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Original Article

Type I cadherins are required for differentiation and coordinated rotation in *Xenopus laevis* somitogenesis

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ABSTRACT In *Xenopus laevis* somitogenesis, somitic blocks undergo coordinated movements resulting in their detachment from the rest of the mesodermal ridge, followed by a 90° rotation of the entire metamere. Here we investigated the function of type I cadherins in somitogenesis. Type I cadherins are Ca^{2+}-dependent cell-cell adhesion molecules concentrated in the adherens junctions and highly expressed in the somitic tissue. We analyzed their role in somitogenesis by overexpressing either the intracellular (ΔE) and the extracellular (C-trunc) dominant-negative forms of cadherin. The resulting phenotype was a downward bend of the anterior-posterior axis in tadpole stage embryos. 12/101 antigen and *X-Myo-D* expression were altered. Microscopy revealed disorganization of the myotomes. Conversely, segmentation was conserved at the microscopic and molecular levels.

KEY WORDS: *Xenopus laevis somitogenesis, myogenesis, adhesion, cadherins*

Introduction

Somite formation in *Xenopus laevis* displays several unique features. A group of cells of the unsegmented mesoderm, lying perpendicular to the notochord, separate from the rest of the mesoderm and form a block, which undergoes rotation resulting in a 90° rotation of the cells. This rotation requires a series of cellular movements that have rarely been investigated. The cells composing the block tissue do not move « *en bloc* » as suggested by Hamilton (Hamilton, 1969), but instead undergo differential movements (Keller, 2000) depending on their position within the block. Each cell senses its location within the block and undergoes morphological changes and cellular rearrangements according to that position. As observed by Youn and Malacinski (Youn and Malacinski, 1981), during their rotation, myoblasts adopt morphology typical of single migratory cells, with lots of protrusions and a defined polarity. Nevertheless, these cells establish cell-cell contacts that are essential for maintenance of the block structure. The adhesive process is essential to the achievement of coherent rotation and may be considered to be the sensor mechanism underlying the « dynamic synchronicity » of cell movements.

The cells of the somitomere begin to rotate after the formation of the intersomitic furrow (Keller, 2000). However the orchestration of these cellular rearrangements has been subject to little investigation (McCaig, 1986; Wilson *et al.*, 1989). The rotation step is a dynamic process involving a set of coordinated cell movements. Based on studies of somitic cell morphology during rotation (Youn and Malacinski, 1981) it could be hypothesized that a leading cell initiates the rotation, inducing the other cells to rotate in a coordinate manner. This hypothesis implies that each cell must follow its own pathway during somite rotation, depending upon its location within the block (Keller, 2000). Furthermore, such movements require continual changes in cell morphology, with the cells resembling single migratory cells with polarized lamellipodia, as observed by Youn and Malacinski (Youn and Malacinski, 1981) and by Wilson *et al.* (Wilson *et al.*, 1989).The process may have features in common with *Drosophila* gastrulation, in which a group of cells stochastically initiates its own invagination, leading to formation of the germ layers (Kam *et al.*, 1991).

Little is also known about the molecular mechanisms underlying this amazing phenomenon of somite rotation; but there appears two defined steps: one concerning segmentation of the presegmental mesoderm (PSM) and the other related to rotation of the segmented mesoderm. These two steps are connected be-

Abbreviations used in this paper: A-P, anterior-posterior; Car, Cardiac actin promoter; DAB, 3-3' Diaminobenzydine; DAPI, 4',6-Diamidino-2-phenylindole; FCS, foetal calf serum; HBDER, hormone-binding domain of the estradiol receptor; N.F., Niewkoop and Faber; PAPC, paraxial protocadherin; PBS, phosphate-bufferd saline; p*SB*, Sleeping Beauty plasmid; PSM, presegmental mesoderm; SEM, Scanning Electron Microscopy; TBS, Tris-buffered saline.

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cause the inhibition of normal segmentation leads to defective somitogenesis (Kim *et al.*, 2000). It has been shown that PSM segmentation depends on the Notch signaling pathway, WRPW-bHLH proteins and the bHLH transcription factors of the Mesp family (Pourquie, 2000; Rawls *et al.*, 2000). It has recently been demonstrated that a cell-cell adhesion molecule, the paraxial protocadherin (PAPC), is also involved in the process (Kim *et al.*, 2000). Therefore we postulated that this process requires intracellular signaling and interactions with the extracellular matrix.

Cadherins are a superfamily of Ca^{2+}-dependent cell-cell adhesion molecules that have been classified into several families, including type I cadherins, type II cadherins, protocadherins, cadherins from desmosomes and cadherin-related molecules (for a review see (Tepass *et al.*, 2000)). We restricted our study to the potential role of type I cadherins during somitogenesis because these molecules are present in large amounts in adherens junctions and because of their developmental expression. Particularly, the major type I cadherins expressed during early development are E-, N- and the maternal C-cadherin. As above mentioned, type I cadherins are one class of molecule involved in cell-cell adhesion and signal transduction. They are composed of a large extracellular domain required for homotypic binding, a transmembrane domain required for lateral clustering (Huber *et al.*, 1999) and a cytoplasmic tail responsible for functional cell adhesion via interaction with β-catenin, which connects cadherins to the actin cytoskeleton. Cadherins are expressed throughout *Xenopus* development and consequently the inhibition of their function in the early phases of development has dramatic effects (Dufour *et al.*, 1994; Levine *et al.*, 1994). C-cadherin (also referred to as EP-cadherin) is the most prevalent type I cadherin in somites. Other type I cadherins are produced in only small quantities or only at later stages in muscle development (Simonneau *et al.*, 1992). C-cadherin is expressed in somitomeres, even before rotation, and is present throughout the development of axial and smooth muscles (Levi *et al.*, 1991).

