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RESEARCH REPORT

Snail2 and Zeb2 repress *P-cadherin* to define embryonic territories in the chick embryo

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ABSTRACT

Snail and Zeb transcription factors induce epithelial-to-mesenchymal transition (EMT) in embryonic and adult tissues by direct repression of *E-cadherin* transcription. The repression of E-cadherin transcription by the EMT inducers Snail1 and Zeb2 plays a fundamental role in defining embryonic territories in the mouse, as E-cadherin needs to be downregulated in the primitive streak and in the epiblast, concomitant with the formation of mesendodermal precursors and the neural plate, respectively. Here, we show that in the chick embryo, *E-cadherin* is weakly expressed in the epiblast at pre-primitive streak stages where it is substituted for by *P-cadherin*. We also show that *Snail2* and *Zeb2* repress *P-cadherin* transcription in the primitive streak and the neural plate, respectively. This indicates that *E-* and *P-cadherin* expression patterns evolved differently between chick and mouse. As such, the Snail1/E-cadherin axis described in the early mouse embryo corresponds to Snail2/P-cadherin in the chick, but both Snail factors and Zeb2 fulfil a similar role in chick and mouse in directly repressing ectodermal cadherin genes to contribute to the delamination of mesendodermal precursors at gastrulation and the proper specification of the neural ectoderm during neural induction.

KEY WORDS: E-cadherin repressors, EMT, P-cadherin, Sip1, Gastrulation, Neural plate

INTRODUCTION

During early embryonic development, the embryo progresses from a single layer of epithelial cells (the epiblast) to a three-dimensional structure composed of several layers and territories. As part of this complex process, embryonic cells integrate environmental cues to acquire positional information, fate specification and control of cell behaviours, resulting in the formation of embryonic layers, either by modelling epithelial sheets or by inducing individual or collective cell migration. The epithelial-to-mesenchymal transition (EMT) enables delamination at the primitive streak to give rise to the definitive endoderm and mesoderm, while cells that remain in the epiblast at gastrulation contribute to the ectoderm (reviewed by Acloque et al., 2009).

The EMT program is triggered by the activation of transcription factors called EMT-TFs that include the Snail and Zeb families (i.e. Nieto et al., 1994; Vandewalle et al., 2009), which directly repress *E-cadherin* transcription, confirming their crucial role in modulating cell adhesion (Batlle et al., 2000; Cano et al., 2000; Eger et al., 2005; Comijn et al., 2001). Snai1 in mammals and *Snail2* in birds are expressed in the ingressing cells at the primitive streak, in neural crest cells delaminating from the neural tube, in the presomitic mesoderm and the lateral plate mesoderm among other EMT territories (Nieto et al., 1994; Acloque et al., 2011; Blanco et al., 2007; Morales et al., 2007; Dale et al., 2006; Niessen et al., 2008). Snail1 mouse mutants maintain high levels of E-cadherin at the primitive streak, do not complete EMT and the resulting defective mesoderm fails to migrate (Carver et al., 2001). In chick and mouse, *Zeb2* (also known as *Sip1*) is expressed in the neural plate and neural tube. Zeb2 does not induce EMT, as these territories remain epithelial all throughout neurulation, but defines the neural versus the non-neural ectoderm (Van de Putte, 2003; Van Grunsven et al., 2007; Vandewalle et al., 2009). Like Snail mutants, *Zeb2* mutant embryos maintain E-cadherin expression in the corresponding territories: the neural plate and the presumptive neural crest. These mice exhibit multiple neural crest defects, fail to specify the neuroepithelium correctly and die right after neurulation, at E9.5 (Van de Putte, 2003). All these data support the importance of E-cadherin repression in the definition of embryonic territories and subsequent tissue differentiation in the mouse. In the chick embryo, L-CAM was proposed to be the functional equivalent of E-cadherin in the mouse because it is expressed in the chick epiblast (Dady et al., 2012; Ohta et al., 2007). However, *L-CAM* is only faintly expressed in the epiblast of pre-primitive and primitive streak chick embryos (Moly et al., 2016 and this work). The chicken genome includes another type I cadherin, located in a cluster adjacent to *L-CAM* (E-cadherin). Because this is reminiscent of the organization of *P-* and *E-cadherin* in mammals, Redies and Müller (1994) proposed that this is the homologue of *P-cadherin* in the chick. Here, we show that the chick embryo mostly expresses *P-cadherin* instead of *E-cadherin* in the epiblast and that, like E-cadherin in the mouse, P-cadherin is downregulated in the mesoderm and in the induced neural plate while it is maintained in non-neural ectoderm. As *P-cadherin* expression is complementary to that of Snail and Zeb genes, we performed gain- and loss-of-function experiments to evaluate whether these epithelial repressors are responsible for the downregulation of *P-cadherin* during primitive streak stages, when *Snail2* is expressed in the streak and in the ingressing mesendoderm, and at neurulation stages, when *Zeb2/Sip1* is expressed in the early neural plate. We find that Snail2 and Zeb2 repress *P-cadherin* expression in the chick embryo as Snail1 and Zeb2 repress E-cadherin in the mouse, indicating a reshuffling in the expression of Snail and cadherin family members and a functional conservation in the mechanism that helps define embryonic territories in vertebrates.

