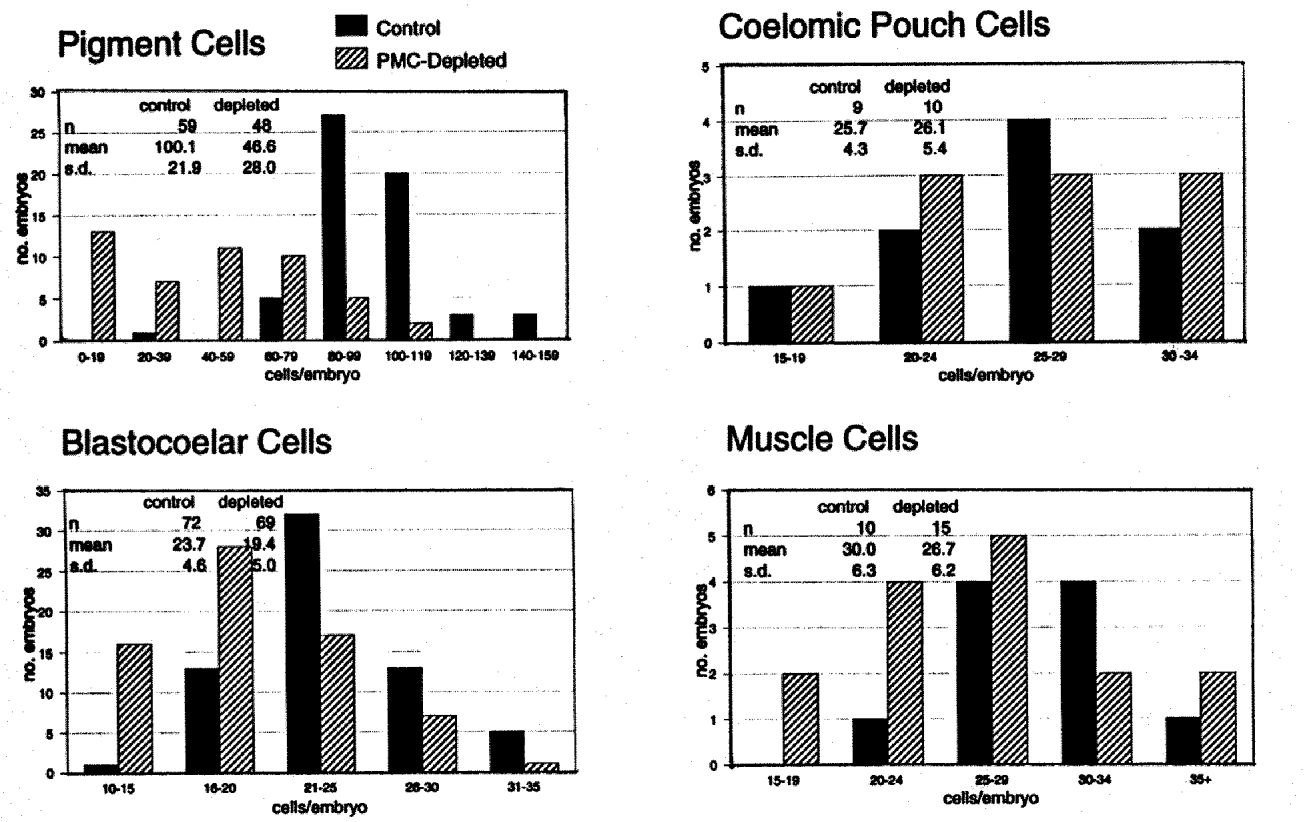

Fig. 4. Counts of SMC-derived cell types in control and PMC(−) larvae. Cell types scored and developmental stages used were as follows: pigment cells and blastocoelar cells (60 hours pluteus larvae), coelomic pouch cells (40 hours pluteus larvae) and myogenic cells (late prism-stage embryos). See Methods for details of the cell counting methods. The differences between control and experimental embryos are significant in the case of pigment cells and blastocoelar cells ($P<0.001$ in each case), but not in the case of muscle cells ($P>0.20$) or coelomic pouch cells ($P>0.80$) when analyzed by two-sided *t*-tests.

muscle cells were not statistically significant (for muscle cells, $P>0.20$; for coelomic pouch cells, $P>0.80$). The most striking difference in SMC-derived cell types in PMC(−) and control larvae was in the number of pigment cells, the most abundant SMC-derived cell type (see also Fig. 2A,B). Control plutei (60 hours postfertilization) have an average of 100.1 pigment cells/embryo (n=59, s.d.=21.9), while PMC(−) larvae at the same stage have an average of only 46.6 pigment cells/embryo (n=48, s.d.=28.0), a reduction of more than 50 cells, or greater than 50% of the total. This difference was highly significant by a *t*-test ($P<0.001$). SMCs conversion therefore leads to a selective decrease in the numbers of pigment cells in late-stage larvae.

