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Nuclear β-catenin is required to specify vegetal cell fates in the sea urchin embryo

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SUMMARY

β-catenin is thought to mediate cell fate specification events by localizing to the nucleus where it modulates gene expression. To ask whether β-catenin is involved in cell fate specification during sea urchin embryogenesis, we analyzed the distribution of nuclear β-catenin in both normal and experimentally manipulated embryos. In unperturbed embryos, β-catenin accumulates in nuclei that include the precursors of the endoderm and mesoderm, suggesting that it plays a role in vegetal specification. Using pharmacological, embryological and molecular approaches, we determined the function of β-catenin in vegetal development by examining the relationship between the pattern of nuclear β-catenin and the formation of endodermal and mesodermal tissues. Treatment of embryos with LiCl, a known vegetalizing agent, caused both an enhancement in the levels of nuclear β-catenin and an expansion in the pattern of nuclear β-catenin that coincided with an increase in endoderm and mesoderm. Conversely, overexpression of a sea urchin cadherin blocked the accumulation of nuclear β-catenin and consequently inhibited the formation of endodermal and mesodermal tissues including micromere-derived skeletogenic mesenchyme. In addition, nuclear β-catenin-deficient micromeres failed to induce a secondary axis when transplanted to the animal pole of uninjected host embryos, indicating that nuclear β-catenin also plays a role in the production of micromere-derived signals. To examine further the relationship between nuclear β-catenin in vegetal nuclei and micromere signaling, we performed both transplantations and deletions of micromeres at the 16-cell stage and demonstrated that the accumulation of β-catenin in vegetal nuclei does not require micromere-derived cues. Moreover, we demonstrate that cell autonomous signals appear to regulate the pattern of nuclear β-catenin since dissociated blastomeres possessed nuclear β-catenin in approximately the same proportion as that seen in intact embryos. Together, these data show that the accumulation of β-catenin in nuclei of vegetal cells is regulated cell autonomously and that this localization is required for the establishment of all vegetal cell fates and the production of micromere-derived signals.

Key words: Catenin, Cell signaling, Axial patterning, Sea urchin, Vegetal cell fate

INTRODUCTION

In the sea urchin embryo, tiers of blastomeres located in invariant positions along the animal-vegetal (A-V) axis are fated predictably to give rise to the ectoderm, the endoderm and the mesoderm (reviewed in Cameron and Davidson, 1991; Cameron et al., 1991; Logan and McClay, 1997; Ransick and Davidson, 1998). These tissues are specified and patterned by the coordinated action of localized, maternally derived determinants and cell-cell signals (reviewed in Davidson, 1989). A number of studies have shown that the cytoplasm along the animal-vegetal axis is qualitatively different and can direct isolated cells to assume fates characteristic of their position (Boveri, 1901; Runnström, 1929; Hörstadius, 1939; Okazaki, 1975; Kitajima and Okazaki, 1980; Maruyama et al., 1985; Henry et al., 1989; Khaner and Wilt, 1990). For example, the micromeres that form at the vegetal pole produce skeletogenic mesenchyme cells both in unperturbed embryos and in culture, suggesting that they are autonomously specified (Okazaki, 1975). Cell autonomous signals have also been shown to regulate the asymmetric expression of several genes in animal, but not vegetal blastomeres of cleavage-stage embryos (Ghilione et al., 1993; Wei et al., 1995; Ghilione et al., 1996; Kozlowski et al., 1996; Wei et al., 1997). In addition to these cell-intrinsic mechanisms, cell-cell signaling plays a role in establishing cell fates along the animal-vegetal axis (reviewed in Hörstadius, 1973; Wilt, 1987; Davidson, 1989, 1990, 1993). Notably, the large micromeres produce a vegetalizing signal that is involved in specifying neighboring blastomeres to differentiate as secondary mesoderm and

endoderm (Hörstadius 1973; Khaner and Wilt, 1990; Ransick and Davidson, 1993, 1995). Micromere-derived signals are also thought to initiate a subsequent series of inductive interactions that position the ectoderm-endoderm boundary and that regulate the diversification of cell types within mesodermal and endodermal tissues (Davidson, 1989; Khaner and Wilt, 1991; Ettensohn, 1992; McClay and Logan, 1996).

Although many studies in the sea urchin have examined the events that direct cell fate choices at the cellular level, little is known about the underlying molecular pathways that carry out these functions. A candidate signaling pathway that may play a role in axial patterning of the sea urchin is the Wnt signaling pathway. Wnts have been shown to regulate a variety of cell fate specification events in both invertebrate and vertebrate embryos (reviewed in Nusse and Varmus, 1992; Klingensmith and Nusse, 1994; Moon et al., 1997). In particular, one component of this pathway, β-catenin, is involved in axial specification processes of deuterostome embryos such as *Xenopus* (Heasman et al., 1994; Schnieder et al., 1996; Larabell et al., 1997), zebrafish (Kelly et al., 1995; Schnieder et al., 1996) and the mouse (Haegel et al., 1995), making it an attractive molecule that may also regulate early axial patterning of the sea urchin embryo.

β-catenin is a multifunctional protein that regulates both intercellular adhesion and the establishment of cell fates (reviewed by Peifer, 1995; Miller and Moon, 1996; Cavallo et al., 1997). The function of β-catenin in cell fate specification is thought to be mediated through its direct association with Lef/Tcf transcription factors and the accumulation of this complex in the nucleus where it regulates gene expression (Cavallo et al., 1997; Willert and Nusse, 1998). For example, β-catenin is required for the development of dorsal fates in *Xenopus* embryos and this activity coincides with the accumulation of β-catenin in nuclei of dorsal blastomeres where it appears to act with XTcf-3 to regulate the expression of dorsal-specific genes (Heasman et al., 1994; Molenaar et al., 1996; Schneider et al., 1996; Brannon et al., 1997; Larabell et al., 1997). Likewise, Armadillo is present in the nuclei of cells that receive Wingless signals in the ventral epidermis of early *Drosophila* embryos where, in combination with dTCF, its function is required for the establishment of segmentally reiterated posterior cell fates (Orsulic and Peifer, 1996; Brunner et al., 1997; van de Wetering et al., 1997). Thus, the localization of β-catenin in nuclei is predictive of its function in regulating cell fate specification through its ability to modulate the expression of target genes.

In a previous study, we reported the cloning of sea urchin β-catenin from *Lytechinus variegatus* and characterized its potential role in modulating cell-cell adhesion during gastrulation (Miller and McClay, 1997a). Here, we show that β-catenin is localized to nuclei of vegetal cells in a pattern consistent with a role for β-catenin in specifying vegetal cell identities. To experimentally test the hypothesis that β-catenin is involved in vegetal specification, we used a combination of pharmacological, embryological and molecular perturbations, and examined the signaling function of β-catenin during early development. We show that the nuclear accumulation of β-catenin in vegetal cells is regulated by a cell autonomous mechanism and that this localization is required for the normal formation of the endoderm and mesoderm. Furthermore, we demonstrate that the presence of nuclear β-catenin in micromeres is necessary for these cells to produce vegetalizing signals and to differentiate as skeletogenic primary mesenchyme cells. These data suggest that β-catenin plays an early, required role in the establishment of vegetal cell fates and provide a starting point for further studies examining the molecular pathways that regulate axial specification in the sea urchin embryo.

MATERIALS AND METHODS

Embryos

Adult *Lytechinus variegatus* animals were obtained from Duke University Marine Laboratory (Beaufort, NC) and from Susan Decker Services (North Hollywood, FL). Gametes were obtained by intracoelomic injection of 0.5 M KCl. Eggs were de-jellied by passage through cheesecloth and were fertilized with a dilute suspension of sperm in 10 mM para-aminobenzoic acid (PABA, Sigma). After 1 hour, embryos were washed into ASW and cultured until the appropriate stages. Embryos were grown in artificial sea water (ASW) at room temperature. Embryos treated with LiCl were incubated from 30 minutes postfertilization to the 7th cleavage stage in 30-50 mM LiCl in ASW.

Antibody staining

Embryos were fixed in 2% paraformaldehyde-ASW for 10 minutes at room temperature and then were passed briefly through 100% MeOH for permeabilization. Manipulated embryos were fixed individually in glass depression slides. Embryos were stained with affinity-purified anti-β-catenin polyclonal sera as previously described (Miller and McClay, 1997a). We also confirmed that localization of β-catenin following hatching was nuclear by co-staining embryos with anti-β-catenin antibodies and a polyclonal antibody raised against a nuclear lamin B that identifies the nuclear membrane (Holy et al., 1995; data not shown). These primary antibodies were detected with Cy3- or FITC-conjugated goat anti-guinea pig (for β-catenin) and Cy-3-conjugated goat anti-chicken (for lamin B) secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Endoderm and mesoderm markers used during this study included 1g8 which is PMC-specific, Endo1 which marks the midgut and hindgut, and EctoV which marks the foregut and oral ectoderm (Wessel and McClay, 1985; Coffman and McClay 1990).

