NF-protocadherin, a novel member of the cadherin superfamily, is required for *Xenopus* ectodermal differentiation

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Background: The assembly of complex tissues during embryonic development is thought to depend on differential cell adhesion, mediated in part by the cadherin family of cell-adhesion molecules. The protocadherins are a new subfamily of cadherins; their extracellular domains comprise cadherin-like repeats but their intracellular domains differ significantly from those of classical cadherins. Little is known about the ability of protocadherins to mediate the adhesion of embryonic cells, or whether they play a role in the formation of embryonic tissues.

Results: We report the isolation and characterization of a novel protocadherin, termed NF-protocadherin (NFPC), that is expressed in *Xenopus* embryos. *NFPC* showed a striking pattern of expression in early embryos, displaying predominant expression within the deep, sensorial layer of the embryonic ectoderm and in a restricted group of cells in the neural folds, but was largely absent from the neural plate and surrounding placodal regions. Ectopic expression in embryos demonstrated that NFPC could mediate cell adhesion within the embryonic ectoderm. In addition, expression of a dominant-negative form of NFPC disrupted the integrity of embryonic ectoderm, causing cells in the deep layer to dissociate, though leaving the outer layer relatively intact.

Conclusions: Our results indicate that NFPC is required as a cell-adhesion molecule during embryonic development, and its function is distinct from that of classical cadherins in governing the formation of a two-layer ectoderm. These results suggest that NFPC, and protocadherins in general, are involved in novel cell–cell adhesion mechanisms that play important roles in tissue histogenesis.

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Background

During early development of the vertebrate embryo, the ectoderm, an epithelium that covers the outside of the embryo, gives rise to different tissue derivatives, most notably epidermal and neural tissue. When ectodermal cells form these derivatives, they acquire the ability to differentially recognize and adhere to other similarly fated cells. This sorting ability was demonstrated by Townes and Holtfreter [1], who dissociated the skin and neural tube from amphibian embryos into single cells and mixed these cells together in explant culture. Over time in culture, the skin cells reassociated and formed an epidermis on the outside of the explant, whereas the neural cells reformed a neuroepithelium characteristic of neural tissue on the inside of the explant. This and subsequent studies have emphasized the importance of differential cell adhesion as one factor governing histogenesis and have led to an investigation of the molecules involved in cell recognition and adhesion [2].

One class of molecules strongly implicated in the differential adhesion of embryonic cells is the cadherin superfamily of calcium-dependent, cell-adhesion molecules [3–7]. The original members of this family, called classical cadherins, have five tandem cadherin-specific repeats in their extracellular domain and a highly conserved cytoplasmic domain that is required for adhesive activity. Classical cadherins are localized to sites of cell–cell contact — the intermediate, or adherens, junction — where they function as dimers, each dimer binding an identical one in an adjacent cell to form a 'cadherin zipper' structure [8,9]. Three intracellular proteins, catenins α, β and γ, promote adherens-junction formation by interacting with a conserved region in the carboxy-terminal cytoplasmic tail, mediating the interaction of cadherins with the actin-based cytoskeleton [10–14]. The role of classical cadherins in histogenesis has been extensively studied. In *Xenopus* embryos, a maternally expressed cadherin, C-cadherin, is present in all cells of the embryo until late neurula stages when the ectoderm forms, after which expression is restricted to the epidermis and skeletal muscle [15–18]. During gastrulation, the dorsal ectoderm begins to express N-cadherin as it forms neural tissue, whereas the ventral ectoderm forms epidermis and expresses E-cadherin. Functional studies indicate that all these cadherins are essential for the structural integrity of the ectoderm and

its derivatives, as was predicted from their key role in the formation of the adherens junction [19,20].

A potentially large group of distantly related cadherin family members, termed protocadherins, has been identified recently in rats and humans [21,22]. Although little is known about the role of protocadherins in embryonic development, their structural properties suggest that they mediate a novel adhesion mechanism. We therefore initiated a screen for protocadherins expressed in *Xenopus* embryos and isolated a cDNA encoding a novel *Xenopus* protocadherin, termed NF-protocadherin (NFPC). The extracellular domain of NFPC contains seven cadherin-specific repeats, and its intracellular domain contains novel sequences that are related to those in human protocadherin-42 (HPC42 or Pchd1). *NFPC* RNA showed a striking pattern of expression in early *Xenopus* embryos, displaying predominant expression within the deep, sensorial layer of the embryonic ectoderm and epidermis, as well as in a restricted group of cells in the neural folds, but was largely absent from the neural plate and the surrounding placodal regions. NFPC could act as a cell-adhesion molecule *in vivo*, inhibiting cell mixing when ectopically expressed in embryos. In addition, expression of a mutant NFPC in which the extracellular domain is deleted resulted in a specific lesion within the deep layer of the embryonic ectoderm and epidermis. These findings indicate that NFPC is required for the histogenesis of the embryonic epidermis, suggesting a role for novel adhesive interactions in the morphological diversification of ectodermal derivatives.

