The Snail genes as inducers of cell movement and survival: implications in development and cancer

Alejandro Barrallo-Gimeno and M. Angela Nieto*

Instituto de Neurociencias de Alicante CSIC-UMH, Apartado 18, Sant Joan d'Alacant, 03550, Spain
*Author for correspondence (e-mail: anieto@umh.es)

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Summary

The functions of the Snail family of zinc-finger transcription factors are essential during embryonic development. One of their best-known functions is to induce epithelial to mesenchymal transitions (EMTs), which convert epithelial cells into migratory mesenchymal cells. In recent years, many orthologues of the Snail family have been identified throughout the animal kingdom, and their study is providing new clues about the EMT-dependent and -independent functions of Snail proteins. Here, we discuss these functions and how they influence cell behaviour during development and during diseases such as metastatic cancer. From these findings, we propose that Snail genes act primarily as survival factors and inducers of cell movement, rather than as inducers of EMT or cell fate.

Introduction

The function that Snail genes are best known for is the induction of a phenotypic change called epithelial to mesenchymal transition (EMT). Snail-induced EMT converts epithelial cells into mesenchymal cells with migratory properties that contribute to the formation of many tissues during embryonic development and to the acquisition of invasive properties in epithelial tumours. Snail-induced EMT is partly due to the direct repression of *E-cadherin* transcription both during development and tumour progression. As the loss of E-Cadherin expression in tumours is considered to be a marker of a poor clinical outcome, E-Cadherin repressors are regarded as markers of malignancy, and as targets for anti-invasive drugs (for reviews, see Nieto, 2002; Thiery, 2002).

Snail genes also have additional cellular functions that sometimes occur independently of the induction of EMT. They protect cells from the death induced either by the loss of survival factors or by direct apoptotic stimuli. In some instances, survival properties emerge concomitant with the induction of EMT, whereas, in others, both the resistance to cell death and the persistence of the epithelial phenotype are required. In addition, there are certain cell movement processes that do not require a full EMT, such as mesoderm formation in *Drosophila* and in anamniotic vertebrate embryos (vertebrate embryos that lack an amniotic membrane, such as amphibian and fish embryos). Interestingly, recent evidence shows that Snail genes also participate in these processes by regulating cell adhesion and migration. Thus, it seems that the prevalent function of Snail might be to regulate cell adhesion rather than to induce EMT. In this context, the triggering of the EMT would be just one of the mechanisms used by these transcription factors to allow cell movement.

Another commonly discussed function of Snail genes is their role as mesodermal determinants. Again, recent data from both invertebrates and vertebrates indicate that their participation in mesoderm formation is more related to their role in regulating cell movement than to their putative function as inducers of cell fate.

Here, we review recent insights into the evolution of the Snail superfamily, and discuss a new unified nomenclature for this family of transcription factors that will facilitate the comparison of family members among distant species. We also discuss recent data on how Snail family members are post-transcriptionally regulated. Finally, on the basis of recent findings, we propose a new model of Snail function, in which Snail genes act as survival factors and as inducers of cell movement, rather than as inducers of mesodermal fate or EMT. This new theory will be discussed within the context of the properties that both embryonic and tumour cells share when they become migratory and invasive.

The Snail superfamily: new members and new nomenclature

snail was first described in *Drosophila melanogaster* (Boulay et al., 1987), where it was shown to be essential for the formation of the mesoderm (Alberga et al., 1991). In the 20 or so years since its isolation, more than 50 family members have been described in metazoans (reviewed by Nieto, 2002) (see below also), with several family members found in different groups.