Loss of sensitivity to the PMC signal during SMC conversion

The above observations indicate that the PMC-derived signal directs a subpopulation of SMCs to express a pigment cell rather than a skeletogenic fate. Previous data indicate that, even in the continuous presence of the PMCs, responding cells do not become committed to a pigment cell fate until late in gastrulation, probably because they are insensitive to the PMC-derived signal until that time (Ettensohn, 1990a). We next considered the question of whether SMCs might remain responsive to the PMC-derived signal even after their expression of a skeletogenic fate. To answer this question, rhodamine-labeled, converted SMCs were microinjected into normal mesenchyme blastula-stage recipient embryos that had the usual complement of 60-65 PMCs (Fig. 5). After 12 hours, the embryos were fixed and stained by indirect immunofluorescence using mAb 6a9b10 and a fluorescein-conjugated secondary antibody. As shown in Fig. 6, under these conditions the converted SMCs continued to express a skeletogenic phenotype. Of 159 total donor cells injected, 130 (82%) migrated to PMC-specific target sites and continued to express msp cell surface proteins 12 hours after transplantation. It is possible that the remaining 18% of the donor cells that were 6a9-negative represent skeletogenic SMCs that altered their fate as a result of an interaction with PMCs. An alternative explanation appears more likely, however. Because the blastocoel is also populated with non-skeletogenic SMCs at this stage, some of which are closely associated with the ring of skeletogenic cells, it is highly likely that a small number of such cells were inadvertently included as donors. This is supported by experiments in which donor cells isolated in the same way were injected into recipient embryos lacking PMCs. In these experiments, a similar proportion of donor cells were found to be non-skeletogenic 12 hours after transplantation despite the absence of any PMC-derived signal (108/129=84%). As has been previously reported in the case of embryos with intermediate numbers

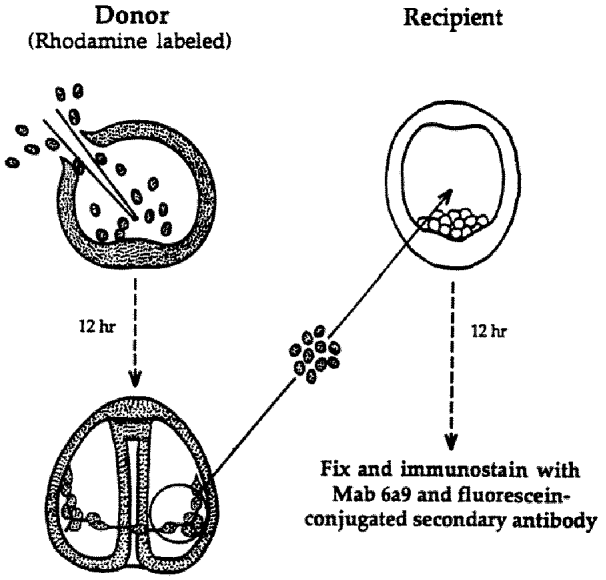

Fig. 5. Experimental protocol used to test the sensitivity of converted SMCs to the PMC-derived signal. Converted SMCs were removed from rhodamine-labeled donor embryos and microinjected into younger (mesenchyme blastula stage) recipient embryos. 12 hours later the recipient embryos were fixed and immunostained with mAb 6a9b10 and a fluorescein-conjugated secondary antibody to determine whether the injected donor cells continued to express a skeletogenic phenotype.

of PMCs (Ettensohn and McClay, 1988), converted SMCs mingled with endogenous PMCs of the host and contributed to all regions of the subequatorial ring pattern and larval skeleton. These observations indicate that during their conversion to a skeletogenic fate, SMCs lose their sensitivity to the PMC-derived signal.

Signaling competence of skeletogenic SMCs

Previous studies have shown that during their switch in fate, SMCs express PMC-specific cell surface molecules, migrate to target sites on the blastocoel wall normally occupied by PMCs and produce spicules (Ettensohn and McClay, 1988). As an independent test of the equivalence of PMCs and converted SMCs, we examined whether these cells acquire the same signaling capacity shown by PMCs, as assayed by the ability of the cells to suppress SMC skeletogenesis when microinjected into PMC(−) embryos. The experimental protocol was identical to that shown in Fig. 5, except that in these studies donor cells were microinjected into PMC(−) recipient embryos. For each set of operations, several PMC(−) embryos were processed in parallel with recipients but were not injected with donor cells. These embryos served as an internal control for any batch-to-batch variability in the numbers of converting cells or in the timing of conversion. These experiments clearly showed that converted SMCs are highly effective at suppressing the conversion of uncommitted SMCs to the skeletogenic fate (Fig. 7). When 20-45 (mean=32) skeletogenic SMCs were injected into recipient embryos, the numbers of SMCs derived from the recipients that expressed a skeletogenic fate was reduced by 80-85%. Therefore, as SMCs adopt a skeletogenic fate, they acquire PMC-specific signaling properties.

