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A snail tale and the chicken embryo

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ABSTRACT Some 25 years ago, a clone was identified that contained the chicken *Slug* sequences (now called *Snail2*). How could we anticipate at that time how much the chick embryo would help us to understand the ins and outs of cell migration during development and in disease? Indeed, the chick embryo helped us identify Snail2 as the first transcription factor that could induce the epithelial-mesenchymal transition (EMT), key for the migration of embryonic and cancer cells.

KEY WORDS: *chicken embryo, neural crest, gastrulation, tumour progression, evo-devo*

When Claudio Stern contacted me to participate in this Special Issue of the *Int. J. Dev. Biol.,* a testimony to the role of the chick embryo as a model in developmental biology, my immediate thought was to reflect on how a cloning expedition that started 25 years ago helped us to understand the epithelial to mesenchymal transition (EMT), its impact on morphogenesis and its importance in biomedicine. In 1992, I was fortunate to be a postdoctoral fellow in David Wilkinson's laboratory at the National Institute for Medical Research in London, working in close contact with Robb Krumlauf, Robin Lovell-Badge, Jim Smith, Andrew Lumsden and Claudio Stern, along with other prominent scientists. It was a fantastic time, during which we identified a host of vertebrate genes that were homologues of genes previously identified in *Drosophila*, and that had been instrumental in teaching us how embryos can develop and establish a body plan. We were embarked on a fishing expedition, to identify genes segmentally-expressed in the hindbrain of the mouse after the discovery of *Krox-20* and the segmentally restricted expression of *Hox* genes (Wilkinson *et al.,* 1989a; 1989b; see Parker and Krumlauf, 2017, for a recent review). Having isolated the mouse Snail homologue (Nieto *et al.,* 1992), we thought that we could better understand Snail function if we were to take advantage of the amenability of the chicken embryo for experimental embryology. As such, and along with Mike Sargent, we set out to identify the chick homologue of Snail by screening a cDNA library that we made from more than 600 HH10 stage chicken embryos obtained over just a few days.

Not only did we find *Snail* but also, another homologue that we initially called "*Zip*" as it was expressed "at the edges of the neural plate during neural tube closure" (as my notes read on June 30th, 1992: see Fig. 1A). With the help of Claudio Stern, who was optimizing the *in situ* hybridization protocol for chicken embryos, we enhanced the sensitivity of this technique protocol and soon realized that this gene was expressed in neural crest cells. We named this gene *Slug*, as it was a paralogue of the chicken *Snail* gene, and interestingly, we found it in both the premigratory and migratory neural crest populations (Fig. 1B). Years later the HUGO Nomenclature committee suggested naming this gene *Snail2* (*Snai2*: see Barrallo-Gimeno and Nieto, 2005). The pattern of *Slug/Snail2* expression was very exciting because at that time there were no markers of the premigratory neural crest and therefore, the appearance of these cells at the top of the neural folds, and their delamination and migration, could only be studied using chick/quail grafts as pioneered by Nicole Le Douarin (Le Douarin, 1973). The migratory crest could be tracked by labelling it with NC-1, an antibody generated against the quail ciliary ganglion by Jean Paul Thiery and that later turned out to recognize the same antigen as HNK-1, that was raised against a human leukemic cell-line (Tucker *et al.,* 1984). Comparing the distribution of *Snail2* and HNK-1 in the 2-day-old chicken embryo we confirmed that *Snail2* was expressed by both the premigratory and early migratory neural crest (Fig. 1C,D). Interestingly, *Snail2* was not expressed in neural crest derivatives, already suggesting that its role could be related to cell movement rather than to cell fate. At this point, at the end of 1992 and having just analysed the expression pattern of this gene, I returned to Spain to set up my laboratory at the Cajal Institute in Madrid.

The epithelial to mesenchymal transition and its connection with tumour progression

Although important and often very useful, patterns of expression do not always help understand gene function, although it was clear that this transcription factor could play an important role in

Abbreviations used in this paper: EMT, epithelial to mesenchymal transition.

