Default neural induction: neuralization of dissociated *Xenopus* cells is mediated by Ras/MAPK activation

Hiroki Kuroda,[1] Luis Fuentealba, Atsushi Ikeda, Bruno Reversade, and E.M. De Robertis[2]

Howard Hughes Medical Institute and Department of Biological Chemistry, University of California, Los Angeles, California 90095-1662, USA

***Xenopus* embryonic ectodermal cells dissociated for three or more hours differentiate into neural tissue instead of adopting their normal epidermal fate. This default type of neural induction occurs in the absence of Spemann's organizer signals and is thought to be caused by the dilution of endogenous BMPs into the culture medium. Unexpectedly, we observed that BMP ligands continue to signal in dissociated cells. Instead, cell dissociation induces a sustained activation of the Ras/MAPK pathway, which causes the phosphorylation of Smad1 at sites that inhibit the activity of this transcription factor. It is this activation of Ras/MAPK that is required for neuralization in dissociated ectoderm.**

Supplemental material is available at http://www.genesdev.org.

Received February 15, 2005; revised version accepted March 24, 2005.

Neural induction in amphibians requires secreted signals, in particular BMP antagonists, produced in the dorsal organizer region of the embryo (Spemann 1938; Harland 2000; De Robertis and Kuroda 2004). However, early work also showed that brain tissue could be obtained in the absence of the organizer, for example, by simply culturing newt ectodermal explants in sub-optimal saline solutions (Barth 1941; Holtfreter 1944). In *Xenopus*, neural differentiation without the organizer can be induced by dissociating ectodermal cells, which would otherwise give rise to epidermis, for 3 h or more (Grunz and Tacke 1989; Sato and Sargent 1989). Addition of Bone Morphogenetic Protein 4 (BMP4) to dispersed *Xenopus* cells can restore epidermal differentiation (Wilson and Hemmati-Brivanlou 1995), leading to the generally accepted proposal that during cell dissociation endogenous BMPs diffuse into the culture medium (Muñoz-Sanjuán and Brivanlou 2002). In this view, the dilution of BMP protein would be the cause of neural differentiation in dissociated cells.

A large body of work also indicates that neural differentiation in vertebrate embryos can be induced by positive signals that act through Receptor Tyrosine kinases (RTKs). Growth factors that signal via RTKs, such as Fibroblast Growth Factor (FGF), Insulin-like Growth Factor (IGF), and Hepatocyte Growth Factor (HGF), are potent neural inducers in vertebrates (Wilson and Edlund 2001; De Robertis and Kuroda 2004; Stern 2004). RTK signaling activates the mitogen-activated protein kinase (MAPK) known as extracellular signal-regulated protein kinase (ERK) via the Ras pathway, and in this way causes neural induction.

Two disparate views dominate the neural induction field at present. Work in the chick embryo has stressed the importance of FGF signaling, whereas work in *Xenopus* has tended to emphasize the requirement for anti-BMPs in neural induction (Harland 2000; Stern 2004). We have argued that these apparently conflicting observations can be reconciled through a molecular mechanism in which Ras/MAPK phosphorylation regulates the BMP transducers Smad1/5/8 (De Robertis and Kuroda 2004). It has been reported that both FGF and IGF can promote neural induction through the phosphorylation, via MAPK, of inhibitory sites located in the linker region of the Smad1 transcription factor (Pera et al. 2003). Linker phosphorylation of BMP-sensitive Smads by MAPK opposes the effect of C-terminal Smad phosphorylation by the BMP receptor serine–threonine protein kinase (Fig. 1A), causing inhibition of the nuclear function of Smad1 and promoting neural development (low BMP/Smad) at the expense of epidermal (high BMP/Smad) fates (Massagué 2003).

It is known that activation of ERK can be triggered by a large number of external stimuli, including cell stress (Wetzker and Böhmer 2003). In *Xenopus* embryos, the simple extirpation of ectodermal explants can activate ERK, causing this kinase to become diphosphorylated (dpERK) (LaBonne and Whitman 1997; Christen and Slack 1999). However, this ERK activation by microsurgery is transient, lasting <30 min. Transient ERK activation after animal cap dissection is without phenotypic effect, and the explants go on to differentiate according to their normal epidermal fate. Since BMP and MAPK signaling are integrated during neural induction, we decided to investigate whether cell dissociation in conditions that cause neural differentiation might induce a stronger, or more prolonged, activation of the Ras/MAPK pathway.

In the present paper we report two findings: first, that, unexpectedly, endogenous BMPs continue to signal in an autocrine fashion in dissociated animal cap ectodermal cells, at the same levels as in undissociated cells; second, that default neural differentiation is mediated by a sustained activation of the Ras/MAPK pathway induced by cell dissociation. The results indicate that the choice between epidermal and neural cell fates is determined by the integration of the opposing activities of BMP receptor and Ras/MAPK at the level of Smad1 phosphorylation.