During metazoan evolution, the generation of gene families is believed to have occurred by gene duplication and by the divergence of an ancestral gene (Ohno, 1970; Ohno, 1999; Furlong and Holland, 2002). Until recently, it was assumed that it was independent gene duplications that gave rise to the four snail genes in insects (*snail*, *escargot*, *worniu* and *scratch*), and to the two in vertebrates (*Snail* and *Slug*). However, from the results of database searching and phylogenetic analyses, it was proposed a few years ago that the Snail superfamily consists of two related, but independent, families: *Snail* and *Scratch* (Manzanares et al., 2001; Nieto, 2002). (See Fig. 1 for an updated version of the Snail family phylogenetic tree,

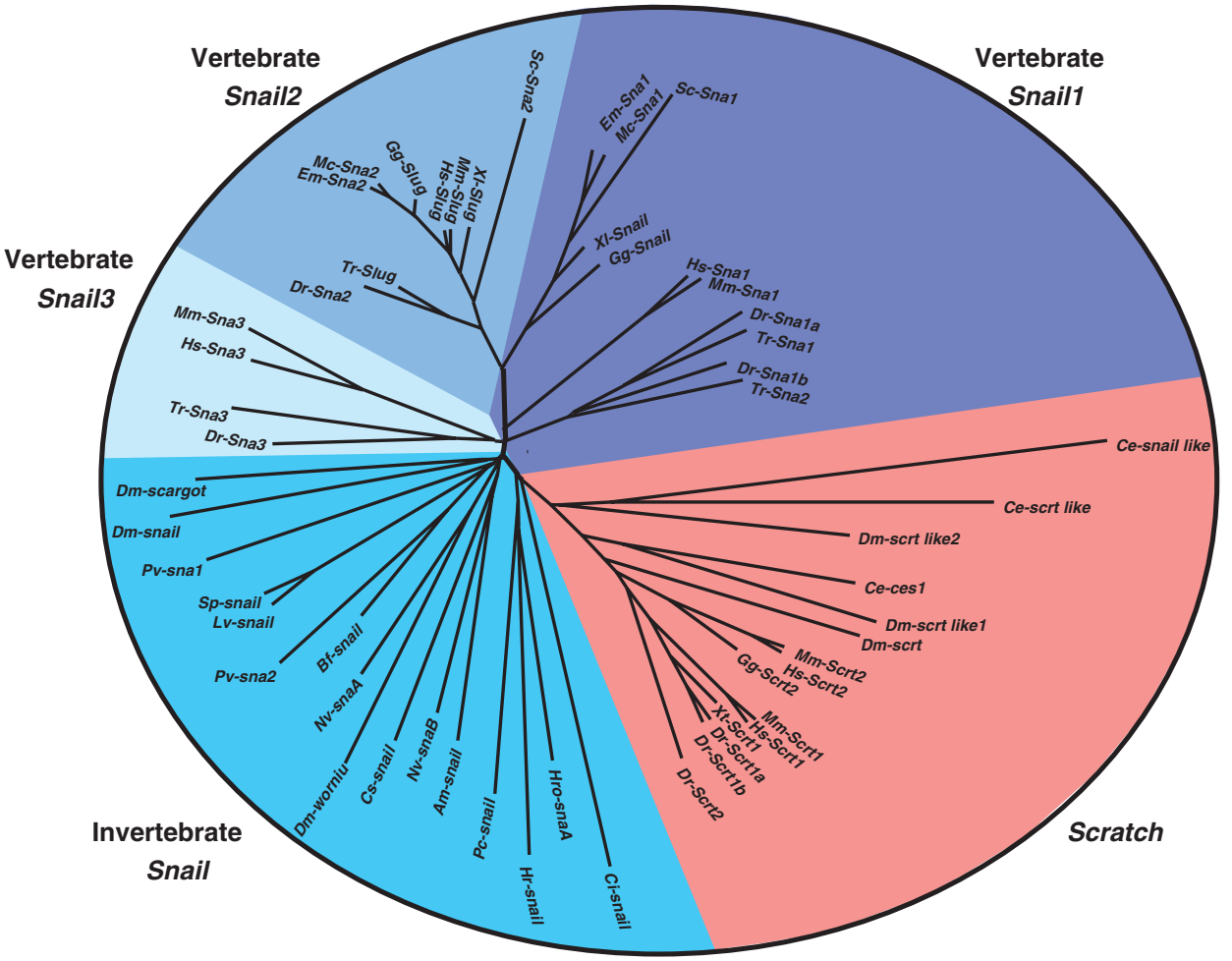

Fig. 1. Phylogenetic tree of the Snail superfamily. This tree is updated from a previous version (Nieto, 2002) with 22 new members. The different family members are grouped into two families: Snail (blue) and *Scratch* (pink). Vertebrates have three Snail members: *Snail1* (previously *Snail*), *Snail2* (previously *Slug*) and *Snail3* (previously *Smuc*). *Am*, *Acropora millepora* (coral); *Bf*, *Branchiostoma floridae* (amphioxus); *Ce*, *Caenorhabditis elegans* (nematode); *Ci*, *Ciona intestinalis* (ascidia); *Cs*, *Cupiennius salei* (spider); *Dm*, *Drosophila melanogaster* (fruitfly); *Dr*, *Danio rerio* (zebrafish); *Em*, *Eublepharis macularius* (gecko); *Gg*, *Gallus gallus* (chicken); *Hr*, *Halocynthia roretzi* (ascidian); *Hro*, *Helobdella robusta* (leech); *Hs*, *Homo sapiens* (human); *Lv*, *Lytechinus variegatus* (green sea urchin); *Mc*, *Mauremys caspica* (turtle); *Mm*, *Mus musculus* (mouse); *Nv*, *Nematostella vectensis* (anemone); *Pc*, *Podocoryne carnea* (jellyfish); *Pv*, *Patella vulgata* (mollusc); *Sc*, *Scyliorhinus canicula* (shark); *Sp*, *Strongylocentrotus purpuratus* (sea urchin); *Tr*, *Takifugu rubripes* (pufferfish); *Xl*, *Xenopus laevis* (African clawed toad); *Xt*, *Xenopus tropicalis* (western clawed frog).