Results

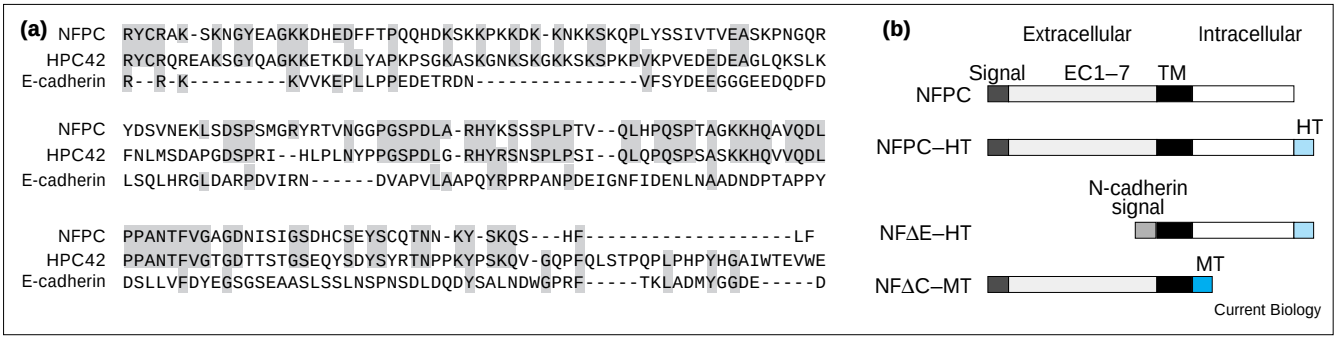
Cloning of *NFPC*

To isolate *Xenopus* protocadherin family members, we used conserved motifs in the extracellular domains of both cadherins and protocadherins to design degenerate oligonucleotides for use as PCR primers. Sequencing of the PCR products identified a novel clone that we then used to isolate a full-length cDNA from a *Xenopus* stage 17 cDNA library. The sequence of this cDNA predicts a 1035 amino-acid transmembrane protein with overall structural similarity to cadherin family members. The extracellular domain of the predicted protein contains seven cadherin-like repeats, rather than the five found in classical cadherins. Like other known protocadherins, the protein does not have a typical pro-sequence nor does its cytoplasmic domain show homology to classical cadherins (Figure 1). Overall, the predicted protein, including the cytoplasmic domain, is most closely related to HPC42 (55% similarity) [22], whereas it is only 14% similar to classical cadherins. On the basis of these structural features, the cDNA we isolated encodes a novel *Xenopus* protocadherin, which we termed NFPC.

Embryonic expression pattern of *NFPC*

To examine the developmental expression pattern of *NFPC*, RNA from pre-gastrulation embryos was collected and analyzed in RNase protection studies. Results indicated that *NFPC* is expressed maternally and is present in the embryo at roughly constant levels from early cleavage to gastrula stages, an expression pattern similar to that of *C-cadherin* (Figure 2). A more precise localization of *NFPC* RNA expression was obtained using whole-mount *in situ* hybridization. Prior to gastrulation, *NFPC* was expressed at low levels throughout the ectoderm; by the end of gastrulation and at early neurula stages, however, *NFPC* RNA could be easily detected in the ectoderm, with strong staining on the ventral side, which gives rise to epidermis, and little or no staining on the dorsal side, which gives rise to neural tissue (Figure 3a). Similarly, at later stages,

Figure 1

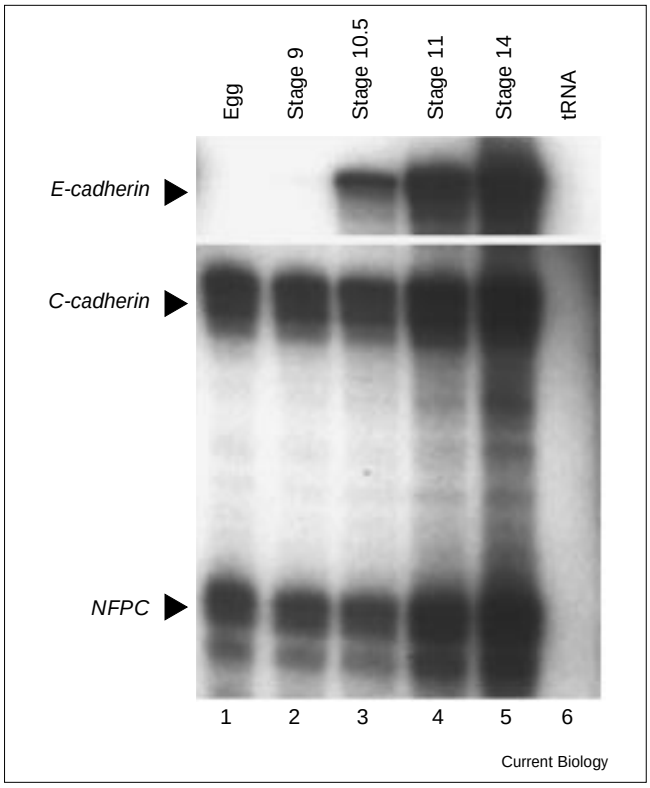


(a) Alignment of the cytoplasmic domain of *Xenopus* NFPC with that of HPC42 and to the classical cadherin E-cadherin. Whereas NFPC contains seven repeat regions within the extracellular domain (EC1–7) that identify it as a member of the cadherin family, the cytoplasmic domain is not homologous to classical cadherins and identifies NFPC as a member of the protocadherin subfamily. Identical residues are shaded. (b) Products of the *NFPC* constructs used in this study. NFPC–HT is the product of the entire coding of region of *NFPC* with six copies of the His-epitope tag (HT) at the carboxyl terminus. In NFΔE–HT, most of the extracellular domain of NFPC–HT, including its signal sequence, was replaced with the signal sequence of N-cadherin, and in NFΔC–MT, the cytoplasmic domain of NFPC was replaced with six tandem Myc-epitope tags (MT). TM indicates the transmembrane domain.

NFPC RNA was expressed at high levels in the differentiating epidermis, but was absent from the neural plate and neural tube (Figure 3b,c). A control *in situ* hybridization with a sense *NFPC* probe showed no detectable staining (data not shown). A comparison with other genes expressed in the embryonic ectoderm revealed that *NFPC* expression closely paralleled that of *E-cadherin* and *epidermal keratin*, two genes involved in epidermal differentiation. Both *E-cadherin* and *epidermal keratin* were expressed after gastrulation in the embryonic ectoderm but during neural induction became excluded from the presumptive neural plate and neural tube (Figure 4c,d) [23,24]. In addition, like the expression of *E-cadherin* and *epidermal keratin*, *NFPC* expression was excluded from the placodal regions, marked in part by the expression of *Xslug*, which labels the cranial neural crest (Figure 4b) [25]. In contrast to *epidermal keratin* and *E-cadherin*, however, the early expression of *NFPC* included a stripe of cells located within the neural folds, apparently marking the point where the converging neural folds will fuse (Figures 3b,3c,4a). In sum, the early expression of *NFPC* followed the differentiation of ectoderm into epidermal tissue but also included a discrete stripe of cells within the neural folds. Because of the striking expression in the neural folds, we named this gene *NF protocadherin*.