Results and Discussion

BMPs continue to signal in dissociated cells

Cells from *Xenopus* animal cap explants develop as epidermis when cultured in saline solution, but when cells

[*Keywords*: Neural induction; Smad1; MAPK; FGF; IGF; BMP]
[1]**Present address: Shizuoka University, Faculty of Education (Biology), 836 Ohya, Shizuoka, 422-8529 Japan.**
[2]**Corresponding author.**
E-MAIL ederobertis@mednet.ucla.edu; FAX (310) 206-2008.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1306605.

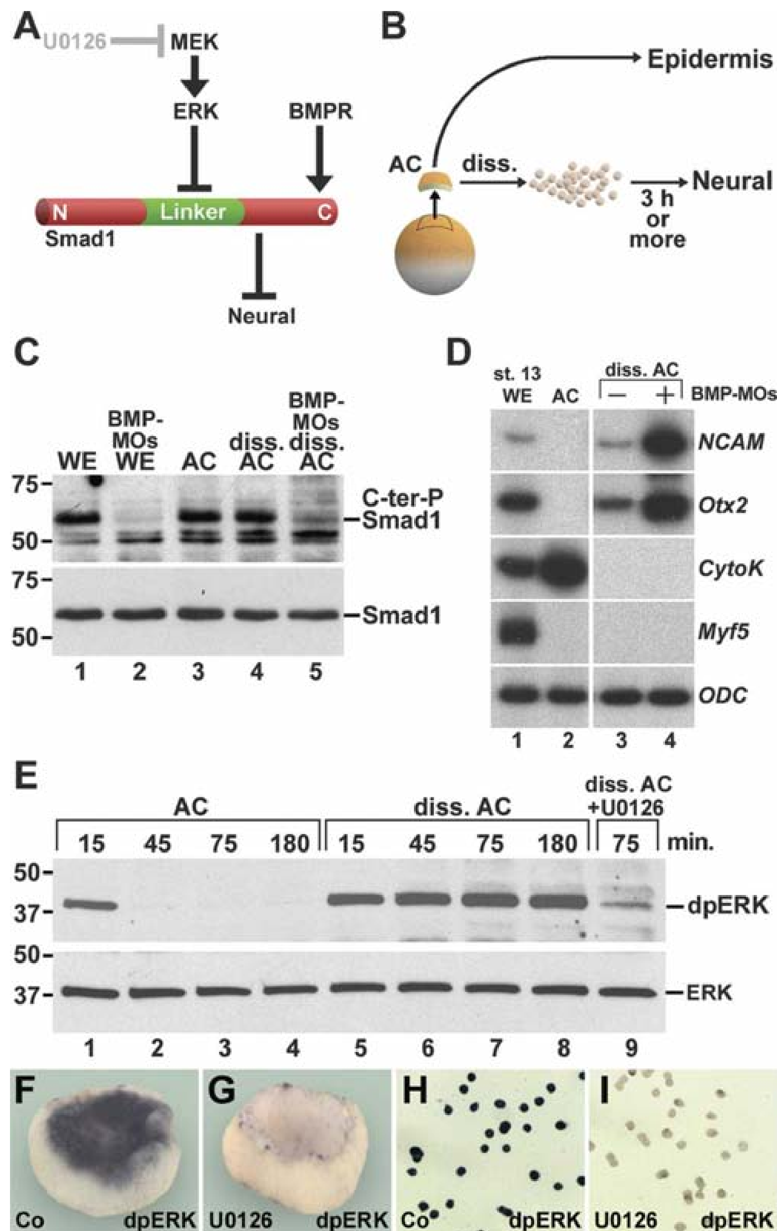

Figure 1. Cell dissociation does not cause BMP depletion by dilution, but rather triggers sustained ERK/MAPK activation in *Xenopus* ectodermal cells. (*A*) Signaling pathway integration at the level of Smad1. (*B*) Default neural induction in dissociated animal caps (AC) cells. (*C*) Western blot assay using anti-C-terminal-phospho-Smad1 (C-ter-P-Smad1) antibody in animal cap cells microinjected with Smad1 mRNA; note that BMPR signaling is not affected by cell dissociation. (WE) Whole embryo; (diss. AC) dissociated animal caps. (*D*) RT–PCR of neural (*NCAM*, *Otx2*), mesodermal (*Myf5*), and loading control (*ODC*) markers, showing a great increase of neural differentiation when Bmp2, Bmp4, and Bmp7 are inhibited with specific MOs. (*E*) Sustained ERK activation revealed by anti-diphospho-ERK (dpERK) antibody in dissociated AC cells. A general ERK antibody serves as loading control. (*F*,*G*) Activation of dpERK in a control animal explant 15 min after excision and its inhibition by addition of the MEK inhibitor U0126 at 40 µM. (*H*,*I*) Activation of dpERK in all cells 75 min after dissociation, and its dependence on MEK.

are dispersed by removing Ca^{2+} and Mg^{2+} for three or more hours neural differentiation ensues (Fig. 1B). This default neural induction is believed to be caused by the diffusion and dilution of endogenous BMPs, but this proposition has not been tested directly. To test whether BMP signals are down-regulated in dissociated animal cap cells, we examined the level of endogenous BMP receptor (BMPR) signaling (Fig. 1A) on the transcription factor Smad1 using a C-terminal phospho-specific antibody (Persson et al. 1998). Surprisingly, the same levels of Smad1 C-terminal phosphorylation were detected in intact or dissociated animal caps (Fig. 1C, cf. lanes 3 and 4).