including all the members isolated in the last four years.) The phylogeny shown in Fig. 1 is compatible with a revised evolutionary history that has been previously proposed (Nieto, 2002) and which is updated in Fig. 2. According to this phylogenetic history, the duplication of a single *snail* gene in the metazoan ancestor gave rise to two genes, *snail* and *scratch*, which, after undergoing independent duplications in the different Cnidaria and Bilateralia, themselves gave rise to a number of genes in each group (Fig. 2).

The increasing number of Snail family members and their phylogenetic analysis has triggered a recent discussion about their nomenclature. As a result, the HUGO gene nomenclature committee has now decided that when there is more than one Snail gene in a species, it should be called *Snail1* to *SnailX* (http://www.gene.ucl.ac.uk/nomenclature/genefamily/snail.htm), the convention being to use the name Snail, followed by a number. For example, in vertebrates, in addition to *Snail* and *Slug*, a third Snail gene has been characterised. According to the HUGO gene nomenclature committee, it has been named *Snail3* (Katoh and Katoh, 2003; Manzanares et al., 2004), and, for consistency, *Snail* now becomes *Snail1*, and *Slug*, *Snail2*. The additional duplicates that originated in the teleost lineage (Postlethwait et al., 1998) should likewise be named with the corresponding number, plus "a" or "b"; for example, *snail1a* and *snail1b* (Fig. 2).

Snail genes: mesodermal determinants or inducers of cell movement?

The evolutionary origin of the mesoderm is still a matter of debate (reviewed by Technau and Scholz, 2003). In order to resolve this question, the homologues of so-called 'mesodermal determinant' genes have been isolated in diploblastic animals (animals with only two germ layers – ectoderm and endoderm), such as in the Cnidarians. *Snail* is

regarded as one of the interesting genes to study due to its classification as a mesodermal determinant gene. This classification explains why, in the last few years, Snail homologues have been identified in Cnidarians, such as the anemone *Nemastostella vectensis* (Fritzenwanker et al., 2004; Martindale et al., 2004), the coral *Acropora millepora* (Hayward et al., 2004) and the jellyfish *Podocoryne carnea* (Spring et al., 2002).

