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Neural crests are actively precluded from the anterior neural fold by a novel inhibitory mechanism dependent on Dickkopf1 secreted by the prechordal mesoderm

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Abstract

It is known the interactions between the neural plate and epidermis generate neural crest (NC), but it is unknown why the NC develops only at the lateral border of the neural plate and not in the anterior fold. Using grafting experiments we show that there is a previously unidentified mechanism that precludes NC from the anterior region. We identify prechordal mesoderm as the tissue that inhibits NC in the anterior territory and show that the Wnt/β-catenin antagonist Dkk1, secreted by this tissue, is sufficient to mimic this NC inhibition. We show that Dkk1 is required for preventing the formation of NC in the anterior neural folds as loss-of-function experiments using a Dkk1 blocking antibody in *Xenopus* as well as the analysis of *Dkk1*-null mouse embryos transform the anterior neural fold into NC. This can be mimicked by Wnt/β-catenin signaling activation without affecting the anterior posterior patterning of the neural plate, or placodal specification. Finally, we show that the NC cells induced at the anterior neural fold are able to migrate and differentiate as normal NC. These results demonstrate that anterior regions of the embryo lack NC because of a mechanism, conserved from fish to mammals, that suppresses Wnt/β-catenin signaling via Dkk1.
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Introduction

The neural crest (NC) is a cell population characteristic of vertebrates that gives rise to a variety of cell types, including neurons and glia in the peripheral nervous system, connective tissues of the craniofacial structures and pigment cells of the skin (LeDouarin and Kalcheim, 1999). From studies in chick, amphibian and zebrafish embryos, some of the signals involved in the induction of the NC have been identified, including BMPs, Wnts, FGF, Notch and RA (Liem et al., 1995; Mayor et al., 1995, 1997; Saint-Jeannet et al., 1997; Marchant et al., 1998; Nguyen et al., 1998; LaBonne and Bronner-Fraser, 1998; Lekven et al., 2001; Deardorff et al., 2001; Villanueva et al., 2002; Garcia-Castro et al., 2002; Tribulo et al., 2003; Endo et al., 2003; Glavic et al., 2004; Lewis et al., 2004; Bastidas et al., 2004, reviewed in Mayor and Aybar, 2001; Knecht and Bronner-Fraser, 2002; Aybar and Mayor, 2002; Basch et al., 2004; Steventon et al., 2005). At early stages of crest induction an intermediate level of BMP may be necessary, neither so high as to specify ventral epidermis nor so low as to specify neural tissue (LaBonne and Bronner-Fraser, 1998; Nguyen et al., 1998; Morgan and Sargent, 1997; Marchant et al., 1998; Barth et al., 1999). In addition, signaling by Wnts, FGF and RA is required for full induction of the NC (Saint-Jeannet et al., 1997; Mayor et al., 1997; LaBonne and Bronner-Fraser, 1998; Lekven et al., 2001; Deardorff et al., 2001; Villanueva et al., 2002).

Despite these important advances in understanding NC induction and the identification of many inductive molecules, we still do not fully understand how NC is patterned within the ectoderm. It has been shown that NC cells originate at the neural

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plate border as a consequence of the interactions between the neural plate and the non-neural ectoderm (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; Mayor et al., 1997). However it is well established that no NC forms in the anterior neural folds, where a neural plate/non-neural ectoderm border also exists. Early work in the amphibian embryo established that the anterior neural ridge (anterior neural fold, ANF) does not produce NC (Hörstadius, 1950). More recent work, using the quail–chick marker system (LeDouarin and Kalcheim, 1999), also supports the conclusion that the ANF does not give rise to NC (Couly and Le Douarin, 1985; Couly et al., 1993). If interactions between neural plate and epidermis are sufficient to induce NC, why is there no NC at the ANF, where the neural plate is also apposed to epidermis?

There are three possible explanations as to why no NC is generated from the ANF. First, NC inductive signals are present only in regions where NC is normally induced, but absent from the ANF or the adjacent epidermis (Fig. 1A). Second, posteriorizing signals cannot reach the anterior region of the embryo and thus the NC is not induced there (Fig. 1B). Recently, it has been shown that signals coming from the posterior region of the embryo, such as Wnts, Fgfs and retinoic acid, are required for NC induction (Villanueva et al., 2002). The activation of NC inductive pathways, such as Wnt or FGF, in the ANF leads to an expansion of NC markers in that region (Villanueva et al., 2002; Monsoro-Burq et al., 2003; Wu et al., 2005; Voigt and Papalopulu, 2006) supporting the notion that the absence of these inductive signals explains the lack of NC at the ANF. Nevertheless, these data have never been discussed in this context before. However, we have observed that anterior neural plate explants conjugated with anterior epidermis can interact and give rise to NC if they are isolated from the embryo (Mancilla and Mayor, 1996). This observation strongly suggests that there is a transient and early induction of NC at the ANF challenging the idea that all the required signals are never present in this region.

Here we propose a third explanation for the absence of NC in the ANF. We show the existence of a NC inhibitory mechanism at the anterior region of the embryo that precludes the NC development there (Fig. 1C). In addition, we identified the tissue that produces this negative signal and the inhibitory molecule secreted by this tissue. This is the first time that an inhibitory signal is described to control neural crest specification.

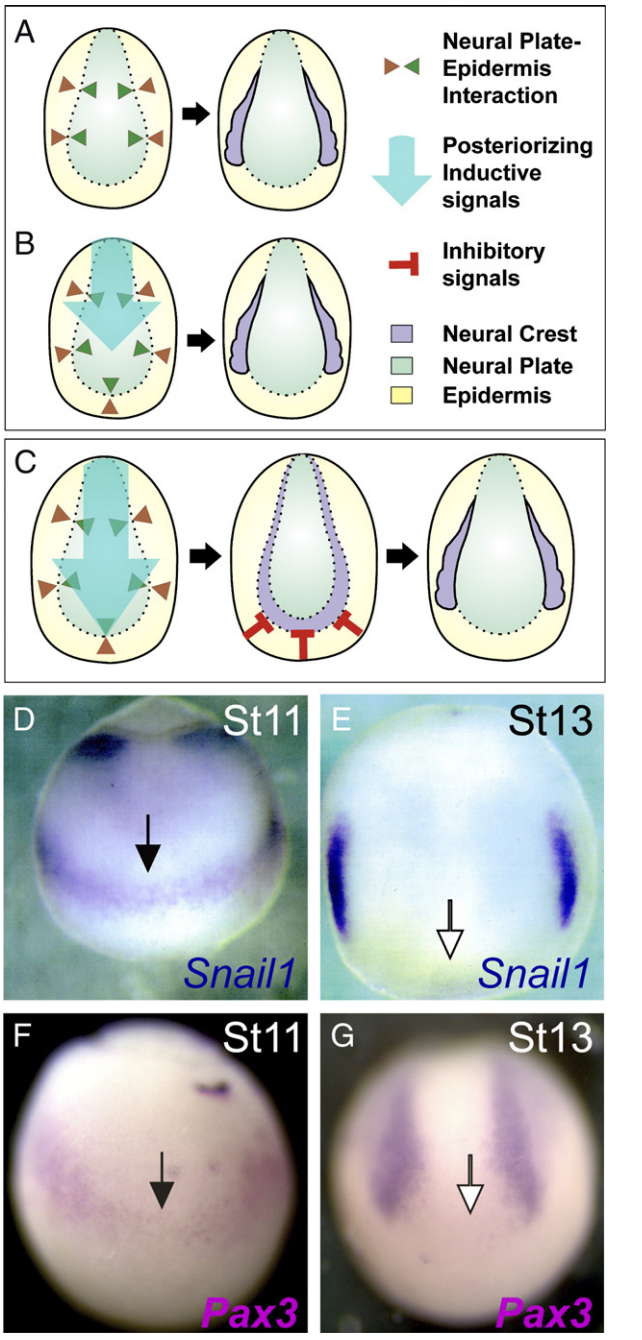

Fig. 1. Alternative models for the absence of NC at the ANF. (A, B) Current models. (A) The NC inductive signals (neural plate/epidermis interaction) are present only where the NC is induced and absent from the ANF. (B) Posteriorizing signals required to induce NC do not reach the ANF. (C) Proposed model: the NC inductive signals are present along the entire border of the neural plate and the NC is induced along the entire neural plate border, but at a later stage a specific NC inhibitory signal is produced at the ANF that restricts NC development to the lateral sides of the neural plate border. (D) Expression of *Snai1* at stage 11, showing expression at the ANF (arrow). (E) Expression of *Snail1* at stage 13, showing absence of expression at the ANF (arrow). (F) Expression of *Pax3* at stage 11, showing expression at the ANF (arrow). (G) Expression of *Pax3* at stage 13, showing absence of expression at the ANF (arrow).