A second line of evidence indicating that BMPs continue to signal in dissociated animal caps was provided by knock-down of endogenous BMP ligands. In the course of studies on the requirement of anti-BMP signals in neural induction, we designed and characterized antisense morpholino oligos (BMP-MOs) that inhibit specifically the activity of BMP2, BMP4, and BMP7 (Fig. 1C, lanes 2,5; B. Reversade, H. Kuroda, H. Lee, A. Mays, and E.M. De Robertis, in prep.). RT–PCR analyses using BMP-MOs showed that endogenous BMP ligands continue to signal in dissociated ectoderm (Fig. 1D, cf. lanes 3 and 4). As expected, uninjected animal cap explants expressed the epidermal-specific marker *Cytokeratin* in the absence of the mesoderm marker *Myf5*. After dissociation, these cells gained expression of the pan-neural marker *NCAM* and the anterior gene *Otx2*, and lost *Cytokeratin* expression (Fig. 1D, lanes 2,3). Remarkably, coinjection of anti-BMP morpholinos caused a large increase in neural differentiation in dissociated cells (Fig. 1D, lane 4). This indicates that although dissociated cells lost *Cytokeratin* expression, they had not yet reached their full neural potential. We conclude that, contrary to current belief, autocrine signaling by endogenous BMP proteins continues to take place, at the same levels as in intact animal cap explants, in dissociated *Xenopus* ectoderm undergoing neuralization.

Cell dissociation causes sustained MAPK activation

We next investigated whether activation of Ras/MAPK, through the molecular mechanism shown in Figure 1A, might provide an explanation for how lowering of Smad1 activity could be achieved in the presence of BMPR signals in dissociated cells. We used a diphospho-specific ERK antibody (dpERK) that only recognizes the active form of the enzyme, to ask whether endogenous ERK signaling was activated by cell dissociation. Surgical excision of the animal cap was sufficient to phosphorylate ERK after 15 min, but this activation was transient and undetectable in whole caps after 45 min in culture (Fig. 1E, lanes 1–4). Remarkably, cell dissociation caused sustained activation of ERK for at least 3 h in culture (Fig. 1E, lanes 5–8). Immunocytochemical staining showed that ERK was phosphorylated in cells neighboring the healing animal cap wound 15 min after excision (Fig. 1F). We note that this is the same region previously found to give rise to neural tissue in newt animal

cap explants in which wound healing was delayed by sub-optimal saline solutions (Holtfreter 1944). ERK diphosphorylation is mediated by MEK (for MAPK/ERK kinase), which can be inhibited by the specific chemical inhibitor U0126 (Fig. 1A) in the micromolar range (Fig. 1E, lane 9). U0126 blocked both the transient ERK activation (Fig. 1F,G) in animal caps, and the sustained dpERK signal seen in dissociated cells (Fig. 1H,I). Interestingly, immunocytochemistry (see Materials and Methods) showed that after dispersal all ectodermal cells were strongly dpERK positive (Fig. 1H). We conclude that cell dissociation causes a sustained activation of the MEK/ERK pathway in *Xenopus* ectoderm.

Ras/MAPK mediates neuralization

We next asked whether MEK/ERK activation was required for default neural induction. As shown in Figure 2A, control animal cap cells dissociated for 5 h and then allowed to reaggregate by addition of Ca^{2+} and Mg^{2+} differentiated into histotypic brain after 2 d in culture. Addition of the MEK inhibitor U0126 to the culture medium during dissociation blocked neural differentiation, causing the histological and molecular differentiation of epidermal tissue instead (Fig. 2B,D,E). In RT–PCR analyses carried out at early neurula (stage 13), cell dissociation eliminated *Cytokeratin* expression and induced expression of the pan-neural markers *Sox2* and *NCAM* (Fig. 2F, lanes 2,3). Addition of U0126 inhibited neural differentiation in dissociated cells and restored epidermal fate (Fig. 2F, lane 4). The MEK inhibitor was effective even if added 75 min after the initial dissociation (Supplementary Fig. 1A), indicating that its effect on neural differentiation was not dependent on the transient ERK activation caused by animal cap excision, but rather on the sustained activation described here for dissociated cells (Fig. 1E). The effect of U0126 on neural differentiation was specific for the ERK pathway (Supplementary Fig. 1B). The results indicate that MEK/ERK activation is required for default neural induction in dissociated *Xenopus* ectodermal cells; when ERK activation is prevented, dissociated cells develop as epidermis instead.