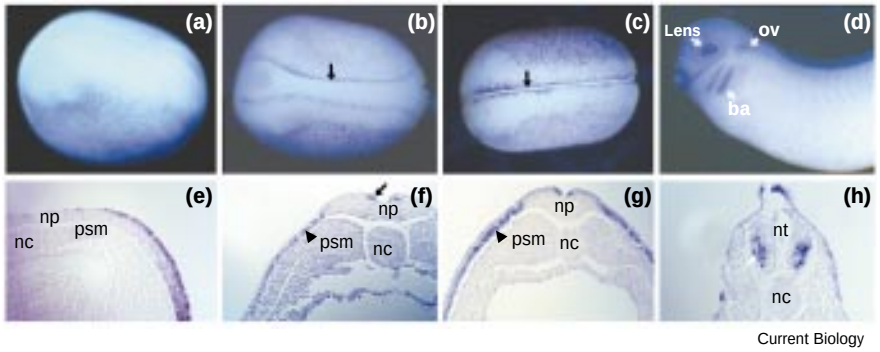
The ectoderm of *Xenopus* is unusual in that it consists of two layers, an inner cell layer (the sensorial layer) and an outer cell layer (the epithelial cell layer). Sections through embryos stained for *epidermal keratin* RNA reveal strongest expression of this RNA in the outer, epithelial layer (Figure 4f), as has also been reported for *E-cadherin* ([23] and data not shown). In contrast, whereas *NFPC* is expressed in both ectodermal layers at stage 14 (Figure 3e), at later stages it is most highly expressed in the deeper, sensorial layer (Figures 3f,3g,4e).

Figure 2



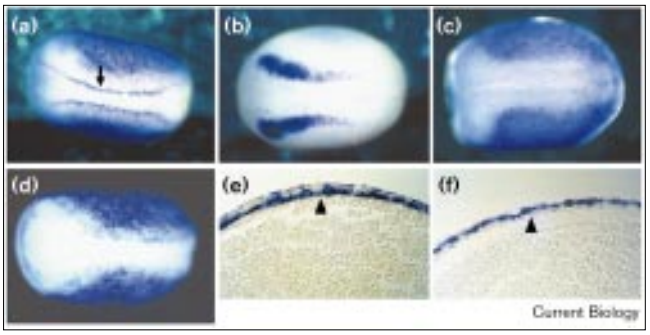
RNase protection analysis of *NFPC* transcripts present in early *Xenopus* embryos. RNA from embryos at various stages was isolated and assayed simultaneously for the presence of *NFPC*, *C-cadherin*, and *E-cadherin* RNA. The positions of the protected fragments are shown on the left and the stages analyzed are indicated. As a negative control, the assay was carried out on tRNA (lane 6). Whereas both *NFPC* and *C-cadherin* were expressed at roughly equal levels in all stages examined, *E-cadherin* expression began at stage 10.5 and increased thereafter.

Figure 3



NFPC is expressed in embryonic ectoderm. Embryos at (a,e) stage 14, (b,f) stage 16, (c,g) stage 18 and (d,h) stage 28 were stained by *in situ* hybridization for *NFPC* and viewed (a–d) in whole mount or (e–h) in cross-section. (a,e) At stage 14 (early neural plate), *NFPC* was restricted to the ventral ectoderm and was not expressed in the neural plate (np). In (e), the right-hand side of the dorsal side of the embryo is shown. (b,f) As the neural plate folds to become the neural tube (stage 16), *NFPC* was expressed in the prospective epidermis and at the tips of the neural folds (arrow), and this pattern continued as the neural folds meet in the dorsal midline (c,g). (f,g) Transverse sections through stained embryos revealed that, within the ectoderm, *NFPC* was more highly expressed in the inner layer (arrowhead). (d,h) At tailbud stages, *NFPC* was expressed in the epidermis, branchial arches (ba), lens, otic vesicle (ov), and also within presumptive motor neurons (white arrow in h) in the caudal neural tube (nt) and in the dorsal fin. Other abbreviations: nc, notochord; psm, pre-somitic mesoderm.

Figure 4



Comparison of *NFPC* expression with known ectodermal markers. Stage 18 embryos were analyzed by *in situ* hybridization for expression of (a,e) *NFPC*, (b) *Xslug*, (c) *E-cadherin*, or (d,f) *epidermal keratin*. *NFPC*, *E-cadherin* and *epidermal keratin* showed similar expression patterns in whole mount, expression being restricted to the ventral ectoderm and excluded from the neural plate and placodal region, as marked by expression of *Xslug* (b). *NFPC* was also uniquely expressed within the neural folds, however; arrow in (a). Transverse sections revealed that, within the ectoderm, *NFPC* was most highly expressed in the sensorial layer whereas *epidermal keratin* was most highly expressed in the outer layer; arrowheads in (e,f).

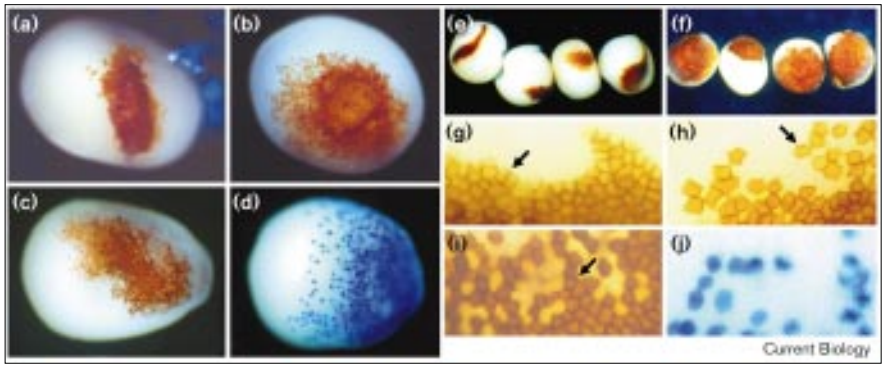
At tailbud stages (stage 28), expression of *NFPC* is still detected in the epidermis, within the developing dorsal fin, and in the embryonic eye, otic vesicles and branchial arches (Figure 3d). Importantly, sections through tailbud-stage embryos reveal that *NFPC* is also expressed in presumptive neuronal cells within the central nervous system (Figure 3h). Staining in neuronal cells is detected within the presumptive motor neurons of the ventral spinal cord as early as stage 22, thus correlating expression with the onset of neurite outgrowth. Similarly, in the eye, staining for *NFPC* can be detected in the ganglion-cell layer as early as stage 35 and in the lens at stage 25 (data not shown).