Diploblasts do not have a mesodermal layer, but still Cnidaria, such as jellyfish, coral and anemona, prominently express snail (Sring et al., 2002; Fritzenwanker et al., 2004; Hayward et al., 2004; Martindale et al., 2004). The main site of snail expression in these animals is the endoderm, which, interestingly, forms by the invagination of the ectoderm. This morphogenetic movement is reminiscent of that occurring during mesoderm formation in *Drosophila*, where Snail plays a pivotal role. Thus, regardless of whether the mesoderm derives in evolutionary terms from the endoderm, or whether it is formed by contributions from the ectoderm and endoderm, the point here is that due to the analysis of snail expression in Cnidaria, the idea that Snail functions as a mesodermal determinant now has to be questioned.

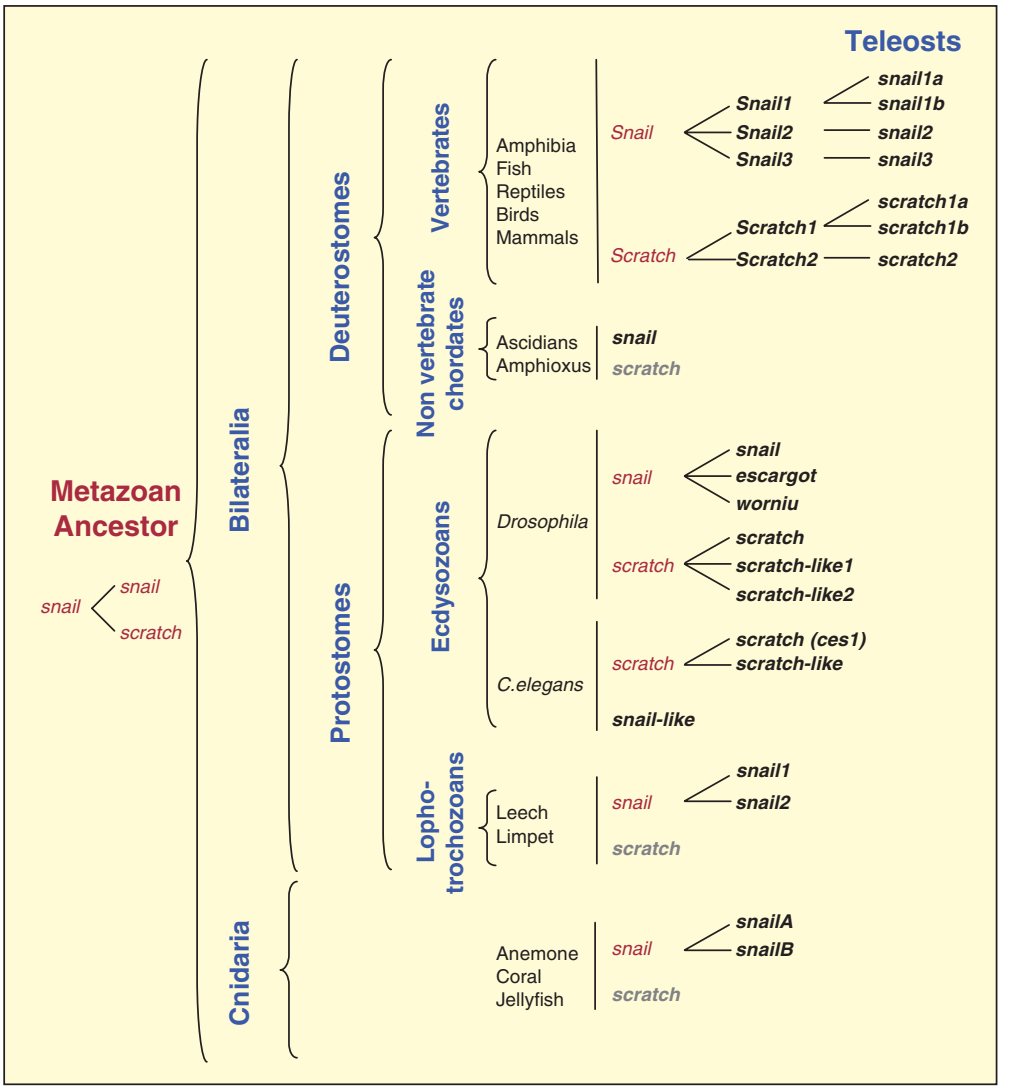

Fig. 2. Evolutionary history of the Snail gene superfamily. Duplication of a Snail gene in the metazoan ancestor probably gave rise to two highly related genes, *Snail* and *Scratch*. Independent duplications in Cnidarians, Protostomes and Deuterostomes gave rise to several family members in each group. Ancestral genes are shown in red and predicted members in grey, while existing genes are in black. Updated from Nieto (Nieto, 2002).