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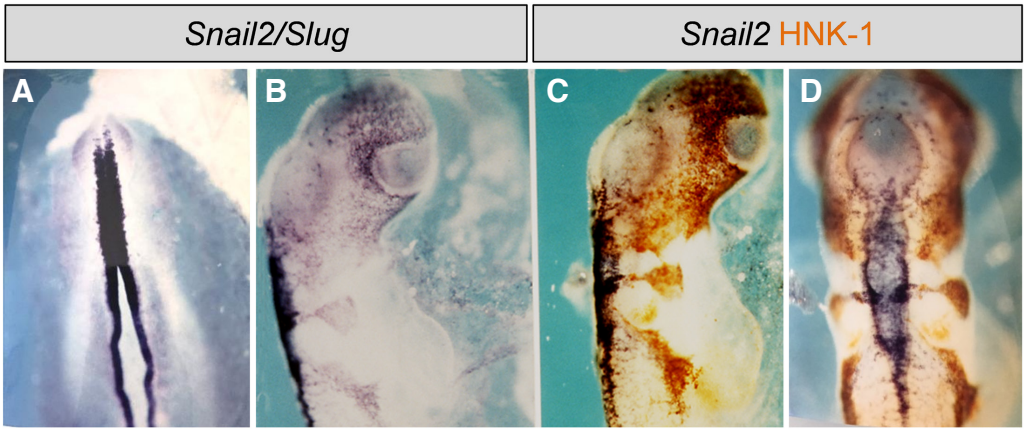

Fig. 1. Expression of *Slug/Snail2* in the chick neural crest. (A) *Early expression at the edges of the neural plate.* **(B)** *Note the expression in both the premigratory and migratory populations.* **(C, D)** *The latter is better assessed when compared with that of the migratory crest marker HNK-1. Adapted from Nieto et al. (1994) and Del Barrio and Nieto (2002).*

the early embryo. Indeed, in addition to the neural crest, it was also expressed strongly in the primitive streak and by the cells that delaminate from it: the early mesoderm and the precursors of the definitive endoderm. I had learned to culture chick embryos with Jonathan Cooke, and when we cultured and incubated them with antisense oligonucleotides to block these genes, we saw the most striking phenotype whereby cells were unable to delaminate and migrate from either the neural tube or the primitive streak (Nieto *et al.,* 1994). Inspired by Ruth Bellairs´ concept of mass migration in development, and her comparison between the neural crest and the primitive streak in the chick embryo (Bellairs, 1987), it was clear that this transcription factor was regulating the so-called epithelial-mesenchymal transition (EMT), first studied by Betty Hay in the 60´s and also in the chick (Hay, 1968; Hay, 1995). Essentially, cells born far from their final destination implement this programme in order to delaminate, migrate and populate different regions of the embryo.

The fact that we could simultaneously observe the premigratory and the migratory crest populations facilitated the description and analysis of the neural crest under different experimental conditions, something that was immediately appreciated by Nicole Le Douarin who was extremely supportive of our work from the very beginning (Le Douarin *et al.,* 1994). We continued our collaboration with Dave Wilkinson and Marianne Bronner using Snail2 alone as a marker (Nieto *et al.,* 1995). Experiments were carried out jointly in Spain, the UK and US, proving to be a truly fantastic example of collaborative work and publishing the results before I had even met Marianne in person. The chick embryo has continued to be a key model in the analysis of developmental processes (Stern, 2005; Gerety *et al.,* 2013) and in particular, of the neural crest (Le Douarin and Dieterlen-Lièvre, 2013; Martik and Bronner, 2017, and references therein), for which Nicole and Marianne have played instrumental roles (see Fig. 2).