Mapping β-catenin-positive nuclei in post-hatching embryos

Mapping the position of β-catenin-positive nuclei required embryos to be compressed in order to image the adherens junctions and the nuclear staining separately. Embryos were placed in micromanipulation chambers between a coverslip and coverslip fragment separated with vacuum grease. Using a glass needle mounted on a Narishige micromanipulator, the vegetal pole was oriented in the direction of the confocal objective. The specimens were flattened by slowly and gently pressing down on the coverslip fragment. Once compressed, embryos were optically sectioned using a confocal microscope. The resulting Z-series images were analyzed by counting and mapping β-catenin-positive and -negative nuclei relative to the position of cell outlines defined by adherens junction β-catenin within the vegetal half of the embryo.

To estimate the total number of *veg1* or *veg2* cells at a given stage, DiI labeling was performed iontophoretically by labeling single *veg1* or *veg2* cells with DiIC16 (Molecular Probes; 5 mg/ml in EtOH) as previously described (Logan and McClay, 1997). The numbers of DiI-labeled progeny were counted and the number of labeled cells was multiplied by eight because there are eight *veg1* or *veg2* cells at the 60-cell stage.

cDNA constructs and RNA injections

A cDNA construct encoding the amino-terminus and signal sequence of sea urchin Notch (Sherwood and McClay, 1997) fused to the transmembrane domain and intracellular domain of sea urchin LvG-cadherin (Miller and McClay, 1997b) was made by standard molecular biology techniques (details of this construction are available upon request). GFP cDNA was used to synthesize a control mRNA (Green Lantern, Gibco). Capped synthetic RNAs were prepared using a mMessage mMachine Kit (Ambion, Austin TX). Eggs were prepared and RNA injections were performed as described by Mao et al. (1996).

Micromanipulation of embryos

Fertilization envelopes were removed immediately preceding blastomere deletions or transplantations by gently passing embryos through a glass pipette with a diameter of approximately 100 μm. Embryos were then immobilized in microinjection chambers for surgery.

For micromere deletions, embryos nearing the end of the fourth cleavage were placed in hyalin extraction medium (McClay, 1986) for 10 seconds prior to loading in chambers. Only two to three embryos were placed in microinjection chambers at one time. A suction pipette (bore size of 5-10 μm) attached to a micromanipulator was used to remove micromeres as they formed from the overlying macromeres. In order to perform micromere transplantations, several host embryos were immobilized at the 4-cell stage in a microinjection chamber containing ASW and were oriented with the animal-vegetal axis perpendicular to the double-stick tape backstop. Since the first two cleavages occur parallel to the animal-vegetal axis, orienting the embryos at the 4-cell stage increased the chances that one would implant micromeres at the animal pole. Two or three 16-cell-stage donor embryos were placed individually in a separate chamber containing only CFSW, and the mesomeres and macromeres were eliminated. The isolated micromere quartets were transferred immediately with a mouth pipette to the chamber containing the host embryos and placed at the animal pole. After several minutes, the micromeres stuck to the host embryos well enough so the specimens could be removed from the chamber and cultured individually. Embryos were examined on an inverted microscope and those cases with micromeres at the animal pole were processed further. When embryos reached the 7th or 10th cleavage stage, they were individually stained with β-catenin antibodies as described above.

Dissociation of embryos and staining of single cells

Embryos were dissociated starting at the 2-cell stage by placing them in calcium-free sea water (CFSW) and cells were stirred at 60 revs/minute with a paddle attached to a motor. Every 20 minutes until the 7th cleavage, the culture was transferred to a beaker and swirled vigorously, taking care not to damage or lyse the cells. Additionally, starting at the 16-cell stage, the dividing cells were gently triturated with a 10 ml pipette following the swirling step to ensure that there were no clumps of dividing cells. A sample of dissociated embryos was examined on a microscope to ensure disaggregation. Cells were collected by gentle centrifugation and were washed once in CFSW to remove any cellular debris. Washed cells were immediately transferred to fixative and processed for staining as described above.

Microscopy

Embryos were mounted on glass slides underneath coverslips using clay feet as separators. Confocal microscopy was performed on a Zeiss Laser Scanning Confocal Microscope (Axiovert, Carl Zeiss Inc., Thornwood, NY) using either a 40× plan-apo oil immersion objective (NA=1.3) or a 63× plan-apo oil immersion objective (NA=1.4).

RESULTS

Pattern of nuclear β-catenin localization during early development

The signaling activity of β-catenin is thought to be linked to its accumulation in the nucleus where it modulates gene expression (Miller and Moon, 1996; Cavallo et al., 1997). Based on this model, we predicted that if β-catenin were involved in signaling events associated with early cell fate specification in the sea urchin embryo, its nuclear accumulation might occur in a pattern predictive of this function. Therefore, we utilized an affinity-purified polyclonal antibody raised against *L. variegatus* β-catenin (Miller and McClay, 1997a) and laser scanning confocal microscopy to determine the subcellular distribution of β-catenin from early cleavage until just prior to archenteron formation.

The pattern of nuclear β-catenin accumulation during early development suggests a role for β-catenin in patterning the animal-vegetal axis. An animal-vegetal asymmetry in the levels of β-catenin is seen in a small percentage of embryos examined at the 16-cell stage (approx. 10-20%; data not shown). In these embryos, the micromeres possess elevated levels of cytoplasmic β-catenin relative to that seen in the macromeres and mesomeres. Following the 5th cleavage division, all embryos exhibit high levels of cytoplasmic and nuclear β-catenin in both the small and large micromeres while lower levels of β-catenin are present in nuclei of the macromeres (Fig. 1A-C). At the sixth cleavage (60-cell stage), both the large and small micromeres retain high levels of cytoplasmic and nuclear β-catenin. However, the meridional division that separates the macromeres into *veg1* and *veg2* tiers is accompanied by a lineage-specific restriction in nuclear β-catenin. β-catenin is present in nuclei of *veg2* cells but is found only at low levels or is absent from nuclei of *veg1* cells (Fig. 1D-F). This difference in levels of nuclear β-catenin becomes accentuated at the 7th cleavage stage; *veg2* cells retain high levels of nuclear β-catenin while staining in *veg1* nuclei becomes barely detectable or is lost completely (Fig. 1G-I). Following the 7th cleavage, nuclear β-catenin persists in micromere and *veg2* descendants (Fig. 1J-L).

Between hatching and the onset of archenteron formation, a dynamic change in nuclear β-catenin occurs and is depicted schematically in Fig. 2B. β-catenin is lost gradually from nuclei of cells at the center of the vegetal plate and accumulates increasingly in nuclei of a ring of cells that lie peripheral to the archenteron (Fig. 2B). To assess the relationship between various cell lineages and the changing pattern of nuclear β-catenin, we mapped the number and location of β-catenin-containing nuclei at different stages following hatching. At all stages examined, the small micromeres possessed very high levels of nuclear β-catenin, providing an unambiguous reference point for the position of the vegetal pole. We compared the distribution of β-catenin-containing nuclei at various stages with the estimated number of micromere, *veg2* and *veg1* progeny in embryos of the same stage (see Materials and methods; Fig. 2A). Nuclear β-catenin was found within the vegetal regions occupied by *veg1* descendants. From this analysis, we conclude that nuclear β-catenin is lost stochastically from the descendants of the large micromeres and *veg2* cells, and becomes progressively localized to nuclei