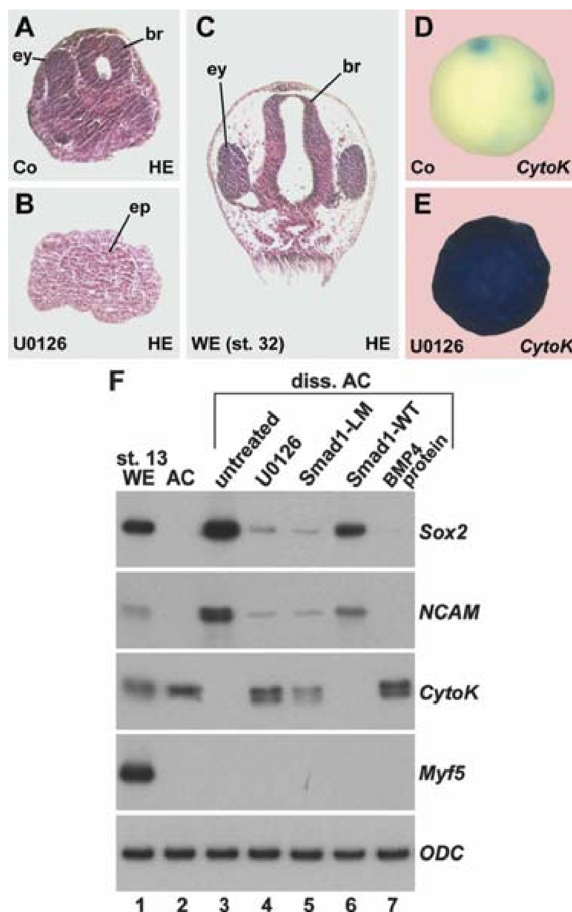

Figure 2. Histological sections, whole-mount in situ hybridizations, and RT–PCR analyses showing that inhibition of ERK blocks neural induction in dissociated animal cap cells. (*A*) Reaggregate from control (Co) untreated cells dissociated for 5 h, showing brain structures. (*B*) Reaggregate from U0126-treated cells differentiated into epidermis. (*C*) Head region of sibling intact stage 32 embryo (Sive et al. 2000). (ey) Eye-like structure; (br) brain; (ep) epidermis. (*D*) Control reaggregates with low-*Cytokeratin* (*CytoK*) by in situ hybridization. (*E*) Strong expression of *CytoK* in reaggregate treated with U0126. (*F*) RT–PCR analysis of dissociated AC cells at stage 13. Pan-neural marker (*Sox2*, *NCAM*) expression was inhibited and epidermal *CytoK* was induced by U0126 treatment, *Smad-LM* (but not *Smad1-WT*) mRNA injection, and 50 ng/mL (2 nM) BMP4 protein.

MAPK-resistant Smad1 inhibits neuralization

We next investigated whether phosphorylation of the linker Smad1 MAPK sites (Fig. 1A) was involved in the choice between epidermis and neural tissue, by comparing the effects of wild-type (WT) and linker mutant (LM) human *Smad1* mRNA. The LM-Smad1 mutant has four serine-to-alanine substitutions at conserved PXSP sites that eliminate MAPK phosphorylation (Kretzschmar et al. 1997; Pera et al. 2003). Microinjection of this MAPK-insensitive *LM-Smad1* mRNA strongly inhibited *Sox2* and *NCAM* expression and restored epidermal (*Cytokeratin*-positive) differentiation of dissociated ectodermal cells (Fig. 2F, lane 5), whereas *WT-Smad1* mRNA at the same concentration failed to block neuralization or cause epidermal differentiation (Fig. 2F, lane 6). Addition of recombinant BMP4 protein to dissociated ectoderm (Wilson and Hemmati-Brivanlou 1995) blocked neural induction and restored epidermal differentiation (Fig. 2F, lane 7). Exogenous BMP4 addition did not affect ERK activation, but caused a large increase in reserve Smad transcriptional activity detected by a BMP response element (BRE) reporter gene in animal cap (Supplementary Fig. 2). Thus, although endogenous levels of Smad1 activity in *Xenopus* animal caps are sufficient to cause epidermal differentiation, a large reserve pool of inactive BMP Smads exists. This spare capacity for additional BMP response explains why the addition of excess BMP4 protein restores epidermal differentiation to dissociated animal cap cells. The antineural effects of Smad1 linker mutants indicate that the inhibitory linker phosphorylation of Smad1 by ERK/MAPK plays a critical role in neural induction by cell dissociation.

Activation of the Ras/MAPK pathway

ERK activation is a late step in the Ras/MAPK signal transduction pathway regulated by RTKs (Fig. 3A). To investigate at which level cell dissociation activates the Ras/MAPK pathway we used, in addition to the MEK chemical inhibitor U0126, a dominant-negative form of Ras (Ribisi et al. 2000) and *Xenopus Sprouty2* mRNA.