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RESULTS AND DISCUSSION

Expression profiles of type I cadherins differ between chick and mouse embryos

It is currently assumed that, as in mammals, E-cadherin (L-CAM) is expressed in the epiblast of the chicken embryo (Bobbs et al., 2012; Thiery et al., 1984). However, *in situ* hybridization for *E-cadherin* reveals very weak expression before Hamburger and Hamilton stage (HH) 9 (Hamburger and Hamilton, 1951) (Fig. 1A,D,G; Moly et al., 2016). To assess whether another cadherin could substitute for *E-cadherin* in the early chick embryo, we examined the expression of other type I cadherins and observed *N-cadherin* strongly expressed in the early mesoderm as previously described (Fig. 1B; Hatta and Takeichi, 1986; García-Castro et al., 2000), but also strong expression of *P-cadherin* in the epiblast (Fig. 1C,F). Real-time RT-PCR on embryos at pre-primitive streak (EGXI-XIII) (pre-primitive streak stages according to Eyal-Giladi and Kochav, 1976; EG), primitive streak (HH4) and neurulation (HH9) stages and in chick embryonic fibroblasts (CEFs) shows that *P-cadherin* is expressed around 10-fold more strongly than *E-cadherin* at EGXI and HH4, and 2-fold more at HH9 and *N-cadherin* is predominantly expressed in CEFs and to a lesser extent at HH4 and HH9 (Fig. S1), all confirmed by *in situ* hybridization at equivalent stages (Fig. 1A-I). Thus, *P-cadherin* is the predominant type I cadherin expressed in the chick embryo epiblast at pre-primitive and primitive streak stages. Double *in situ* hybridization for *P-* and *N-cadherin* in gastrulating embryos (Fig. 1J) shows patterns reminiscent of those described in the mouse for *E-* and *N-cadherin*, respectively (Radice et al., 1997). In the mouse, *P-cadherin* is not expressed in the epiblast and appears in the extra-embryonic ectoderm and in the visceral endoderm, and later in various embryonic epithelia (Nose and Takeichi, 1986; Hirai et al., 1989; Palacios et al., 1995; Lin and DePhilip, 1996; Xu et al., 2002). Our data suggest an exchange between the expression of *P-* and *E-cadherin* in chicken and mouse, reminiscent of the swap that occurred between the two transcription factors, *Snail1* and *Snail2*, during evolution (Locascio et al., 2002). *Snail2* in the chick shows a pattern of expression reminiscent of that of *Snail1* in the mouse.

Snail2 represses *P-cadherin* in the epiblast of primitive streak stage chick embryos

As expected, because of its role as an *E-cadherin* repressor, the pattern of *Snail1* expression is complementary to that of *E-cadherin* in the mouse embryo (Cano et al., 2000). Because in early chick embryos *P-cadherin* is mostly expressed in the same way as mouse *E-cadherin*, we examined whether *Snail2* and *P-cadherin* are expressed in complementary patterns during gastrulation (Fig. 1K,L). *P-cadherin* is mostly expressed in the ectoderm and its expression decreases in *Snail2* positive cells at the primitive streak and after ingression (Fig. 1M), compatible with the idea that Snail2 may be a repressor of *P-cadherin*. To test this, we overexpressed Snail2 in the anterior epiblast of stage HH3 embryos. Embryos showed a decrease of *P-cadherin* expression in the electroporated area when compared with the control side (Fig. 2D-F, n=14/14) or with embryos electroporated with a GFP-only control construct (Fig. 2A-C, n=11/11). Conversely, knockdown of *Snail2* using double-stranded RNA (dsRNA) (Pekarik et al., 2003) expands the territory of *P-cadherin* expression up to the midline of the embryo at the primitive streak, where it is normally downregulated (Fig. 2G-I, n=4/6; see Fig. S2 to assess Snail2 downregulation, n=3). These data indicate that Snail2 represses *P-cadherin* transcription in the chick epiblast.