Fig. 2. Meeting during the FEBS-EMBO 2014 Conference in Paris. *The conference celebrated the 50th anniversaries of FEBS and EMBO and the 100th anniversary of the French Society for Biochemistry and Molecular Biology. The author with Marianne Bronner (left) and Nicole Le Douarin (middle).*

When we first described the phenotype of *Slug/Snail2* defective chick embryos, we proposed that "pathological activation of Slug or of functionally related genes could contribute to the onset of the invasive or metastatic phenotype during the progression of cancers of epithelial origin, because the ability to break through an epithelial basement structure is reminiscent of the mechanism by which mesoderm and the neural crest originate" (Nieto *et al.,* 1994). Although it took us several years, along with Amparo Cano we finally showed that Snail was indeed activated in dedifferentiated carcinomas, and at the same time as Antonio Garcia de Herreros in Barcelona, that Snail acted as a repressor of *E-Cadherin* transcription (Cano *et al.,* 2000; Batlle *et al.,* 2000; Blanco *et al.,* 2002), the loss of which was known to be fundamental for the transition from adenoma to invasive carcinoma (Behrens *et al.,* 1989; Perl *et al.,* 1998). Snail proteins could induce a cellular transition compatible with the EMT (Fig. 3) and reminiscent of that observed in a new fibroblastic cell type originated from tumour-derived mammary epithelial cells (Dulbecco *et al.,* 1981) and when epithelial carcinoma bladder cells were incubated with epidermal growth factor (EGF: Boyer *et al.,* 1988; 1992). Other transcription factors from different gene families have been later shown to induce EMT and they may be reactivated at the invasive front of carcinomas of different aetiologies (see Ye and Weinberg, 2015; Nieto *et al.,* 2016, for recent comprehensive reviews).

The dynamics of the EMT is complex and it is a transient process; tumour cells reacquire epithelial traits to engage in metastatic colonization, as do migratory embryonic cells upon reaching their destination. Moreover, it has proven difficult to generate appropriate animal models that can follow the whole process. Accordingly, it has been extremely difficult to study how EMT influences the progression of carcinomas. For instance, it has not been possible to reliably observe tumour cells all the way from the primary tumour to the metastatic site, fuelling discussion

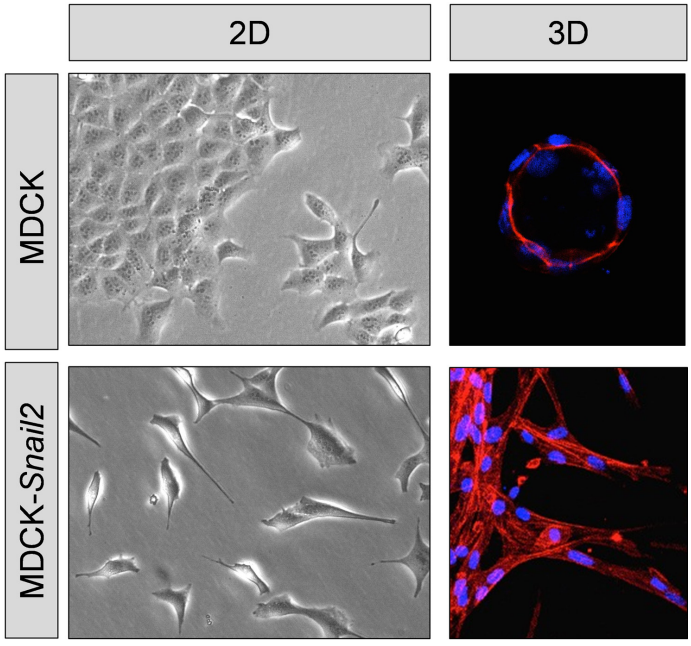

Fig. 3. Epithelial cells (MDCK cell line) undergo epithelial to mesenchymal transition (EMT) upon activation of Snail2/Slug. *Images show cells grown in 2D or embedded in Matrigel (3D). In Matrigel, MDCK cells form epithelial ducts, whereas Snail2-expressing MDCK cells form mesenchymal networks.*

and encouraging those working in the field to design models better optimized to understand these processes. Indeed, the EMT and its significance in the initial steps of the metastatic cascade remain the subject of some debate (Brabletz *et al.,* 2017). Ironically, as early as 1890 Cajal already noticed this cellular transition and its implication in the invasive potential of breast carcinoma (Ramón y Cajal, 1890a; Text and Fig. 48 in the first Edition). Interestingly, and in the context of this special issue dedicated to the chick embryo, Cajal's neuronal theory, that neurons were individual entities, was formulated on the basis of his studies of the axons sprouting from spinal commissural neurons in the chick embryo (Ramón y Cajal, 1890b).