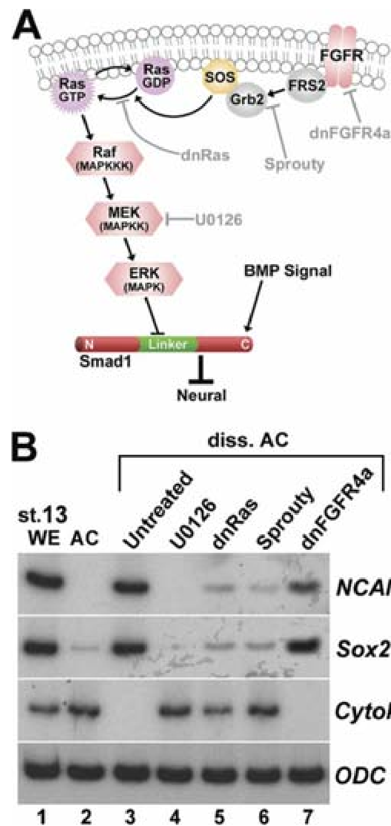

Figure 3. Dissociation activates much of the Ras/MAPK pathway. (*A*) Diagram of ERK pathway components. Dimerized FGF receptors (FGFR) become phosphorylated and recruit the Grb2–SOS complex via FGFR substrate protein 2 (FRS2). SOS activates Ras by exchanging GDP for GTP, allowing Ras to bind to and activate Raf (MAPKKK), which in turn activates MEK (MAPKK), and finally ERK (MAPK). (*B*) RT–PCR analysis of dissociated AC cells treated with U0126 or derived from *dn-Ras*, *Xsprouty2*, or *dnFGFR4a* mRNA-injected embryos. Inhibitors of the Ras/MAPK pathway, except for dnFGFR4a, blocked neural induction by cell dissociation, causing epidermal differentiation. The amount of *dnFGFR4a* mRNA (50 pg/blastomere at the four-cell stage) was sixfold less than that used by other authors (Hongo et al. 1999), as higher concentrations were toxic to the embryos.

Sprouty2 is a phosphoprotein that binds to the Grb2 adaptor (Nutt et al. 2001; Hanafusa et al. 2002), inhibiting the pathway just downstream of RTKs (Fig. 3A). All three inhibitors were able to block neural and restore epidermal markers in dissociated cells (Fig. 3B, lanes 3–6). Dominant-negative FGF receptor-4a (*dnFGFR4a* mRNA) (Hongo et al. 1999) only slightly inhibited neuralization and was unable to cause epidermal differentiation (Fig. 3B, lane 7), suggesting that Ras/MAPK activation in dissociated cells might involve other RTKs or be ligand-independent. We conclude that most of the Ras/MAPK pathway, from Grb2 on down, is activated by cell dispersal.

Cell dissociation phosphorylates endogenous Smad1

To determine whether Smad1 itself is phosphorylated in dissociated *Xenopus* ectodermal cells, a phospho-specific antibody directed against a Smad1 MAPK site in the linker region was generated. Four conserved PXSP MAPK phosphorylation sites exist in *Xenopus* and human Smad1; the antibody targeted the most C-terminal site in the linker region. Western blots of cell lysates showed that sustained linker phosphorylation of endogenous *Xenopus* Smad1 was, indeed, elicited by cell dispersal (Fig. 4A, lanes 1–8). Smad1 linker phosphorylation correlated with the time course of ERK activation (Fig. 1E) and could be blocked by inhibiting MEK/ERK activity with U0126 (Fig. 4A, lane 9). These experiments on Smad1 linker phosphorylation reflect in vivo protein levels and did not involve overexpression of Smad1. We conclude that an inhibitory site on the endogenous Smad1 transcription factor becomes phosphorylated when *Xenopus* animal cap cells are dissociated.

Choosing between neural and epidermal fates

The default neural induction model has provided a very important paradigm for understanding how neural tissue is induced in *Xenopus* (Muñoz-Sanjuán and Brivanlou 2002). In this model, BMP signaling in the ectoderm plays a critical role in the choice between two alternative fates, epidermis or neural tissue (Harland 2000; De Robertis and Kuroda 2004). Dissociation of ectodermal cells induces anterior neural tissue and was believed to act through the dilution of endogenous BMPs, which would diffuse into the culture medium. Here we tested this proposal and found that autocrine BMP signals continue to function in dissociated cells at the same levels as those of animal cap explants that develop into epidermis.