NFPC as an adhesion molecule

The expression pattern of *NFPC* described above indicates that NFPC could be involved in the formation of the embryonic epidermis. Although NFPC lacks the conserved tripeptide HAV (in single-letter amino-acid code) cell-binding domain and the catenin-binding domain of classical cadherins, other studies have demonstrated a weak adhesive function for protocadherins *in vitro* [22]. Thus, we asked whether NFPC might contribute to epidermal differentiation by acting as a cell-adhesion molecule. To test the ability of NFPC to mediate cell–cell adhesion *in vivo*, we employed a cell-mixing assay used previously to examine the adhesive properties of the *Xenopus* proteins N-cadherin and F-cadherin [26]. In this assay, RNA encoding NFPC tagged with the His epitope (NFPC–HT) was injected into a single, animal blastomere of a *Xenopus* embryo at the 8–16-cell stage. Embryos were allowed to develop until the neural plate stage (stage 14), at which time they were fixed and processed for immunohistochemistry with an antibody against the His epitope. In control embryos injected with cytoplasmic *lacZ* RNA, cells derived from the injected blastomere intermingled with cells from neighboring blastomeres, showing extensive cell mixing at the borders of the patch of cells expressing β galactosidase (Figure 5d,j). In contrast, embryos injected with *NFPC–HT* exhibited a more cohesive clone of immunostained cells, revealing little cell mixing: most immunostained cells were in contact with another immunostained cell (Figure 5a,e,g). In addition, antibody staining for NFPC–HT was concentrated at sites of cell–cell contact, suggesting that NFPC is binding homotypically.

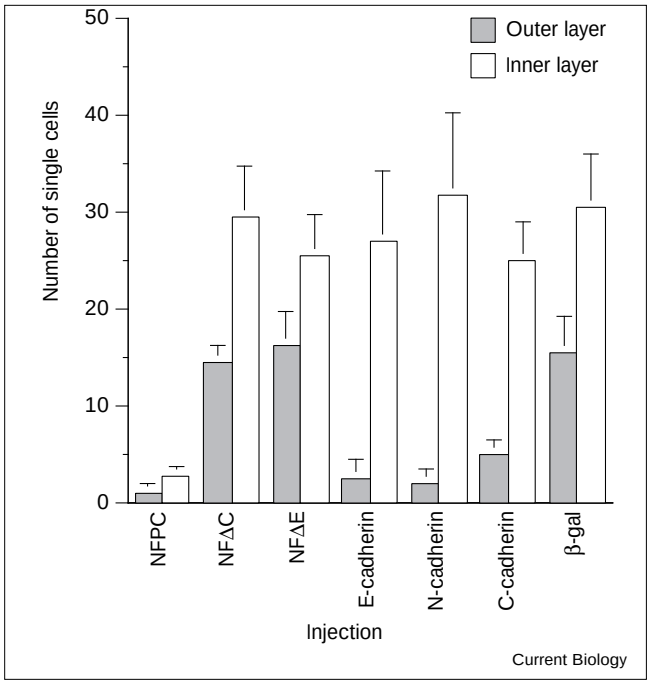
Quantification of cell mixing was obtained from serially sectioned immunostained embryos by counting the number of labeled single cells in both the inner and outer layers that were not in contact with other labeled cells.

Figure 5



Ectopic expression of NFPC can inhibit ectodermal cell mixing. Embryos were injected with RNA encoding NFPC–HT or with various mutant constructs, fixed at stage 14, and examined by immunohistochemistry for the His or Myc epitope tag. (a,e,g) Embryos injected with *NFPC–HT* showed a cohesive cluster of expressing cells, indicating little cell mixing between progeny of injected and uninjected blastomeres. In contrast, embryos injected with (b,f,h) *NFΔE–HT*, and (c,i) *NFΔC–MT* showed extensive cell mixing with neighboring, non-expressing cells, in a similar way as (d,j) control embryos injected with RNA encoding cytoplasmic β galactosidase. Only embryos injected with *NFΔE–HT* exhibited ectodermal blistering (b,f). (g–j) High-power photomicrographs of the same embryos showed that whereas NFPC–HT (g), NFΔE–HT (h), and NFΔC–MT (i) were all correctly expressed at the cell membrane, only NFPC–HT and to a lesser extent NFΔC–MT were concentrated at sites of cell–cell contact – compare site indicated in (g) and (i) to the one in (h) – suggesting that NFPC acts as a homophilic adhesion molecule.

Figure 6

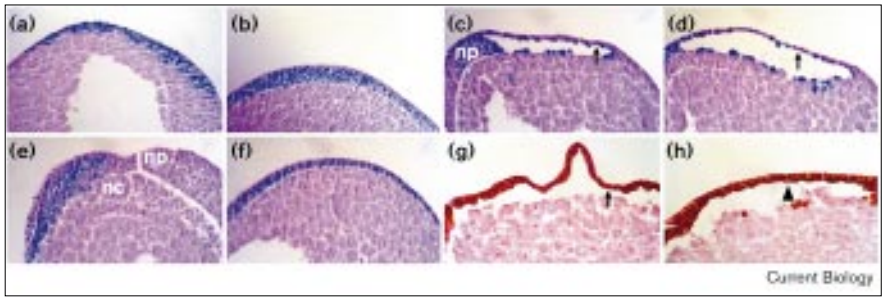


NFPC inhibits cell mixing in both the inner and outer ectodermal layers. To quantify cell-mixing assays, embryos injected as described in Figure 5 with RNAs encoding the indicated proteins were immunostained, serially sectioned (10 μm thick sections), and analyzed for single, labeled cells that were surrounded by non-expressing cells. Single, labeled cells were counted and the mean number and SEM determined for 10–15 embryos for each RNA injected. As a negative control, embryos were injected with *nlacZ* RNA and stained for β-galactosidase activity (β-gal). In general, cells within the inner layer showed more extensive cell mixing than did outer-layer cells; ectopic NFPC expression inhibited cell mixing in both layers, however. In contrast, embryos injected with RNA for the classical cadherins N-cadherin, C-cadherin and E-cadherin showed reduced cell mixing amongst only the outer-layer cells.