Materials and methods

Micromanipulation, whole-mount in situ hybridization, DiI and cartilage staining

Dissections and grafts were performed as described by Mancilla and Mayor (1996). For in situ hybridization, antisense digoxigenin or fluorescein labeled RNA probes were used. Specimens were prepared, hybridized and stained using the method of (Harland, 1991), and NBT/BCIP or BCIP alone was used as a substrate for the alkaline phosphatase. The genes analyzed were for *Xenopus*: *Snail* (Mayor et al., 1993); *Snail2* (Mayor et al., 1995); *Wnt8* (Christian and Moon, 1993); *Bf1* (Hardcastle and Papalopulu, 2000); *Cpl1* (Richter et al., 1988); *Krox20* (Bradley et al., 1993), and *Otx2* (Pannese et al., 1995); for mouse: *Sox10* (Southard-Smith et al., 1998); for zebrafish: *FoxD3* (Kelsh et al., 2000); *Rx3* (Chuang et al., 1999). Proliferating cells were labeled with BrdU essentially as described by Hardcastle and Papalopulu (2000). To distinguish the injected from the control side of the embryos, we combined the BrdU immunohistochemistry with a second immunostaining against fluorescein used

as a lineage tracer. In these experiments the number of BrdU-positive cells in the injected side was compared with that of the uninjected control side. DiI labeling was performed at the neurula stage as described in Linker et al. (2000) and for cartilage staining an adaptation of the zebrafish protocol previously described was used (Barrallo-Gimeno et al., 2004). NC and ANF in vitro migration assays were performed as described before (De Calisto et al., 2005).

RNA synthesis in vitro, microinjection of mRNAs or morpholino oligonucleotides and dexamethasone activation

All plasmids were linearized and transcribed as described (Harland and Weintraub, 1985), using SP6 or T7 RNA polymerases, and the GTP cap analogue (New England Biolabs). The RNA was co-injected with FLDx (fluorescein dextran; Molecular Probes) or *β-gal* mRNA using 8–12 nl needles into 2- or 8-cell embryos, or at the D1.1 blastomere of 16-cell and A1 of 32-cell embryos as described before (Aybar et al., 2003). The constructs used were: *Dkk1* (Glinka et al., 1998), *β-catenin-GR* (Domingos et al., 2001), *Wnt8* (Christian et al., 1991), *Crescent* (Pera and De Robertis, 2000) and *Cerberus* (Bouwmeester et al., 1996). Treatment with dexamethasone was performed as we described previously (Tribulo et al., 2003). *Tcf3a* morpholino was used as described before (Kim et al., 2000).

Dkk1 protein and antibody treatment

Heparin coated acrylic beads (Sigma) were incubated overnight with 40 μg/ml of Dkk1 protein (Dkk1 human recombinant, Calbiochem) or BSA as controls and grafted into embryos at the appropriate stage. The Dkk1 antibody was injected into the blastocoel cavity as described in Kazanskaya et al. (2000). Treatment of explants with Dkk1 was performed as described in Kazanskaya et al. (2000).

Results

Inhibition of NC at the ANF by the prechordal mesoderm

We hypothesize that the absence of NC from the ANF is due to an inhibitory mechanism that restricts them only to the lateral border of the neural plate. As a first approach to test this idea, we analyzed the expression of the earliest NC-specific genes *Snail1* and *Pax3*. Surprisingly, an undescribed early expression at the ANF was found at the beginning of gastrulation (Figs. 1D, F). This expression was lost by the end of gastrulation (Figs. 1E, G) when these markers were restricted to the lateral border of the neural plate.

To test whether neural crest development is actively inhibited at the ANF or not, we grafted NC from embryos at different neurulation stages into the ANF region of stage 14 host embryos and analyzed them at late neurula stage by in situ hybridization (Fig. 2A). These NC explants were already specified as NC as they maintained the expression of NC-specific genes (such as *Snail2*) when cultured alone (60/60; Fig. 2B). Surprisingly, when these NC explants from early neurula stage embryos were grafted in the ANF of the host, the NC-specific gene expression was completely lost (48/48; Figs. 2C, D). If the same experiment is repeated but with NC taken from later embryos, the ability of the anterior portion of the embryo to inhibit NC was gradually decreased and lost at stage 17 (Fig. 3D, black bar). This inhibitory effect was due the existence of an active mechanism located specifically at the anterior region of the embryo because no NC inhibition was observed on explants of any stage when grafted on other parts of the embryo (10/10; Fig. 2E).

Then we decided to identify the tissue responsible of this NC inhibition. Two main tissues are close to the ANF at the early neurula stage, the prechordal mesoderm (PM) and the anterior ectoderm (AE). Therefore, we decided to graft explants of these tissues laterally to normal crests and analyzed if they were able to inhibit NC (Fig. 2F). When pieces of AE were grafted near the NC, no effect on *Snail2* or other NC-specific genes was observed (24/24; Fig. 2G). On the contrary, a strong inhibition of *Snail2* was observed when pieces of PM were grafted near the crest region, (27/35; Fig. 2H). To confirm this, we conjugated specified NC explants with pieces of AE or PM, both from early neurula embryos. The conjugates were cultured until the equivalent of late neurula stage and then analyzed by in situ hybridization (Fig. 2I). Similarly to the previous experiment, when NC explants were conjugated with pieces of AE, no *Snail2* inhibition was observed (23/23; Fig. 2J); however, when a similar NC explant was co-cultured with PM, a strong inhibition of *Snail2* was observed (30/38; Fig. 2K). These results also show that the PM is sufficient to restrict NC development.

Altogether, these results reveal the existence of a previously unidentified signal of NC inhibition at the anterior region of the embryo. This signal is likely to be produced by the PM as this structure is sufficient to inhibit NC development.

Dkk1 is an inhibitor of NC induction at the ANF

Next we tried to identify the signaling molecule involved in this inhibition. We developed an in vitro assay to test the ability of candidate molecules to inhibit NC development. We screened candidate molecules for the following criteria: to be expressed in the prechordal mesoderm and to be a secreted molecule related to the known signaling pathways in NC induction. mRNA encoding the molecule of interest was injected at the one-cell stage. The embryos were cultured until blastula stage, when animal caps were dissected and conjugated with NC taken from early neurula stage embryos (stage 15). After culture, the expression of the NC marker *Snail2* was analyzed (Fig. 3A). Control caps injected with FLDx alone did not have the ability to inhibit *Snail2* expression in NC explants (67/67; Fig. 3B). Nevertheless, caps injected with the canonical Wnt/β-catenin antagonist Dickkopf1 (Dkk1, Glinka et al., 1998) completely abolished *Snail2* expression in conjugated NC explants (51/51; Fig. 3C). NC conjugates with animal caps expressing other PM molecules showed inconclusive results or no effect (noggin, chordin, BMP4, Cyp26, Raldh2, Sprouty and Spred, data not shown).

As we observed that grafts of late neurula NC were not inhibited as much as early neurula NC, we decided to test if something similar happened with the response of NC to Dkk1. Consequently, we conjugated Dkk1 expressing animal caps with NC coming from embryos at different stages (form stage 13 to 17). *Snail2* expression was analyzed by in situ hybridization. Interestingly, the NC response in the in vivo experiments (Fig. 2A) and in vitro experiments with Dkk1 (Fig. 3A) showed a very similar kinetic, i.e. strong *Snail2* inhibition at early neurula and low or no effect later in development. Fig. 3D summarizes the results of both experiments.