In the dissociated animal cap system, anterior neural

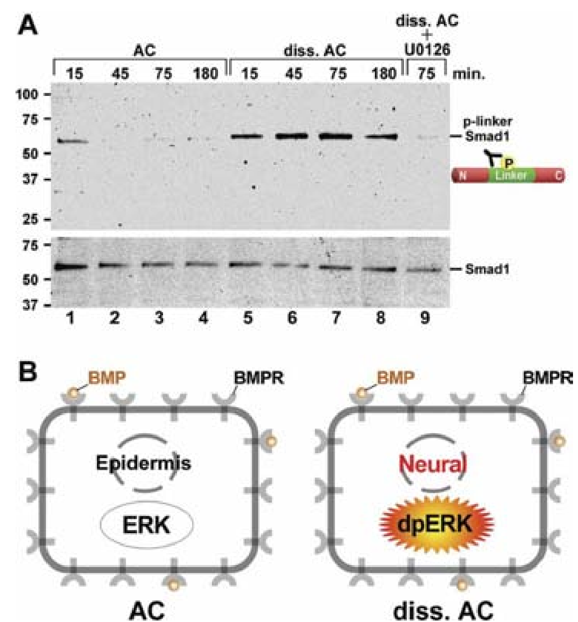

Figure 4. Cell dissociation causes linker-specific phosphorylation of Smad1 in *Xenopus* ectoderm. (*A*) Western blot time course using a linker-specific phospho-Smad1 antibody that reacts with endogenous normal levels of Smad1. Cell dissociation induced sustained linker phosphorylation of Smad1, which was blocked by the MEK inhibitor U0126. A general Smad1 antibody serves as loading control. (*B*) Model for neural induction in dissociated AC cells. Ectodermal BMPs signal in autocrine fashion, but in dissociated cells their effect is counterbalanced by activation of the Ras/MAPK pathway, which causes an inhibitory phosphorylation in Smad1, lowering its activity.

differentiation is entirely dependent on the activation of the Ras/MAPK pathway (Fig. 4B). This was shown by inhibition of MEK/ERK pathway components (by *Sprouty2*, *dn-Ras*, or U0126) or injection of mutant forms of Smad1 resistant to phosphorylation by MAPK, which inhibit neutralization and result in epidermal differentiation. Injection of *dnFGFR4a* mRNA only slightly inhibited neural differentiation in our study, although strong inhibitory effects for anterior neural differentiation have been noted by others in *Xenopus* embryos (Hongo et al. 1999; Delaune et al. 2005). Low levels of FGF signaling are sufficient for anterior neural induction (Delaune et al. 2005); perhaps the amount of *dnFGFR4a* mRNA injected was insufficient to block FGF signaling. Another possibility is that many other RTKs for FGF, IGF, and other growth factors exist on surface of embryonic cells. The molecular mechanism by which a sustained activation of Ras/MAPK is induced by cell dissociation is unknown. It could involve, for example, the activation of multiple RTKs by increasing the rate of dimerization of these receptors in the cell membrane. Transient versus sustained activation of Ras/MAPK is known to cause distinct phenotypic effects in other systems; for example, in cultured pheochromocytoma PC12 cells EGF causes transient activation of Ras/MAPK and cell proliferation, while NGF causes sustained activation and neuronal differentiation (Marshall 1995).

Cell dissociation during the late blastula stage induces neuralization of ectodermal cells in *Xenopus*. However, work in the chick has suggested that BMP inhibition is required only as a late step during gastrulation (Linker and Stern 2004). The situation appears to be different in *Xenopus* embryos, in which injection of multiple BMP-MOs or dnBMPRs can greatly increase neural marker expression (Yamamoto et al. 2001; B. Reversade and E.M. De Robertis, in prep.). In addition, the effects on neural differentiation of manipulating the BMP and FGF pathways are maximal at pre-gastrula stages in *Xenopus* (Delaune et al. 2005; Wawersik et al. 2005). This timing correlates with the observation that blastula cells on the dorsal side of the embryo, where the CNS will form, have high ERK and low BMPR signaling (Schohl and Fagotto 2002). The early BMP inhibition in the *Xenopus* blastula is achieved through transcriptional down-regulation of BMP4 and the expression of secreted BMP antagonists, such as Chordin and Noggin, in the future CNS by the action of an early β-catenin signal (Baker et al. 1999; Kuroda et al. 2004). The second critical step, which is the principal cause of neural differentiation in dissociated cells, is the activation of Ras/MAPK. Because endogenous BMP signals are still active in animal cap cells after dissociation (Fig. 1C,D), this system has an absolute requirement for sustained Ras/MAPK activity in order to attain the very low levels of endogenous BMP/Smad1 signals required for neural differentiation. We conclude that anterior neural differentiation in *Xenopus* ectoderm results from the balanced integration of MAPK and BMPR signals at the level of Smad (Fig. 1A).

Classically, neural induction is considered the primary embryonic induction (Spemann 1938). As such, it has been the subject of intense investigation since it was discovered in 1924 that Spemann's organizer could induce neural tissue. An unresolved mystery in neural induction research, dating from the 1930s, is why dead organizer tissue and many heterologous substances such as fatty acids, sterols, methylene blue, and even sand particles were able to induce neural differentiation in newt embryonic ectoderm (Spemann 1938; Holtfreter and Hamburger 1955; De Robertis and Kuroda 2004). In future, it will be interesting to reinvestigate these observations in light of the essential role played by Ras/MAPK activation in default neural differentiation.