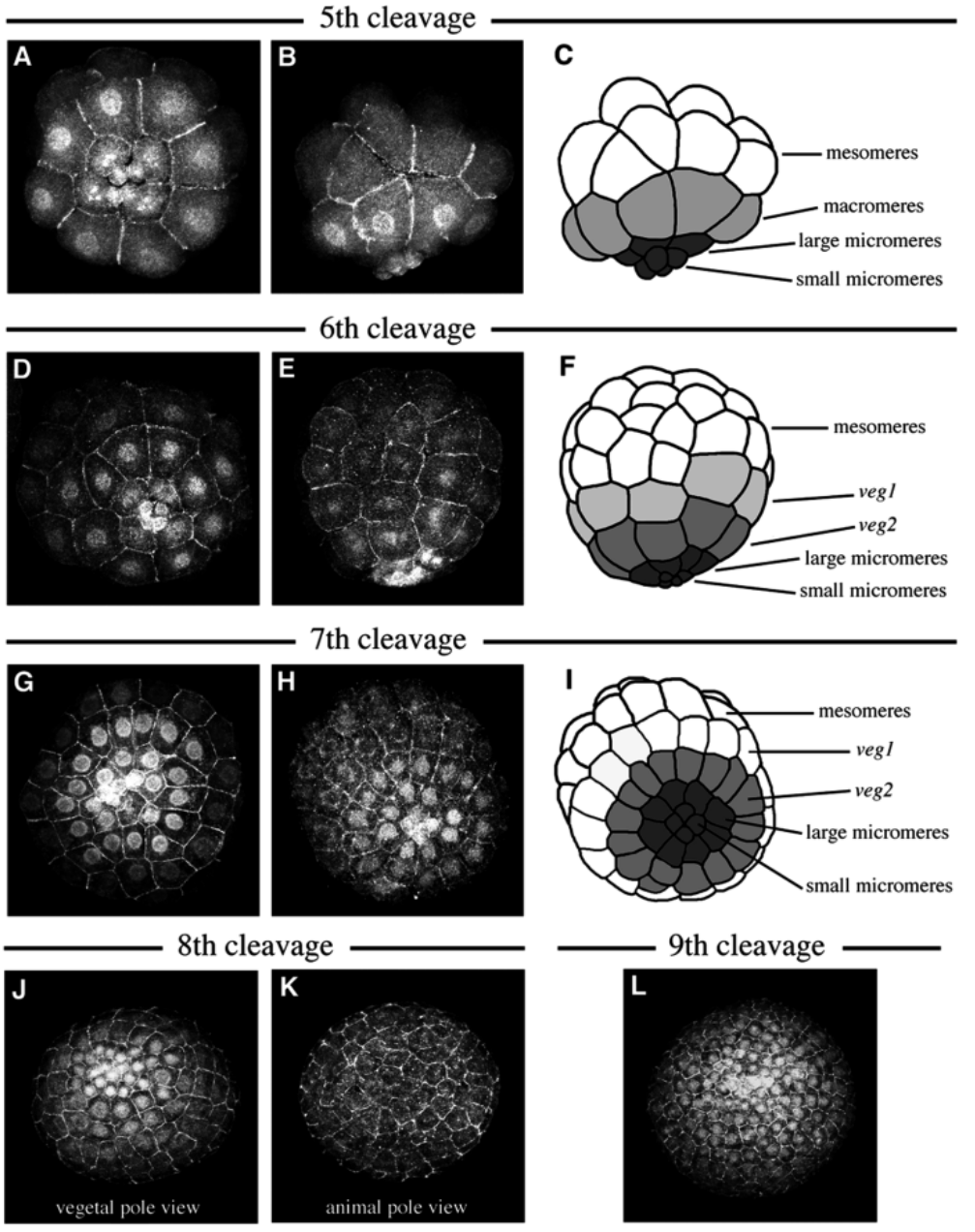

Fig. 1. Pattern of nuclear β-catenin accumulation during cleavage. (A-C) At the 5th cleavage stage, β-catenin is found in nuclei of both the large and small micromeres and the macromeres, but is absent from nuclei of mesomeres. (D-F) At the 60-cell stage, β-catenin accumulates to high levels in the nuclei of all micromere-derived blastomeres. The equatorial cleavage that forms the *veg2* and *veg1* tiers is marked by a difference in the levels of nuclear β-catenin seen in these cells. Specifically, β-catenin accumulates in nuclei of all *veg2* cells while lower levels are found in nuclei of *veg1* cells. (G-I) The difference between the levels of nuclear β-catenin found in nuclei of *veg2* cells compared to that seen in nuclei of *veg1* cells increases at the 7th cleavage stage. The descendants of the micromeres retain high levels of nuclear β-catenin at this stage. (J-L) At the 8th and 9th cleavage stages, the pattern of nuclear β-catenin is unchanged and high levels of nuclear β-catenin are found in progeny of the micromere and *veg2* lineages (J,L) and is absent from progeny of the *veg1* and mesomere lineages (K).

of a subset of *veg1* cells that form a narrowing ring in the vegetal half of the embryo (Fig. 2A).

Since *veg1* progeny contribute to both the ectoderm and endoderm (Logan and McClay, 1997), we asked whether the accumulation of β-catenin in *veg1* cells coincides with the developmental segregation of this lineage into these distinct cell fates. By comparing the number and location of *veg1* cells possessing nuclear β-catenin with the number of prospective endoderm and mesoderm cells that lie in the vegetal plate prior to archenteron invagination (Ruffins and Ettensohn, 1996), we determined that β-catenin accumulates in *veg1* nuclei that are predicted to become endoderm. This same conclusion was reached independently in an analysis of Notch distribution relative to the pattern of nuclear β-catenin (D. R. Sherwood and D. R. M., unpublished data). These data suggest that β-catenin is localized to nuclei of vegetal cells that give rise to the mesoderm and endoderm during early development of the sea urchin embryo.

β-catenin is involved in the specification of vegetal cell fates

The pattern of nuclear β-catenin is responsive to LiCl-induced vegetalization

The pattern of nuclear β-catenin observed during early development suggests that β-catenin may play an important role in specifying vegetal cell fates. We tested this hypothesis by assessing whether changes in the pattern of nuclear β-catenin caused by experimental perturbations would correlate with changes in the distribution of cell fates along the animal-vegetal axis. Since many cell fate decisions along the animal-vegetal axis are thought to be specified initially during cleavage (reviewed in Davidson, 1989), we restricted our

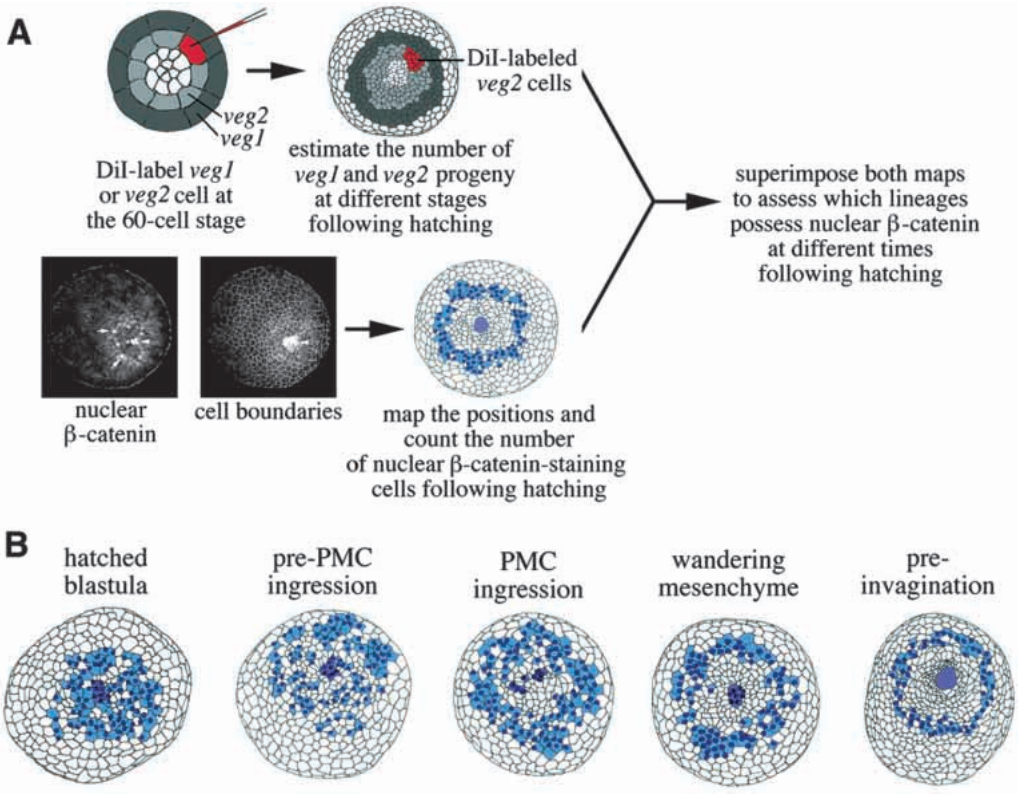

Fig. 2. Pattern of nuclear β-catenin in post-hatching embryos. (A) Schematic showing how we generated stage-specific maps of nuclear β-catenin and determined their relationship to cell lineages. The number of *veg1* or *veg2* progeny at a given stage was determined by DiI labeling a single *veg1* or *veg2* cell at the 60-cell stage and by counting the number of labeled descendants at each of several stages after hatching. Since there are eight *veg1* or *veg2* cells at the 60-cell stage, the total number of *veg1* or *veg2* cells was estimated by multiplying the number of DiI-labeled cells by 8. These lineage maps were compared to maps of nuclear β-catenin to determine whether the pattern of nuclear β-catenin showed any relationship to micromere, veg2 and veg1 lineages. The small micromeres (arrowheads) were used as a marker of the vegetal pole. (B) Nuclear β-catenin distribution in post-hatching embryos (shown as dark-blue nuclei). Drawings of embryos show a representative pattern of nuclear β-catenin at each of five stages between hatching and invagination of the archenteron. The drawings are vegetal pole views. The position of the small micromeres is indicated by dark-blue cells and the location of β-catenin-positive cells are marked by light-blue cells. Following hatching, progeny of the large micromeres and *veg2* cells stochastically downregulate levels of nuclear β-catenin such that all of the PMCs and *veg2* cells lack nuclear β-catenin following ingression. This process occurs as β-catenin accumulates in a subset of *veg1* cells that form a ring 3-5 cells wide at the wandering mesenchyme stage. Prior to invagination of the archenteron, the circle of *veg1* cells that possess nuclear β-catenin narrows to a ring approximately 2 cells wide.

experimental analyses in this study to cleavage-stage embryos.