We describe here the effects of two dominant-negative forms of type I cadherins (see Fig. 1) on *Xenopus* somitogenesis. The first, which we named ΔE, consisted of the cytoplasmic tail and transmembrane domain of E-cadherin. It exerts its dominant-negative effect by recruiting all the cytoplasmic β-catenin, thereby inhibiting all type I cadherins and probably other cadherins too. The second, called C-trunc, consisted of the extracellular domain of C-cadherin.

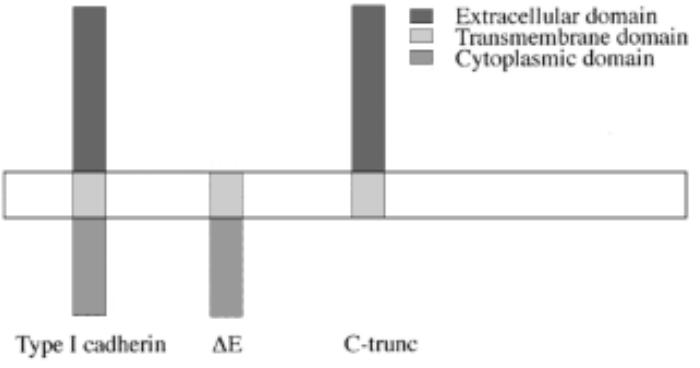

Fig. 1. Schematic representation of the structure of type I cadherins and the dominant-negative forms used in these experiments.

This molecule competes with the extracellular domain of endogenous C-cadherin, thereby selectively inhibiting the homotypic binding of this cadherin. The targeted expression of synthetic mRNA or plasmid DNA constructs encoding these molecules in somitic tissue resulted in significant morphological defects. These abnormalities in somitogenesis were mostly due to the inability of somitic cells to undergo coherent rotation. Further analysis, at the cellular level, confirmed changes in cell organization and revealed a loss of the adhesive phenotype. Segmentation did not appear to be affected because metameric units were still present and *X-Delta-2* expression was not altered. In contrast, *X-Myo-D* expression was repressed by the dominant-negative cadherins, suggesting that type I cadherins have a pivotal role in muscle differentiation as well as somite rotation.

Results

ΔE-Cadherin causes Morphological Defects in Embryos at the Tailbud and Tadpole Stages

In this study, we investigated whether alterations in the type I cadherin adhesive system had an effect on the morphogenic processes involved in somitogenesis. ΔE-cadherin, the intracellular dominant-negative form of cadherin exerts its function by titering all endogenous β-catenin, thereby inactivating the endogenous type I cadherin adhesion mechanism. This mutant may also disturb the Wnt signaling pathway, of which β-catenin is the key component, as shown in cadherin overexpression experiments (Fagotto *et al.*, 1996). It may also disturb β-catenin accumulation at the boundaries between each somite and between somites and notochord (Fagotto and Gumbiner, 1994).

The most abundant type I cadherin is produced ubiquitously. It was therefore necessary to direct the ΔE-construct to somitic territories in a time-specific manner. This was achieved by injecting the construct into blastomeres B2B3B4C2C3C4 B2'B3'B4'C2'C3'C4' in accordance with the somite fate maps previously described (Dale and Slack, 1987; Moody, 1987). Temporal specificity was achieved by using a construct encoding the inducible form of the protein (Kolm and Sive, 1995). We constructed a fusion protein, consisting of the intracellular domain of E-cadherin fused to the ligand-binding domain of the estradiol receptor (HBDER). Briefly, in physiological conditions, the binding site for β-catenin is concealed by HSP90 which forms a complex with HBDER. If β-estradiol is added, it binds to HBDER and displaces HSP90, freeing the β-catenin binding site, enabling the hybrid protein to exert its dominant negative effect (Giacomello *et al.*, 2001).

The activation of ΔE at the tailbud stage in embryos injected with ΔE-HBDER mRNA resulted in a downward kink in the anterior-posterior (A-P) axis. The position of this kink depended on the precise time of induction (Fig. 2, A,B), and the degree of bending depended on the last somite to be correctly formed before induction. The later the induction, the closer to the posterior was the kink. In contrast, injected embryos without β-estradiol induction (Fig. 2C) did not show any deformation confirming that the phenotype was due to ΔE effect and not to the RNA injection.

In order to identify the step in somitogenesis that was affected, embryos were unilaterally injected with ΔE mRNA and treated with β-estradiol at stage 14 N.F., prior segmentation. The embryos were analyzed at the tailbud stage by whole-mount immunostaining with