Materials and methods

Embryo manipulations

Xenopus laevis embryos were obtained by in vitro fertilization (Sive et al. 2000). AC cell dissociations were performed in Ca^{2+}, Mg^{2+} free 1× Steinberg's solution (CMFSS: 58 mM NaCl, 0.67 mM KCl, 4.6 mM Tris-HCl at pH 7.4, 100 mg/L kanamycin) containing 0.1% Bovine serum albumin (BSA) at stage 9 (7 h after fertilization at 22°C). The outer layer of cells was discarded, and the inner layer disaggregated into a single-cell suspension by gentle pipetting. All steps were carried out in 35 × 10-mm plastic plates (Fisher) coated with 6% Poly (2-hydroxyethyl methacrylate) in ethanol (Polysciences) and allowed to dry for 30 min. For cell reaggregations, cells from 10 explants were transferred 5 h after dissociation into 1× Steinberg's solution (SS) containing 0.83 mM $MgSO_4$ and 0.34 mM $Ca(NO_3)_2$ in 1.5 mL of NoStick Hydrophobic Microtubes (GENEMATE), centrifuged at 1000 rpm for 10–15 sec; then fresh SS was added and the cells were cultured until sibling embryos reached stage 14 for in situ hybridization and stage 32 for histological assays. Hematoxylin solution (Gill's formulation #3, Fisher) and eosin solution containing 0.2% eosin Y (Sigma) in 70% ethanol containing 0.02% acetic acid were used for histological staining. RT–PCR conditions and primers, as well as the protocol for whole-mount in situ hybridization, are described at http://www.hhmi.ucla.edu/derobertis/index.html.

RNA and morpholino injections

To generate synthetic mRNAs, plasmids pCS2-*smad1-WT*, pCS2-*smad1-LM*, pCS2-*Xsprouty2*, and pCS2-*dnFGFR4a* were linearized with NotI and transcribed with SP6 RNA polymerase, and pSP64T-*dnRas* with EcoRI and SP6 RNA polymerase (Hongo et al. 1999; Pera et al. 2003). After initial titration, the doses of mRNA were microinjected into each blastomere at the four-cell stage: 50 pg for human *Smad1-WT*, 50 pg for human *Smad1-LM*, 500 pg for *Sprouty2*, 50 pg for *dn-FGFR4a*, and 250 pg for *dnRas* mRNAs. The 25-bp morpholino antisense oligomers for Bmp2, Bmp4, and Bmp7 targeted regions lacking mismatches with any of the allelic forms in the *Xenopus* EST database were obtained from Gene Tools, LLC and analyzed for biochemical and biological activity (B. Reversade, H. Kuroda, H. Lee, A. Mays, and E.M. De Robertis, in prep.). They consist of the following sequences: Bmp2-MO, 5′-GATCCCAGCGACCATTGTCAACCTG-3′; Bmp4-MO, 5′-CAGCATTCGGTTACCAGGAATCATG-3′; Bmp7-MO, 5′-TTACTGTCAAAGCATTCATTTTGTC-3′. Control morpholinos of randomized sequence were without effect. Morpholinos were resuspended in sterile water to a concentration of 1 mM each, then further diluted to give a working solution of 0.25 mM (Bmp2-MO:Bmp4-MO:Bmp7-MO:H_2O = 1:1:1:1), and 2 nL was injected into each blastomere at the four-cell stage.

Western blots

Explant or cell lysates were prepared in PhosphoSafe Extraction Buffer (Novagen), lipids were removed by extracting once with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (Kodak), and proteins were separated by 4%–15% gradient SDS-PAGE gels (Bio-Rad). Western blots were performed using polyclonal rabbit antibodies against ERK1/2 (1:1000; Cell Signaling), Smad1 (1:1000; Upstate), and C-terminal phospho-Smad1 (1:4000; kind gift of C. Heldin, Ludwig Institute, Uppsala, Sweden) and monoclonal mouse antibody against diphospho-ERK1/2 (anti-dpERK-YT antibody, 1:1000; Sigma). To detect Smad1 C-terminal phosphorylation, embryos injected with *Smad1-WT* mRNA (500 pg) were used. The rabbit polyclonal antibody against phospho-linker Smad-1 was prepared by Covance Research Products and affinity-purified over a column of SSDPGS(PO_3)PFQMPADT (Princeton BioMolecules Corporation) after negative selection with the unphosphorylated peptide. The antibody stains phospho-Smad-1 specifically in Western blots at 1:200,000 dilution (L. Fuentealba, A. Ikeda, and E.M. De Robertis, in prep.). Unphosphorylated peptide (6 nM) was added as a competitor.

Immunostaining

Dissociated cells were allowed to attach for 1 min to uncoated plastic tissue culture dishes in CMFSS and fixed with MEMFA for 2 h at room temperature (Sive et al. 2000), washed with PBS, refixed (Christen and Slack 1999) with 0.1 M $K_2Cr_2O_7$ in PBS for 1 h, washed three times with PBS for 5 min, dehydrated into methanol, treated with 5% hydrogen peroxide in methanol for 1 h, and washed twice with methanol for 5 min; the animal caps were stored at −20°C until used. Samples were rehydrated into PBS, washed three times with PBS for 10 min, incubated with BSA buffered Triton (BBT: 1% BSA and 0.1% Triton-X in PBS) buffer for 2 h, incubated with 1:10,000 diluted anti-dpERK-YT antibody (Sigma) in BBT at 4°C overnight, washed four times with BBT at room temperature for 1 h, treated with 1:1000 diluted anti-mouse IgG-conjugated to alkaline phosphatase (Novagen) in BBT at 4°C overnight, washed with BBT for 1 h, washed with PBS-Tween (PBSw: 0.1% Tween-20 in PBS) for 4 h, washed twice with alkaline phosphatase buffer (APB: 0.1 M Tris-HCl at pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for 5 min, and stained with BM Purple substrate (Roche) diluted 1:10 in APB at 4°C overnight (Sive et al. 2000). The staining reaction was stopped by washing twice with PBS. All steps were performed in plastic dishes for dissociated AC cells and in 15 × 45-mm glass vials (Fisher) for AC explants.