An analysis of Snail expression and function in triploblasts (animals with three germ layers), among representatives of the Lophotrochozoans, Ecdysozoans and Deuterostomes, supports a role for Snail in regulating cell movement. For instance, both in the mollusc *Patella vulgata* (Lespinet et al., 2002) and in the spider *Achaearanea tepidariorum* (Yamazaki et al., 2005), snail homologues are not expressed in the mesoderm, but rather in ectodermal tissues that undergo changes in cell shape or morphogenetic movements. Similarly, Snail is expressed in the developing skin of the mouse when skin cells lose E-cadherin expression and invaginate to form the hair follicule buds (Jamora et al., 2005). Furthermore, it is worth noting that Snail-mutant mice die at gastrulation because of defects in the EMT, which is needed in amniotes for mesoderm development (Carver et al., 2001). Interestingly, mesoderm forms in these mutants and expresses well-known mesodermal markers, such as brachyury, despite being unable to downregulate E-cadherin and migrate. To conclude, these data together therefore suggest that Snail regulates cell movement and adhesion rather than cell fate.

Snail genes at the crossroads of the EMT

The first indication that the Snail gene family had a role in triggering EMT came from *Snail2* loss-of-function experiments carried out in chick embryos (Nieto et al., 1994); a role that was later confirmed in cell lines and in other vertebrate embryos (reviewed by Nieto, 2002; De Craene et al., 2005). EMT is crucial for the formation of many different tissues and organs during embryogenesis, such as for the development of the mesoderm in amniotes, the neural crest in all vertebrates, as well as the heart cushions and the palate, among others. Interestingly, Snail genes are expressed in all EMT processes where they have been studied (reviewed by Nieto, 2002). EMT can be triggered by different signalling

molecules, such as by epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), transforming growth factor β (TGFβ), bone morphogenetic proteins (BMPs), WNTs and Notch. In agreement with the involvement of Snail in all studied processes of EMT, these signalling molecules have been shown to induce Snail genes in different cellular contexts (see Fig. 3 for a summary of the signalling pathways that can activate Snail genes) (for a review, see De Craene et al., 2005).

With respect to the TGFβ superfamily, TGFβ1 induces *Snail1* in hepatocytes (Spagnoli et al., 2000; Valdés et al., 2002), in the palate (Martinez-Alvarez et al., 2004) and in epithelial (Peinado et al., 2003) and mesothelial cells (Yáñez-Mo et al., 2003; Margetts et al., 2005). TGFβ2 induces *Snail1* in the developing mouse skin (Jamora et al., 2005) and *Snail2* during heart development (Romano and Ruyan, 2000); and BMP4 induces *Snail2* during neural crest development (Dickinson et al., 1995; Liem et al., 1995).

EGF is also linked to the induction of Snail and EMT in several ways. It reduces E-Cadherin function by promoting its caveolin-dependent endocytosis, which leads to subsequent Snail induction and to the triggering of EMT (Lu et al., 2003). Another recent connection between EGF, Snail and EMT has been described in mice that express the EGF family member Crypto in the mammary gland. A full EMT process accompanied by the induction of Snail expression is observed both in the hyperplasias and tumours derived from these mice (Strizzi et al., 2004). Furthermore, the EGFR pathway can activate STAT3, which can upregulate Snail function via activation of the zinc-finger transporter LIV1 (Yamashita et al., 2004), which has also been associated with the progression of breast tumours (Manning et al., 1994). In this work, LIV1 was described as being a target of the estrogen receptor (ER) pathway. Interestingly, recent data have also related this signalling pathway with Snail expression (Fujita et al., 2003). The metastasis-associated protein 3 (MTA3) is an ER target that has been identified as being a component of the repressor complex that directly inhibits *Snail* transcription in breast epithelial cells. Indeed, the absence of ER signalling or MTA3 leads to aberrant Snail expression and EMT. This could, at least in part, explain why a poor prognosis is associated with ER-negative breast tumours (Lapidus et al., 1998).