First, we asked whether the accumulation of β-catenin in nuclei of vegetal cells is sufficient to promote vegetal development by taking advantage of the recent convergence of classical embryological analyses with modern molecular data. Treatment of embryos with the classical vegetalizing agent LiCl causes an expansion of vegetal cell fates at the expense of animal cell fates (Herbst, 1892; von Ubisch, 1929; Livingston and Wilt, 1989, 1990; Nocente-McGrath et al., 1991; Cameron and Davidson, 1997). Recently, LiCl has been shown to inhibit glycogen synthase kinase 3 (GSK-3), a negative regulator of β-catenin stability and signaling activity, providing a potential link between the action of LiCl and the signaling function of β-catenin (Yost et al., 1996; Klein and Melton, 1996; Stambolic et al., 1996; Hedgepeth et al., 1997). Thus, we reasoned that the vegetalizing effect of LiCl might be due to increased and/or ectopic activity of β-catenin during cleavage.

Embryos were vegetalized by exposure to 30 or 50 mM LiCl from the 2-cell stage through the 7th cleavage stage and the resulting pattern of nuclear β-catenin was compared to that seen in untreated embryos (Fig. 3). In control embryos, nuclear β-catenin is present at high levels in the micromeres and at moderate levels in *veg2* cells, but is either absent or found at very low levels in nuclei of *veg1* cells at the 7th cleavage stage (Figs 1G-I, 3E-H). In contrast, LiCl treatment causes an increase in nuclear β-catenin levels present in *veg2* cells and promotes the ectopic accumulation of β-catenin in nuclei of *veg1* cells (Fig. 3A-D). This effect of LiCl on nuclear β-catenin levels in vegetal cells appears to be concentration dependent. Treatment with 30 mM LiCl elevates nuclear β-catenin in *veg2* cells to that seen in micromeres and also causes the accumulation of low to moderate levels of β-catenin in nuclei of all *veg1* cells (Fig. 3A,B). In the presence of 50 mM LiCl, a dramatic enhancement in the levels of nuclear β-catenin was found in nuclei of *veg2* and *veg1* cells (Fig. 3C,D). Specifically, β-catenin accumulated in nuclei of all *veg2* and *veg1* cells to levels similar to that seen in progeny of the micromeres. β-catenin was never observed in nuclei of mesomeres following LiCl treatment, even at high levels of LiCl (50 mM). This concentration-dependent effect of LiCl on the accumulation of β-catenin in nuclei of vegetal cells mimics the concentration-dependent action of LiCl on the development of vegetal cell fates (Nocente-McGrath et al., 1991). Since one effect of LiCl is to inhibit GSK-3, which negatively regulates β-catenin signaling, these data suggest that the effects of LiCl action on the patterning of cell fates along the animal-vegetal axis may be due to the ectopic and increased levels of nuclear β-catenin in vegetal cells. These results are consistent with the hypothesis that the accumulation of β-

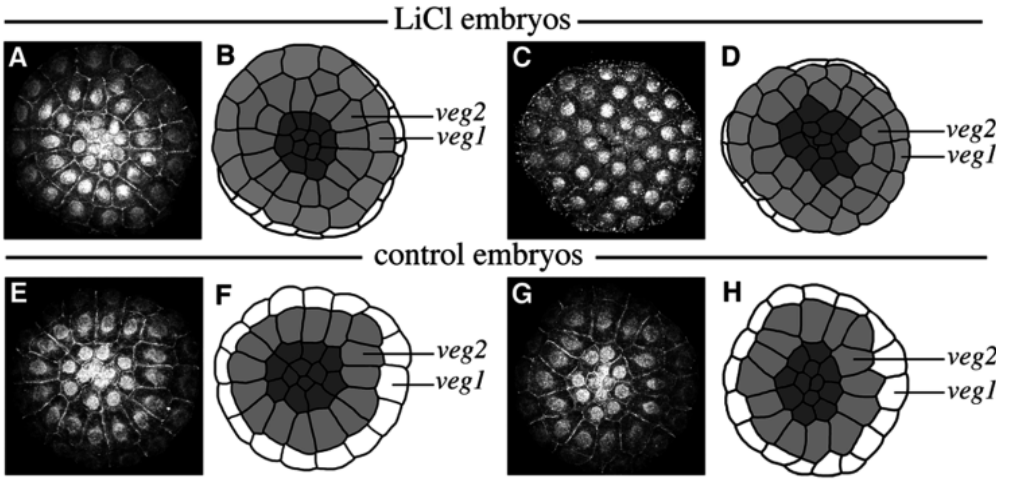

Fig. 3. LiCl treatment enhances the accumulation of β-catenin in nuclei of vegetal cells during cleavage. (A-D) Embryos reared in the presence of LiCl display increased levels of nuclear β-catenin in progeny of micromeres and *veg2* cells and the ectopic accumulation of β-catenin in nuclei of all *veg1* cells. This effect of LiCl is concentration dependent: 30 mM LiCl causes an increase in the levels of nuclear β-catenin found in *veg2* cells to that seen in micromeres and causes low to moderate levels of β-catenin to accumulate in nuclei of *veg1* cells (A,B); 50 mM LiCl causes levels of nuclear β-catenin seen in both *veg2* cells and *veg1* cells to increase dramatically such that levels in *veg2* and *veg1* cells are similar to that observed in the micromeres (C,D). This pattern contrasts that seen in control embryos which lack nuclear β-catenin in *veg1* cells (E-H).

catenin in early blastomere nuclei plays an important role in directing early blastomeres towards vegetal cell fates.

Inhibition of nuclear β-catenin localization abolishes vegetal development

To test more rigorously whether β-catenin function is required for the development of vegetal cell fates, we used RNA overexpression to prevent the accumulation of β-catenin in cell nuclei. Previous studies in *Xenopus* embryos have shown that overexpression of cadherin can sequester β-catenin at the cell membrane, thereby blocking its ability to act as a signaling molecule and to direct the development of dorsal fates (Fagotto et al., 1996). Therefore, we overexpressed the cytoplasmic portion of a sea urchin cadherin, LvG-cadherin (Miller and McClay 1997b), to examine the requirement of β-catenin function for the differentiation of vegetal cell types.

The overexpression of a synthetic mRNA encoding the transmembrane and intracellular domains of LvG-cadherin (ΔLvG-cadherin) showed a dose-dependent effect on the localization of β-catenin to nuclei in embryos at the 7th cleavage, and on vegetal differentiation of embryos scored at the 48-hour pluteus stage. Injection of 0.01 pg produced no effect on either the levels of nuclear β-catenin (compare Fig. 4A,B) or the development of endodermal and mesodermal tissues (Fig. 4A′,B′). However, injection of 0.03-0.05 pg of ΔLvG-cadherin RNA inhibited nuclear β-catenin accumulation in micromeres and *veg2* cells, although cytoplasmic staining remained high in the small micromeres (Fig. 4C). Sibling embryos receiving the same dose of ΔLvG-cadherin RNA and raised to 48 hours lacked all vegetal tissues as judged both morphologically (Fig. 4C′) and by staining with endoderm and mesoderm markers (data not shown). Notably, injected embryos lacked skeletogenic mesenchyme cells, a cell type thought to be autonomously specified (reviewed in Davidson, 1989). Wikramanayake et al. (1998) have also demonstrated that injection of a *Xenopus* C-cadherin construct in sea urchins produces animalized embryos that lack expression of several molecular markers. Injected embryos cultured until the 72-hour pluteus stage never formed endoderm or mesoderm, demonstrating that the lack of vegetal tissue differentiation is not simply due to a developmental delay and that these embryos lack a capacity to regulate for missing endoderm (data not shown). Injection data were obtained from at least 400 embryos that were collected from at least three different females and represent the phenotypes exhibited by >90% of specimens. Injection of a control RNA encoding green fluorescent protein at the same doses of ΔLvG-cadherin had no effect on nuclear β-catenin accumulation or on the differentiation of vegetal tissues (data not shown). From these analyses, we conclude that the accumulation and signaling activity of β-catenin in nuclei of vegetal cells is an early, required step in the development of all vegetal tissues.