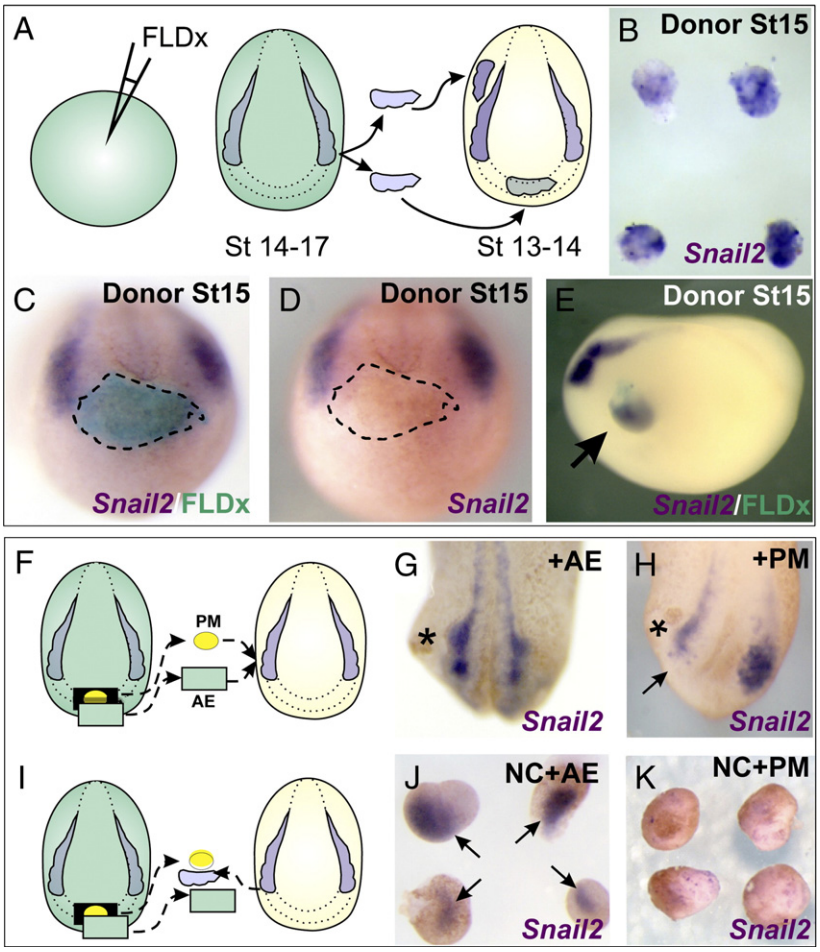

Fig. 2. The NC is inhibited at the ANF by the PM. (A–G) Inhibition of NC induction at the ANF. (A) Embryos were injected at the 1-cell stage with FLDX, at stages 14–18 the NC were dissected and grafted into the ANF, or lateral epidermis of a stage 13/14 control embryos, or cultured in vitro until stage 18/19, when the expression of *Snail2* was analyzed. (B) NC dissected at stage 15 and cultured in vitro until stage 18 showing expression of *Snail2* ($n=60$, 100% of expression). (C) Anterior view of an embryo containing a graft of stage 15 NC as described. Dotted line shows the green color of the FLDx graft. (D) Same embryo as in C, showing the absence of *Snail2* in the graft (dotted line). (E) Lateral view of an embryo in which NC taken from a stage 15 embryo was grafted in lateral epidermis, showing expression of *Snail2* in the graft. Approximately 50 embryos were analyzed for each case. (F–K) PM inhibits NC induction. (F) Anterior ectoderm (AE, green square) or prechordal mesoderm (PM, yellow circle) was dissected from a stage 15 embryo and grafted next to the NC from a control embryo, after culture until stage 18, the expression of *Snail2* was analyzed. (G) No effect on *Snail2* expression was observed with the AE graft. Asterisk: graft, $n=24$, 100% of expression. (H) Inhibition of *Snail2* expression with PM graft. Asterisk: graft, $n=35$; 78% of grafted embryos exhibited inhibition of *Snail2* expression. (I) PM and NC were dissected from stage 15 embryos, conjugated in vitro and the expression of *Snail2* was analyzed at the equivalent of stage 18. (J) NC cultured in vitro showing normal expression of *Snail2* (arrow); $n=23$; 100% of expression. (K) Conjugate of NC and PM showing inhibition of *Snail2* expression; $n=38$, 80% of conjugates with inhibition of *Snail2* expression.

To confirm that Dkk1 can indeed inhibit NC induction in vivo, we soaked heparin beads either in a human Dkk1 recombinant protein solution or in BSA as a control and then grafted them next to the NC of an early neurula embryo (Fig. 3E). As expected, beads soaked in BSA did not affect NC induction (20/20; Fig. 3F), while Dkk1 was a clear inhibitor of *Snail2* (13/14; Fig. 3G).

Dkk1 is expressed in the prechordal (head) mesoderm of other vertebrates such as mouse (Glinka et al., 1998) and zebrafish (Caneparo et al., 2007). If Dkk1 acts as an NC inhibitor, we expected that its expression should be complementary to the expression of NC markers. This was indeed the case when the expression of *Pax3* was compared to the *Dkk1* expression (Figs. 3H–M). Interestingly before *Pax3* expression was precluded from the ANF (stage 11, Fig. 3H), *Dkk1* expression had not yet reached the anterior region (Figs. 3I, J). On the contrary, at stage 13 the expression of *Dkk1* (Fig. 3L) correlated perfectly with the absence of *Pax3* in the ANF (Figs. 3K, M). Taken together, these results show that *Dkk1* is sufficient to inhibit NC induction and its expression pattern makes it a good candidate to be the signal that limits the anterior edge of the NC.

Dkk1 loss of function is sufficient to transform the ANF into NC

Although we have demonstrated that Dkk1 can inhibit NC in vitro and in vivo, we have not shown whether this is the actual mechanism of precluding NC from the ANF. To address this issue, we performed some Dkk1 loss-of-function experiments. First, in *Xenopus* embryos, we injected an antibody against Dkk1, which has been characterized as a specific inhibitor of

Dkk1 activity, into the blastocoel (Kazanskaya et al., 2000). Embryos injected with a control antibody never expressed *Snail2* at the ANF (0/40; Fig. 4A), while embryos injected with Dkk1 antibody showed strong expression of NC-specific genes in the entire ANF (52/82; Fig. 4B, black arrow). In addition, we were able to block the inhibitory activity of Dkk1 in vitro. Stage 15 NC was conjugated with PM that has already been specified (stage 15) and the expression of NC markers analyzed at stage 20, as described in Fig. 2I. As expected, no conjugate cultured in control media expressed *Snail2* (0/7, Fig. 4C). However, when conjugates were cultured in a media containing the Dkk1 antibody, most of them showed a strong *Snail2* expression (6/8, Fig. 4D). This shows that the PM is inhibiting NC via Dkk1.

Finally, the expression of the NC marker *Sox10* was analyzed in *Dkk1* null mutant mouse embryos (Mukhopadhyay et al., 2001). This mutation displays defects in forebrain development but NC markers have not been analyzed. Wild type (*Dkk1*+/+) embryos show the characteristic absence of *Sox10* expression in the ANF (arrow, Fig. 4E), while homozygous mutants (Dkk1−/−) exhibit a strong expression of *Sox10* in the ANF (10/12; Fig. 4F), similar to the phenotype observed in *Xenopus* embryos injected with anti-Dkk antibody (Fig. 4B). These experiments indicate that Dkk1 activity is not only sufficient to inhibit NC but also required to preclude NC from the ANF.

As Dkk1 is known to work as a Wnt inhibitor (Glinka et al., 1998) we tested whether a similar transformation of the ANF into NC could be achieved by activation of the Wnt pathway. In *Xenopus*, we restricted the effect by injecting mRNA coding elements of the Wnt pathway in the A1 blastomere of a 32-cell morula, which is fated to form the ANF (Fig. 5A). Accordingly with our prediction, the injection of Wnt8 mRNA provoked a complete transformation of the ANF into a tissue expressing NC-specific genes like *Snail2* (28/50; Figs. 5C, D). This effect is notoriously similar to the Dkk1 inmunodepletion effect (Fig. 4B). Because Wnt8 is a diffusible molecule the effect was not only restricted to the injected cells but to all the cells at the ANF (Fig. 5D). This is an interesting observation because it shows that only the neural plate border can transform its fate to NC in response to this treatment. Nonetheless, this diffusible feature of Wnt8 opens the possibility to explain this transformation as an indirect phenotype caused by the effect on other regions like the mesoderm. To exclude this possibility we repeated the same experiment but this time injecting mRNA coding β-catenin, an intracellular component of this pathway. As shown in Figs. 5E and F, a similar transformation of the ANF occurs but in this case it is restricted only to the injected cells present at the neural plate border demonstrating that it is a cell autonomous effect (33/49).