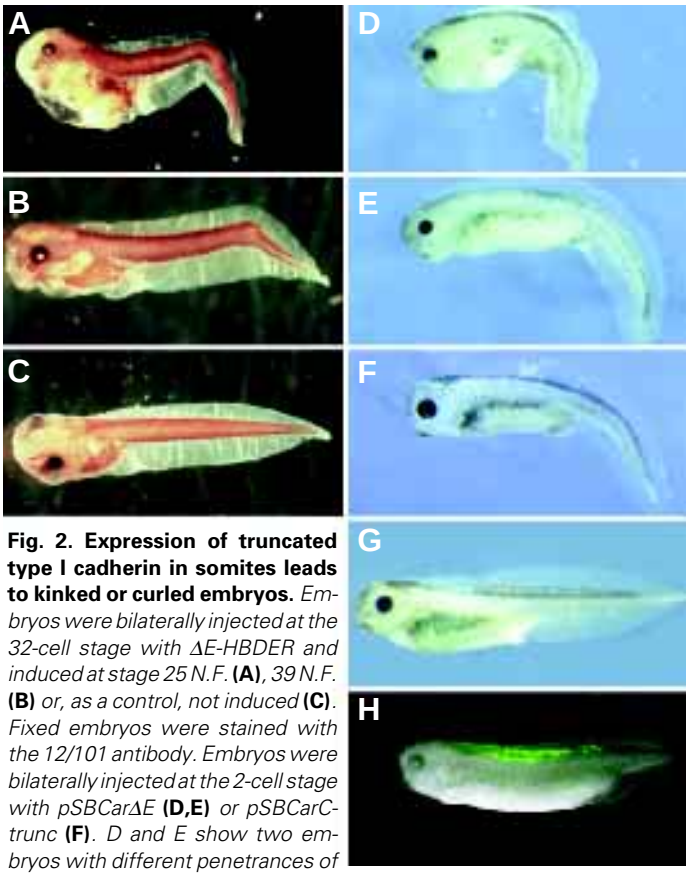

Fig. 2. Expression of truncated type I cadherin in somites leads to kinked or curled embryos. *Embryos were bilaterally injected at the 32-cell stage with ΔE-HBDER and induced at stage 25 N.F.* **(A)**, *39 N.F.* **(B)** *or, as a control, not induced* **(C)**. *Fixed embryos were stained with the 12/101 antibody. Embryos were bilaterally injected at the 2-cell stage with pSBCarΔE* **(D,E)** *or pSBCarC-trunc* **(F)**. *D and E show two embryos with different penetrances of the phenotype.* **(G)** *shows an uninjected embryo, while* **(H)** *shows a control embryo injected with the control plasmid pSBCarGFP at stage 35.*

the muscle-specific 12/101 antibody (Fig. 3A). Perturbation at this early stage of somitogenesis led to extensive disorganization of the somitic tissue, which lost its canonical pattern and was less abundant. Segmentation did not seem to be affected. Some repetitive units were visible on the left side of the embryo (Fig. 3A), but there were clearly fewer cells in each block than on the uninjected side; antibody staining was weaker and showed the structure to be diffuse.

We then exploited another experimental system, which allowed the injection of more embryos. It is based on the high stability of the *Sleeping Beauty* plasmid (p*SB*), as previously described in Vallin *et al.* (2001). The ΔE mutant coding sequence was inserted into p*SB* under the control of the cardiac actin promoter, which becomes active at stage 12 N.F. and drives specific expression in the somites and heart. Plasmid DNA was injected into two-cell stage embryos, into one or both cells, with p*SB* CarGFP used as the tracer. Embryos were allowed to grow to the appropriate stage and only embryos displaying uniform fluorescence were analyzed. The embryos into which the construct was injected were examined at the tadpole stage, when they were found to have a curved anterior-posterior (A-P) axis (Fig. 2 D,E). The results were reproducible but penetrance differed between embryos (compare Fig. 2D and 2E). Phenotypic variation may have resulted from differences in the amount of dominant-negative protein expressed or mosaicism due to the p*SB* injection. Embryos into which p*SB* CarΔE was injected on one side only (Fig. 3B, the injected side is indicated by an arrow) were analyzed at the tailbud stage and 70% (n=100) of these embryos presented defects. This result is consistent with that obtained for mRNA injections. Control embryos (n>100) were injected with equimolar amount of p*SB* CarGFP and none of them presented any default in the somitic phenotype (Figs. 2H and 3D).

The two phenotypes at tadpole stage may have raisen from the differences in the temporal expression of the two ΔE constructs. The severity of the "kink" phenotype could be due to the sudden production of large amounts of dominant-negative protein. The phenotype differences at tailbud stage may have raisen from differences in protein quantity between the two techniques, because mRNA injections usually results in higher level of exogenous protein than plasmid injections.

The C-Trunc Cadherin Mutant causes the Same Defects as ΔE-Cadherin

We used another mutant, C-trunc, to investigate the possible direct role of type I cadherins in disturbing adhesion rather than signaling. The C-trunc mutant consists of the extracellular domain of C-cadherin and exerts its function by competing with the extracellular domain of endogenous C-cadherin molecules. As C-cadherin is produced in large amounts in somitic tissue, we expected the mutant to cause massive inhibition of cadherin function but no direct interference with β-catenin signaling. Embryos at the tailbud stage were stained with the 12/101 antibody (Fig. 3C): somite perturbation was observed in 57% of the samples analyzed (n=102). The phenotype in these cases was similar to that observed in embryos injected with ΔE-cadherin: the somitic tissue is extensively disorganized but the segmentation is still maintained. In the injected side of the embryo the expression of 12/101 antigen appeared to be weaker. The observation raises questions about the differentiation state of injected myotomal cells. Embryos injected with C-trunc constructs had the same curled

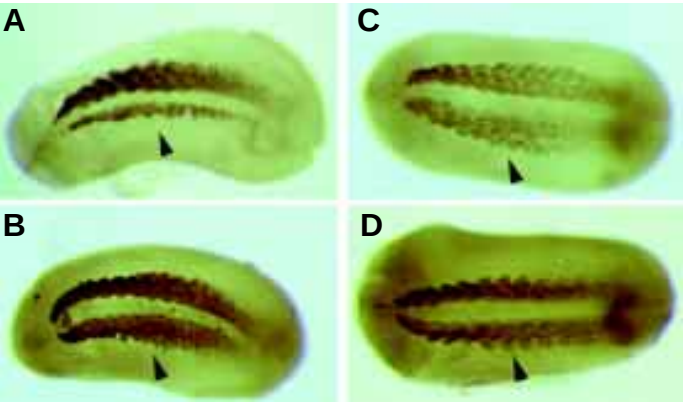

Fig. 3. Dominant-negative forms of type I cadherins cause perturbations in the somitic tissue. *RNA injections: embryos were injected with* ΔE-HBDER *mRNA at the 32-cell stage* **(A)**. *The injections were made into blastomeres B2B3B4C2C3C4 and protein activity was induced at stage 14 N.F. Plasmid injections: embryos were unilaterally injected at the 2-cell stage with* pSBCarΔE **(B)**, pSBCarC-trunc **(C)** *or* pSBCarGFP **(D)**. *Embryos were fixed at the tailbud stage and whole-mount immunostained with the 12/101 antibody. The injected side (anterior is on the left, embryos were injected in the left side, arrow) presented somite abnormalities; these structures were not properly organized, the 12/101 staining was less pronounced in injected sides. The injection of the control plasmid* pSBCarGFP *did not cause any alteration.*