Overexpression in *Xenopus* of cadherin constructs similar to that used has been shown to affect cell-cell adhesion (Kintner, 1992), raising the possibility that the lack of vegetal cell types is due to a reduction in cell-cell adhesion that perturbs the morphogenetic movements of gastrulation. We also observed an effect on cell adhesion following injection of high doses of LvG-cadherin RNA (0.10-0.12 pg). At these doses, embryos showed discontinuous junctional β-catenin staining (Fig. 4D), accumulated loose, opaque cells in the blastocoel (Fig. 4D′), and eventually became dissociated into single cells (Fig. 4D′ inset). However, doses of ΔLvG-cadherin RNA (0.03-0.05 pg) that were sufficient to eliminate endoderm and mesoderm produced embryos that remained epithelial and cells never appeared loose or non-adherent (Fig. 4C′). In addition, junctional β-catenin staining was found at high levels and appeared continuous in these embryos (compare Fig. 4A and C). Thus, we conclude that the effect of overexpression of intermediate levels of ΔLvG-cadherin on vegetal cell fates is due to its ability to prevent the nuclear accumulation of β-catenin and is not due to the disruption of cell adhesion and morphogenesis.

Nuclear localization of β-catenin imparts micromeres with the ability to differentiate as PMCs and to signal to neighbors

Classical embryological experiments have shown two important characteristics of the micromere lineage. Micromeres are thought to be autonomously specified to form skeletogenic cells

(Okazaki, 1975), and micromere-derived signals have been shown to play an important role in the initial specification of the vegetal plate and the positioning of spicule initiation sites (Ransick and Davidson, 1993, 1995). Since overexpression of ΔLvG-cadherin blocks development of all vegetal cell types including the PMCs, we predicted that β-catenin might be involved in regulating both micromere fate and signaling. To test this hypothesis, we prevented the nuclear localization of β-catenin in micromeres by overexpressing ΔLvG-cadherin mRNA at quantities ranging from 0.03 to 0.05 pg (Fig. 5A). Then, we transplanted micromeres from these injected embryos to the animal pole of uninjected, 8-cell-stage hosts, and asked whether the ectopic micromeres could form PMCs and/or induce neighboring blastomeres to form vegetal tissues (Fig. 5A). To follow nuclear β-catenin-deficient micromeres, donor embryos were labeled with rhodamine-isothiocyanate (RITC) (Fig. 5A). In all experiments, over 100 injected embryos were also cultured in parallel for 48 hours to confirm that donor embryos lacked endoderm and mesoderm (Fig. 5B).

Prevention of nuclear β-catenin accumulation in micromeres blocks both PMC differentiation and production of micromere-derived signals. When ΔLvG-cadherin-injected donor micromeres were transplanted to the animal poles of uninjected hosts, the transplanted micromeres never ingressed and remained epithelial within the plane of the ectoderm (*n*=13/13; Fig. 5C-E). To confirm that the absence of donor PMCs was due to a lack of PMC differentiation and not due to a lack of ingression of these cells, the manipulated embryos were stained with the PMC-specific marker 1G8. The donor cells within the ectoderm did not stain positively, suggesting that the ΔLvG-cadherin-injected micromeres were unable to differentiate as PMCs (*n*=3; data not shown). The injected donor micromeres also did not induce secondary gut-like tissues or ectopic spicule patterning sites (*n*=13/13; Fig. 5C-E). In contrast, when uninjected micromeres were transplanted to the animal poles of uninjected hosts, the micromeres always gave rise to PMCs, which ingressed and distributed themselves in a ring at the animal pole of the embryo (*n*=11/11; Fig. 5F-H). In addition, the control donor micromeres were able to induce a secondary archenteron or a small flattened region of thickened cells that exhibited an epithelial buckling as well as secondary spicule initiation sites (*n*=11/11; Fig. 5F-H). These data suggest that at least one of the functions of nuclear β-catenin during the early cleavage stages is to provide the micromeres with the ability to differentiate as PMCs and to produce a vegetalizing signal that induces neighboring cells to adopt vegetal cell identities.

The accumulation and maintenance of nuclear β-catenin during cleavage is not regulated by micromere-derived signals

Our analysis of the effects of ΔLvG-cadherin overexpression suggest that β-catenin is required for vegetal development and plays a role in both micromere fate specification and signaling. However, since overexpression of ΔLvG-cadherin is predicted to block the earliest activity of β-catenin, these data do not eliminate the possibility that β-catenin may also be induced in *veg2* cells by micromere-derived signals and promote vegetal development. Therefore, we asked whether nuclear β-catenin accumulation in *veg2* cells is regulated by micromere-derived cues.

First, we tested the requirement of micromeres to cause the localization of β-catenin in nuclei of *veg2* cells by removing the micromeres immediately following their formation at the 16-cell stage. The resulting embryos were cultured to the 7th cleavage stage or hatched blastula stage at which time they were fixed and stained with antibodies to β-catenin (Fig. 6A). We chose these two stages to determine whether the absence of the micromeres would affect the establishment and/or the maintenance of nuclear β-catenin accumulation in *veg2* cells. In micromere-deleted embryos, nuclear β-catenin was found in *veg2* cells at the 7th cleavage stage (Fig. 6B,C; *n*=11/11) and this pattern persisted through the hatched blastula stage (*n*=9/9 data not shown). Further, levels of nuclear β-catenin seen in *veg2* cells of manipulated embryos were comparable to that seen in control embryos. Micromere-deleted embryos that were allowed to continue developing displayed approximately a 9-hour delay in the initiation of gastrulation and impaired endodermal and mesodermal development, indicating that the removal of the micromeres had perturbed vegetal cell fate specification (*n*=21/21, data not shown). The persistence of nuclear β-catenin in *veg2* cells of micromereless embryos suggests that a micromere-derived signal is not involved in promoting or maintaining the accumulation of β-catenin in the nuclei of *veg2* cells during cleavage.

Since it is possible that the transfer of micromere-derived cues is so rapid that it cannot be prevented by microsurgical deletion of the inductive source, we transplanted the micromeres to the animal pole of a host embryo at the 8-cell stage to test whether micromere signals are sufficient to induce nuclear β-catenin localization in neighboring cells (Fig. 6D). Transplantation of the micromeres to the animal pole has been shown to result in the induction of a secondary vegetal axis (Ransick and Davidson, 1993). Thus, if nuclear β-catenin is induced by micromere signals, transplantation of micromeres to the animal pole would be expected to induce ectopic accumulation of nuclear β-catenin in mesomeres. However, transplantation of micromeres to the animal pole was not accompanied by an accumulation of nuclear β-catenin in neighboring mesomeres at the 7th cleavage stage (arrows in Fig. 6E,F; *n*=12/12). The ectopic micromeres retained high levels of nuclear β-catenin (arrowheads in Fig. 6E,F) and transplantation did not affect the endogenous pattern of nuclear β-catenin in vegetal cells of manipulated embryos (Fig. 6E,F). As expected, manipulated embryos that were allowed to develop to the pluteus larva stage possessed a secondary gut confirming that the transplanted micromeres induce neighboring mesomeres to assume vegetal cell fates (*n*=12/12, data not shown). These data demonstrate that micromere-derived signals do not promote the ectopic accumulation of nuclear β-catenin in mesomeres just as micromere-derived signals do not appear to induce the endogenous accumulation of nuclear β-catenin in *veg2* cells. These results provide further evidence that β-catenin acts upstream of micromere-derived signaling. In addition, these data suggest that micromere-derived cues can induce neighboring cells to adopt vegetal cell fates via a β-catenin-independent mechanism.

The nuclear localization of β-catenin is regulated by a cell autonomous mechanism