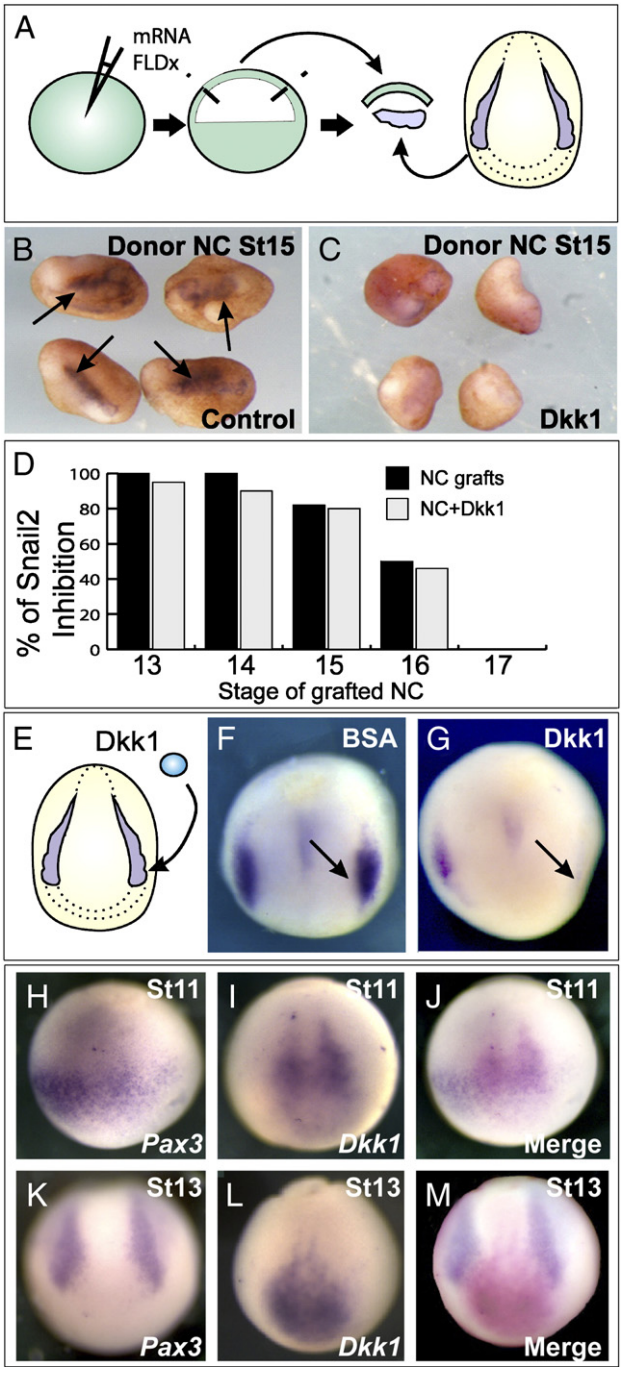

Fig. 3. Assay to identify NC inhibitors in vitro. (A) Embryos were injected at the 1-cell stage with mRNA coding for the tested molecules, at stage 9 animal caps were dissected and conjugated with NC taken from stages 13–17 control embryo; the expression of *Snail2* was analyzed. (B) Stage 15 NC conjugated with control animal caps shows normal *Snail2* expression; $n=67$, 100% of expression. (C) Stage 15 NC conjugated with AC expressing Dkk1 shows absence of *Snail2* expression. (D) Summary of expression of *Snail2* in grafts into the ANF of NC taken from embryos at different stages (as described in Fig. 2A; black bars) and Dkk1 expressing animal caps (as described in panel A; light gray bars). Note a similar trend on NC inhibition in the graft and in the Dkk1 conjugates. Approximately 50 embryos were analyzed for each stage. (E) Beads soaked with BSA or Dkk1 protein were grafted next to the NC of a stage 15 embryo. (F) Control bead soaked with BSA (arrow) shows no effect on *Snail2* expression; $n=20$, 100% of expression. (G) Bead soaked with Dkk1 shows inhibition of *Snail2* expression; $n=14$, 92% of inhibition. (H–M) Analysis of *Pax3* and *Dkk1* expression. (H) Expression of *Pax3* in a stage 11 embryo. (I) Expression of *Dkk1* in stage 11 embryo. (J) Overlapping of pictures shown in panels I and J after artificial color change. (K) Expression of *Pax3* in a stage 13 embryo. (L) Expression of *Dkk1* in stage 13 embryo. (M) Overlapping of pictures shown in panels K and L after artificial color change. Note the correlation between *Dkk1* expression and *Pax3* restriction.

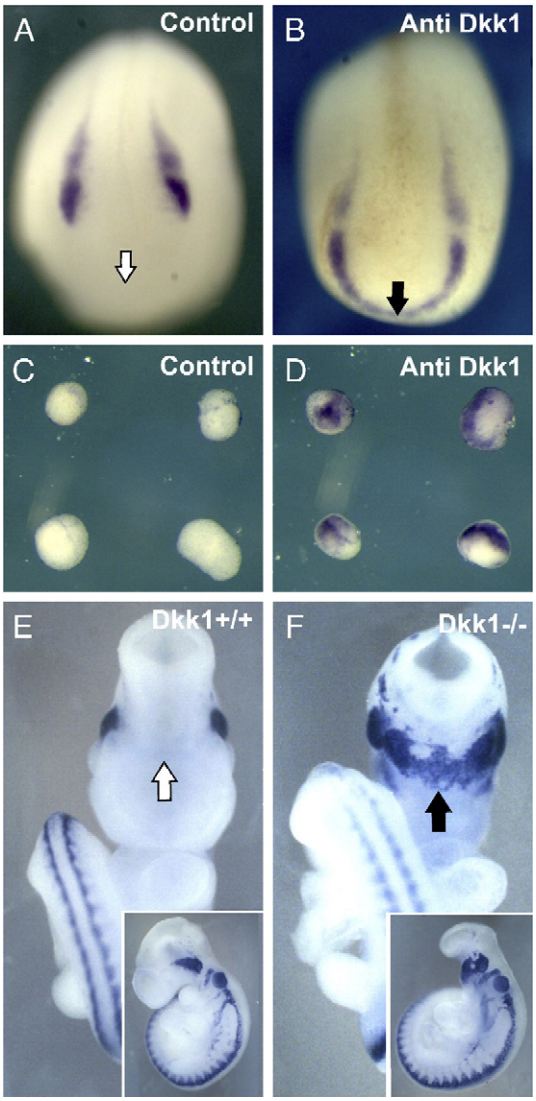

Fig. 4. Dkk1 is essential for NC inhibition at the ANF. (A–D) Effect on *Xenopus*. Embryos are shown in dorso-anterior view. (A) Embryo injected at the blastula stage with an anti-prolactin antibody as control, it shows absence of *Snail2* expression at the ANF (arrow); $n=40$, 0% of expression at the ANF. (B) Embryo injected at the blastula stage with anti-Dkk1 antibody showing *Snail2* expression at the ANF (arrow); $n=86$, 60% of expression. (C) Control NC/PM conjugates (0% of *Snail2* expression, $n=7$). (D) NC/PM conjugates cultured with the anti-Dkk1 antibody (75% *Snail2* of expression, $n=8$). (E, F) Effect on mouse. (E) Anterior view of wild type mouse embryo (E9.5) showing normal expression of *Sox10*, and no expression at the ANF (arrow). Inset: lateral view of the same embryo. (F) Anterior view of Dkk1 mutant mouse embryo (E9.5) showing expression of *Sox10* at the ANF (arrow). Inset: lateral view of the same embryo; $n=12$, 83% of embryos showing expression of *Sox10* at the ANF. Note that the expression of *Sox10* is normal in the midbrain, hindbrain and trunk NC, suggesting more NC rather than a truncation.

We wanted to further corroborate the epistasic relationship between Dkk1 and the canonical Wnt pathway in establishing the limits of the NC. For that, we injected zebrafish embryos with morpholino oligonucleotides that specifically block the translation of *Tcf3a* (*Tcf3aMO*). This treatment has been shown to activate Wnt signaling in the anterior region of the embryo (Kim et al., 2000). As expected, an equivalent transformation of ANF into NC was produced in the injected embryos (Fig. 5H), which is never observed in control embryos (Fig. 5G). Taken together, we have shown that these three different treatments that activate Wnt signaling lead to the same transformation of the ANF into NC, suggesting that Dkk1 works as a Wnt inhibitor in the process precluding NC induction at the ANF.

Temporal analysis of ANF transformation into NC

Is it possible to dissociate the ANF transformation from other processes like the NC expansion observed in other reports where Wnt activity was also increased? (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998). This expansion is different from the Dkk1 loss-of-function effect where the ANF cells were transformed but no border expansion was observed along the medio-lateral axis (Fig. 4B). Although we could phenocopy this effect by increasing Wnt activity (Figs. 5C–H, 6A), we observed some embryos showing a medio-lateral expansion accompanying the transformation (Fig. 6B). Note that in the embryo with the transformation, the NC looks normal lateral to the plate; but in the embryo with the expansion the NC is affected laterally to the neural plate as much as it is frontally to the neural plate (Figs. 6A, B). We thought it might be possible to separate these two effects by modulating either the time or level of β-catenin activity. To manipulate the timing, we used an inducible β-catenin construct in which the time of activation could be controlled (Domingos et al., 2001). Different doses of this construct were injected in A1 blastomere and the embryos were analyzed after activation of β-catenin at different stages (Fig. 6C). Embryos injected with the same dose of β-catenin but activated at different times clearly showed differences on the penetrance of the two phenotypes (Fig. 6D). Embryos in which the β-catenin was activated at stage 9 showed less transformation than the embryos in which the β-catenin construct was activated between stage 10 and 12 (Fig. 6D). On the contrary, the expansion phenotype was favored when β-catenin was activated at the earliest stages and decreased later (Fig. 6D, inset).

Interestingly, when we tried injecting different doses of β-catenin, we found that even when activated at stages where the transformation effect was predominant (stages 10–11, Fig. 6D) it was possible to get a dominance of the expansion phenotype if the dose was increased enough (inset in Fig. 6E). These results show that it is possible to phenocopy the Dkk1 loss-of-function phenotype by locally increasing Wnt activity. In addition, they show that is possible to dissect it from other Wnt-dependent effects like the previously described NC expansion. These results show the importance of the time, space and dose control in order to get a clean transformation, and they suggest that the NC inhibition at the ANF takes place slightly later than the process of NC induction.