Acknowledgments

We thank E. Amaya, H. Okamoto, and C. Heldin for materials; A. Mays and D. Greenberg for technical assistance; and S. Millard, X. Zhan, J. Kim, H. Lee, E. Eivers, and R. Nishinakamura for comments and advice on the manuscript. This work was supported by the NIH (R37 HD21502-19). H.K. is an Associate and E.M.D.R. an Investigator of the Howard Hughes Medical Institute.

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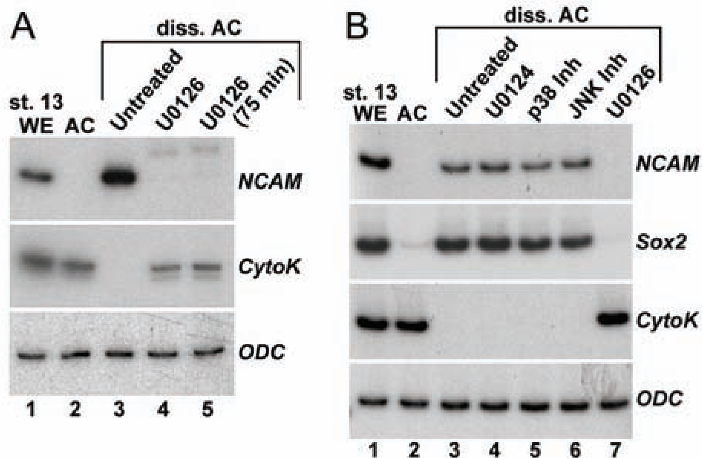
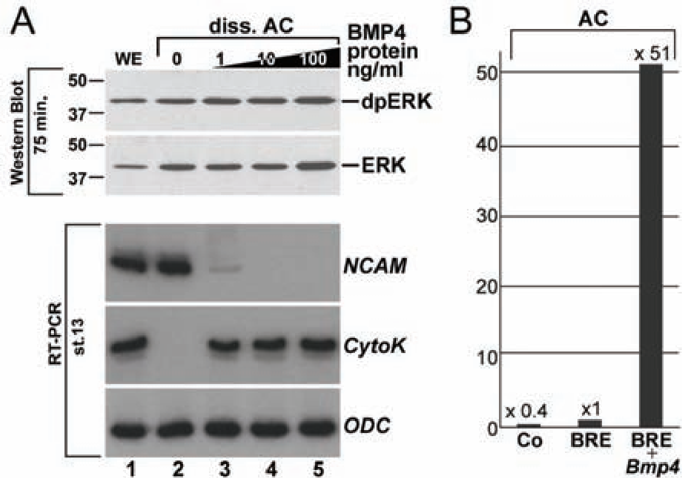


Supplementary Figure 1. Default neural induction requires sustained activation of Ras/MAPK in dissociated animal cap cells, but not the activation of JNK or p38 MAPKs.

(*A*) U0126 treatment blocks the neural differentiation caused by cell dissociation (lanes 3 and 4). When U0126 treatment was started after 75 min culture, neural induction was still blocked (lane 5); this demonstrates that ERK activation during the first 75 min is not required for neuralization.

(*B*) Neural induction is blocked by 40 μM ERK inhibitor U0126 in dissociated AC cells (lane7), but not by 40 μM U0124 (negative control for U0126), 20 μM p38 inhibitor, and 50 μM JNK inhibitor SP600125 (lanes 3-6). All reagents were from Calbiochem Co and used at concentrations active in cultured cells.



Supplementary Figure 2. BMP4 protein inhibits neural differentiation in dissociated animal cap cells without affecting Ras/MAPK activity; *Xenopus* ectodermal cells contain a large reserve of functional Smad that can be activated by BMPR-dependent signals.

(*A*) The top two rows show western blot analyses using anti-diphospho-ERK antibody (dpERK) and a general anti-ERK antibody (ERK) used as a loading control. ERK activity (75 min after dissociation) was not affected by addition of several concentrations of BMP4 protein. The bottom three rows show RT-PCRs demonstrating that these amounts of BMP4 protein were effective in blocking neural induction in dissociated AC cells at stage 13.

(*B*) Luciferase assay in injected AC explants using a specific BMP response element (BRE) DNA fragment from the promoter region of the *Id1* gene (Korchynskyi, O. and ten Dijke, P. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J. Biol. Chem. *277*, 4883-4891, 2002). The response of *BRE-Id1-luciferase* (50 pg of DNA per embryo) in animal cap explants injected with 50 pg of *Xenopus Bmp4* mRNA (indicated as BRE+*Bmp4*) was stimulated 51-fold. Background signal of cells from control embryos (Co) not injected with BRE reporter is also indicated.