Numbers of SMCs with skeletogenic potential

Removal of the entire complement of PMCs results in the conversion of 60-70 SMCs to the skeletogenic phenotype (Ettensohn and McClay, 1988). It is not known whether these 60-70 cells represent the limit of the embryo's regulative capacity, or whether additional SMCs (or unrelated populations of cells) might also possess skeletogenic potential. The most direct way to test this hypothesis would be to eliminate the converted SMCs specifically and assay for the appearance of new skeletogenic cells. Unfortunately, we have not developed methods for eliminating skeletogenic SMCs from embryos once these cells have switched fate, when they are dispersed throughout the blastocoel. An alternative strategy, shown in Fig. 8A, was therefore adopted. Rhodamine-labeled, PMC(−) embryos were created by the usual methods and allowed to develop to the late gastrula stage, at a time when SMC fate-switching was beginning but while most of these cells were still associated with the archenteron. Using a beveled, siliconized micropipette, 30-40 SMCs were removed from the tip of the archenteron. Because these cells presumably represented a mixture of SMC subtypes, it was not possible to determine how many prospective skeletogenic SMCs were removed from any given embryo. Therefore, all SMCs that had been removed from a donor embryo were microinjected into a younger, PMC(−) recipient. The recipient embryo served as a neutral environment lacking any skeletogenic cells (PMCs or converted SMCs) that might restrict the ability of the donor cells to express a skeletogenic fate. The donor and recipient embryos were allowed to develop for 6-7 hours and then processed together as a pair for immunofluorescent staining with mAb 6a9. Recipient embryos were fixed and analyzed before endogenous SMCs had begun to switch fate. The sum of the rhodamine-labeled, mAb 6a9-positive cells in the recipient and the 6a9-positive cells in the donor represented the total number of potentially skeletogenic cells in the original donor embryo at the late gastrula stage. For each trial, several PMC(−) donor embryos that had not been used for cell transplantations were allowed to develop alongside experimental embryos and provided an internal standard, as described above. As shown in Fig. 8B, the average number of skeletogenic cells in each embryo pair closely matched the average number of skeletogenic cells in control PMC(−) embryos, indicating that the removal of SMCs did not result in any increase in the number of skeletogenic cells. We therefore conclude that, by the late gastrula stage, the number of potentially skeletogenic SMCs is restricted to 60-70 cells.

DISCUSSION

The SMCs are a heterogeneous population of cells with several fates. There has been no general agreement even on the classification of these cells; some workers consider all non-skeletogenic mesoderm, including the coelomic pouches and esophageal myoblasts, to be derived from the secondary mesenchyme (Cameron et al., 1991; Ettensohn,