Transformation of ANF into NC by Wnt activation is not dependent on anterior–posterior (AP) patterning of the neural plate or inhibition of placodal fates

A possible alternative explanation of this transformation of the ANF into NC is that there is a truncation of the anterior neural plate produced by a general disruption of the anterior–

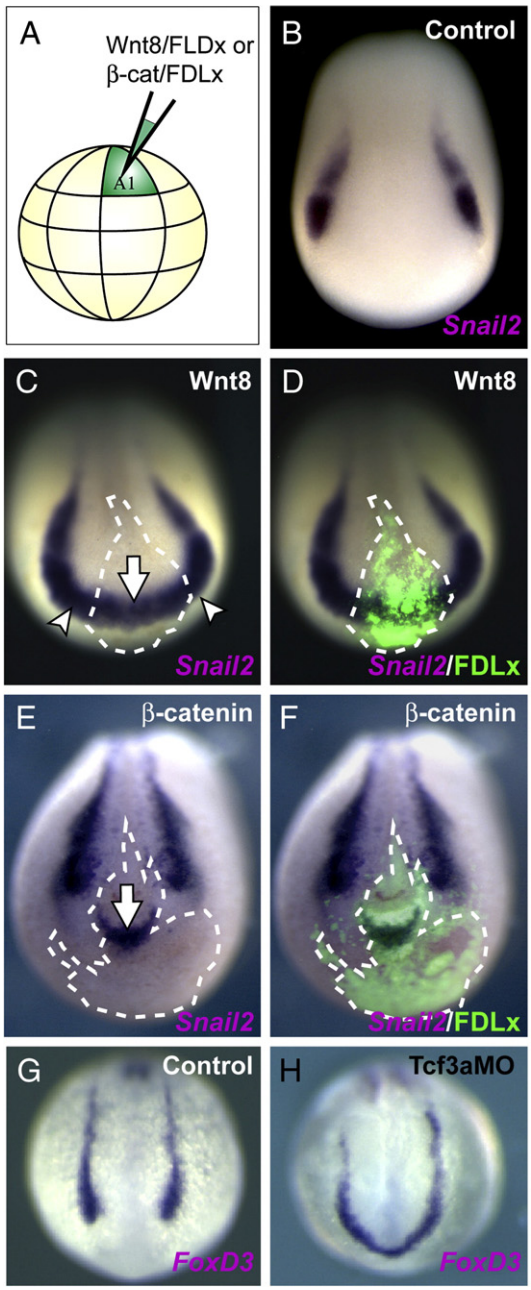

Fig. 5. Activation of Wnt signaling leads to transformation of ANF into NC. (A–F) Effect on *Xenopus*. Embryos are shown in dorso-anterior view. (A) Diagram showing where the injections were performed. (B) Control embryo showing normal *Snail2* expression. (C, D) Embryos injected with 2 ng of Wnt8 mRNA and FLDx into A1 blastomere of a 32-cell stage embryo. Green cells in panel D correspond to the injected cells, which are indicated by the dotted line in panels C and D. Note expression of *Snail2* in all the ANF (arrows and arrowheads); $n=50$, 55% of embryos showing ectopic *Snail2* expression. (E, F) Embryos injected with 130 pg of β-catenin-GR mRNA and FLDx into A1 blastomere of a 32-cell stage embryo, induced at stage 10.5. Green cells in F correspond to the injected cells which are indicated by the dotted line in E. Note expression of *Snail2* at the ANF but this time restricted to the injected cells (arrow); $n=67$, 56% of embryos showing ectopic *Snail2* expression. (G, H) Zebrafish embryos showing expression of *Foxd3*. (G) Control embryo. (H) Embryo injected with 0.01 pmol of *Tcf3a* MO.

posterior (AP) axis. As a result of the absence of anterior structures, the more posterior and lateral regions could be fused, including the two lateral domains of the NC. That would mean that there is an abolition of the ANF rather than a transformation. To address this possibility we analyzed the transformation of the ANF into NC under very controlled conditions of space, time and levels of Wnt signaling. We used the conditions that favored the transformation phenotype (130 pg of β-catenin mRNA and activation at stage 10.5, see Fig. 6) and analyzed the expression of anterior–posterior neural plate and placodal markers. No shift in the expression of the AP markers *Krox20* (2/40) or *Otx2* (10/45) was observed (Figs. 7A, B), suggesting that the general AP pattern of the neural plate was not affected. Nevertheless, this does not rule out the possibility of the anterior truncation as more anterior regions of the neural plate could be absent. Therefore, we analyzed the expression of the most anterior markers *Bf1* and *Cpl1* under the same conditions and found that they are not affected (5/41, 10/42; Figs. 7C, D). Interestingly, when we injected higher concentrations of β-catenin an effect on the AP markers was observed (Fig. S1A) as has been reported elsewhere (Braun et al., 2003; Kiecker and Niehrs, 2001; McGrew et al., 1997; Erter et al., 2001, Itoh and Sokol, 1997; Niehrs, 2001; Nordstrom et al., 2002). Under conditions where the *Tcf3a*MO can produce the transformation of ANF into NC in zebrafish embryos, (Figs. 7E, F), no effect was observed on the expression of the anterior neural plate marker *Rx3* (30/40; Figs. 7G, H). Of note, this effect was achieved by standardizing the concentrations in a similar way to the one described above for *Xenopus* experiments; if higher levels of *Tcf3a*MO are injected, an inhibition of *Rx3* was observed confirming previous observations (Figs. S1C, D; Kim et al., 2000).

Another possible tissue that could be affected by the transformation of the ANF into NC is the preplacode field, as it has been shown that inhibition of Wnt signaling is required for its specification (Brugmann et al., 2004; Litsiou et al., 2005). When the preplacodal-specific gene *Six1* (23/48; Fig. 7I) and the preplacodal domain of *Sox3* (20/45; Figs. 7J, K) were analyzed, a marked anterior–ventral shift was observed (compare the white lines in the control and injected side in Figs. 7I, K); however the level of placodal marker expression was hardly affected. When higher levels of Wnt activity were used preplacodal markers were usually inhibited (Fig. S1B). Taken together, these results show that the transformation of the ANF into NC is not an indirect effect of an anterior truncation of the embryo. In addition, they indicate that this transformation can be at least experimentally dissociated from the general AP patterning of the embryo; however, this does not mean that they work as two different processes in a normal embryo.

Importantly, we are not affecting the development or migration of the prechordal mesoderm as injected embryos show normal Dkk1 expression (36/40; Fig. 7L). This is an important control because, as we have shown here, the prechordal mesoderm contains the inhibitory signals that preclude the expression of NC markers from the ANF. A schematic representation of the effect of these experiments on the ectoderm is displayed in Fig. 7M.