phenotype at the tadpole stage (Fig. 2F) as was observed for ΔE-injected embryos. In addition to bending of the A-P axis, some late tadpole embryos injected with ΔE or C-trunc cadherin had swimming problems, which may have resulted from physical impediment due to the kink and/or the presence of defective muscles. In some cases the phenotype with the C-trunc mutant was less severe than the one observed with the ΔE mutant: this effect may be due to the inhibition of C-cadherin function only, allowing the others to still be able to contribute to somitogenesis.

Truncated Cadherins modify X-Myo-D *Expression*

The expression of the muscle specific marker 12/101 appeared lower in either ΔE or C-trunc injected embryos. We therefore investigated whether the two cadherin mutants interfered with the myogenic signaling pathway. We analyzed the expression pattern of *X-Myo-D*, the earliest marker of muscle cell differentiation. Activation of *X-Myo-D* expression requires the community effect (Gurdon, 1988), operating *via* the interactions of a minimum number of mesodermal cells. It has been shown *in vitro* that dominant-negative forms of cadherins may affect *MyoD* expression in explants (Holt *et al.*, 1994). Similar result were obtained following the injection of the two dominant-negative cadherin constructs. The injected side of the embryo displayed lower levels of *X-Myo-D* expression (Fig. 4 A,B,D,E, arrows show the injected side). However, *X-Myo-D* expression was not completely abolished because the *X-Myo-D* promoter is active before the cardiac actin promoter (Hopwood and Gurdon, 1991) and therefore presumably expression was not immediately affected. However, the loss of cell-cell contacts and the decreased number of cells within the somites may have attenuated the community effect, causing a lack of induction of *X-Myo-D* expression and changes to the myogenic differentiation program. It seems likely that the tadpoles are unable to swim because they lack fully differentiated muscle cells.

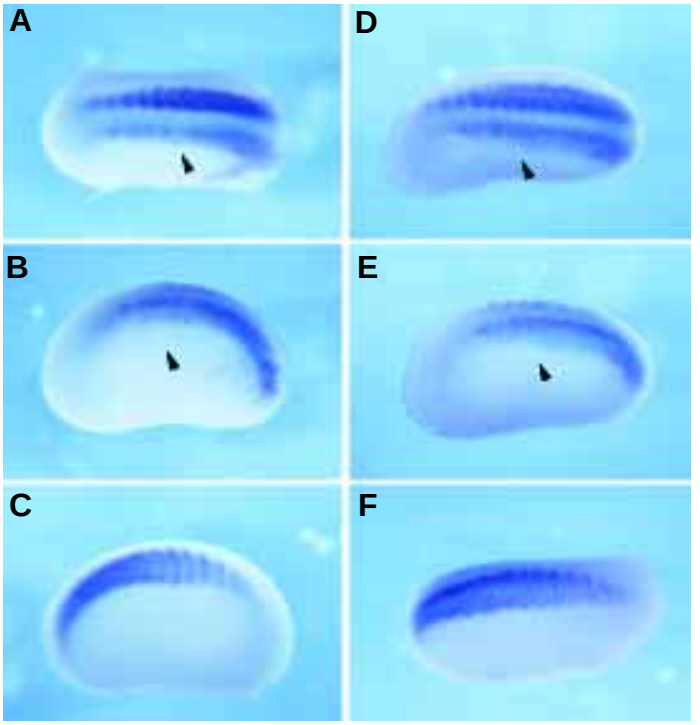

Fig. 4. The two mutant cadherins downregulate *X-Myo-D* expression. In situ *hybridization of unilaterally injected embryos showed lower levels of* X-Myo-D *transcripts in the injected side (left side indicated by an arrow) for ΔE* **(A,B)** *(anterior is on the left) and C-trunc* **(D,E)** *(anterior is on the left).* **(C,F)** *show the uninjected side (anterior is on the right).*