In summary, the importance of the EMT in tumour progression and in other diseases would have surely remained elusive without the contribution of the instrumental studies carried out on chicken embryos. These studies defined the cellular events associated with the EMT and many of the fundamental elements involved. Going back to the *Snail* genes, unexpected findings often made them somewhat difficult to work with, issues that over time have developed into interesting evo-devo and tissue-specific stories, the nature of which I will outline below.

Were the test tubes switched or is evolution playing tricks on us?

After finding that in the chick embryo, Snail2/Slug was required by cells in the neural tube and primitive streak to delaminate, and having connected this event with cancer, it was clear that we needed to study this process in mammals. We set out to better characterize Snail and Slug in the chick and mouse, and we were frankly dismayed when we studied their expression patterns, as we seemed to have somehow swapped the test tubes in the lab. Indeed, the pattern of *Slug* expression in the mouse was not only different from what we had seen in the chicken but significantly, it was very similar to that of *Snail* in the chick (Fig. 4). Eventually, it dawned on us that the main expression domains of *Snail* and *Slug* were interchanged between the chick and mouse (Sefton *et al.,* 1998). As such, we predicted that the *Slug* mutant mice would probably not have a gastrulation or strong neural crest phenotype, as this gene was rather expressed in subpopulations of migratory mesoderm and neural crest cells. This was confirmed by Tom Gridley (Jiang *et al.,* 1998), who later also showed that it was in fact the *Snail* rather than the *Slug* gene that was required for EMT in the gastrulating mouse embryo (Carver *et al.,* 2001). By then, we had already drawn the connection between E-cadherin and cancer cells, also confirming that Snail was the prominent family member for EMT in mammalian cells (Cano *et al.,* 2000).

After observing how the expression domains of chick and mouse *Snail* and *Slug* had been swapped around, we started another fishing expedition to isolate *Snail* genes in representatives of all the vertebrate groups. We found an unprecedented degree of reshuffling in the expression domains of these genes, some of which could be explained by neofunctionalisation or subfunctionalisation, whereas others did not fit the models of tissue-specific

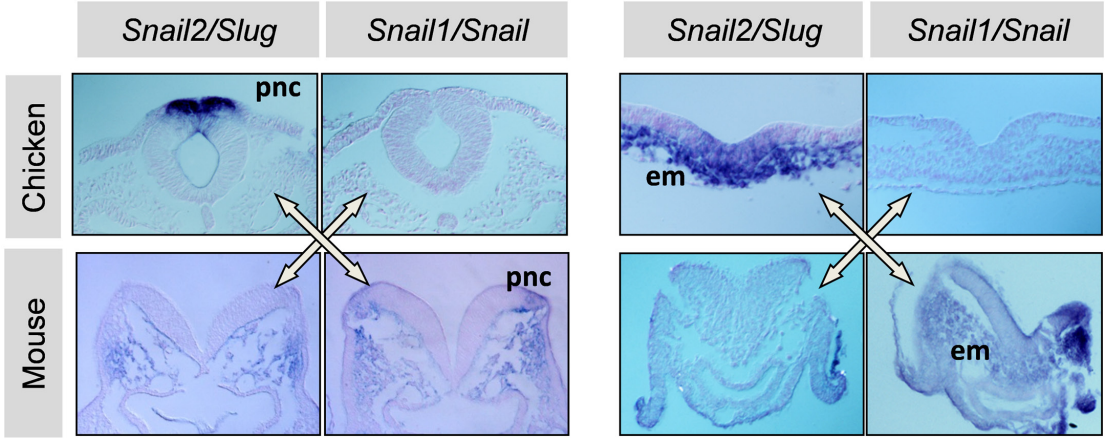

Fig. 4. The expression patterns of *Snail1* and *Snail2* are interchanged in the premigratory neural crest and the early mesoderm in the chick and the mouse. *Adapted from Sefton* et al. *(1998) and Nieto (2002).*