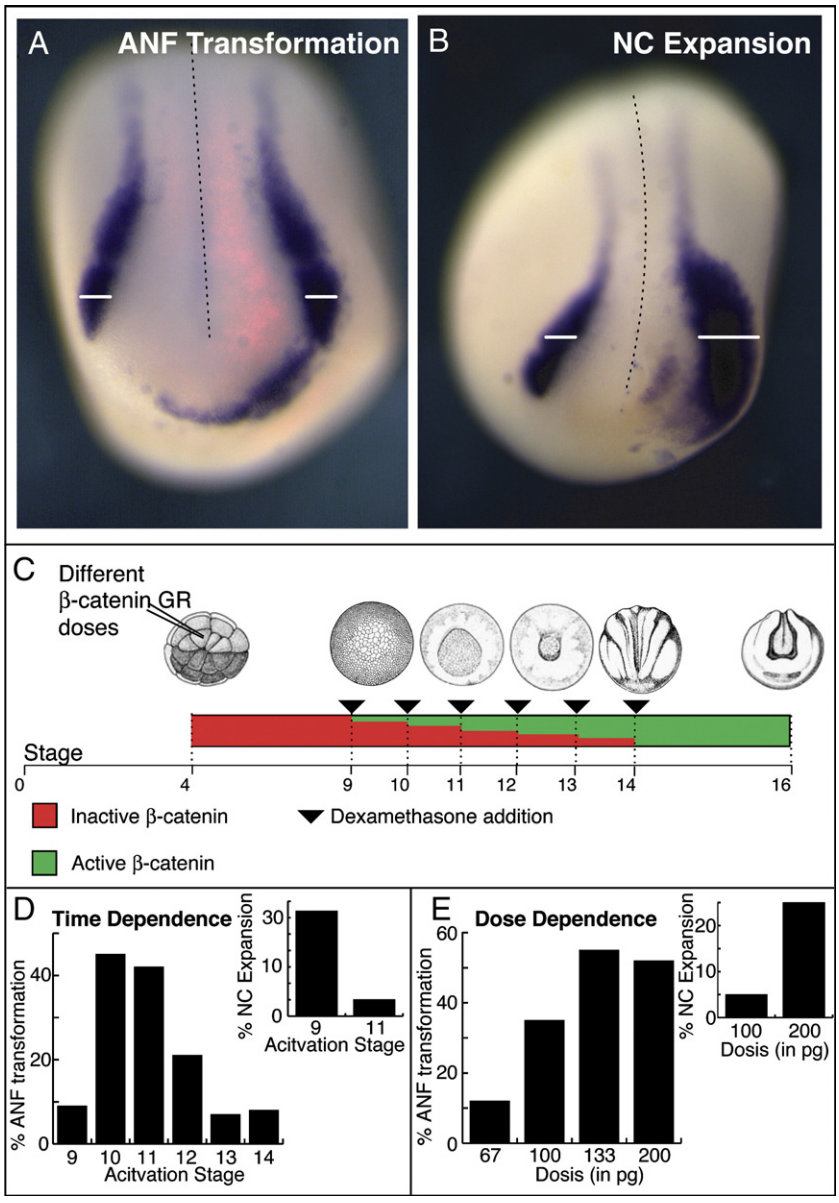

Fig. 6. Temporal analysis of transformation of ANF into NC. (A) Representative embryo showing transformation phenotype monitored by the expression of the NC-specific gene *Snail2*. Note the transformation of the ANF into NC with almost no effect in the medio-lateral axis. (B) Representative embryo showing the expansion phenotype monitored by the expression of *Snail2*. Note that the expansion of the NC is as much in the anterior–posterior axis as it is in the medio-lateral axis. (C) Diagram to show the experimental design used in panels D and E. Embryos were injected with different doses of β-catenin-GR mRNA at the 32-cell stage and treated with dexamethasone at different stages (green bar), the expression of NC markers was analyzed at stage 16. (D) Analysis of the ANF transformation phenotype and NC expansion phenotype (inset) after injection of 100 pg of β-catenin-GR and activation at different stages. Note that the highest ANF transformation is reached with activation at stage 10–11 while the highest NC expansion phenotype is reached with activation at stage 9. (E) Analysis of the ANF transformation phenotype and NC expansion phenotype (inset) after injection of different doses of β-catenin and activation at stage 11. Note that the higher doses of β-catenin can still induce NC expansion at later stages (inset).

According to these results, the ectopic induction of NC is not at expenses of anterior neural tissue or placodes. Because usually the injected embryos have a thicker ectoderm, we hypothesized that this could be due to an increase in cell number. To test this, we analyzed the levels of cell proliferation on β-catenin injected embryo by BrdU staining. A significant increase ($p<0.05$) on the number of BrdU-positive nuclei was observed in injected regions (Figs. 7N, O). This difference was most obvious in the ANF region (Fig. 7N, black arrowhead). These experiments suggest that, in parallel to the ANF transformation, the activation of the Wnt signaling might increase the cell number by promoting proliferation. This can account for the coexistence of NC and other populations on the transformed ANF.

NC cells induced at the ANF are able to migrate and differentiate as normal NC cells

It is possible that some treatments can transiently change the expression profile of a cell population without changing its fate and behavior at later stages. Can NC cells induced at the ANF by

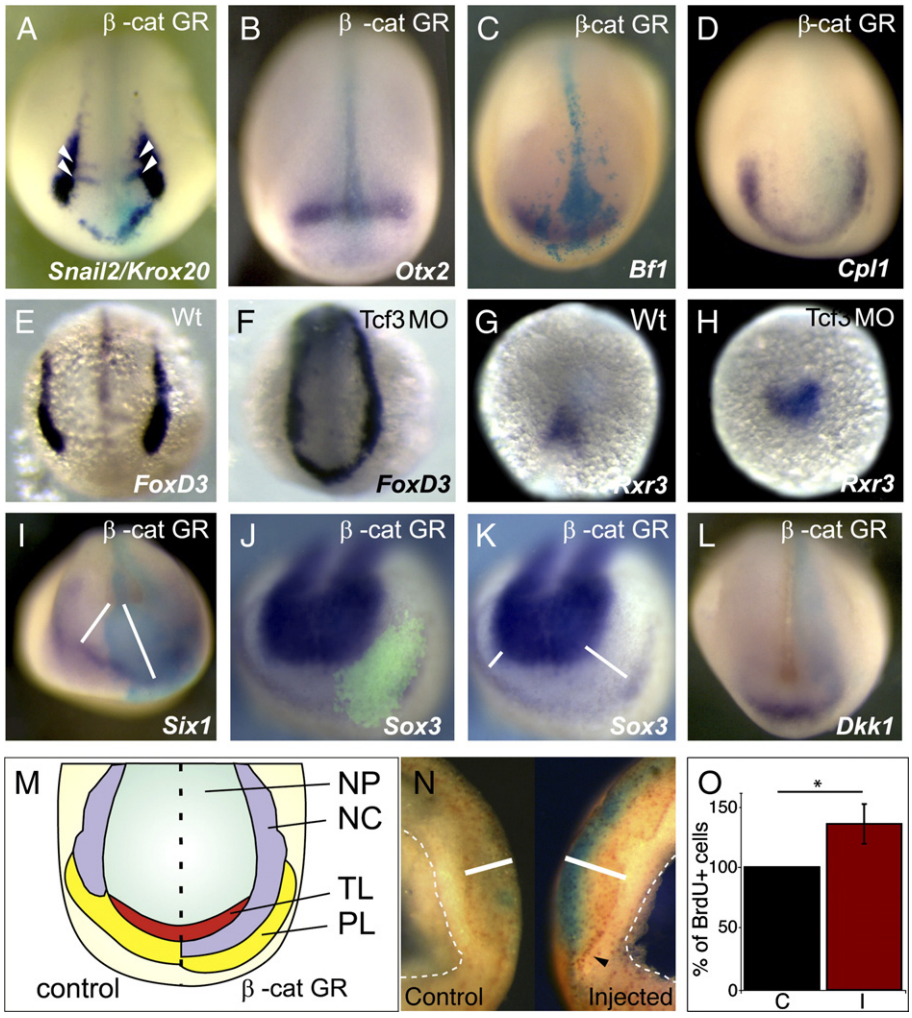

Fig. 7. Analysis of neural plate and placodal markers in embryos with NC at the ANF. (A–D, I–L) *Xenopus* embryos were injected with 130 pg of β-catenin mRNA in A1 blastomere at 32-cell stage, treated with dexamethasone at stage 10.5, and the expression of different markers was analyzed at stage 16. All embryos are shown in anterior views; injected side is to the right and recognized by FLDx or β-gal staining (blue color). (A) *Snail2*/*Krox20*. Note expression of *Snail2* (52%, $n=50$) at the ANF and normal expression of *Krox20* (white arrowheads, 95%, $n=40$). (B) *Otx2*. Note no effect on *Otx2* in the injected side (23%, $n=45$). (C) *Bf1*. No effect on *Bf1* at the injected side (12%, $n=41$). (D) *Cpl1*. No effect on *Cpl1* at the injected side (17%, $n=42$). (E, F) Zebrafish embryos. (E) Wild type embryos showing expression of *FoxD3*. (F) Embryo injected with 0.01 pmol of Tcf3a MO. Note the expression of *FoxD3* at the ANF (100%, $n=50$). (G, H) Sibling embryos of panels E and F, respectively analyzed for *Rxr3* expression. Note the normal expression of *Rxr3* in wild type and injected embryo (75%, $n=40$). (I–L) Placodal markers. White lines indicate the distance between the neural grove or neural plate and the placodal markers. (I) *Six1* expression. Note the anterior–ventral shift in the expression of *Six1* at the injected side (47%, $n=48$). (J, K) *Sox3* expression. Note the shift in the preplacodal domain of *Sox3* expression (45%, $n=45$), while the neural plate domain is not affected. (L) Dkk1 expression; note similar expression in the injected and uninjected side (99%, $n=40$). (M) Summary of anterior markers. NP: neural plate; NC: neural crest; TL: telencephalon; PL: placodal field. (N) BrdU staining. Left: control side; right: injected side. Bar indicates the thickness of the ectoderm which is higher in the injected side; black arrowhead shows the ANF where there is a particularly higher number of stained nuclei in the injected side. (O) Summary of BrdU staining. BrdU-positive nuclei in the injected ectoderm compared with the ones on the contralateral uninjected region.

inhibition of Dkk1 or activation of Wnt behave like normal NC cells? The most prominent characteristics of NC cells are their abilities to migrate and to differentiate into derivatives such as melanocytes, cartilaginous and bony elements in the head, among others. To test whether the ectopically induced NC cells are able to migrate, β-catenin mRNA was co-injected with nuclear GFP under conditions that induce ectopic expression of NC markers in the ANF. At the neurula stage (stage 16) the ANF was labeled with DiI on both the injected and control side (Fig. 8A). The embryos were cultured and cell migration was analyzed by looking at the DiI fluorescence. Immediately after DiI labeling the fluorescence was similar in the control and β-catenin injected sides (Fig. 8B). In the control side at the late neurula stage the DiI labeling remains in the anterior region of the head (Fig. 8D), while the β-catenin injected cells have moved backwards (Fig. 8C, black arrowhead) and start to migrate, joining the normal streams of NC cells (Fig. 8E). This observation shows not only that the NC cells are able to migrate but also that they are able to follow the NC in their normal migratory pathway. In some few cases the ectopically induced NC cells migrated in the head region and did not join the endogenous, more caudal, NC. A second experiment was performed to show the migratory ability of the induced NC cells. When normal NC cells are cultured on fibronectin they are able