Quantification of control embryos injected with nuclear *lacZ* RNA (*nlacZ*) showed that extensive cell mixing occurred within the outer layer (15.5 single cells per embryo) and to a greater extent within the inner cell layer (30.5 single cells per embryo; Figure 6). Ectopic expression of a classical cadherin markedly reduces mixing of outer-layer cells. For example, expression of N-cadherin within the ectoderm resulted in a reduction of outer layer cell mixing to two single cells per embryo; no classical cadherin had an effect on mixing of inner-layer cells, however. In contrast, ectopic expression of NFPC–HT inhibited cell mixing within both outer and inner layers, resulting in an average of 1 and 2.6 single cells, respectively. Thus, ectopic expression of NFPC can suppress cell mixing in both ectodermal layers, implying that NFPC can act as a cell-adhesion molecule *in vivo*.

To examine the adhesive properties of NFPC further, and to determine whether the adhesive activity of NFPC required the extracellular and/or intracellular domains, two mutant forms of NFPC were constructed (Figure 1b): one in which the majority of the extracellular domain is deleted (NFΔE–HT), and one in which the entire cytoplasmic domain is deleted (NFΔC–MT). Both forms were tested in the cell-mixing assay described above. Not surprisingly, NFΔE–HT did not have an effect on cell mixing, implying that the cytoplasmic domain alone cannot mediate cell adhesion (Figures 5b,6). Interestingly, NFΔC–MT was not able to mediate cell adhesion either, suggesting that the cytoplasmic domain of NFPC is required for adhesion activity in this assay (Figures 5c,6). Examination of the border regions around immunostained cells revealed that NFΔC–MT was correctly localized to the cell membrane at the regions that contact another expressing cell, although to a lesser extent than the full-length His-tagged NFPC (Figure 5g,i). In contrast,

Figure 7



Ectopic expression of NFPC and NFΔE in ectoderm. Embryos were co-injected with RNA encoding either (a) NFPC, (b) NFΔC–MT or (c,d) NFΔE–HT together with *nlacz* RNA, fixed at stage 14, stained for β-galactosidase activity and sectioned. Compared to (f) control embryos injected with *nlacZ* RNA, embryos injected with (a) *NFPC* and (b) *NFΔC–MT* were relatively normal, with occasional thickening of the ectoderm observed with *NFPC* (a). In contrast, *NFΔE–HT*-injected embryos had ectodermal blisters, with the ectodermal inner-layer cells reduced or missing; arrow in (c) and (d). (e) *NFΔE–HT*-injected embryos in which ectopic expression was targeted to the neural plate region were relatively normal, with no ectodermal blistering nor loss of inner-layer cells observed, demonstrating that the lesions observed with NFΔE are restricted to the ectodermal region that normally expresses *NFPC*. To rescue NFΔE-induced blisters, embryos were (g) injected with RNA encoding NFΔE–HT or (h) co-injected with RNA encoding NFPC and NFΔE–HT, immunostained for the His epitope and sectioned. Co-expression of NFPC with NFΔE–HT resulted in rescue of the inner cell layer over much of the ectoderm; arrow in (g) compared with arrowhead in (h). Abbreviations: np, neural plate, nc, notochord.

Table 1

Rescue of NFΔE-induced blisters by NFPC.

RNA injected	Number of embryos examined	Number with blisters	Blisters* (%)
NFΔE–HT	97	67	69.0
NFΔE–HT + *NFPC*	66	13	19.7
NFΔE–HT + *E-cadherin*	28	15	53.6
NFΔE–HT + *N-cadherin*	35	24	68.6
NFΔE–HT + *C-cadherin*	18	6	33.3

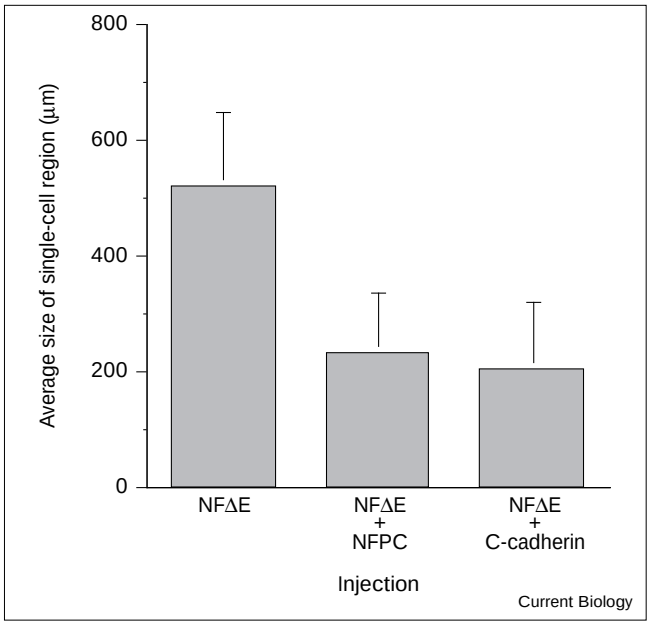
Embryos were either injected with RNA encoding NFΔE–HT or co-injected with *NFΔE–HT* RNA and four times the amount of *NFPC*, *E-cadherin*, *N-cadherin* or *C-cadherin* RNAs. Embryos were immunostained for the His-epitope tag and scored for the appearance of ectodermal blisters. *Percentage of embryos with ectodermal blisters.

NFΔE–HT localized to the entire plasma membrane and was not found preferentially at the contact regions between expressing cells (Figure 5h). This suggests that NFPC can act as a homophilic adhesion molecule, requiring the extracellular domain to interact with another cell and the intracellular domain to mediate adhesion.