enhancer rearrangement during evolution (Locascio *et al.,* 2002). In fact, more recently we also observed that the role played by E-cadherin at gastrulation stages in the mouse is likely played by P-cadherin in the chick (Acloque *et al.,* 2017).

Another recent example of this phenomenon is the case of L/R asymmetry and organ positioning. Perhaps it should be of no surprise that the chick embryo has been at the centre stage of research into organ positioning for many years. The seminal experiments describing transient activation of the Activin receptor-Shh-Nodal-Pitx2 pathway on the left side of the embryo were performed in the chick (Levin *et al.,* 1995; Logan *et al.,* 1998; Piedra *et al.,* 1998; Ryan *et al.,* 1998; Yoshioka *et al.,* 1998). Moreover, Snail was shown to be expressed more strongly on the right side of the chick embryo, where it repressed Pitx2 expression (Patel *et al.,* 1999). Therefore, the model was that organ positioning, and heart *situs* in particular, was driven by conferring left-handed information to the left side of the embryo and excluding it from the right. However, although the expression of Pitx2 in the chick and mouse embryos is very similar and restricted to the left lateral plate mesoderm, Pitx2 mouse mutants do not display heart looping defects but rather, they develop cardiac right isomerism –the absence of a left-hand side and the presence of a mirror image duplication of right-hand morphological features (Lin *et al.,* 1999; Campione *et al.,* 2001). Indeed, Pitx2 is crucial to impart left-handed information (Raya and Izpisua-Belmonte, 2006) and for axis formation (Torlopp *et al.,* 2014), yet not for heart looping. Importantly, heart looping seems to be independent of Nodal in the zebrafish (Noël *et al.,* 2013). In addition, the development and position of the proepicardium, a transient right-specific structure in frogs and avians, is not affected by aberrant bilateral Pitx2 expression (Schlueter and Brand, 2009). Together, these data suggest that as well as the left-handed pathway, an additional instructive pathway might exist that conveys information from the right hand side of the embryo. As such, Prrx1, another transcription factor containing an OAR transactivation domain like Pitx2, is activated by BMP more prominently on the right flank and its downregulation induces mesocardia in the zebrafish (Ocaña *et al.,* 2017: Fig. 5). Interestingly, like Snail, Prrx induces EMT in embryos and cancer cells (Ocaña *et al.,* 2012), suggesting that there might be some common features between the EMT and the mechanisms that drive heart looping. In fact, L/R asymmetric Prrx1 expression and accordingly, L/R asymmetric EMT, drives asymmetric cell movements and forces that produce a leftward displacement of the posterior pole of the heart and hence, normal looping (Ocaña *et al.,* 2017). Once again, it seemed strange that *Prrx1* mutant mice did not display heart-positioning defects (Bergwerff *et al.,* 2000). However, the expression of Prrx1 in the region relevant for heart looping in the fish had been substituted by that of Snail1 in the mouse. This explains the lack of a heart laterality phenotype in the *Prrx1* mutants and the heart looping defects described in *Snail1* mutant embryos (Murray *et al.,* 2006). Here is when the chick embryo helped us again. Both Prrx1 and Snail1 contribute to heart looping in the chick, as they are expressed in complementary patterns in the relevant territories, an intermediate situation to that found in the fish and mouse. Functional analysis in the three model systems confirmed that an asymmetric L/R EMT, more prominent on the right, drives heart looping in vertebrates, significantly through a conserved cellular morphogenetic process driven by different EMT inducers over the course of vertebrate evolution (Ocaña *et al.,* 2017). This has important implications, particularly since heart looping is crucial for the concordance between the heart and the vasculature. Defects in L/R asymmetry arise in 1/10,000 humans, and the associated morbidity and mortality are usually associated with congenital heart defects (CHDs: Lin *et al.,* 2014; Ramsdell *et al.,* 2005).