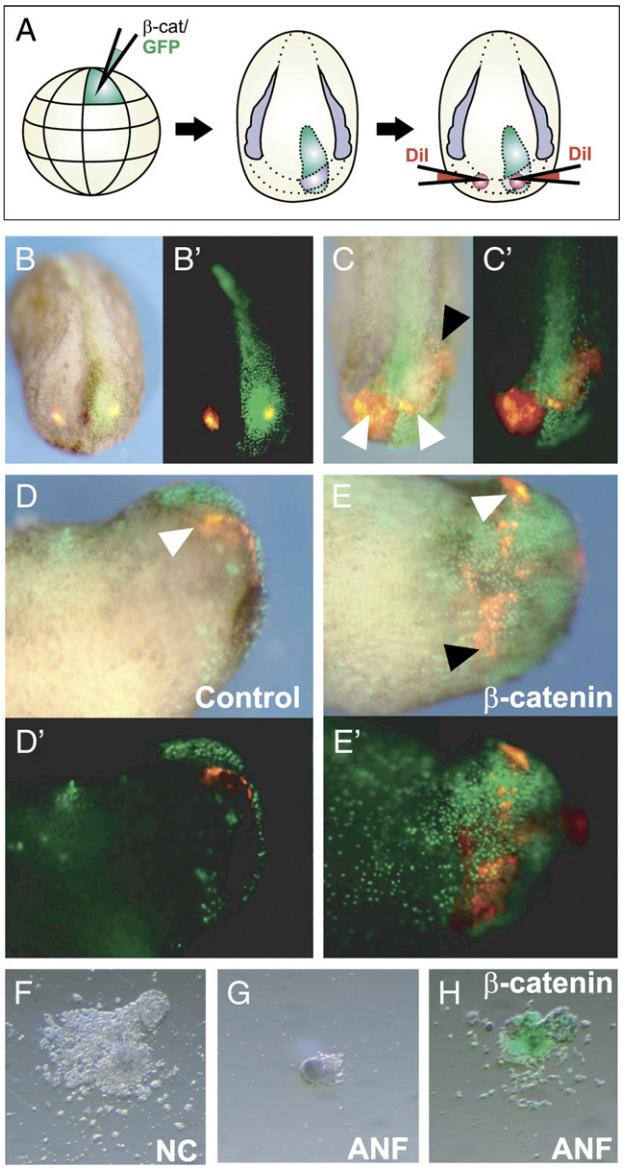

Fig. 8. NC induced at the ANF is able to migrate. (A) Embryos were injected with β-catenin-GR and nuclear GFP in A1 blastomere at 32-cell stage and activated at stage 10.5. At stage 16 symmetrical points at the injected and uninjected ANF were DiI labeled, the embryos were cultured until stage 28, when the migration of the DiI labeled cells was analyzed. (B) Dorso-anterior view of an embryo immediately after the DiI labeling; note the symmetric distribution of both DiI spots and that one overlaps with the GFP. (C) Dorso-anterior view of an embryo at stage 25. Note that both DiI spots have enlarged, but the one that overlaps with GFP has moved backwards and has started to move ventrally (black arrowhead); white arrowhead: initial position of DiI. (D, D′) Control side of a stage 28 embryo, showing a small expansion of the DiI label but no ventral migration; $n=24$, 0% of embryos with migration. (E, E′) Injected side of a stage 28 embryo, showing ventral migration of the DiI labeled cells; $n=24$, 79% of embryo with migration. (F) Control NC was dissected from a stage 15 embryo and cultured on fibronectin for 16 h. Note the migration of NC cells; $n=5$, 100% of migration. (G) ANF dissected from a stage 15 embryo and cultured on fibronectin for 16 h. No migration is observed, $n=5$, 0% migration. (H) ANF dissected from a stage 15 embryo, previously injected with β-catenin into the ANF region. Note the migration of the cells similar to the NC cells; $n=5$, 100% of migration.

to migrate in vitro (Fig. 8F; Alfandari et al., 2003); as expected, cells from the ANF cultured under the same conditions are never able to migrate (Fig. 8G). However, an equivalent ANF taken from an embryo injected with β-catenin under the previous conditions shows migratory activity undistinguishable from normal NC cells, indicating that this is a cell autonomous property (Fig. 8H). In conclusion, the ANF that expresses NC markers is able to migrate in vivo and in vitro as normal NC cells.

A second characteristic of the NC is its ability to differentiate into specific cell types. When embryos injected with β-catenin were left to develop at later stages several NC derivatives were increased or in some case ectopic NC derivatives were observed. Meckel, ceratohyal and ceratobranchial cartilage were expanded in β-catenin injected embryos (Figs. 9A–D); melanocytes were more numerous in the head and sometimes produced a completely black head (Figs. 9E, F). The dorsal fin which in control embryos ends up at the posterior limit of the head (Fig. 9G, arrowhead) was usually extended to the most anterior tip of the head (Fig. 9H, arrow), and sometime ectopic fin-like structures were observed in the head (not shown). Very frequently we observed gill-like structures, which are present only in the branchial arches region of control embryos (Figs. 9I, J), also in the anterior region of the head in the injected embryos (Figs. 9K, L). We should mention that cephalic cartilage and melanocytes are clearly NC derivatives, while dorsal fin and gill are not only NC derived. Taken together, these observations show that the NC induced at the ANF behave as normal cranial NC cells in their migratory and differentiation potential.

Discussion

Fate map studies show that NC originated from the neural plate border except from the most anterior neural fold (Couly and Le Douarin, 1985; Couly et al., 1993). The expression pattern of NC markers (*Snail2*, *Snail*, *FoxD3*, etc.) also supports this conclusion (Essex et al., 1993; Mayor et al., 1995; Sasai et al., 2001; Schlosser and Ahrens, 2004). These studies revealed that the ANF contributes to the forebrain and some placodal cells. However, it was not known by which mechanism the ANF is deprived of NC. Here we present evidence that an active inhibition of NC cells takes place at the ANF. First, grafts of NC into the ANF prevent them from expressing NC markers, which are otherwise expressed when they are cultured in vitro (Fig. 2); second, juxtaposition of NC with prechordal mesoderm (PM) is sufficient to inhibit NC formation (Fig. 2); third, Dkk1, which is produced by the PM, can mimic this NC inhibition (Fig. 3). Finally, loss-of-function experiments of Dkk1, using a functional blocking antibody in *Xenopus* or a *Dkk1* null mouse mutant, transform the ANF into NC (Fig. 4). To our knowledge, this is the first time that an inhibitory signal has been implicated in NC formation.

As shown in transplant experiments (Fig. 2), the highest level of NC inhibition is reached with NC at stage 14 or younger, while hardly any inhibition can be obtained after stage 17. This timing of NC inhibition in vivo is similar to the inhibition seen when using Dkk1 mRNA (Fig. 3D), showing that Dkk1 activity can also mimic the temporal aspects of inhibition of NC formation in vivo.