NFΔE as a dominant-negative inhibitor of cell adhesion

Embryos injected with *NFΔE–HT* RNA were often observed to have large ectodermal blisters (Figure 5b,f). These blisters co-localized with the domains of ectopic NFΔE–HT expression and were not seen in embryos injected with *NFPC–HT*, *NFΔC–MT* or *lacZ* RNAs. To study this further, a single blastomere at the 16-cell stage was injected with RNA encoding NFPC, NFΔC–MT or NFΔE–HT along with *nlacZ* to mark the site of injection. Embryos were allowed to complete gastrulation, fixed at stage 14–15 and processed for β-galactosidase activity. Sections through *NFPC*-injected embryos showed that the ectoderm was relatively normal in morphology, although in some cases the sensorial layer appeared to be slightly thicker than normal (Figure 7a). Similarly, although embryos injected with *NFΔC–MT* showed extensive cell mixing comparable to controls, sections through these embryos appeared relatively normal (Figure 7b). In contrast, in embryos injected with *NFΔE–HT*, the outer layer of ectodermal cells appeared relatively intact, but the inner layer was usually missing or reduced (Figure 7c,d). Interestingly, *NFΔE–HT* expression altered the morphology of epidermal tissue but did not appear to affect the morphology of tissue in the placodal regions or in the neural plate (Figure 7e). Thus, the disruptive effect of ectopic *NFΔE–HT* expression is limited to the regions that normally express *NFPC*, indicating that NFΔE–HT might have a dominant-negative action, inhibiting the normal function of NFPC during epidermal histogenesis.

Figure 8



Ectopic expression of NFPC or C-cadherin inhibits the loss of inner-layer cells caused by NFΔE. Embryos were injected with RNA encoding either NFΔE–HT alone or co-injected with RNA encoding NFΔE–HT and NFPC or C-cadherin, as in Table 1. Immunostained embryos were serially sectioned (10 μm thick sections) and analyzed for rescue of the inner-layer cells by counting the number of consecutive sections in which the inner layer had been lost. Results shown are the mean of 10–15 embryos analyzed for each RNA injection. Thus, co-expression of either NFPC or C-cadherin with NFΔE can inhibit blister formation by partially rescuing the loss of inner-layer cells.

NFΔE-induced blisters are rescued by NFPC

To establish whether NFΔE–HT has a true dominant-negative action, inhibiting the normal function of NFPC, we asked whether co-injection of full-length *NFPC* RNA prevents the blisters produced by NFΔE–HT. As summarized in Table 1, co-injection of *NFΔE–HT* and *NFPC* RNA reduced the incidence of blisters observed with *NFΔE–HT* RNA alone. To quantify the effect of NFPC in rescuing blister formation by preventing or reducing the loss of the ectodermal inner cell layer, embryos co-injected with *NFΔE–HT* and either *NFPC* or *C-cadherin* RNA were sectioned, and the average size of a continuous single-cell ectodermal layer was determined from several sectioned embryos. Whereas *NFΔE–HT* injection resulted in the loss of most of the ectodermal inner cell layer, co-expression of NFPC and NFΔE–HT could rescue this phenotype, ameliorating the loss of inner-layer cells (Figure 7g,h). As shown in Figure 8, injection of *NFΔE–HT* RNA resulted in a single-cell ectodermal region averaging 520 μm,

whereas co-injection of *NFΔE–HT* and *NFPC* could reduce the area of a single-cell ectoderm by 55% to an average of 233 μm, due to the rescue of the inner cell layer over much of the injected region (Figure 7h). This ability of NFPC to at least partially rescue the defect induced by NFΔE–HT indicates that NFΔE–HT does indeed have a dominant-negative action, inhibiting the normal function of NFPC in the ectoderm. Interestingly, the loss of inner-layer cells induced by NFΔE–HT could also be partially rescued by co-injection of *C-cadherin* RNA (Figure 8), indicating that a loss in cell adhesion mediated by C-cadherin may play a role in NFΔE–HT-induced blister formation. In contrast, RNA encoding *Xenopus* N-cadherin or E-cadherin could not rescue NFΔE–HT-induced blister formation. Thus, not all cadherin family members could rescue the blisters induced by NFΔE–HT, implying that simply increasing adhesion by overexpressing a functional cadherin is not sufficient to rescue the ectodermal disruption.

Discussion

The histogenesis of epithelial tissues such as the embryonic ectoderm and its derivatives is known to depend on differential cell adhesion [1]. This adhesion is mediated in part by the classical cadherins, which, as integral components of adherens junctions, are critical for holding embryonic cells together during tissue morphogenesis. Here, we provide evidence that, in addition to the classical cadherins, the differentiation of the ectoderm requires a novel adhesion mechanism mediated by the protocadherin NFPC.

NFPC as an adhesion molecule

Cell-mixing assays demonstrate that NFPC can function as a homophilic cell-adhesion molecule *in vivo*. Indeed, in these assays NFPC can mediate cell adhesion as efficiently as the classical cadherins, N-cadherin, C-cadherin and E-cadherin (Figure 6). These observations appear to be at odds with previous studies in which the adhesive activity of the protocadherins PC42, PC43, and Pchd3 was measured as calcium-dependent cell aggregation after transfection into culture cells. Although classical cadherins promote adhesion strongly in these assays, protocadherins are much less effective [21,22]. Typically, the *in vitro* cell-aggregation assays use non-adherent fibroblasts, and these fibroblasts already express the catenins that are required for adhesion mediated by classical cadherins. Thus, one interpretation of these results is that the protocadherins perform better in embryos than *in vitro* because they require co-factors that are present in embryonic cells but absent from the cultured cell lines used in aggregation assays.

Several other lines of evidence support the idea that co-factors are required for NFPC-mediated adhesion and that these co-factors are different from the catenins. Firstly, removing the intracellular domain of NFPC disrupts its ability to promote adhesion in the cell-mixing assays even though NFΔC is still expressed at the cell surface and accumulates at sites of cell–cell contact. Thus, like the classical cadherins, the cytoplasmic domain of NFPC is essential for cell adhesion. Secondly, expressing the intracellular domain of NFPC (NFΔE) produces a cell-dissociation phenotype that differs significantly from that produced by similar forms of the classical cadherins. Whereas NFΔE disrupts only the inner-layer cells in the ectoderm and epidermis, corresponding to the region of highest *NFPC* expression, ectopic expression of the cytoplasmic domain of E-cadherin or N-cadherin produces severe lesions in both layers of the ectoderm and in both epidermis and neural tissue [19]. Finally, as the cytoplasmic domain of NFPC is not homologous to classical cadherins, it is likely that NFPC does not interact with catenins but requires other cellular factors to mediate adhesion. Indeed, affinity purification of NFPC–HT does not co-purify β-catenin (data not shown), suggesting further that the cytoplasmic domain of NFPC must interact with other, potentially novel, intracellular effectors of cell adhesion, which must be present in both inner-layer and outer-layer cells.