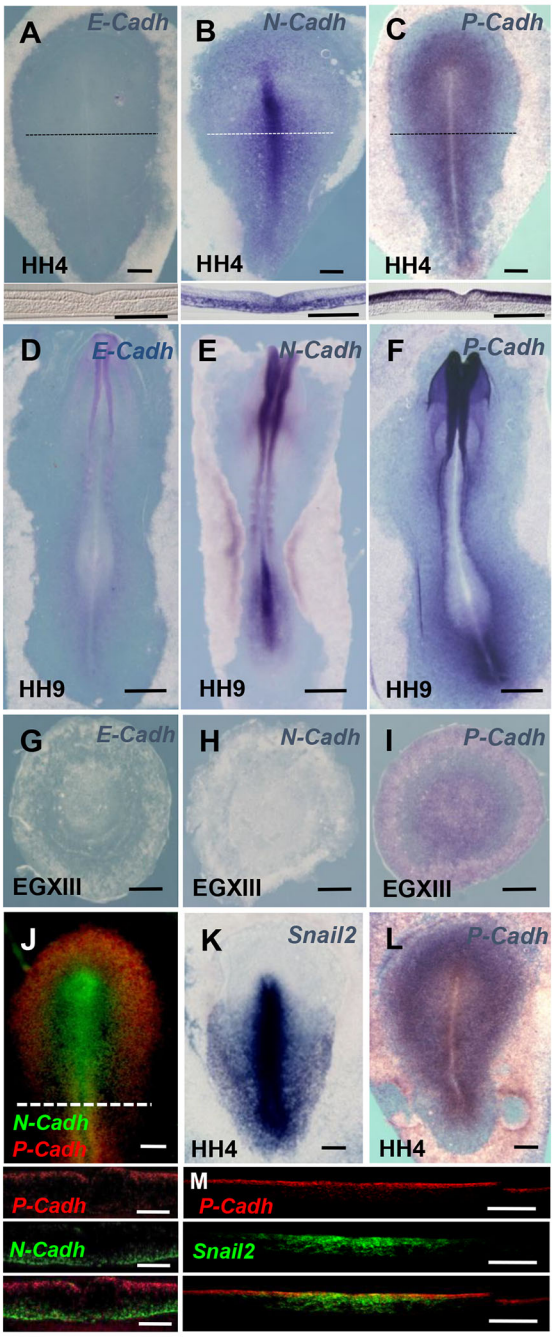

Fig. 1. Expression of type I cadherins relative to *Snail2* in the early chick embryo. Whole-mount *in situ* hybridization for *E-cadherin* (L-CAM), *N-cadherin* and *P-cadherin* (also called K-CAM or B-cadherin) at HH4 (A-C), HH9 (D-F) and EGXIII (G-I). (J) Double *in situ* hybridization confirms the complementary expression of *P-* and *N-cadherin* in primitive streak stage embryos. (K-M) Complementary expression of *P-cadherin* and *Snail2*. The dotted lines indicate the level of the tissue sections. Scale bars: 500 µm in A-C, K-M; 250 µm in J; 1 mm in D-I.

We have previously shown that overexpression of Snail2 in the chick epiblast induces ectopic EMT along with downregulation of cadherin protein and disruption of the basement membrane, and that a similar mechanism operates in the mouse for Snail1 (Acloque et al., 2011). Although a recent study proposed that downregulation of P- and E-cadherin are not necessary for EMT to occur in the chick (Moly et al., 2016), our data, in addition to

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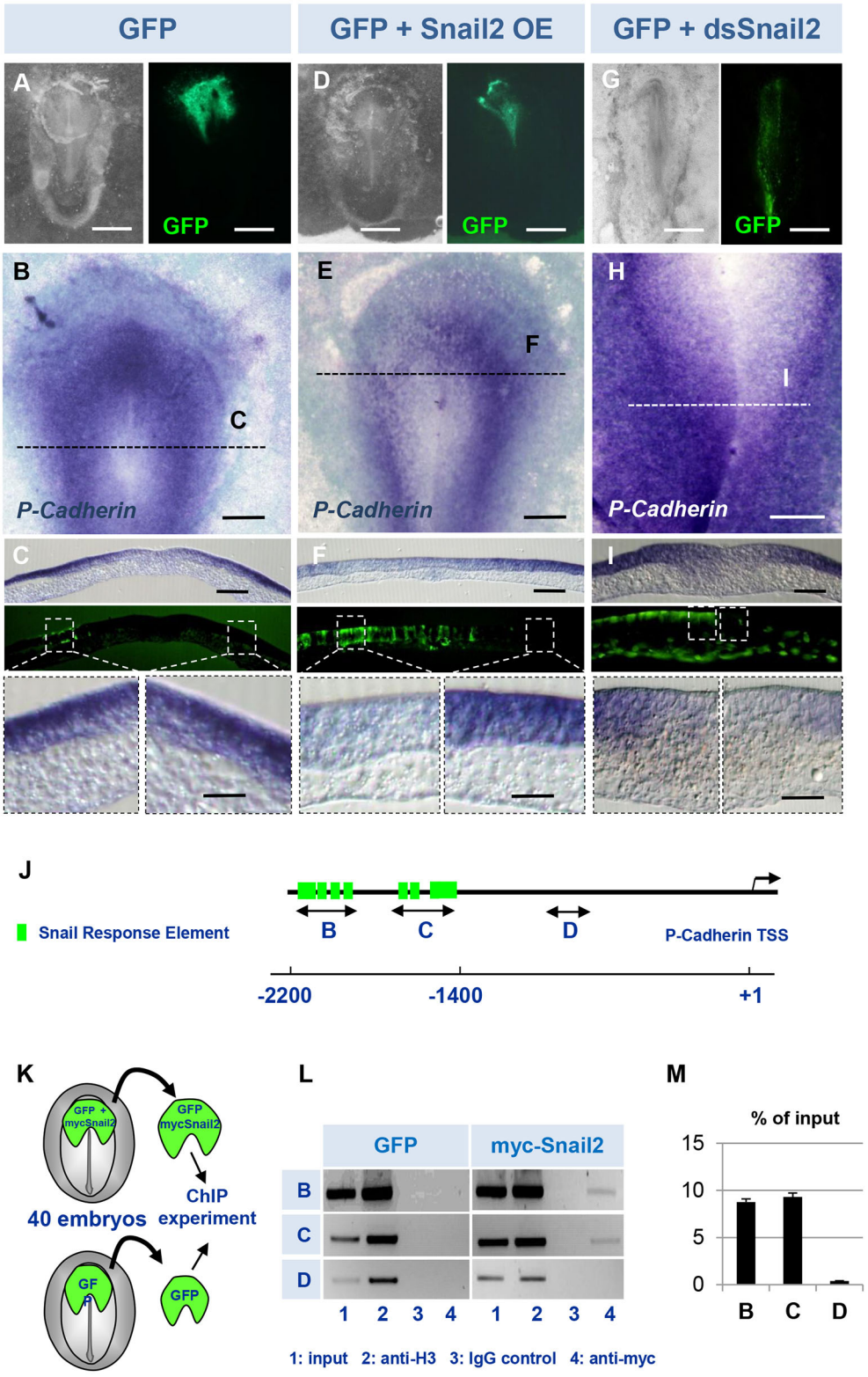