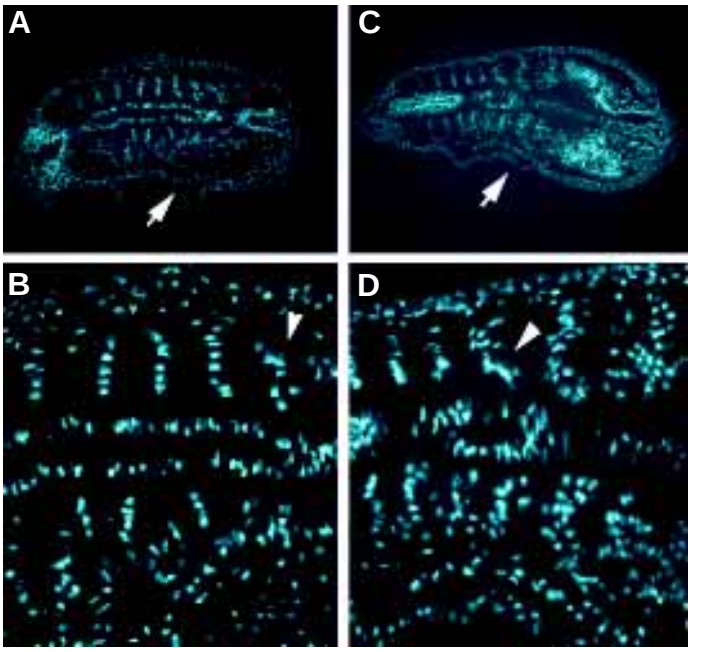

Fig. 5. Analysis of myotome arrays. *Embryos were unilaterally injected at the 2-cell stage with* pSBCarΔE **(A,B)** *or* pSBCarC-trunc **(C,D)**. *At the tailbud stage, embryos were fixed, sectioned and stained with DAPI. Anterior is on the left of the panels; injections were made in the left side of the embryo. In A and C (10x), a lack of organization on the injected side (indicated by an arrow) may be observed, and this is emphasized at higher magnifications (B and D, 30x). The arrowheads in B and D show a normal somite in the process of rotation.*

Cell Organization is altered in Somites injected with ΔE or C-Trunc

In each somite, myotomal cells are typically stacked in blocks with their nuclei dorso-ventrally aligned. Thus, horizontal sections of normal somites display a pattern, with nuclei forming regularly spaced stripes (one stripe corresponds to one somite, see Fig. 5 panels A,B,C,D, uninjected side in the upper part of the each panel) and a random pattern in unsegmented mesoderm. The curved arrangement of the nuclei facilitates the recognition of somites undergoing rotation (Fig. 5 B,D, the rotating somite is indicated by an arrowhead). This observation reflects the dynamic synchronicity of the movements.

The perturbation of cell-cell contacts, by the injection of a ΔE or C-trunc construct, caused dramatic disorganization of the typical myotome arrays in injected embryos: the nuclei were not arranged in ordered stripes and it was not possible to distinguish somites undergoing rotation from those that had already been formed (Fig. 5 B,D show the difference between the injected (lower part of the panel) and uninjected (upper part of the panel) sides at higher magnification). Double staining with 12/101 antibody and DAPI showed that cell orientation was indeed strongly affected in areas

in which the nuclei were randomly distributed (Fig. 6 shows an embryo injected with ΔE; similar results were obtained for C-trunc, not shown). This is probably the consequence of the loss of order during the cell rearrangements required for the rotation of myotomes. Moreover Fig. 6 B gives prominence to the difference of 12/101 antigen expression between the uninjected and injected side of the embryos. In fact, with the immunofluorescence technique, it is possible to better observe that the uninjected side of the embryos displayed a strong reactivity for 12/101 antibody while the injected cells displayed lower reactivity and their cell membranes were less defined.

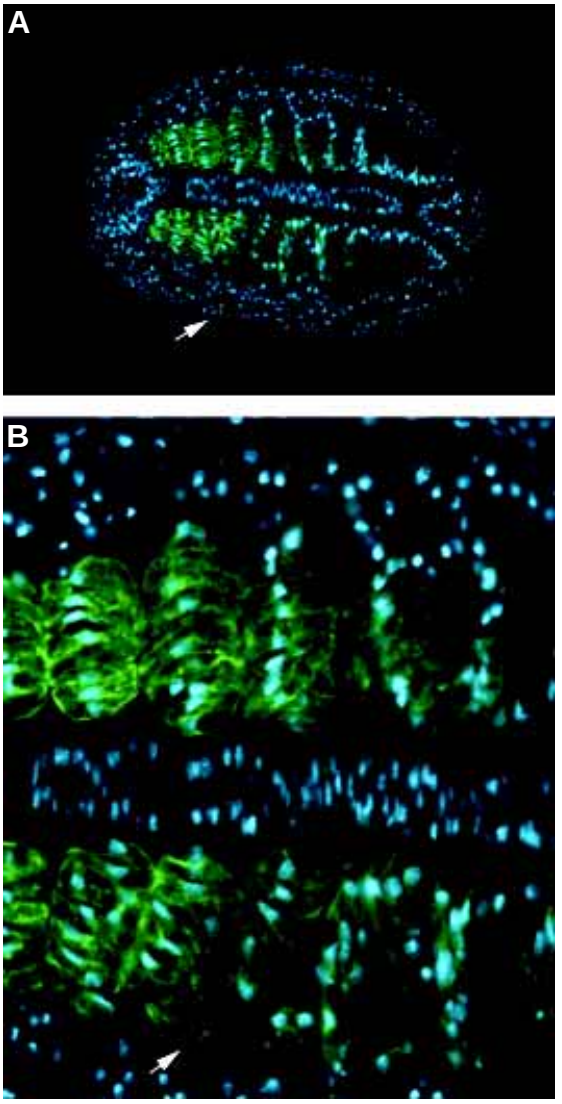

Fig. 6. Nuclear disorganization is accompanied by cellular disorganization. *Sections were obtained from a tailbud embryo injected with* pSBCarΔE. *Double-staining with DAPI (blue) and the 12/101 antibody (green) showed that the loss of nuclear organization was associated with cellular disorganization.* **(A)** *The difference in nuclear symmetry between the injected (the anterior is on the left, the embryo was injected in the left side, arrow) and uninjected sides (X10).* **(B)** *A clear loss of cellular order. The injected side is indicated by an arrow (similar results were obtained for the injection of pSBCarC-trunc).*