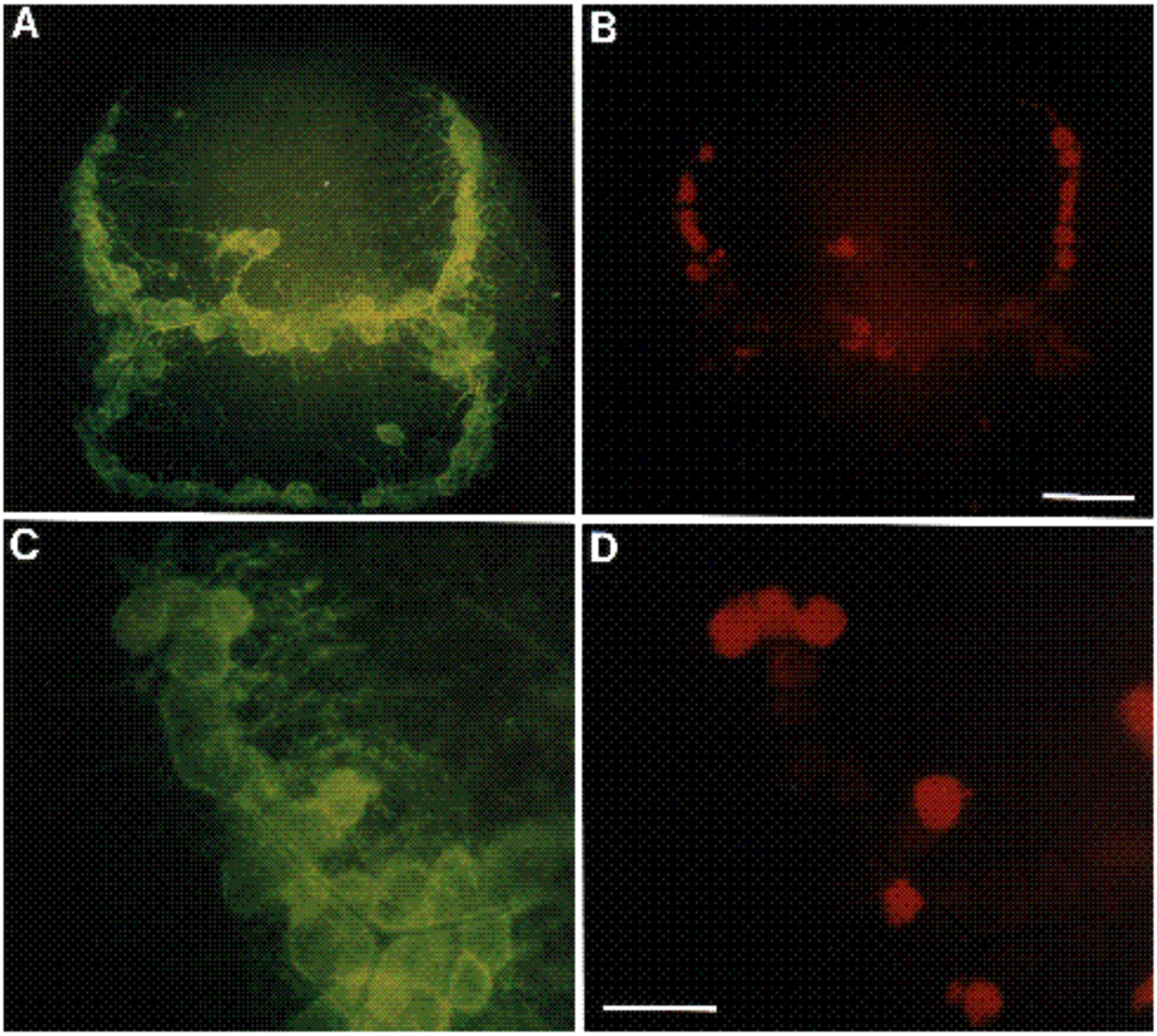

Fig. 6. Skeletogenic SMCs are insensitive to the PMC-derived signal. Two experimental embryos prepared as indicated in Fig. 5 are shown, one at low magnification (upper pair) and one at high magnification (lower pair). (A,C) Immunostaining with mab 6a9 showing the distribution of all skeletogenic cells in the recipient embryos. (B,D) Rhodamine fluorescence showing the position of donor cells (converted SMCs). The donor cells continue to express PMC-specific cell surface proteins and join the subequatorial ring pattern of the host embryo, cooperating with endogenous PMCs in forming the skeleton. Scale bars, 50 μm (A, B) and 20 μm (C, D).

1992) while others exclude the coelomic pouches and myogenic cells that arise from them, as these appear relatively late in development, and because many coelomic pouch cells never delaminate from the epithelium (Gustafson and Wolpert, 1963; Burke and Alvarez, 1988). For the purposes of this study, we have considered all non-skeletogenic mesoderm to be secondary mesenchyme and have not excluded any subpopulations of SMCs as potential sources of skeletogenic cells.

Taken as a whole, our observations constitute the most detailed census of SMC-derived cell types within a single species. In *L. variegatus*, at the late, prefeeding larval stage (55-60 hours postfertilization), it can be estimated that there are 150-200 SMCs (mean=180), of which 56% are pigment cells, 13% blastocoelar cells, 14% coelomic pouch cells and 17% muscle cells. Because there is no information concerning cell division by SMCs, this census is accurate only at the late, pre-feeding larval stage and does not reflect the numbers of precursor cells at earlier stages. Moreover, because counts of circumesophageal muscle cells and coelomic pouch cells were made using relatively young embryos, these estimates do not take into account any division of these cells during the pluteus stage, and therefore may represent underestimates of the actual numbers of these two SMC derivatives.