Fig. 2. Snail2 directly represses *P-cadherin* transcription in the epiblast of primitive streak stage chick embryos. (A-C) GFP ectopic expression does not affect *P-cadherin* transcripts in the epiblast of early chick embryos, as observed in whole embryos and sections. (D-F) Snail2 overexpression causes downregulation of *P-cadherin* expression in the epiblast (compare the electroporated with the control side in E,F). (G-I) Knock down of *Snail2* extends *P-cadherin* expression to the midline, adjacent to the primitive streak (H, sections in I). (J) Specific regions enriched for Snail-binding sites (E-boxes with the consensus CASSTG), are located between 2.2 kb and 1.4 kb upstream of the *P-cadherin* TSS (regions B and C). (K) Electroporated prospective neural plate regions ectopically expressing either GFP or GFP together with a Myc-tagged Snail2 were dissected and subjected to chromatin immunoprecipitation (ChIP) assays. (L,M) DNAs from the chromatin-enriched fraction for the regions described in J were amplified by PCR. Amplification from input DNA is shown in lane 1, and positive and negative controls using either a pan-H3 antibody or an IgG control are shown in lanes 2 and 3, respectively. When Myc-Snail2 was expressed in the epiblast, regions B and C were specifically enriched in the immunoprecipitated fractions, detected with an anti-Myc antibody (compare the GFP control conditions shown in lane 4). The quantification of enrichment is shown in M. Data represent mean±s.d. of three independent experiments. Scale bars: 1 mm in A,D,G; 400 µm in B,C,E,F,H,I; 150 µm in the magnified panels of the sections.

previous studies in various models (Ramkumar et al., 2016; Schäfer et al., 2014; Rogers et al., 2013; Carver et al., 2001; Wu and McClay, 2007; Oda et al., 1998), confirm the downregulation of *E-* and *P-cadherin* transcripts at sites of EMT, and support a model in which their transcriptional downregulation is necessary for the transition towards a mesenchymal tissue arrangement. As the half-life of E-cadherin and β-catenin proteins at adherens junction can exceed 25 h in epithelial cell lines (Lozano and Cano, 1998), additional mechanisms favouring E-cadherin endocytosis and players such as Rho modulators, Crumbs2 or the MAP-kinase pathway are fundamental to speed the turnover and removal of E-cadherin in remodelled embryonic epithelia (Nakaya et al., 2008; Ramkumar et al., 2016; Moly et al., 2016; Zohn et al., 2006).

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Snail2 binds to the *P-cadherin* promoter *in vivo*

Snail factors repress *E-cadherin* transcription directly by binding to specific E-boxes located in the *E-cadherin* promoter (Cano et al., 2000; Batlle et al., 2000). We therefore examined whether Snail2 binds to the *P-cadherin* promoter. As the sequence of P-cadherin is incomplete at the 5′ end of the gene in the public databases, we used RNA-seq data together with 5′-RACE and ESTs alignments to define a putative transcription start site (TSS) of the *P-cadherin* gene. After combining 5′-RACE and genomic DNA PCR amplification to obtain additional sequences, we aligned RNA-seq data on this reconstructed *P-cadherin* locus and confirm the genomic structure of the *P-cadherin* gene as containing 15 exons over 5.7 kb (Fig. S3), encompassing the full coding region (2466 bp) including the signal peptide (Brasch et al., 2012, Fig. S2B). Our study completes the sequence of the chick *P-cadherin* gene from earlier studies (Sorkin et al., 1991; Napolitano et al., 1991) and supports the idea that *P-cadherin* and *E-cadherin* have undergone tandem duplication in birds and mammals.

Snail factors bind the consensus sequence CASSTG, which is over-represented (see RNA-seq analysis in the Materials and Methods) in two regions upstream of the *P-cadherin* promoter at positions between −1400 bp and −2200 bp from the TSS (Fig. 2J). To test whether Snail2 can bind to these response elements in epiblast cells, we electroporated GFP together with either a Myc-tagged Snail2 or a myc-tagged control construct in the anterior epiblast of stage HH3 chick embryos. Ten hours after electroporation, GFP-positive epiblast regions were dissected and processed for ChIP (Fig. 2K). Myc-tagged Snail2 overexpression led to a specific enrichment of regions B and C of the *P-cadherin* promoter after ChIP, as assessed by precipitation with an anti-Myc antibody followed by PCR with specific primers (Fig. 2L-M). Positive (panH3 antibody) and negative (rabbit anti-IgG antibody) controls confirmed the specificity of the Myc-antibody. The absence of enrichment for a control region (D) that does not contain Snail response elements confirms the specificity of Snail2 binding to regions B and C. Together, these results indicate that Snail can directly bind to and repress transcription of the *P-cadherin* gene *in vivo*.