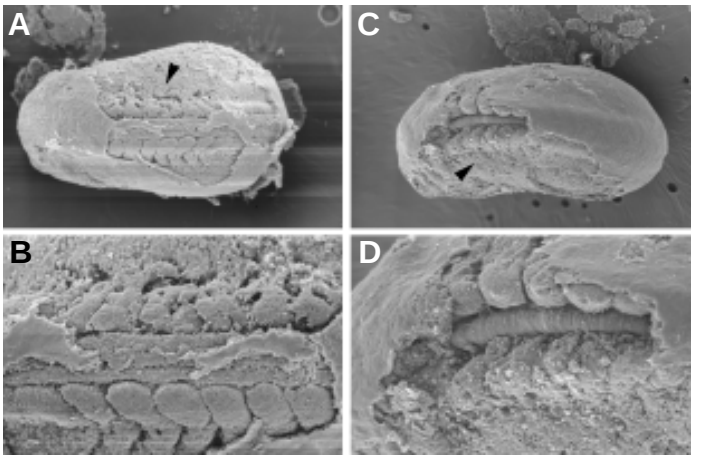

Fig. 7. SEM analysis of embryos injected with *pSBCarΔE* or *pSBCarC-trunc.* *2-cell stage embryos were unilaterally injected (site of injection indicated by an arrow). They were then allowed to develop to the tailbud stage, when they were fixed and processed.* **(A,B)** *show embryos injected with the ΔE construct on the right side (anterior on the left, A: 60x, B: 150x).* **(C,D)** *show embryos injected with the C-trunc construct on the left (C: 60x, D: 150x).*

In some cases, we observed that the first somites formed on the injected side of the embryo were almost normal, possibly because smaller amounts of exogenous protein were produced at early stages of somitogenesis.

We then used scanning electron microscopy (SEM) to analyze the defects induced by the dominant-negative constructs in more detail. Once more it was evident that the injected side of the embryos was highly perturbed following the injection of ΔE-cadherin (Fig. 7 A,B, the embryo was injected in the right side, the anterior is on the left) or C-trunc cadherin (C and D, injected in the left side). The somites of the injected side had a less well-defined shape than those on the control side. They seemed to be only partly developed and did not have the chevron-like or arrowhead shape typical of somites at this stage of development. However, some metameric structures were distinguished. In Fig. 8 (panels A,B,C and D show non-injected embryos, panels E,F,G and H show ΔE-injected embryos, same results were obtained for C-trunc embryos), the somites were observed from the ventral side; myotomes of already formed somites were not typically stacked (compare panels A,B and E,F) confirming what above observed with DAPI staining. It was evident that the rotation movements found in the uninjected embryos were not maintained in the rotating somitomeres of injected embryos. Cells undergoing rotation were different when injected with the dominant negative cadherins (compare panels C,G): they did not have the elongated shape as found with the uninjected cells; and above all, they did not follow the same curved trajectory and were randomly distributed. This erratic distribution gave the appearance of a sort of “extra-somite” indicating that some cells in the somite were somehow taking their own trajectory independently from the rest of the “group”. We also analyzed the effects of the exogenous protein in the single myotomes performing fractures inside the somites. The injected cells were found to be more rounded (panel H), and cell-cell contacts and membrane protrusions were much less frequent than in the control (panel D). It could be hypothesized that injected cells still preserve their migratory ability but their migration process is not coordinated. The

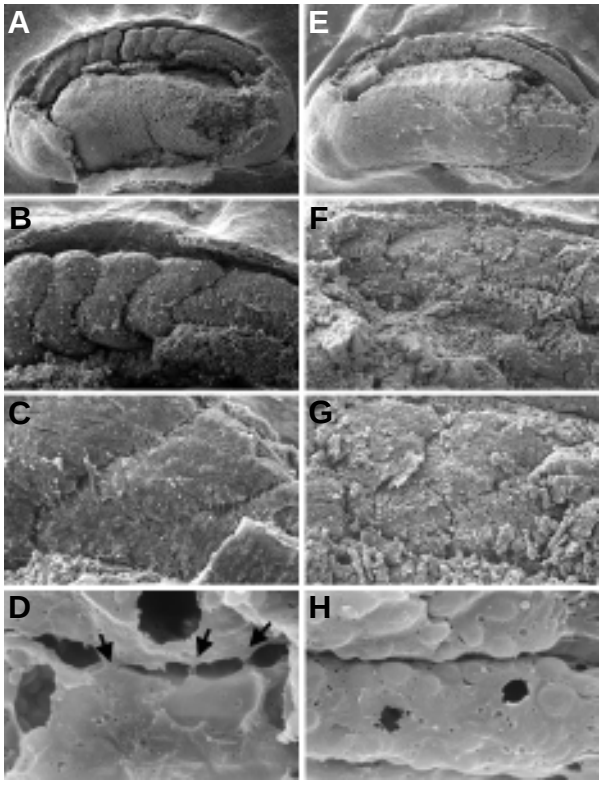

Fig. 8. SEM analysis of embryos injected with *pSBCarΔE* observed from the ventral side. (A) *(60x),* **(B)** *(200x),* **(C)** *(500x) and* **(D)** *(4000x) were obtained from non-injected embryos.* **(E)** *(60x),* **(F)** *(200x),* **(G)** *(500x) and* **(H)** *(4000x) were obtained from* pSBCarΔE *injected embryos (same results were obtained with the C-trunc construct). The three arrows in D show the protusions present in the uninjected somitic cells which were not present in the injected cells (H).*

loss of cell-cell contacts alters the transmission throughout the somitic unit of the forces and the information necessary for a coordinated movement. This hypothesis is in agreement with that of Keller who states that a cell at the interior of the somite has to crawl on the other cells to rotate. Moreover the presented data are consistent with previous studies showing that cadherins play a crucial role in several cell movements during *X. laevis* embryogenesis (Broders *et al.*, 1995; Delarue et *al.*, 1998; Tepass, 1999).