Given that the nuclear localization of β-catenin during the early

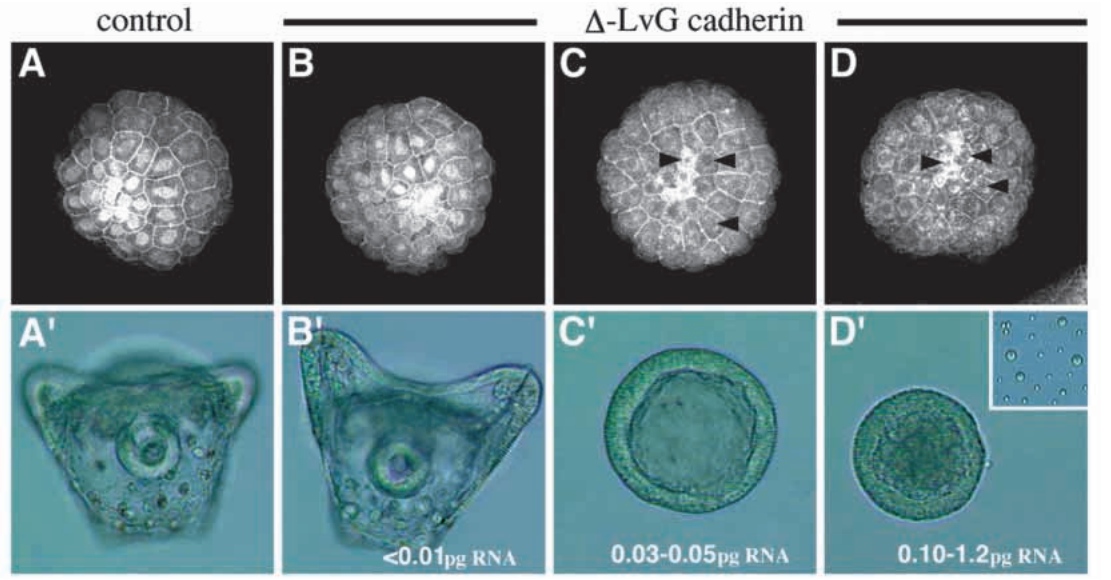

Fig. 4. Overexpression of ΔLvG-cadherin prevents nuclear localization of β-catenin and inhibits endoderm and mesoderm formation. (A-D) Confocal images of embryos at the seventh cleavage stained with anti-β-catenin antibodies. All embryos are oriented with the vegetal pole towards the viewer. (A′-B′) Bright-field images of embryos cultured until the 48-hour pluteus stage. (A,A′) Uninjected embryos. Nuclear β-catenin is found in vegetal nuclei, and the resulting embryos possess endoderm and mesoderm. (B,B′) Embryos injected with less than 0.01 pg of cadherin mRNA. Nuclear β-catenin is still evident during early cleavage and the pluteus larvae contain differentiated endoderm and mesoderm (C,C′) Embryos injected with 0.03-0.05 pg mRNA do not exhibit nuclear β-catenin (arrowheads) and do not form endoderm or mesoderm. (D,D′) At the highest concentrations of cadherin mRNA (0.10-1.2 pg), nuclear β-catenin is lost from both nuclei (arrowheads) and cell-cell junctions contain an opaque mass of cells in the blastocoel. Many of these embryos eventually become dissociated into single cells (D′, inset).

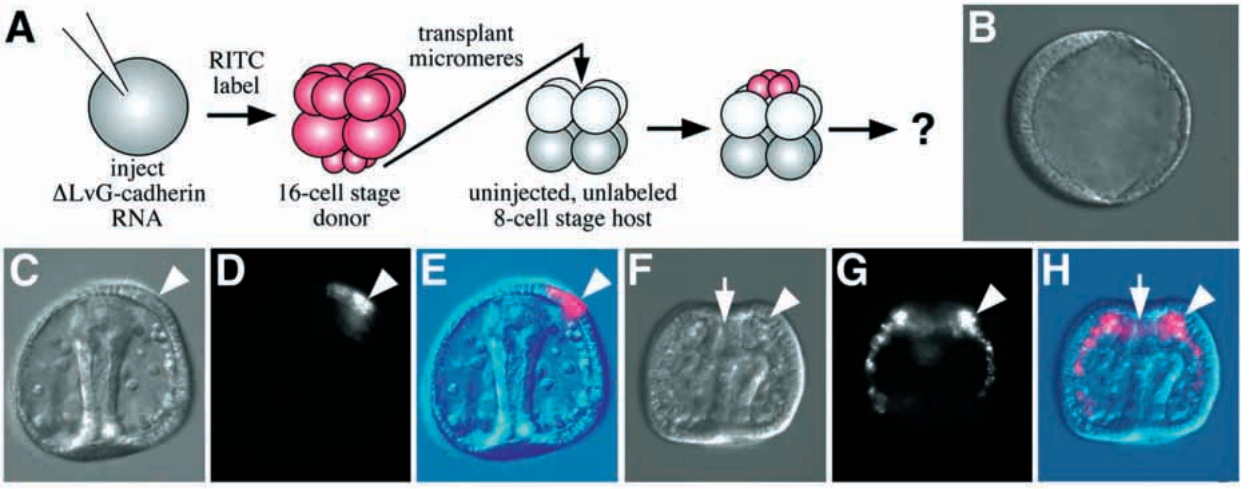

Fig. 5. Micromeres require nuclear β-catenin in order to differentiate as PMCs and to exhibit inductive capacities. (A) The requirement of nuclear β-catenin in micromeres was tested by overexpressing ΔLvG-cadherin (0.03-0.05 pg mRNA) in donor embryos and transplanting their micromeres to uninjected hosts. The donors were labeled with RITC in order to follow the micromere progeny. (B) Example of an unmanipulated donor embryo showing that the overexpression of ΔLvG-cadherin reliably produced embryos that lacked endoderm and mesoderm. (C-E) An embryo in which ΔLvG-cadherin RNA-injected micromeres were implanted at the animal pole (arrowhead). The animal pole is up; the vegetal pole is down. The donor micromeres remained in the plane of the epithelium and did not ingress into the blastocoel. (F-H) An embryo in which uninjected donor micromeres were implanted at the animal pole (arrowhead). The animal pole is up; the vegetal pole is down. The donor micromeres ingressed to form a secondary ring of PMCs at the animal pole (arrowheads in F-H) and induced a secondary gut at the animal pole (arrow in F,H).

cleavage stages does not appear to involve micromere-derived signaling, an alternative possibility is that the nuclear localization of β-catenin is regulated by a cell autonomous mechanism. A test of this hypothesis is to dissociate embryos and determine whether β-catenin will localize to nuclei of blastomeres cultured in isolation, in the absence of cell-cell interactions. Accordingly, embryos were dissociated at the 2-cell stage and at each successive cell division until the seventh

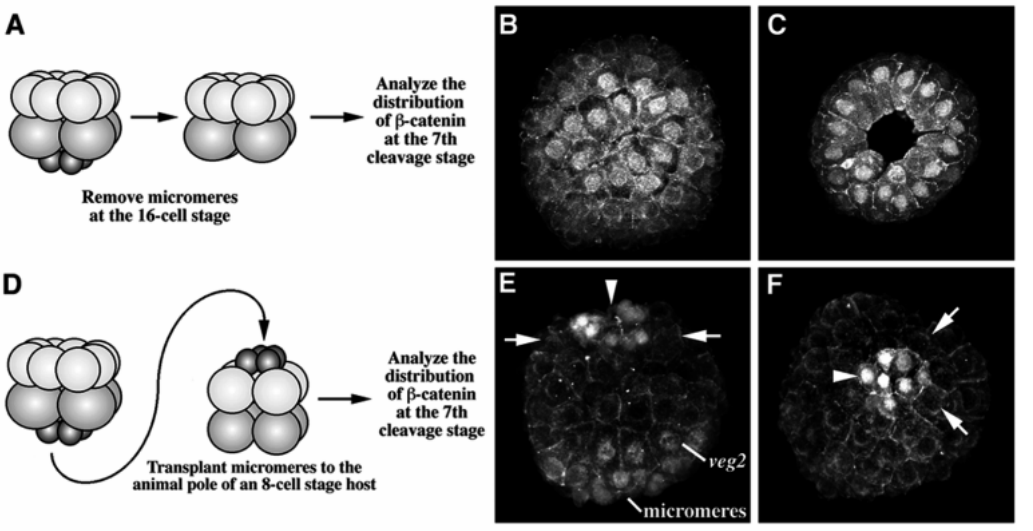

Fig. 6. Nuclear localization of β-catenin is not regulated by micromere-derived signals. (A-C) Deletion of the micromeres does not affect the accumulation of β-catenin in *veg2* cells. (A) Micromeres were removed immediately following their formation at the 16-cell stage. Micromereless embryos were cultured to the 7th cleavage stage when they were fixed and stained with anti-β-catenin antibodies and the distribution of nuclear β-catenin was determined by confocal microscopy. (B,C) In the absence of micromeres, β-catenin still accumulates to high levels in all *veg2* cells. (D-F) Transplantation of the micromeres to the animal pole does not induce the ectopic accumulation of β-catenin in nuclei of mesomeres. (D) A quartet of micromeres were removed from donor embryos at the 16-cell stage and placed at the animal pole of host embryos at the 8-cell stage. Manipulated embryos were cultured to the 7th cleavage stage when they were fixed and stained with anti-β-catenin antibodies and the distribution of nuclear β-catenin was determined by confocal microscopy. (E,F) β-catenin does not accumulate in nuclei of mesomeres in response to micromere-derived signals. In addition, the transplanted micromeres (arrows) retain high levels of nuclear β-catenin and the normal accumulation of β-catenin in nuclei of micromeres and *veg2* cells in the vegetal third of the embryo is unaffected (arrowheads).

cleavage, at which time the dissociated cells were fixed and stained with anti-β-catenin antibodies. We observed that a proportion of the dissociated cells contained nuclear β-catenin, indicating that the accumulation of β-catenin in nuclei of cleavage-stage cells is regulated at least in part by a cell autonomous mechanism (Fig. 7).