P-cadherin expression is downregulated in the neural plate concomitantly with activation of Zeb2

After gastrulation, *P-cadherin* expression decreases in the developing neural plate but remains expressed at its border (Fig. 3A). To identify putative transcription factors involved in this repression, we looked at EMT-related TFs expressed at neurulation stages in the chick embryo (Fig. 3B-E) and confirmed that *Zeb2* expression is expressed in the very early neural plate (Fig. 3E; Sheng et al., 2003), concomitant with the decrease of *P-cadherin* expression. Double *in situ* hybridization comparing *Zeb2*, *P-cadherin* and *Sox2* (a marker of the neural ectoderm) shows that *P-cadherin* is expressed in the non-neural ectoderm and in the neural plate border (Fig. 3F,G). *Zeb2* is expressed in the neural

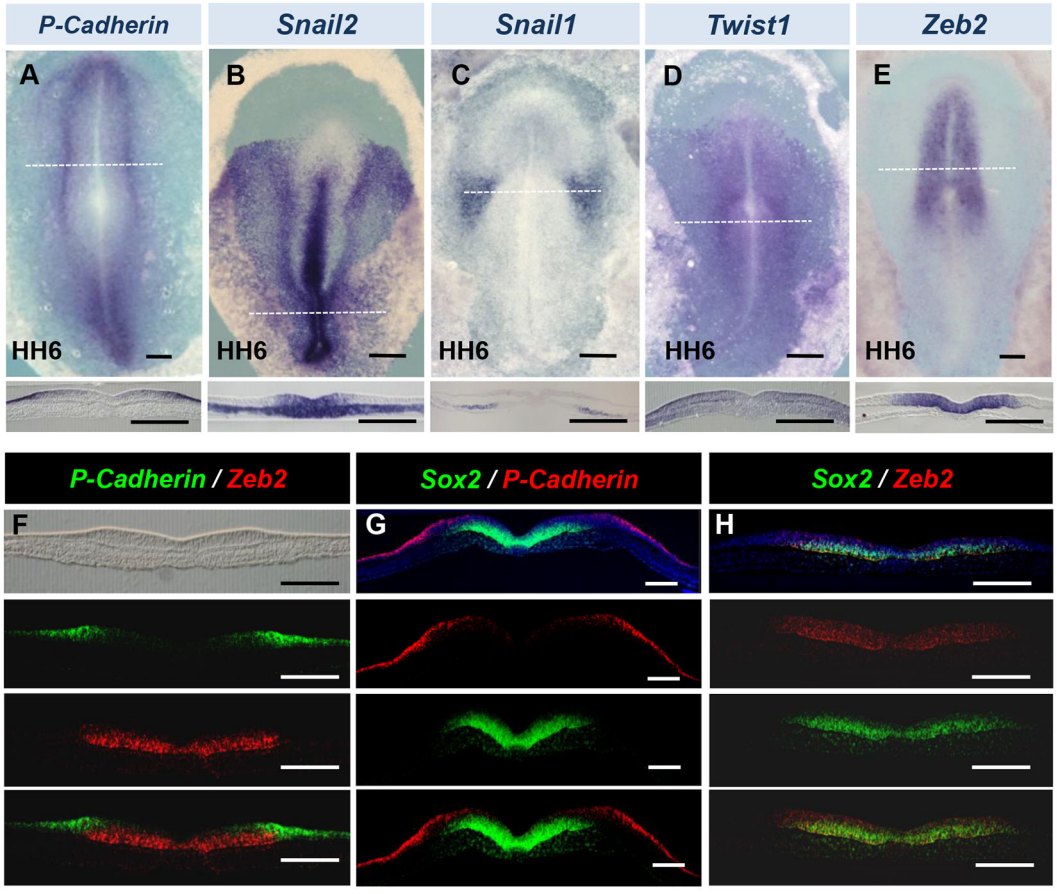

Fig. 3. Expression of EMT inducers relative to *P-cadherin* at early neurulation stages. (A) *P-cadherin* is downregulated in the early neural plate. (B,C) Snail genes are mostly expressed in the mesoderm. (D) *Twist* is weakly expressed in the lateral mesoderm and the ectoderm. (E) *Zeb2* is strongly expressed in the early neural plate, at sites where *P-cadherin* is downregulated. (F-H) Expression of *Zeb2* and *P-cadherin* relative to *Sox2*. P-cadherin is expressed in the non-neural ectoderm and at the border of the neural plate, whereas *Zeb2* transcripts are specifically detected in a *Sox2*-positive region of the neural ectoderm devoid of *P-cadherin* expression. The dotted lines indicate the level of the tissue sections. Scale bars: 500 µm in A-E; 300 µm in F-H.