Although typical somitic organization was lost in injected embryos, the segmentation of the mesoderm did not seem to be affected. Thus, dominant-negative cadherins do not seem to interfere with mesoderm segmentation, allowing the formation of somitomeres, but disrupt the conversion of these somitomeres into ordered mature somites.

X-Delta-2 *Expression is not perturbed by Dominant-Negative Forms of Cadherins*

We investigated the expression of *X-Delta-2* in injected embryos to confirm the presence of metameric units observed with 12/101 whole mount immunostaining and with the SEM analysis. *X-Delta-2*, the ligand of Notch, is one of the most well known genes in mesoderm segmentation. Its expression is uniform in unsegmented mesoderm, reduced to the anterior half in somitomeres and lost in mature somites (Jen *et al.*, 1999). This results in a characteristic pattern with three stripes corresponding to the prospective somites. Kim *et al.* have reported that PAPC protocadherin plays an important role during segmentation linked to the Notch pathway (Kim *et al.*, 2000). *In toto in situ* hybridization revealed (Fig. 9) that the expression pattern of *X-Delta-2* was conserved in the injected side of the embryo, whether that embryo was injected with ΔE (panel A) or C-trunc (panel B). Thus, the segmentation signal was not perturbed by the alteration of cell-cell adhesive interactions and our results confirm that the changes induced by dominant-negative cadherins occurred downstream from or independent of the segmentation-signaling pathway.

Discussion

In this paper we demonstrated that perturbation of type I cadherin function affected somite rotation and muscle differentiation. These phenotypic changes were demonstrated using two different dominant negative cadherins and exploiting different experimental approaches.

Injected embryos analyzed at the tailbud stage, had abnormal somites, smaller in mass than normal somites and with morphology not resembling the typical chevron-like conformation. The loss of disorganized arrangement of cells in somite was demonstrated with different experimental methods. At high resolution, electron microscopy showed that the morphology of cells expressing exogenous protein, regardless of whether this protein was the intracellular or the extracellular mutant, differed from that of “wild-type” cells. Cells lost protrusions and contacts with their neighbors suggesting they were no longer migrating normally. It is also likely that the lack of cell-cell contacts may have abolished the sensing mechanisms required to induce these relatively coordinate movements. The cells probably maintain their migratory capability; but without the intercellular contacts, they loose the ability to crawl each across other as proposed by Keller (Keller, 2000). Therefore the cells, lacking cell-cell contacts, are unable to transmit the physical information necessary for their movements. This results in the loss of synchronicity and in the random movement of each single myotomal cell. Unfortunately the difficulties involved in trying to observe the rotation step *in vivo* make it difficult to dissect out the possible mechanisms. Preliminary video microscopy analysis showed that rotation is accompanied by a propagated contraction wave along the entire length of the axis (Morali, unpublished). This implies the necessity for a perfect interplay of forces, which is disrupted in somites expressing the two dominant-negative cadherins. Fundamentally, because the mutated cadherins were targeted to the somites, the results add

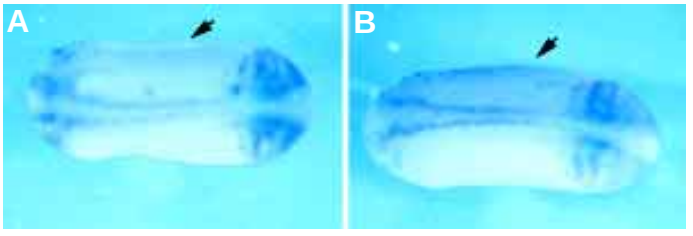

Fig. 9. *X-Delta-2* expression is preserved in embryos injected with the ΔE or C-trunc constructs. *Embryos were unilaterally injected with* pSBCarΔE **(A)** *or* pSBCarC-trunc **(B)** *in the right side (anterior is on the left, the injected side is indicated by an arrow).* X-Delta-2 *expression was detected by* in situ *hybridization, and expression was not altered by either of the mutant proteins.*

more evidence to indicate that the cells within the somites are responsible for the rotation of somite.

The expression of truncated cadherin affected rotation, size and organization of somites but not segmentation. The morphological observations were confirmed by analysis of *X-Delta-2* expression, which was not affected by the production of dominant-negative type I cadherins. This suggests that cadherin function in the developing somite is not directly linked to the signaling pathway involved in segmentation and that segmentation can occur even in the absence of correct somite rotation.

Another major effect of ΔE and C-trunc mutants was the inhibition of muscle differentiation. It consisted of the lowered reactivity of 12/101 antibody and the underexpression of *X-Myo-D,* both differentiation markers of muscle differentiation. Elsewhere *X-Myo-D* expression was found to be dependent on cadherin function (Holt *et al*., 1994). Consistent with these observations, we showed that both of the mutant cadherins studied downregulated *X-Myo-D* expression *in vivo*, whereas Holt *et al.* used only the intracellular mutant (corresponding to ΔE-cadherin). It is likely that the loss of adhesion leads to a loss of the community effect, probably resulting in a decrease in the expression of *X-Myo-D* and consequently of those genes involved in myotube differentiation that are strictly dependent on *X-Myo-D* expression. Hence, the observed difficulties of embryos to swim may have been due to the loss of properly differentiated muscles.