In marked contrast to the classical cadherins, which appear to mediate adhesion in the outer ectodermal layer only, NFPC is also a potent mediator of cell adhesion among inner-layer cells. Together with the evidence that NFPC interacts with novel intracellular proteins, this implies that NFPC could be a component of a new adhesive complex, distinct from classical cadherins, that plays an important role in tissue histogenesis.

NFPC is required within the embryonic ectoderm

Several lines of evidence indicate a role for NFPC in the formation of the embryonic ectoderm and the subsequent differentiation of the ectoderm into epidermal tissue. *NFPC* is maternally expressed and upregulated in the ectoderm during gastrulation, becoming restricted to ventral ectoderm that will give rise to epidermis, and is absent from the dorsal neural plate. Significantly, the expression of *NFPC* within the ectoderm follows its differentiation into epidermis, but in contrast to most genes associated with epidermal differentiation, including *E-cadherin* and *epidermal keratin*, the expression of *NFPC* is primarily restricted to the deeper, sensorial layer. The role of NFPC in the ectoderm was tested by expressing a dominant-negative form of NFPC, NFΔE, which lacks an extracellular domain. Whereas NFΔE had no effect on the histogenesis of the neural plate and tube, suggesting that NFPC does not have an essential function in dorsal ectoderm, NFΔE did cause specific defects in the inner sensorial layer, implying that NFPC plays an important role in the formation of this ectodermal layer.

The histogenesis of different ectodermal derivatives

The properties of NFPC are consistent with the fact that in *Xenopus* embryos the ectoderm is organized into two

distinct layers each with different characteristics. Whereas the ectodermal outer layer forms an occluding epithelium, the inner-layer cells remain loosely organized. At late blastula and early gastrula stages, as the ectoderm thins in the process of epiboly, these two layers undergo different programs of histogenesis. The outer layer spreads by a change in cell shape and cell division, whereas the inner cells spread primarily by radial intercalation of deeper cells [27,28]. Finally, once formed, the inner and outer layers contribute separately to the differentiating epidermis. Thus, NFPC activity can account for the differences observed in the two layers of the ectoderm; these differences could previously be explained only by C-cadherin, which is expressed ubiquitously prior to gastrulation, and by E-cadherin, which is expressed in both layers of the ectoderm after gastrulation.

Although we conclude that NFPC plays an essential role in the histogenesis of the epidermal inner cell layer, the exact stage at which it is required is not yet clear. As *NFPC* expression occurs throughout early embryogenesis, it could be required early, during epiboly, when the deep ectodermal cells begin to rearrange by intercalation. Conversely, NFPC may not be required until later, to maintain the inner layer after it forms, presumably by mediating adhesion of the inner-layer cells to each other and/or to the outer layer. We note that expression of NFΔE results in a loss of the inner layer, rather than a mere separation of the two layers, and that this defect was detected as early as stage 11, soon after ectoderm begins to spread during epiboly. These observation indicate that NFΔE prevents deep cells from ever forming a distinct inner layer, suggesting a mechanism whereby NFPC functions in the intercalation of the inner-layer cells that occurs during gastrulation and epiboly. Thus, NFPC may contribute to the formation of the two cell layers, and its activity may explain the differences between these two layers in terms of their morphology, behavior during epiboly, and contribution to the epidermis.

Rescue experiments

Our results suggest strongly that the inner and outer layers of the ectoderm have different requirements for the distinct adhesive mechanisms represented by the classical cadherins and NFPC. In ectopic expression studies, NFPC mediated adhesion in both inner and outer layers, whereas classical cadherins mediated adhesion in only the outer layer. Dominant-negative mutants of classical cadherins caused both layers to dissociate, whereas the dominant-negative *NFΔE* construct caused a dissociation of just the inner cell layer. Moreover, although this defect could be partially rescued by NFPC, it was not rescued by co-expression of N-cadherin or E-cadherin, indicating that loss of NFPC cannot be compensated by simply increasing cellular adhesion in the ectoderm. These observations support the idea that NFPC and classical cadherins play different roles in the formation of the ectoderm, reflecting the different properties of the inner and outer layer of the ectoderm.

Interestingly, NFΔE-induced blisters were partially rescued by co-injection of C-cadherin, suggesting that although they represent distinct adhesion mechanisms, NFPC and C-cadherin may interact. Both NFPC and C-cadherin, but not N-cadherin or E-cadherin, are maternally expressed and are present in the ectoderm at the time NFΔE-induced blisters form. Lee and Gumbiner [29] have shown that expression of a dominant-negative C-cadherin, consisting of the extracellular and transmembrane domains, results in ectodermal disruptions that begin with a loss in adhesiveness of the inner cell layer and progress to a larger lesion in both inner and outer cell layers. Thus, C-cadherin may also be involved in formation of the inner cell layer and it is possible that both NFPC and C-cadherin act in concert in epidermal histogenesis. There does not, however, appear to be a direct association between NFPC and C-cadherin, as affinity purification of NFPC–HT does not co-purify C-cadherin (data not shown).

Expression of *NFPC* in neural tissue

Although the analysis of NFPC has so far focused on its role in the ectoderm, we note several other sites of *NFPC* expression that may also indicate a role in tissue histogenesis. One of these sites is a discrete group of cells within the neural folds, corresponding to the site where the folds fuse during neural-tube closure. Preliminary results suggest that although ectopic expression of *NFΔE* can effect the integrity of the neural folds, it cannot prevent neural-fold fusion (data not shown). How this group of cells arises, and whether NFPC plays a role in its localization within the neural folds, are important issues to address in the future.

Another important site of *NFPC* expression is within the differentiating neurons of the neural tube. The onset of *NFPC* expression in the spinal cord appears to correspond roughly to the outgrowth of axons from both sensory and motor neurons. In particular, we have observed that *NFPC* expression in the eye occurs in ganglion cells, appearing around stage 35, when these cells first begin to differentiate and send axons to the optic tectum (data not shown). Thus, it will be of interest to determine whether NFPC is required for axonal outgrowth, as found previously for classical cadherins [30].