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ectoderm where *P-cadherin* expression is absent (Fig. 3F-H). The neural plate border and the anterior part of the neural plate maintain *P-cadherin* expression and do not express *Zeb2*. These expression patterns highlight embryonic territories that are becoming different from each other (reviewed by Acloque et al., 2012). The complementary expression of *P-cadherin* and *Zeb2* is consistent with the idea that *Zeb2* may act as a *P-cadherin* repressor in the developing neural plate.

Zeb2 binds to the *P-cadherin* promoter *in vivo*

To assess whether Zeb2 represses *P-cadherin* expression in the neural plate, we overexpressed Zeb2 in the epiblast of stage HH3 embryos (Fig. 4A-L). *P-cadherin* transcripts were downregulated in the electroporated cells (Fig. 4D-F, n=18/20). Conversely, blocking Zeb2 expression with a morpholino antisense oligonucleotide previously described (Rogers et al., 2013) maintained the expression of *P-cadherin* in the neural plate at the time when is being downregulated in the control side (Fig. 4J-L, n=7/13). ChIP assays to assess whether Zeb2 can bind the E-boxes present in the *P-cadherin* promoter to repress its activity confirm that, as described above for Snail2, overexpression of a HA-tagged Zeb2 construct (HA-Zeb2) followed by ChIP shows binding to regions B and C of the *P-cadherin* promoter (Fig. 4M,N). Efficiency and specificity of this experiment were evaluated in a similar manner to that shown in Fig. 2. As for Snail2, these results indicate that Zeb2 can directly bind to and repress transcription of the *P-cadherin* gene *in vivo*.

Snail2 and Zeb2 directly repress *P-cadherin* expression in the developing chicken embryo

Once shown that both Zeb2 and Snail2 bind *P-cadherin in vivo* at regions where *P-cadherin* expression is downregulated, we examined whether they could directly repress promoter activity by transfecting a *P-cadherin* promoter reporter construct in the presence or absence of Zeb2 and Snail2 in COS cells (Fig. 4O). This confirmed that both Zeb2 and Snail2 can directly repress *P-cadherin* transcription. It is worth noting here that Zeb2 does not repress *E-cadherin* expression whereas Snail2 does (Fig. S4A-I). This is consistent with the absence of P-cadherin and the presence of E-cadherin protein in the neural tube (Dady et al., 2012) and also with the finding that Zeb2 overexpression is not sufficient to induce EMT in the neural plate cells. Instead, its role in the neural tube is modulating the border between neural and non neural ectoderm (Fig. S4J-R). This indicates that Zeb2 contributes to the definition of neural versus non-neural ectoderm.

Together, our data show that, in primitive streak stage chick embryos, P-cadherin is the functional homolog of E-cadherin in mammals, and that the sequential activation of different EMT-TFs to repress type I cadherins in the primitive streak and the neural tube is conserved and contributes to the definition of embryonic territories in vertebrates.

MATERIALS AND METHODS

Chick embryos and explant cultures

Fertilized hens' eggs were purchased from Granja Gilbert (Tarragona, Spain). The eggs were incubated, opened and the embryos explanted for EC culture as described previously (Flamme, 1987; Chapman et al., 2001). Embryos were staged according to Eyal-Giladi and Kochav (1976) (EG stage) and Hamburger and Hamilton (1951) (HH stage).

Chick embryos electroporation

Explanted embryos at HH2-HH3 were placed, vitelline membrane and filter paper down, in an electroporation chamber (NEPAGEN) connected to the negative pole of a current pulse generator. A solution containing expression plasmids (2 mg/ml in PBS with 0.1% Fast Green and 6% sucrose), dsRNA (Pekarik et al., 2003) or morpholinos (MOs at 1 µM in PBS together with 1 µg/µl pCX plasmid, 0.1% Fast Green and 6% sucrose) was injected between the vitelline membrane and the epiblast. An anodal electrode was placed over the ventral side of the embryo to cover the injected area. A train of electric pulses (5 pulses, 4 V, 50 ms, 0.5 Hz) was applied using an Intracept TSS10 pulse stimulator (Intracel). In all experiments, the non-electroporated right side of the embryo was used as a control.

DNA constructs

pCX-Snail2, pCX-GFP and pCX-mycSnail2 expression vectors were described previously (Morales et al., 2007; Acloque et al., 2011). Full-length Zeb2 was amplified using degenerate primers from sequence alignment of the ATG region of *Xenopus*, human and mouse orthologues 5′-ACCATGAAGCARSNGATCATG-3′ and a previously published sequence of the C-terminal region (Sheng et al., 2003). Full-length Zeb2 and HA-Zeb2 were sequenced and cloned in pCX at the *Eco*R1 restriction site.

The *P-cadherin* promoter was amplified by PCR using primers described in Table S1 using KAPA High Fidelity HotStart polymerase and then subcloned at the *Kpn*I restriction site of pGL2-basic (Promega) to produce the p1821-luc plasmid. The whole *P-cadherin* gene sequence was deposited in GenBank with the Accession Number KY120274.