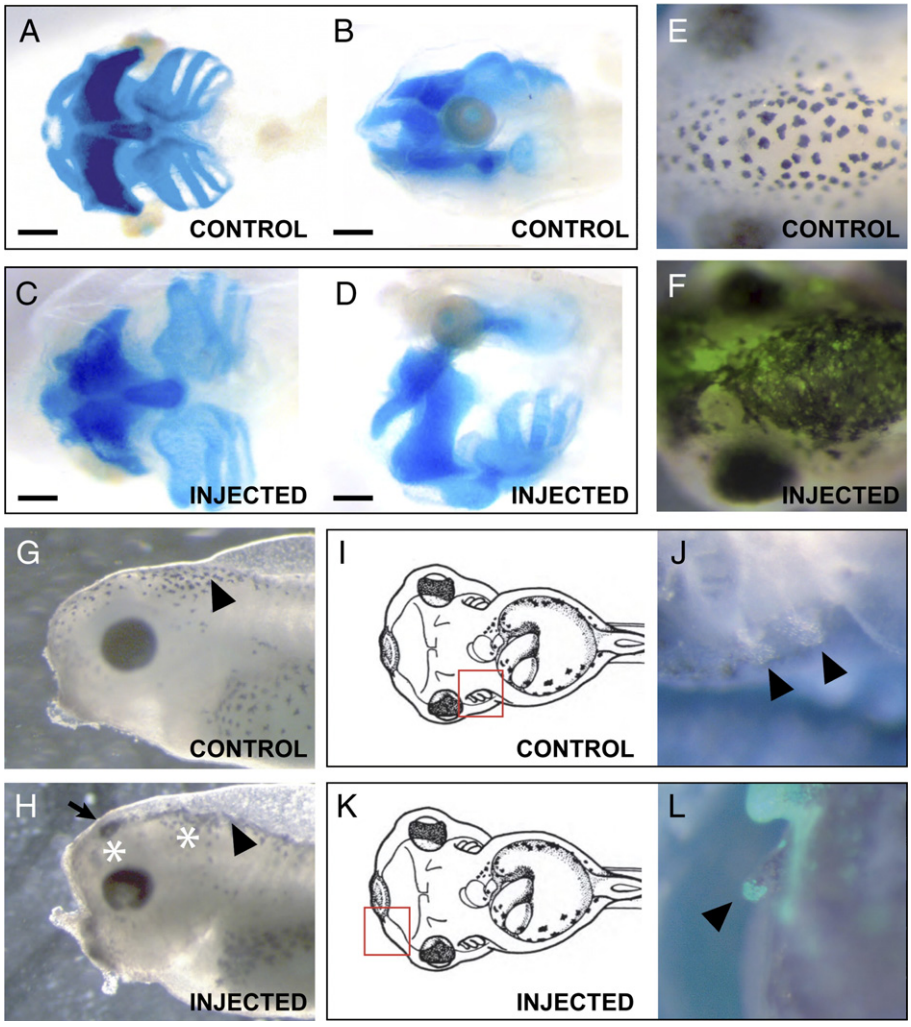

Fig. 9. NC induced at the ANF is able to differentiate. (A–D) Cartilage staining of embryos at stage 40. Scale bar: 150 μm. (A, B) Control embryos. (A) Ventral view; (B) lateral view. (C, D) Embryo injected with β-catenin in the ANF. (C) Ventral view; (D) lateral view. Note the expansion on the cartilages (30–40%). (E) Dorsal view of the head of a stage 40 embryo, showing the normal melanocyte pattern. (F) Dorsal view of the head of an embryo stage 40 injected with β-catenin in the ANF, note the increase in the number of melanocytes (50–60%). (G) Lateral view of the head of a stage 35 embryo; arrowhead indicates the anterior end of the dorsal fin. (H) Lateral view of the head of a stage 35 embryo injected with β-catenin in the ANF; note that the normal position of the anterior limit of the dorsal fin (arrowhead) has moved anteriorly (arrow, 40–50%). Note also a mild increase on the number of dorsal melanocytes (white asterisks). (I–L) Analysis of gill-like structures. (I, J) Gills of a normal embryo, localized in the branchial region (arrowhead). Square in panel I corresponds to the region shown in panel J. (K, L) Ectopic gill-like structures formed in the anterior region of the head in an embryo injected with β-catenin in the ANF (50–60%). Square in panel K corresponds to the region shown in panel L. Approximately 100 embryos were analyzed.

The experiments using the Dkk1 antibody and the *Dkk1* null mutant show the essential role of Dkk1 for the inhibition of NC formation at the ANF. However, it is well established that in the *Dkk1* null mutant there is a dramatic reduction of forebrain development (inset in Fig. 4F and Mukhopadhyay et al., 2001), and the expression of the NC markers at the ANF could be only a morphological consequence of the absence of forebrain tissue which in normal embryos separates the two lateral NC domains. However, more detailed observation of the embryos suggests that this is not the case as the most anterior domain of *Sox10* expression is larger in the mutant than in wild type embryos, indicating an increase in the number of crest cells in the anterior region. This observation is confirmed in *Xenopus* where we can have a better control of Wnt signaling, in space and time, and in which we were able to transform the ANF into NC without affecting forebrain specification. This improved control of Wnt signaling was reached by local expression of inducible β-catenin, a downstream target of Wnt. In addition to forebrain development, Wnt signaling has been implicated in placodal and anterior–posterior patterning (Braun et al., 2003; Kiecker and Niehrs, 2001; McGrew et al., 1997; Erter et al., 2001, Itoh and Sokol, 1997; Niehrs, 2001; Nordstrom et al., 2002; Brugmann et al., 2004; Litsiou et al., 2005). However, as we can control the space, time and level of β-catenin activation we have been able to dissociate the transformation of the ANF into NC from the effect of Wnt signaling on placodal development and AP patterning, in addition to forebrain development. Thus, our embryos show ectopic expression of NC markers in the ANF, and normal levels in the expression of forebrain, placodal and AP markers (Fig. 7M). This observation that no

anterior markers are lost at the expense of NC cells can be explained by the increase in cell number after activation of Wnt signaling. In fact, we observed an increase in cell proliferation in the ANF that was accompanied by the expression of NC markers (Figs. 7N, O).

We show that the ANF transformation into NC is not the result of lack of anterior structures in injected embryos as anterior markers do not disappear. We were able to experimentally dissociate the ANF transformation from AP patterning but we cannot say that ectodermal patterning and NC inhibition at the ANF are actually two completely different processes in the developing embryo. Nonetheless, our results suggest that at least different levels of Wnt activity are required to subdivide the anterior neural plate region, the preplacodal field and the NC, the latter being the most sensitive. If higher levels of Wnt signaling are reached at the ANF, an inhibition of forebrain and placodal markers is observed (Fig. S1), as has been already described for the Dkk1 mouse mutant, the *headless* zebrafish mutant and by activation of Wnt signaling in *Xenopus* and chick embryos (Kim et al., 2000; Mukhopadhyay et al., 2001; Brugmann et al., 2004; Litsiou et al., 2005). An interesting observation is the anterior–ventral shift of the placodal markers produced by the transformation of ANF into NC. This suggests that the induction of NC at the ANF is not at the expense of placodal ectoderm as the ectopically induced NC cells "push" the preplacodal field away.

It has been shown by many laboratories that Wnt activity is required for NC induction (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Lekven et al., 2001; Deardorff et al., 2001; Villanueva et al., 2002; Garcia-Castro et al., 2002; Tribulo et al., 2003; Lewis et al., 2004; Bastidas et al., 2004). Therefore, it is possible that the transformation of ANF into NC described here is part of the process of NC induction. However, our results suggest that these are two different processes. Competence of ectoderm to Wnt signaling to expand the NC territory is almost lost after the beginning of gastrulation, while the ability of the same signal to transform the ANF into NC reaches its peak during gastrulation; only treatments producing high levels of β-catenin are able to generate both phenotypes after gastrulation (Fig. 6).

Another interesting observation is that inhibition of Wnt signaling by Dkk1 in cells that are already specified as NC is able to reverse the specification state and blocks the expression of NC markers (Fig. 3); this is something that had not been tested before as all the published experiments blocked Wnt signaling before the stage at which the NC begins to express any of its characteristic markers. The inhibition of NC in vitro by Dkk1 suggests that the NC produces its own secreted Wnt, which can be blocked by Dkk1. We have also observed that Wnt8 is expressed in the NC of early neurula embryos (C. C-F. and R. M. unpublished), which further supports this idea. This observation suggests that NC cells require a continuous source of Wnt signaling and that part of the mechanism of NC specification could be the production of Wnts by the NC itself.

The activation of Wnt signaling in the anterior neural plate leads to a very discrete expression of NC markers only in the ANF. This suggests that additional and earlier factors control the competence of this region to respond to the Wnt signals and produce NC cells. As already discussed, induction of NC appears to be an earlier event as inhibition of NC development at the ANF. These observations suggest that, in an early step of NC specification, several signals (including Wnt and BMPs) specify the entire border of the neural plate as prospective NC, and then Dkk1 inhibits NC development at the ANF as a later step (Fig. 1C).

Here we provide evidence that Dkk1 activity is essential for this inhibition but we cannot rule out the possibility that additional molecules, which may or may not be related to Wnt or other NC inductive signals, could also be involved. It is interesting to note that inhibition of Cullin-1, a protein that targets β-catenin for degradation, leads to a similar transformation of ANF into NC (Voigt and Papalopulu, 2006). We should emphasize that, although the transformation of ANF into NC has been reported before by treatments that activate Wnt, FGF or retinoic acid signaling (Villanueva et al., 2002; Monsoro-Burq et al., 2003; Wu et al., 2005; Voigt and Papalopulu, 2006), this is the first time that an active process of neural crest inhibition has been described (Fig. 2). Thus, the simple observation that activation of a signaling pathway at the ANF leads to the expression of NC markers in that region, as it has been described (Villanueva et al., 2002; Monsoro-Burq et al., 2003; Wu et al., 2005; Voigt and Papalopulu, 2006), does not prove that there is inhibition of those signals at the ANF. Additional experiments will be required to analyze whether a similar inhibitory mechanism acts on other neural crest inducing signals in the ANF.

Our results show that the induced ANF cells are proper NC cells, with the ability to migrate in vitro and in vivo, to follow the normal migratory pathways and to differentiate into many normal cranial NC derivatives, such as cartilage, melanocytes, dorsal fin and gill-like structures (Figs. 8, 9). We have observed that when ectopic NC is induced at the ANF some few cells remain at that position and differentiate to produce ectopic NC derivatives in the anterior region of the head. However, most of the ectopically induced NC cells join the normal migratory pathways and contribute to enlarged NC derivatives. The ability of the NC to join the correct migratory pathways could suggest the existence of repulsive molecules in the ectopic location. The role for negatives cues that repress NC migration, such as ephrins, Slit/Robo and semaphorins, has been very well documented (Krull et al., 1997; Smith et al., 1997; De Bellard et al., 2003; Kawasaki et al., 2002). Alternatively, positive signals from the NC or the NC migratory pathways might also contribute to guide the ectopic NC cells to normal migratory paths.

Finally, we have shown a similar transformation of the ANF into NC in fish, amphibian and mammals, indicating that the inhibition of NC at the ANF has been conserved during evolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:doi:10.1016/j.ydbio.2007.07.006.

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