Since not all cells showed nuclear β-catenin, we asked whether the number of cells possessing nuclear β-catenin in dissociated cultures was similar to the number of cells with nuclear β-catenin in unperturbed embryos. A correlation between these numbers would provide stronger evidence that cell autonomous cues are responsible for regulating the pattern of nuclear β-catenin during cleavage. We counted the number of cells possessing nuclear β-catenin by visually assessing levels of nuclear staining found in dissociated cells. Confocal microscopy was used to optically section the cells, and specimens in which nuclei stained more brightly or at the same levels as the surrounding cytoplasm were scored as β-catenin positive; cells in which the nucleus appeared darker than the surrounding cytoplasm were scored as β-catenin negative. In most cases, nuclei were distinguishable from the surrounding cytoplasm by the difference in intensity of staining with the β-catenin antibody. In normal embryos at the seventh cleavage, 28/108, or 26% of the cells are nuclear β-catenin positive (*veg2* cells and small and large micromeres). In dissociated cultures, 1098/5928 or 18.5% of the cells possessed nuclear β-catenin. Although this value is less than that predicted from intact embryos, during the course of our experiments we also observed that smaller sized cells, which correspond to the large and small micromeres, tended to be under-represented in dissociated cultures due to the apparent loss of these cells during the fixation and staining procedure. When we re-calculate the number of nuclear β-catenin-positive and -negative cells that would be found in an undissociated embryo if the micromeres were absent or reduced, we obtain values that range from 16.6 to 20%, which approximates the value of 18.5% seen in dissociated cultures. The simplest interpretation of these data combined with the observation that dissociated cells exhibit clear nuclear β-catenin staining is that nuclear localization of β-catenin in micromeres and *veg2* cells is regulated by a cell autonomous mechanism.

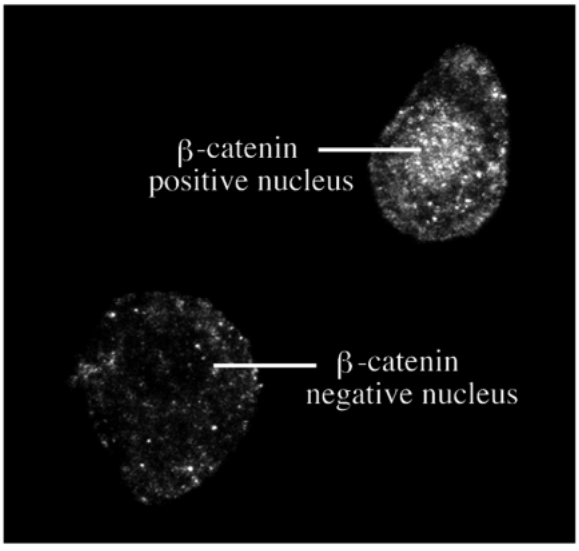

Fig. 7. Examples of single cells following dissociation at the 2-cell stage and cultured continuously in isolation until the 9th cleavage stage. Single cells following the 9th cleavage stained with anti-β-catenin antibodies. Shown is a single cell that possesses nuclear β-catenin and a single cell that lacks nuclear β-catenin.

DISCUSSION

Classical embryological studies have shown that specification of cell types along the animal-vegetal axis in the early sea urchin embryo is dependent on a combination of cell autonomous and cell non-autonomous mechanisms. The egg is pre-formed with an animal-vegetal asymmetry (reviewed in Davidson, 1989), and cell autonomous information localized at the vegetal pole both directs the micromeres to form skeletogenic mesenchyme (Okazaki, 1975) and imparts them with the ability to trigger the initial specification of vegetal plate tissue in adjacent blastomeres (reviewed in Hörstadius, 1973; Ransick and Davidson, 1993, 1995). Later, signals, passed between the *veg2* and *veg1* tiers, between the *veg1* tier and mesomeres, and among the mesomeres, function to refine cell identities along the animal-vegetal axis (reviewed in Hörstadius, 1973; Henry et al., 1989; C. Y. L. and D. R. M, unpublished data). In this paper, we examined the role of β-catenin, a known signaling molecule, in establishing cell fates along the animal-vegetal axis. From these studies, we conclude the following. (1) Nuclear β-catenin is required for development of vegetal cell fates. (2) Nuclear localization of β-catenin in vegetal cells is regulated cell autonomously, making it a component of the primary animal-vegetal asymmetry that had been identified previously by embryological analyses. (3) β-catenin is required for micromere differentiation and signaling. (4) In *veg1* and *veg2* cells, β-catenin may act in concert with additional signals to induce the full range of mesodermal and endodermal tissues.

Nuclear β-catenin functions in vegetal specification

The hypothesis that nuclear β-catenin promotes the development of vegetal cell fates is supported by several lines of evidence. First, the cell autonomous accumulation of nuclear β-catenin occurs in vegetal blastomeres at times when these cells are known to acquire vegetal identity. This observation is important because nuclear localization of β-catenin has been shown to be predictive of its function as a signaling molecule (Funayama et al., 1995; Miller and Moon, 1997), suggesting that β-catenin is performing a signaling role in vegetal cells. Second, LiCl treatment causes the enhancement and expansion of nuclear β-catenin accumulation in vegetal cells. This effect on the pattern of nuclear β-catenin correlates with an increase in the number of cells fated to become endoderm and mesoderm. Third, overexpression of ΔLvG-cadherin prevents the normal accumulation of nuclear β-catenin in vegetal cells and completely inhibits the development of endodermal and mesodermal cell types. Although we cannot completely eliminate the possibility that phenotypes generated at the RNA concentrations used here are caused by perturbations in cell-cell adhesion (see Results), we argue that this is unlikely because junctional β-catenin staining and the integrity of the epithelium appear to be preserved. In addition, β-catenin-depleted micromeres transplanted to the animal pole incorporate into host embryos and remain tightly associated with surrounding cells. Therefore, these data provide evidence that nuclear β-catenin plays a role in directing vegetal cells to adopt endodermal and mesodermal cell fates.

The idea that β-catenin-mediated signaling functions to specify vegetal cell fates is also supported by recent studies that manipulate levels of β-catenin and GSK-3 and assay their effects on specification of cell fates along the animal-vegetal axis in sea urchin embryos. Wikramanayake and colleagues (1998) showed that overexpression of an 'activated' form of *Xenopus* β-catenin causes vegetalization of embryos and can induce endoderm formation by isolated animal caps, which normally only make ectoderm. Similarly, overexpression of a dominant-negative form of GSK-3 also promotes the expansion of vegetal cell fates (Emily-Fenouil et al., 1998). Like LiCl treatment, the vegetalizing effects of activated β-catenin and dominant-negative GSK-3 are predicted to cause nuclear β-catenin to accumulate ectopically and thereby direct animal blastomeres to adopt vegetal cell fates. Conversely, overexpression of wild-type GSK-3, a treatment predicted to destabilize β-catenin thereby preventing its nuclear accumulation in nuclei, results in inhibition of vegetal development (Emily-Fenouil et al., 1998). Similar to our experiments here, Wikramanayake et al. (1998) demonstrated that *Xenopus* C-cadherin can prevent endoderm and mesoderm formation in injected embryos. These studies, together with our data linking vegetal development with nuclear β-catenin localization, support the idea that β-catenin function is required for the specification of vegetal cell fates in the sea urchin embryo.

The presence of nuclear β-catenin in the small micromeres, the large micromeres and the *veg2* cells, all of which have distinct fates, raises the question of how β-catenin regulates the differentiation of these vegetal cell layers. One possibility is that the timing of nuclear β-catenin accumulation is important such that β-catenin signaling exerts distinct effects depending on when it localizes to different vegetal nuclei. We observe nuclear β-catenin first in the micromeres and later in *veg2* cells. If we postulate that there are changes in the availability of promoters of target DNA during cleavage, the difference in timing of nuclear β-catenin localization could account in part for the different fates of the large and small micromeres and *veg2* cells.

A second possibility is that nuclear β-catenin acts in a concentration-dependent manner to exert its effects on different blastomere tiers. In normal embryos, the levels of nuclear β-catenin seen in the small micromeres, the large micromeres and the *veg2* cells are graded such that the small micromeres>large micromeres>*veg2* cells. It is possible that these differences in levels are sufficient to drive cells along different developmental pathways. In support of this idea, the treatment of embryos with LiCl causes an increase in the levels of nuclear β-catenin in *veg2* cells to levels similar to that seen in large micromeres and the accumulation of nuclear β-catenin in *veg1* cells to levels normally seen in *veg2* cells. These changes in the relative levels of nuclear β-catenin correlate with an increase in the number of cells fated to become PMCs and endoderm following LiCl treatment (Livingston and Wilt, 1989; Nocente-McGrath et al., 1991) and the transformation of *veg1* cells to *veg2*-like fates (Cameron and Davidson, 1997; C. Y. L. and D. R. M., unpublished data). In addition, low levels of injected 'activated' β-catenin RNA can affect patterning of the ectoderm whereas high levels can respecify animal cells to form endoderm (Wikramanayake et. al., 1998). Hence, different concentrations of β-catenin may influence the ability of cells to adopt distinct identities.