Cell transfections and promoter activity assays

Cell transfections were carried out as described by Acloque et al. (2004) in COS7 cells (free of mycoplasma contamination) and using pRL-TK to normalize for transfection efficiency. Reporter p1821-luc plasmid (300 ng) and 300 ng of empty pCX plasmid, pCX-Snail2 or pCX-Zeb2 were used. Firefly and Renilla luciferase luminescence assays were successively performed using a Dual Luciferase Assay (Promega) as described by the manufacturer.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was carried out as described previously (Nieto et al., 1996) but omitting the proteinase K treatment. Digoxigenin-labelled probes were synthesized from the partial or full-length chicken cDNAs of *Snail1*, *Snail2*, *L-CAM*, *N-cadherin*, *Sox2* (Nieto et al., 1994; Sefton et al., 1998) and from expressed sequence tags (EST; Boardman et al., 2002) for *P-cadherin* (ChEST913f11) and *Twist1* (ChEST613g12). The Zeb2 probe was generated by RT-PCR and subsequent cloning in pGEMT-easy (Promega). For whole-mount fluorescent *in situ* hybridization, embryos were processed as previously described (Acloque et al., 2008). Peroxidase activity was successively detected with the TSA-plus Cy3 and fluorescein kits (Perkin Elmer). In some cases, embryos were subjected to immunostaining with anti-GFP antibody (A6455, Thermo Scientific, 1:1000). After *in situ* hybridization, embryos were photographed and subsequently embedded in gelatin, sectioned at 40 µm and photographed using a Leica DMR microscope under Nomarski DIC optics.

Chromatin immunoprecipitation (ChIP)

Chick embryos were electroporated with GFP and control myc-Tag or control HA-Tag, with GFP and myc-Snail2 or with GFP and HA-Zeb2 expression plasmids. Eight hours after electroporation, GFP-positive tissues were dissected from HH5 embryos. ChiP assays were performed as previously described (Acloque et al., 2011). For each assay, we used a pool of 40 embryos (corresponding to ~3×10^5 cells). The following antibodies were used for chromatin immunoprecipitation: anti-myc ChIP grade (ab9132, Abcam), anti-HA ChIP grade (ab9110, Abcam), anti-H3 ChIP grade (ab1791, Abcam) or rabbit IgG control (C15410206, Diagenode) using 1 µg of antibody for each tissue lysate. DNA was amplified by PCR and quantified using H3 samples as a reference.

PCR and real-time PCR

DNA obtained from the ChIP experiments was amplified using primers corresponding to regions B, C and D of the *P-cadherin* promoter (see Table S1 for sequences). Efficiency of primers designed for real-time PCR

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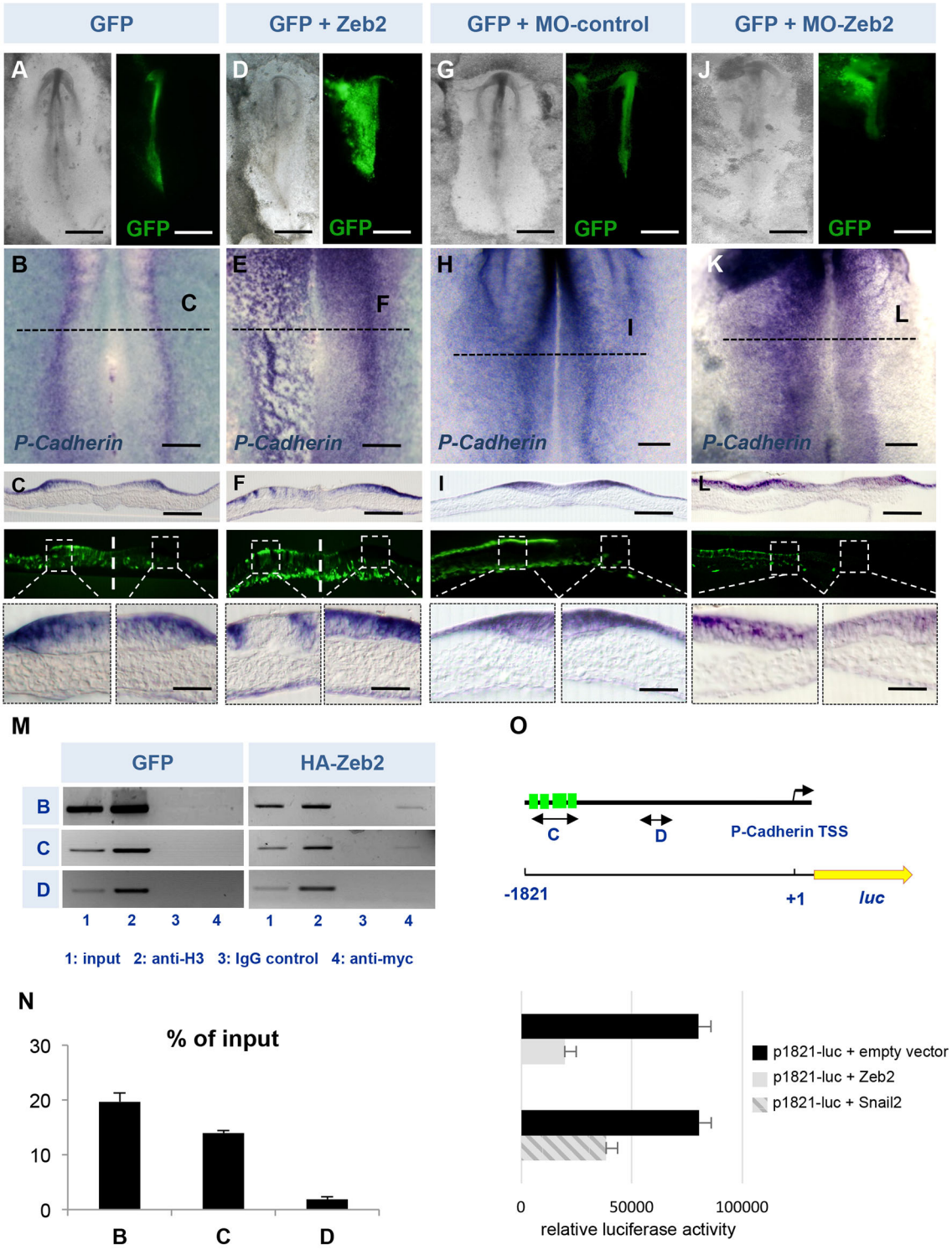