SMC conversion results in a significant and selective reduction in the number of pigment cells in the embryo. Based upon this observation, we propose that the convert-

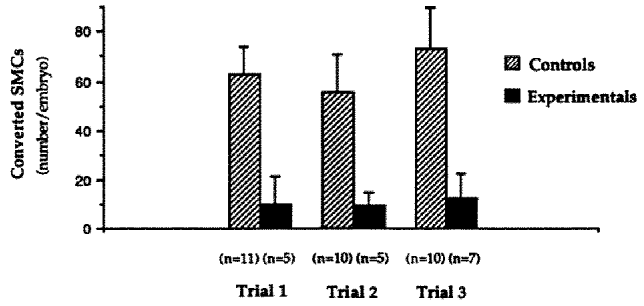

Fig. 7. Converted SMCs acquire the ability to transmit the PMC signal. Cell transplantations were carried out according to the protocol shown in Fig. 5, except that all PMCs were removed from recipient embryos prior to the injection of donor cells. 20-45 (mean=32) skeletogenic cells were injected into each donor embryo. For each trial, PMC(−) recipient embryos that did not receive injections of cells were allowed to develop in parallel with experimental embryos and served as an internal standard. In each of the three trials, injection of skeletogenic SMCs greatly reduced the numbers of host cells that switched fate.

ing cells represent a population of prospective pigment cells. An alternative interpretation of our findings is that pigment cells are dependent in some other way upon PMCs for their normal differentiation but do not give rise to the converting cells, which are instead derived from a different source. This possibility seems unlikely for two reasons. First, pigment cells are by far the most numerous of any SMC-derived cell type (56% of the total), while blastocoelar, coelomic pouch and muscle cells each constitute only 20-30 cells of the larva. For any of the latter subclasses to comprise a large proportion of converting SMCs (60-70 cells) would require a significant stimulation of cell growth and division. Second, because no SMC-derived cell type other than pigment cells shows a major reduction in number in PMC(−) embryos, if other subclasses of SMCs contribute to the population of converting cells, then a complex system of regulative interactions must result in a restoration of the normal numbers of these SMCs. It should be noted that because the number of blastocoelar cells also decreases slightly in PMC(−) larvae, we cannot rule out the possibility that a small number of these cells may also contribute to the population of converting cells. This may, in part, explain the fact that the observed decrease in the number of pigment cells in PMC(−) embryos (53 cells/embryo) is smaller than the number of skeletogenic SMCs (60-70 cells/embryo). The simplest interpretation of our observations, however, is that most of the cells that switch fate in response to PMC ablation would otherwise express a pigment cell fate.

Although the number of pigment cells is reduced in PMC(−) larvae, some pigment cells (an average of 47) nevertheless differentiate in these embryos. Previous time-lapse studies showed that, in PMC(−) embryos of *L. variegatus*, a population of SMCs ingresses early in gastrulation, and that many of these cells penetrate the basal lamina and migrate into the ectoderm (Ettensohn and McClay, 1988). Early ingressing pigment cells have been described in other sea urchin species (Gustafson and Wolpert, 1967; Gibson