A third mechanism is that β-catenin acts in conjunction with either PMC and/or endoderm/SMC-specifying factors to provide the different blastomere tiers with the ability to generate

distinct cell types along the animal-vegetal axis. Since nuclear β-catenin appears to be found in both mesodermal and endodermal precursors, nuclear β-catenin may act to specify a regional vegetal identity while additional localized factors or signaling molecules whose expression depends on nuclear β-catenin function might act in this background to specify distinct cell fates. These various mechanisms are not mutually exclusive and the characterization of nuclear β-catenin targets will help us to understand how vegetal specification and cell type specification are coordinately regulated during development.

β-catenin and the establishment of micromere identity

The micromere lineage is characterized by two traits: (1) the micromeres are autonomously specified (Hörstadius, reviewed in 1939; Okazaki, 1975; Armstrong and McClay, 1994), and (2) the micromeres are a source of a potent vegetalizing signal (Hörstadius, 1939; Ransick and Davidson, 1993, 1995). From data presented in this paper, we propose that nuclear β-catenin functions upstream of both micromere differentiation and signaling. The micromeres are specified very early during development by a cell autonomous mechanism. Thus, any signaling molecule expected to play a role in specifying micromere fate must act early and be regulated by a cell autonomous mechanism. The timing of β-catenin localization and the cell autonomous accumulation of β-catenin in nuclei support this prediction. More direct evidence linking β-catenin function to the specification of micromere fates comes from blocking nuclear β-catenin accumulation by overexpression of ΔLvG-cadherin. Inhibition of nuclear β-catenin accumulation in micromeres inhibits PMC formation, indicating that nuclear β-catenin is a component of the molecular pathway that specifies the micromeres to a skeletogenic fate. This appears to be a direct effect on the micromeres, since nuclear β-catenin-deficient micromeres transplanted to the animal pole of a host embryo fail to form primary mesenchyme cells. Nuclear β-catenin also appears to provide micromeres with the ability to signal to neighboring blastomeres, because nuclear β-catenin-depleted micromeres transplanted to the animal pole of a host embryo fail to induce a secondary gut or ectopic sites of spicule growth.

Although nuclear β-catenin is involved in regulating micromere signaling, it does not appear to be necessary for subsequent signaling events in the blastomeres responding to micromere-derived cues. Transplantation of micromeres to the animal pole of a host embryo does not cause the accumulation of nuclear β-catenin in the responding mesomeres even though it induces the development of a secondary gut and spicules. It is formally possible that transplanted micromeres induce levels of nuclear β-catenin in mesomeres that are undetectable by our antibodies but that are sufficient to drive endoderm formation. However, we favor the model that nuclear β-catenin is required in micromeres for the expression of signaling molecules that in turn, induce mesomeres to form archenteron tissues. Therefore, nuclear β-catenin is not required in the mesomeres because they are responding to micromere-derived signals whose activity is regulated by β-catenin. The cloning and characterization of β-catenin target genes will allow us to test this hypothesis directly. Together, these data indicate that β-catenin is an early-acting component of a maternally regulated machinery that acts to direct micromere development.

Functions of β-catenin in the *veg1* and *veg2* lineages

β-catenin and establishment of *veg2* identity

Previous studies have demonstrated that micromere-derived signals are involved in promoting the development of mesoderm and endoderm by *veg2* progeny (Ransick and Davidson, 1993, 1995). Because experiments involving micromere deletions cannot completely eliminate the possibility that there is some transfer of micromere-derived cues prior to their removal, it has been unclear whether micromere signaling is the only cue required for specification of *veg2* cells as endoderm or whether there is also some cell autonomous information localized in *veg2* cells that are required for endoderm formation. The observations that β-catenin localizes to *veg2* nuclei following micromere removal, that nuclear accumulation of β-catenin is cell autonomous and that β-catenin is sufficient to specify endoderm in animal caps (Wikramanayake et al. 1998) suggest that β-catenin does play some cell autonomous role in *veg2* cells. However, ΔLvG-cadherin overexpression both blocks the production of micromere-derived signals and eliminates *veg2*-derived secondary mesoderm and endoderm. Therefore, we cannot distinguish between whether inhibition of nuclear β-catenin accumulation in vegetal nuclei affects *veg2* differentiation indirectly by preventing micromere signaling or whether it acts cell autonomously within the *veg2* lineage. The mechanism by which veg2 cells are specified to form endoderm is therefore still an important unanswered question.

Similar to its role in micromeres, however, nuclear β-catenin may play a role in veg2 development by promoting the production of inductive signals in *veg2* cells, which can induce endoderm, pattern the archenteron and induce spicule initiation sites. Blastomere recombination studies have shown that *veg2* cells can induce overlying cells to form endoderm (Hörstadius, 1973; C. Y. L. and D. R. M., unpublished data) and that *veg2* cells appear to provide signals that can pattern the gut (C. Y. L. and D. R. M., unpublished data). Furthermore, descendants of the macromeres can induce neighboring ectoderm to form ectopic sites of spicule initiation, demonstrating that these cells can serve as an inductive source (Benink et al., 1997). Therefore, *veg2* cells produce inductive signals that may be similar to the inductive cues produced by micromeres. If nuclear β-catenin is involved in providing *veg2* cells with an ability to signal to neighbors, then one might predict that cells experimentally induced to localize β-catenin to their nuclei would recruit neighboring cells to form endoderm or to generate sites of skeletogenesis. Consistent with this prediction, the ectopic accumulation of nuclear β-catenin in *veg1* cells following LiCl treatment can cause *veg1* cells to assume *veg2*-like fates (Cameron and Davidson, 1997; C. Y. L. and D. R. M., unpublished data) and mesomeres are subsequently recruited into the gut, as shown by cell labeling studies (C. Y. L. and D. R. M., unpublished data). Future embryological studies combined with overexpression and mis-expression studies will provide a more direct test of this hypothesis.

β-catenin during post-hatching stages

The observation that nuclear β-catenin undergoes a dynamic re-localization to descendants of the *veg1* tier after hatching suggests that β-catenin possesses later developmental functions.

Cell marking experiments have shown that the limits of the endoderm do not coincide with any cleavage boundaries, indicating that refinement of the boundary between the ectoderm and endoderm relies on cell-cell signaling (Logan and McClay, 1997). Given the localization of β-catenin to the endodermal side of the ectoderm-endoderm junction, one possible role of β-catenin in veg1 cells might be to regulate the extent of vegetal differentiation in the embryo by participating in cell-cell signaling that occurs near the ectoderm-endoderm boundary. We cannot examine the role of later nuclear β-catenin directly, since cadherin overexpression perturbs the earliest function of β-catenin and prevents all vegetal development. However, as more sophisticated experimental approaches are developed, we will be able to examine the potential function of nuclear β-catenin in *veg1* cells after hatching.

Conservation of pathways that establish early axial asymmetries among deuterostomes

The asymmetry of nuclear β-catenin localization along the animal-vegetal axis of the sea urchin embryo is strikingly similar to the polarized distribution of nuclear β-catenin along the dorsal-ventral axis of other deuterostome embryos such as *Xenopus* and zebrafish (Schneider et al., 1996; Larabell et al., 1997). In *Xenopus*, β-catenin is thought to establish a late blastula-stage organizer, called the Nieuwkoop center, that induces the gastrula-stage Spemann organizer (reviewed in Lemaire and Kodjabachian, 1996; Heasman, 1997). The Spemann organizer, in turn, is thought to be the source of signals that pattern both the dorsal mesoderm and neural ectoderm. A number of genes that are expressed in the Spemann organizer and serve as useful markers include *brachyury* (T) and *forkhead/HNF-3β* (Smith et al., 1991; Ruiz i Altaba and Jessell, 1992; Ruiz i Altaba et al., 1993). Interestingly, both *brachyury* and *forkhead* are expressed in vegetal cells at the onset of gastrulation in sea urchin embryos (Harada et al., 1995, 1996). The vegetal expression of these genes supports the idea that the presence of nuclear β-catenin in micromeres and *veg2* cells during cleavage may establish a blastula-stage signaling center that induces the expression of 'organizer' genes in vegetal cells following hatching. These similarities between the distribution of nuclear β-catenin in both *Xenopus* and sea urchins indicates a possible conservation of the molecular networks that regulate axial patterning events between echinoderms and chordates (see also Wikramanayake et al. (1998), Emily-Fenouil et al. (1998)). Thus, it will be of great interest to identify and characterize other genes involved in animal-vegetal patterning in the sea urchin. These future studies will not only further our knowledge of how the sea urchin develops, but will also provide new and interesting insights into the evolution of developmental networks that regulate axial patterning in deuterostomes.

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