In summary, in some of the experiments described here, evolution seems to have been playing with us. In one case, different gene family members, generated following whole genome duplications at the base of the vertebrate lineage (Mclysaght *et al.*, 2002), play the same role in different species. For heart positioning, the cellular process is conserved but the transcription factor used belongs to a different gene family. Importantly, all of the above, has taught us that if we really want to understand what particular genes do and how they are integrated into a developmental or pathological process, we need to study different models before we can extrapolate data obtained from a single species or vertebrate group to others. This is particularly important when translating studies performed in animal models into medical research. We now try to address our questions simultaneously in the chicken, mouse and fish, even though this implies a more significant effort. As a result, in all our projects we can guarantee that the chick embryo will always be there to help us tell the complete tale.

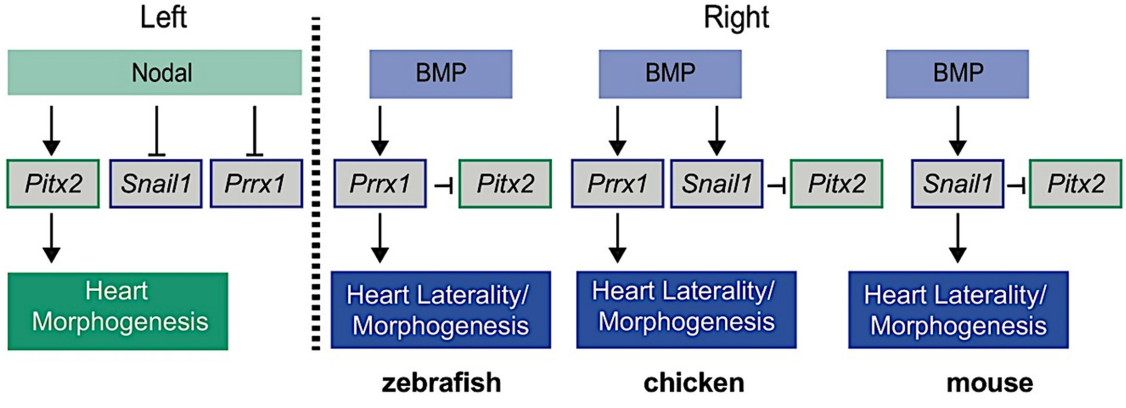

Fig. 5. A differential left-right EMT regulates heart laterality in vertebrates. *In addition to the well-known left-specific Nodal-Pitx2 axis, a BMP-induced EMT program, more prominent on the right side, triggers the leftward displacement of the posterior pole of the heart. The EMT-TFs used vary in different vertebrate groups. Adapted from Ocaña* et al., *(2017).*

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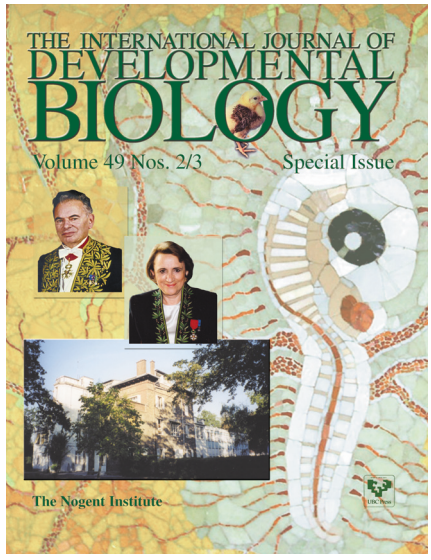

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