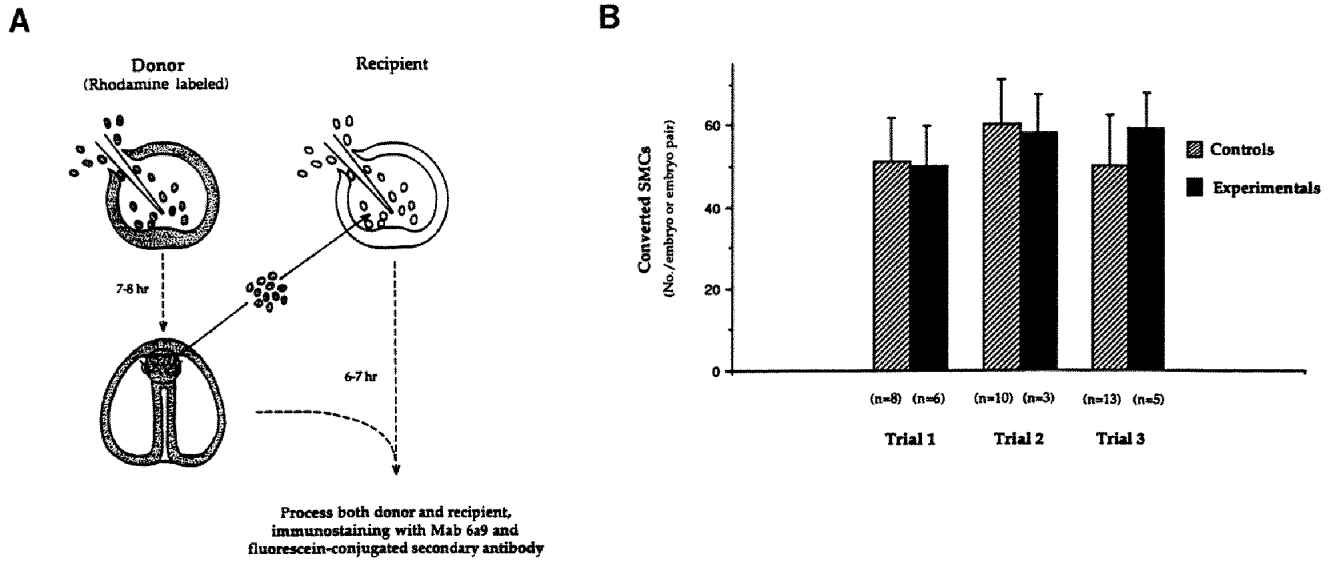

Fig. 8. The number of SMCs with skeletogenic potential is restricted by the late gastrula stage. (A) Experimental protocol. SMCs were removed from the tip of the archenteron of rhodamine-labeled, late gastrula-stage embryos and microinjected into younger, PMC(−) recipients. The recipients served as a permissive environment that allowed the conversion of skeletogenic donor cells. Donor and recipient embryos were then processed as pairs for immunofluorescent staining with MAb 6a9. The sum of the rhodamine-labeled, 6a9-positive cells in the two embryos represented the total number of skeletogenic cells in the donor embryo at the late gastrula stage. (B) Experimental results. For each trial, PMC(−) donor embryos that were not used for cell transplantations were allowed to develop in parallel with experimental embryos and served as an internal standard. In each of the three trials, the average number of converted SMCs in the control embryos was similar to the sum of the skeletogenic cells in the donor/host embryo pairs (the differences in the means are not statistically significant by two-sided *t*-tests, P>0.30). Ablation of SMCs therefore does not lead to the recruitment of any additional cells to a skeletogenic fate.

and Burke, 1985), and it seems reasonable to conclude that the early ingressing SMCs in *L. variegatus* are also presumptive pigment cells, as these are the only macromere-derived cells known to migrate into the ectodermal layer (Cameron et al., 1991) These observations, taken together with the cell counts described above, suggest that there might be two populations of prospective pigment cells in *L. variegatus*, one that ingresses early in gastrulation and differentiates independently of the PMCs, and another that ingresses later in gastrulation and is sensitive to the PMC-derived signal.

The results of fate-mapping studies show that the secondary mesenchyme is derived from the small micromeres and the veg_2 blastomeres of the 64-cell-stage embryo (Hörstadius, 1973; Endo, 1966; Pehrson and Cohen, 1986). BrdU-labeling experiments show that the small micromeres, although they are the closest lineal relatives of the PMCs, do not contribute to the population of converting cells. This observation is relevant with regard to models of regional specification during early development that postulate the existence of skeletogenic factors in the vegetal cytoplasm of the unfertilized egg (Davidson, 1989). Our results suggest that putative skeletogenic regulatory factors may be excluded from the vegetal pole cytoplasm (or inactivated there) before fertilization and are almost certainly absent/inactivated in that region by the fifth cleavage division, when the potential for skeletogenic specification becomes strictly segregated between the large and small daughter cells of the micromeres. This conclusion is consistent with the recent blastomere recombination experiments of Khaner and Wilt (1991), which have shown that when various vegetal cells are combined with mesomeres, spicule-forming properties are restricted to the large micromeres (the progenitors of the PMCs) and the veg_2 blastomeres (the progenitors of the SMCs), while small micromeres show little or no capacity to give rise to skeletogenic cells.

During their switch in fate, SMCs express cell surface molecules unique to skeletogenic cells, migrate to PMC-specific target sites and synthesize a correctly patterned skeleton (Ettensohn and McClay, 1988; Ettensohn, 1991a). We have shown here that skeletogenic SMCs also acquire another functional property of the PMCs - the ability to suppress the skeletogenic potential of uncommitted SMCs. Although the relative effectiveness of PMCs and converted SMCs in this regard has not been carefully analyzed, previous experiments provide some basis for comparison. When 30-40 PMCs are present in the blastocoel, an average of 7 SMCs (n=4, mean=6.8, s.d.=5.0) switch fate (Ettensohn and McClay, 1988). Since in the present study, injection of 20-45 (mean=32) skeletogenic SMCs led to the conversion of an average of 10 cells to a skeletogenic fate, it appears that converted SMCs and PMCs are of approximately equal effectiveness on a per-cell basis at suppressing SMC skeletogenesis. The ability of converted SMCs to block fate-switching by uncommitted cells raises the possibility that interactions among SMCs could serve to limit the numbers of cells that switch fate in PMC(−) embryos, and that the number of SMCs with skeletogenic potential might actually be greater than the 60-70 cells that switch fate following removal of the PMCs. SMC ablation experiments, however, rule out this possibility and show clearly that by the late gastrula stage the number of SMCs with skeletogenic potential is restricted to 60-70 cells.