Fig. 4. Zeb2 directly represses *P-cadherin* transcription in the neural plate. (A-C) GFP electroporation does not affect *P-cadherin* expression in the neural plate of HH7 embryos. (D-F) Zeb2 overexpression represses *P-cadherin* expression in the neural ectoderm (compare the electroporated versus the control side). (G-I) Control morpholino (MO-control) electroporation does not affect *P-cadherin* expression in the neural plate of HH7 embryos. (J-L) Zeb2 downregulation after electroporation of a morpholino directed against the ATG (MO-Zeb2) increases *P-cadherin* expression and extends it to the midline of the embryo. (M) Zeb2 binds to *P-cadherin* promoter *in vivo.* Chromatin immunoprecipitation (ChIP) from electroporated neural plates overexpressing either GFP alone or together with a HA-tagged Zeb2. DNA from the chromatin-enriched fraction was amplified by PCR. Amplification from input DNA is shown in lane 1, and positive and negative controls using either a panH3 antibody or an IgG control are shown in lanes 2 and 3, respectively. Regions B and C are specifically enriched using an antibody directed against HA when HA-Zeb2 was electroporated in the epiblast (compare GFP with HA-Zeb2 conditions in lane 4). (N) Quantification of the enrichment shown in M. Regions B, C and D are described in Fig. 2. Data represent mean±s.d. of three independent experiments. (O) The reporter construct (*P*1821-luc) used to quantify *P-cadherin* promoter activity. COS7 cells were transfected with p1821-luc together with an empty expression vector (black bars), with a Zeb2 expression vector (grey bar) or with a Snail2 expression vector (striped grey bar). Data represent mean±s.d. of two independent experiments. Scale bars: 1 mm in A,D,G,J; 500 µm in B,C,E,F,H,I,K,L; 150 µm in the higher magnification panels of the sections.

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amplification of *P-cadherin*, *E-cadherin*, *N-cadherin*, *ACTB* (Voiculescu et al., 2007), *GAPDH* (Voiculescu et al., 2007) and *RS17* (Lavial et al., 2007) was estimated by standard curve production (Table S1). Reverse transcription of total RNA from EGX-XII, HH4 and HH9 embryos, and chicken embryonic fibroblasts (CEFs) was performed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific), according to the manufacturer's guidelines. Real-time PCRs were performed using SYBR Green mix (Thermo) in a StepOnePlus thermocycler (Thermo). Relative gene expressions were determined using the ΔΔCt method corrected for primer efficiencies with the StepOne Software v2.3 (Thermo), using *RS17* as reference gene (Fig. S3B,C) and HH9 as reference sample (Fig. S3C).

RNA-seq analysis

Data from Frésard et al. (2014) (NCBI SRA Accession Number: SRP033603) were used for analysis. Transcript sequences from two chicken embryos (d4.5) were aligned using Tophat 2.0.5 (http://ccb.jhu.edu/software/tophat/index.shtml) on the reconstructed *P-* and *E-cadherin* regions. Data were visualized on IGV2.3. Transcription start site was defined by the limit of read alignment at the 5′ end of the first exon. Frequency of CASSTG was calculated as follows: random frequency corresponds to one CASSTG site each 1024 bp (1/4*4*2*2*4*4). We observed ten CASSTG in 800 bp for B and C regions upstream of the P-cadherin promoters, a 12-fold higher frequency than expected at random.

Nomenclature

The three chicken type I cadherins were previously named *L-CAM* for *E-cadherin* (*CDH1*), *N-cadherin* (*CDH2*) and *K-CAM* or *B-cadherin* for *P-cadherin* (*CDH3*). To avoid confusion, we use *E-cadherin* for *L-CAM* (*CDH1*), *N-cadherin* for *CDH2* and *P-cadherin* for *K-CAM*/*B-cadherin*/*CDH3*.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

H.A. designed and performed the majority of experiments, analysed the data and wrote the manuscript; O.H.O. contributed to electroporation experiments; D.A. performed expression studies; C.D.S. interpreted the data, hosted H.A. for the completion of some experiments and contributed to the writing of the manuscript; M.A.N. conceived the project, designed experiments, interpreted the data and wrote the manuscript.

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Data availability

The whole P-cadherin gene sequence was deposited in GenBank with the Accession Number KY120274.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.142562.supplemental

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