Another pathway that has been associated with *Snail* expression in different contexts is the Notch pathway. From *Drosophila* to vertebrates, Snail seems to position Notch signalling in the embryo to determine the localisation of different tissues (Cowden and Levine, 2002; Cornell and Eisen, 2002; Morel et al., 2003; Endo et al., 2002; Glavic et al., 2004). One tissue in which this relationship has been studied extensively is the neural crest, which originates in the dorsal region of the neural tube, where Snail genes play a crucial role in inducing EMT. In zebrafish, chick and *Xenopus*, Notch signalling is involved in the localisation of the neural crest in the neural/non-neural border (Cornell and Eisen, 2002; Endo et al., 2002; Glavic et al., 2004). However, the molecular mechanism by which this positioning is achieved has been interpreted in different ways depending on the system. In zebrafish, Cornell and Eisen have shown that Delta/Notch signalling promotes neural crest formation by inhibiting neurogenesis (Cornell and Eisen, 2002). Meanwhile, Glavic and colleagues (Glavic et al., 2004) propose that, in *Xenopus*, Snail plays this role by repressing the Notch ligand Delta, as it does in the fly (Cowden and Levine, 2002). However, Endo and co-workers (Endo et al., 2002) have proposed that, in the chick, it is Snail2 that is a downstream target of Notch signalling. Interestingly, it has been suggested that Snail1 is a target of Notch signalling during the EMT needed for the formation of the cushions in the developing heart in mice, where it inhibits VE-Cadherin expression (Timmerman et al., 2004).

In this section, we have only highlighted some recent data regarding the numerous extracellular factors that induce the expression of Snail family members in the context of EMT. The conclusion that can be drawn is that Snail gene induction