Based upon our present findings as well as previous work, we propose the following overview of the PMC-SMC interaction. The PMC-derived signal regulates a developmental switch, directing SMCs to express a pigment cell phenotype rather than a skeletogenic fate. Because SMCs adopt a skeletogenic phenotype in the absence of this signal, this pathway of differentiation can be considered to be the 'default' developmental program of the cells. By the late gastrula stage, the number of cells in the embryo with the potential to express this fate is limited to 60-70 SMCs. These cells are derived from the veg2 blastomeres of the late cleavage-stage embryo and are among those SMCs that normally leave the tip of the archenteron late in gastrulation. The fate of this subpopulation of SMCs remains labile until the late gastrula stage, probably because they are unresponsive to the PMC-derived signal until that time (Ettensohn, 1990a). During ingression, SMCs sample their environment, possibly by means of filopodial exploration (Ettensohn, 1992). In the absence of the PMC-derived signal, SMCs activate the default developmental program, resulting in major changes in their pattern of migration and gene expression. As this occurs, converting cells lose their responsiveness to the PMC-derived signal, as shown by the stability of the skeletogenic phenotype upon injection into mesenchyme blastula-stage embryos, while they acquire the ability to transmit the same signal.

Competition between cells of similar developmental potential for a particular fate has been termed 'lateral specification' or 'lateral inhibition' (see Simpson, 1990; Greenwald and Rubin, 1992). Expression of this primary fate by one cell within a group, perhaps by a stochastic process, leads to a suppression of that fate in neighboring cells (lateral inhibition), which are directed into alternative developmental pathways. Examples of such cellular systems include what have been termed 'equivalence groups' (Kimble, 1981; Nishida and Satoh, 1989; Martindale and Shankland, 1990; Simpson, 1990). Lateral specification has been distinguished from induction, during which embryonic cells are directed into one of several possible developmental programs in response to a signal from a neighboring cell or tissue, which has an unrelated fate (reviewed by Gurdon, 1987; Jessell and Melton, 1992). The PMC-SMC interaction lies somewhere between these two simplified schemes. This interaction can be considered to be a kind of 'lateral inhibition' in the sense that SMCs and PMCs compete for a common primary (skeletogenic) fate. As discussed elsewhere (Ettensohn, 1991a), however, these cells cannot be considered an equivalence group, primarily because the developmental potential of the PMCs is clearly different (more restricted) than that of the SMCs. In addition, the initial steps of fate specification in this system are not stochastic, as the fate of the PMCs is rigidly specified very early in development.

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REFERENCES

Burke, R. D. and Alvarez, C. M. (1988). Development of the esophageal muscles in embryos of the sea urchin *Strongylocentrotus purpuratus. Cell Tissue Res.* **252**, 411-417.

Cameron, R. A., Fraser, S. E., Britten, R. J. and Davidson, E. H. (1991). Macromere cell fates during sea urchin development. *Development* **113**, 1085-1091.

Cox, K. H., Angerer, L. M., Lee, J. J., Davidson, E. H. and Angerer, R. C. (1986). Cell lineage-specific programs of expression of multiple actin genes during sea urchin embryogenesis. *J. Mol. Biol.* **188**, 159-172.

Davidson, E. H. (1989). Lineage-specific gene expression and the regulative capacities of the sea urchin embryo: a proposed mechanism. *Development* **105**, 421-445.

Endo, Y. (1966). Fertilization, cleavage and early development. In *Contemporary Biology: Development and Differentiation* (ed. Y. Isemura), Vol. 4, pp. 1-61. Iwanami Shoten, Tokyo.

Ettensohn, C. A. (1990a). Cell interactions in the sea urchin embryo studied by fluorescence photoablation. *Science* **248**, 1115-1118.

Ettensohn, C. A. (1990b). The regulation of primary mesenchyme cell patterning. *Dev. Biol.* **140**, 261-271.

Ettensohn, C. A. (1991a). Mesenchyme cell interactions in the sea urchin embryo. In *Cell-Cell Interactions in Early Development* (ed. J. Gerhart), *49th Symp. Soc. Dev. Biol.*, pp. 175-201. New York: Wiley-Liss.

Ettensohn, C. A. (1991b). Primary mesenchyme cell migration in the sea urchin embryo. In *Gastrulation: Movements, Patterns, Molecules* (ed. R. Keller, W. H. Clark and F. Griffin), pp. 289-304. New York: Plenum Press.

Ettensohn. C. A. (1992). Cell interactions and mesodermal cell fates in the sea urchin embryo. *Development* (in press).