Both the dominant negative cadherins gave rise to the same phenotype. The intracellular mutant affects β-catenin functions by sequestering this protein. Hence the links between actin filaments and endogenous cadherin (normally mediated by β-catenin) may have been disrupted. This would then imply a breakdown in the adhesive properties of these cadherins. In addition to its primary role in connecting cadherin to the actin filaments β-catenin is involved in signaling and thought to be important for the formation of tissue boundaries because it is present at the borders between somites, notochord and neural tube (Fagotto and Gumbiner, 1994). The extracellular dominant negative C-trunc inhibits the formation of cell-cell contacts but does not interfere with β-catenin function. Observing the same effects either with ΔE and C-trunc mutants we showed indirectly that the observed phenotype did not depend on perturbation of the β-catenin signaling pathway or of boundaries. Without excluding other roles for β-catenin during somitogenesis, these experiments emphasize a primary role for cell-cell contacts.

The different penetrance in the phenotype observed at tadpole stage, could be due to the different levels of available protein and its localization in the somitic tissue. Also, mosaic protein expression patterns is a feature with plasmid injections (Vallin *et al*., 2001) and presumably causes variability between embryos. The “kink” phenotype observed in mRNA injected tadpoles probably arose from the temporal activation of the dominant negative ΔE-cadherin. The somitogenesis is perturbed only after hormone induction, allowing a correct formation of the A-P axis prior to this. The “curled” phenotype observed in plasmid injected tadpoles, is the consequence of an early perturbation of the cadherin function.

The present paper shows the importance of cadherins in somitogenesis. The inhibition of their function in the somitic tissue causes two major effects: the loss of co-ordination in cell movements and the inhibition of differentiation. They can be differently described as a physical effect, consisting of a loss of adhesion, and a signaling effect, consisting of the repression of *X-Myo-D* expression.

Materials and Methods

Eggs and Embryos

X. laevis eggs were obtained from females injected with human chorionic gonadotrophin. Eggs were artificially fertilized and dejellied by incubation in MMR-1% cysteine. They were transferred to MMR-5% Ficoll, injected, at the 2- or 32-cell stage, with the cadherin constructs and were allowed to develop at 14 or 18°C until the appropriate stage. Embryos were staged as described by Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

DNA and RNA Constructs

The inducible form of the dominant-negative E-cadherin (ΔE) was produced by PCR amplifying the transmembrane and intracellular domain. The PCR product was cloned, sequenced and subcloned in-frame with the HBDER receptor sequence in the PSPN-tag vector, a modified pSP35 vector created for *in vitro* mRNA synthesis (Dufour *et al*., 1994). The same intracellular domain of E-cadherin was also inserted into p*Sleeping Beauty* (p*SB*) under the control of the cardiac actin promoter (Car).

The dominant-negative form of C-cadherin (C-trunc, kindly provided by Prof. B. Gumbiner), consisting of the extracellular domain of C-cadherin was inserted into p*SB* under the control of the cardiac actin promoter.

Injections

ΔE-cadherin-HBDER mRNA for injections was synthesized with the MEGAscript Kit (Ambion). RNA was resuspended in water with GFP mRNA added as a tracer. 2 nl (corresponding to 3.75 ng of ΔE-HBDER mRNA) of this solution were injected at 32 cell-stage embryos in B2B3B4C2C3C4 and B’2B’3B’4C’2C’3C’4 blastomeres or B2B3B4C2C3C4 blastomeres only, depending on the experiment. The dominant-negative effect of the protein was induced (at stages 14, 25 and 39 N.F.) by carefully removing the vitelline membrane of embryos and adding 10 μM β-estradiol to the medium. 370 pg of plasmid DNA corresponding to the ΔE-cadherin or C-trunc construct into 2 cell-stage embryos. Injections were made into one or both cells, and 185 pg of p*SB*CarGFP was coinjected to act as a tracer. In parallel, embryos were injected withequimolar amounts of p*SB*CarGFP as negative control.

Whole-Mount Immunostaining

Embryos at the tailbud or tadpole stage were fixed by incubation for two hours in Dent fixative (20% dimethylsulfoxide, 80% methanol), rinsed and stored in methanol at –20°C. Embryos were then thoroughly washed in TBST (Tris-buffered saline with 0.1% Triton X-100), incubated overnight at 4°C with the 12/101 antibody, washed thoroughly in TBST and incubated overnight at 4°C with the secondary antibody, conjugated with horseradish peroxidase. The staining has been revealed after washing the secondary antibody, by 3-3’ diaminobenzydine (DAB). The embryos were then observed in benzyl alcohol/benzyl benzoate 1/2.

Nuclear Staining and Immunofluorescence

Nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI). Embryos were embedded in paraffin and sectioned. Sections (8 μm) were then incubated in xylene to remove the paraffin, rehydrated by incubation in a graded series of ethanol concentrations, washed in PBS (phosphate-buffered saline) and incubated with DAPI (1μg/ml in PBS) at room temperature for 30 minutes. For immunofluorescence sections were incubated overnight at 4°C with 12/101 antibody diluted in PBS-10% foetal calf serum (FCS), washed twice in PBS-FCS and incubate for 1 hour at room temperature with a secondary antibody coupled to fluorescein. Sections were then stained with DAPI as above described. Sections were thoroughly washed, mounted in Immunomount and observed with a Leica epifluorescence microscope.

In Situ *Hybridization*

In situ hybridization was performed as described by Harland (Harland, 1991), but with minor modifications as described by Mayor *et al.* (Mayor *et al.*, 1995). *X-Delta-2* and *X-Myo-D* RNAs were obtained from Dr. C. Kintner and Prof. J. B. Gurdon, respectively.

Scanning Electron Microscopy

The vitelline membrane was removed from injected tailbud embryos, which were then fixed by incubation in 100mM-cacodylate buffer and 1.5% glutaraldehyde. The embryos were then gradually dehydrated in ethanol. Critical point drying was performed in ethanol and liquid nitrogen. Dorsal epithelium was peeled away to show the somites and fractures were performed at various levels. Finally, the samples were coated with gold.

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