Conclusions

Each new tissue derivative that forms during embryonic development has its own characteristic cytoarchitecture, which is thought to depend in part on factors that produce differential cell adhesion [1]. Thus, a major goal in developmental biology is to determine how cells in different tissues, as well as within a tissue, adhere to each other during tissue histogenesis. In this study, we have examined the role of NFPC, a new member of the protocadherin

family, as a mediator of differential cell adhesion during the histogenesis of epidermal tissue in *Xenopus* embryos. Our results provide strong evidence that the correct cytoarchitecture of epidermal tissue requires NFPC, which acts to mediate adhesion among the deep, sensorial cells. Although NFPC can act as a homophilic adhesion molecule *in vivo*, it appears to represent a novel cell-adhesion complex that is distinct from that of classical cadherins. Thus, the role of NFPC in mediating cell adhesion during differentiation of the epidermis has important implications regarding how different mechanisms of cell adhesion might contribute to tissue morphogenesis.

Materials and methods

Isolation of NFPC *cDNA*

Degenerate oligonucleotides corresponding to conserved repeat regions in cadherin extracellular domains were used as PCR primers to amplify sequences from cDNA prepared from *Xenopus* stage 20 neural tubes. The 5′ primer was 5′-AC|GC|C/TC|C/TT|GAT/CCG|GA-3′ and the 3′ primer was 5′-AA|GC|GG|GCA/GTTA/GTCA/GTT-3′. Forty rounds of amplification were performed, denaturing at 94°C for 1.5 min, annealing at 45°C for 2 min and polymerizing at 72°C for 3 min, using Amplitaq DNA polymerase (Perkin Elmer). PCR products were subcloned into pBluescript II KS (Promega) and sequenced. A novel 450 bp PCR product was chosen to screen a λgt10 *Xenopus* stage 17 cDNA library [24] under high-stringency conditions, yielding a full-length cDNA encoding NFPC (GenBank accession number AF043643). Alignment between NFPC, HPC42 and E-cadherin sequences was made using the Megalign program (DNAStar).

Constructs

A 4.5 kb *Eco*RI fragment containing the entire NFPC coding region was subcloned into CS2+ (gift of D. Turner) to give NFPC/CS2. A 1.4 kb *Eco*RV–*Hind*III fragment encoding approximately 20 extracellular amino acids, the transmembrane domain and the cytoplasmic domain of NFPC was ligated to the N-cadherin signal sequence [19] and cloned into CS2+ to create NFΔE/CS2. Carboxy-terminal His-tagged constructs were generated by inserting an oligonucleotide encoding the sequence RGS–His_6 into the *Xho*I/*Xba*I site of the above constructs, to give *NFPC–HT* and *NFΔE–HT*, respectively (Figure 1b). The *NFΔC–MT* construct encodes the extracellular and transmembrane domains of NFPC, with the entire cytoplasmic domain replaced with the Myc-epitope tag from CS2+MT (gift of D. Turner). Recombinants were verified by sequencing. N-cadherin [31] and E-cadherin [20] were cloned into CS2+, and *Xenopus* C-cadherin in SP64T was a generous gift of B. Gumbiner [29]. The cytoplasmic β-galactosidase construct CS2–*lacZ* and nuclear β-galactosidase construct CS2–*nlacZ*, used as controls, were gifts of D. Turner [32].

RNase protection assay

Total RNA was isolated from early embryos and analyzed as described [33]. Transcripts encoding NFPC were detected using a 500 bp probe corresponding to the NFPC intracellular domain, C-cadherin was detected using a probe corresponding to a PCR fragment generated to the C-cadherin intracellular domain, and E-cadherin was detected with a probe encoding the third and fourth extracellular domains [5].

In situ *hybridization and histology*

In situ hybridization was carried out according to the procedure of Harland [34]. *NFPC in situ* hybridizations utilized a 6 kb probe corresponding to the entire coding region and approximately 3 kb untranslated sequence. The *E-cadherin* and *epidermal keratin in situ* probes have been described [5,24]. The *Xslug* probe is a 880 bp fragment, corresponding to the entire coding region cloned into pBluescript (generous gift of A. Bang). After *in situ* hybridization, embryos were embedded in paraplast, serially sectioned into 10 μm thick sections, and photographed under a phase-contrast microscope.

RNA synthesis and injection

Capped RNA was synthesized *in vitro* from linearized DNA template using SP6 RNA polymerase (Promega) as described [31]. A single animal hemisphere blastomere at the embryo 16-cell stage was injected with 20 pg of *NFPC* RNA along with 5 ng *nlacZ* RNA to mark the site of injection.

Immunocytochemistry

Injected embryos were fixed at stage 14 in MEMFA (100 mM MOPS, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde, pH 7.4) [34] and stained in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to indicate β-galactosidase activity [31] or processed for His-epitope or Myc-epitope immunocytochemistry as follows: embryos were blocked in PBS-T (phosphate-buffered saline, pH 7.4, 0.1% Triton X-100) containing 20% heat-inactivated goat serum and incubated overnight at 4°C in a 1:250 dilution of mouse anti-RGS–His_6-tag antibody (Qiagen) or a 1:10 dilution of mouse anti-Myc hybridoma supernatant 9E10 [35]. The embryos were then washed in PBS-T, incubated overnight in a 1:250 dilution of horseradish-peroxidase-conjugated goat anti-mouse (Jackson Immunochemical), washed in PBS-T and developed in diaminobenzidine (DAB, Sigma) substrate.

Cell-mixing assay

Injected and immunostained embryos were serially sectioned and examined under a microscope (160×) for the presence of labeled cells in the inner and outer ectodermal layers. Immunostained cells that were not in contact with any other labeled cell were counted and an average obtained for 10–15 embryos for each RNA injected.

NFΔE rescue experiments

For rescue experiments, 5 ng *NFΔE–HT* RNA was co-injected with four times the amount of either *NFPC, E-cadherin*, *N-cadherin* or *C-cadherin* RNA. Embryos were immunostained as before and analyzed for the appearance of NFΔE-induced blisters. To quantify the NFΔE rescue, injected embryos were serially sectioned and examined for the appearance of a single-cell ectodermal layer that occurs at an NFΔE-induced blister. The number of continuous sections in which a single cell ectoderm was observed was then counted for 10–15 embryos and the average multiplied by 10 μm (section diameter).

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