Ettensohn, C. A. and McClay, D. R. (1986). The regulation of primary mesenchyme cell migration in the sea urchin embryo: Transplantations of cells and latex beads. *Dev. Biol.* **117**, 380-391.

Ettensohn, C. A. and McClay, D. R. (1988). Cell lineage conversion in the sea urchin embryo. *Dev. Biol.* **125**, 396-409.

Farach, M. C., Valdizan, M., Park, H. R., Decker, G. L. and Lennarz, W. L. (1987). Developmental expression of a cell surface protein involved in calcium uptake and skeleton formation in sea urchin embryos. *Dev. Biol.* **122**, 320-331.

Fukushi, T. (1962). The fates of isolated blastoderm cells of sea urchin blastulae and gastrulae inserted into the blastocoel. *Bull. Marine Biol. Stat. Asamushi* **11**, 21-30.

Gibson, A. W. and Burke, R. D. (1985). The origin of pigment cells in embryos of the sea urchin *Strongylocentrotus purpuratus. Dev. Biol.* **107**, 414-419.

Greenwald, I. and Rubin, G. M. (1992). Making a difference: The role of cell-cell interactions in establishing separate identities for equivalent cells. *Cell* **68**, 271-281.

Gurdon, J. B. (1987). Embryonic induction - molecular prospects. *Development* **99**, 285-306.

Gustafson, T. and Wolpert, L. (1963). Studies on the cellular basis of morphogenesis in the sea urchin embryo. Formation of the coelom, the mouth, and the primary pore-canal. *Exp. Cell Res.* **29**, 561-582.

Gustafson, T. and Wolpert, L. (1967). Cellular movement and contact in sea urchin morphogenesis. *Biol. Rev.* **42**, 442-498.

Hörstadius, S. (1973). *Experimental Embryology of Echinoderms*. London: Oxford Univ. Press.

Ishimodo-Takagi, T., Chino, I. and Sato, H. (1984). Evidence for the involvement of muscle tropomyosin in the contractile elements of the coelom-esophagus complex in sea urchin embryos. *Dev. Biol.* **105**, 365-376.

Jessell, T. M. and Melton, D. A. (1992). Diffusible factors in vertebrate embryonic induction. *Cell* **68**, 257-270.

Khaner, O. and Wilt, F. (1991). Interactions of different vegetal cells with mesomeres during early stages of sea urchin development. *Development* **112**, 881-890.

Kimble, J. (1981). Alterations in cell lineage following laser ablation of cells in the somatic gonad of *Caenorhabditis elegans. Dev. Biol.* **87**, 286-300.

Langelan, R. E. and Whiteley, A. H. (1985). Unequal cleavage and the differentiation of echinoid primary mesenchyme. *Dev. Biol.* **109**, 464-475.

Leaf, D. S., Anstrom, J. A., Chin, J. E., Harkey, M. A., Showman, R. M. and Raff, R. A. (1987). Antibodies to a fusion protein identify a cDNA clone encoding msp130, a primary mesenchyme-specific cell surface protein of the sea urchin embryo. *Dev. Biol.* **121**, 29-40.

Martindale, M. Q. and Shankland, M. (1990). Neuronal competition determines the spatial pattern of neuropeptide expression by identified neurons of the leech. *Dev. Biol.* **139**, 210-226.

Nishida, H. and Satoh, N. (1989). Determination and regulation in the pigment cell lineage of the ascidian embryo. *Dev. Biol.* **132**, 355-367.

Parr, B. A., Parks, A. L. and Raff, R. A. (1990). Promoter structure and protein sequence of msp130, a lipid-anchored sea urchin glyocprotein. *J. Biol. Chem.* **265**, 1408-1413.

Pehrson, J. R. and Cohen, L. H. (1986). The fate of the small micromeres in sea urchin development. *Dev. Biol.* **113**, 522-526.

Simpson, P. (1990). Lateral inhibition and the development of the sensory bristles of the adult peripheral nervous system of *Drosophila. Development* **109**, 509-519.

Tamboline, C. R. and Burke, R. D. (1992). Secondary mesenchyme of the sea urchin embryo: Ontogeny of blastocoelar cells. *J. Exp. Zool.* **262**, 51-60.

Tanaka, S. and Dan, K. (1990). Study of the lineage and cell cycle of small micromeres in embryos of the sea urchin, *Hemicentrotus pulcherrimus*. *Dev. Growth and Differ.* **32**, 145-156.

Wessel, G. M., Zhang, W. and Klein, W. H. (1990). Myosin heavy chain accumulates in dissimilar cell types of the macromere lineage in the sea urchin embryo. *Dev. Biol.* **140**, 447-454.

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