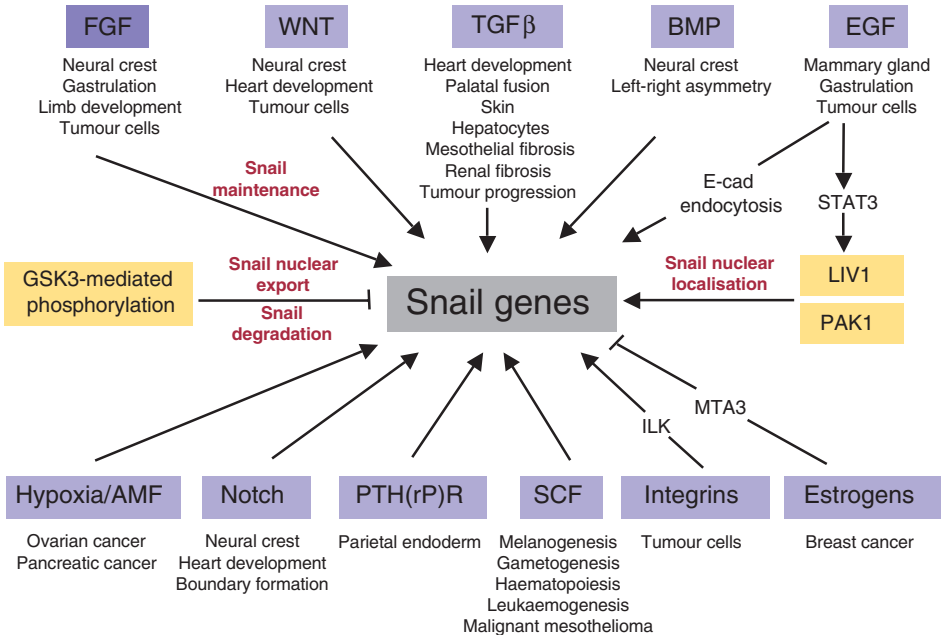

Fig. 3. Snail genes are a convergence point in EMT induction. Numerous signalling pathways induce the epithelial to mesenchymal transition (EMT), and all have been shown to activate the expression of Snail genes. Below each extracellular signal are the tissues and processes in which they have been studied. In addition to being tightly regulated at the transcriptional level, Snail activity is also regulated by its subcellular localisation, which is governed by at least two kinases GSK3 and PAK1, and by the zinc-finger transporter LIV1. (See text for details.) AMF, autocrine motility factor; E-cad, E-cadherin; EGF, epidermal growth factor; FGF, fibroblast growth factor; BMP, bone morphogenetic protein; ILK, integrin-linked kinase; MTA3, metastasis-associated protein 3; PAK1; p21-activated kinase; TGFβ, transforming growth factor β; PTH(rP)R, parathyroid hormone related peptide receptor; SCF, stem cell factor.

is a central convergence point for the factors that induce this cellular event.

EMT-related and EMT-independent Snail functions

Although Snail seems to be required for all processes of EMT that have been studied, this does not necessarily mean that the induction of EMT is the prevalent role of Snail genes. As such, they have additional roles that sometimes operate independently of the induction of EMT, which we discuss below.

One EMT-independent role that is fulfilled by all Snail superfamily members is the protection of cells from cell death. In *C. elegans*, the repression of the Scratch homologue, CES-1 (Metzstein and Horvitz, 1999), promotes the physiological death of a particular class of neurons. In humans, a translocation converts the repressor hepatic leukemic factor (HLF; a putative CES-2 homologue) into an activator that, in turn, induces Snail2 and leads to aberrant cell survival and to the development of leukaemia (Inukai et al., 1999). Furthermore, haematopoietic progenitors in *Snail2* null-mutant mice show an increased sensitivity to death induced by gamma-irradiation (Inoue et al., 2002; Pérez-Losada et al., 2003). Snail1 is also a potent survival factor: *Snail1*-expressing cells survive being deprived of survival factors; are resistant to the action of direct apoptotic stimuli that signal through the death receptor; and are resistant to DNA damage (Kajita et al., 2004; Martinez-Alvarez et al., 2004; Vega et al., 2004).

In some circumstances, the survival properties that are conferred to cells by the expression of the Snail genes are acquired concomitantly with the induction of EMT, as in fetal hepatocytes (Spagnoli et al., 2000; Gotzmann et al., 2002; Valdés at al., 2002) and the neural crest (Vega et al., 2004; Tribulo et al., 2004). However, in the palate, Snail-induced resistance to cell death occurs despite the epithelial architecture of the tissue remaining intact. The Snail-mediated survival of the epithelial cells that lie at the medial edge of the developing palate takes place when the two palatal shelves do not fuse. This occurs in some pathological situations, for example in cleft palate defects in mammals, and where cleft palates have evolved as the normal condition, such as in avians (Martinez-Alvarez et al., 2004). Interestingly, Snail1 is the family member involved in this process in the mouse and Snail2 plays this role in the chick. This is another example of the functional interchange that has been described for the family members during evolution in other processes, such as mesoderm and neural crest development (Locascio et al., 2002).

The cleft palate condition reveals a situation in which Snail genes are expressed and are active in epithelial cells and still do not induce EMT or changes in cell adhesion. The mechanism by which EMT induction is prevented in this case is not known, and as one can always evoke differences in cellular context, it will be extremely interesting to decipher these differences. They could be related to the absence of Snail co-factors or of particular Snail downstream targets. This point takes us back to the origin of the neural crest. This is because *Snail* is expressed in the dorsal region of the neural tube in non-vertebrate chordates (ascidians and amphioxus) (Langeland et al., 1998; Wada and Saiga, 1999), where neural crest cells form in vertebrates. However, ascidian and amphioxus do not have a proper neural crest. Interestingly enough, it has been recently reported that in the ascidian embryo, some cells from the anterior part of the neural tube do have migratory abilities characteristic of neural crest cells (Jeffery et al., 2004). These cells might thus represent the link between neural crest specification and the acquisition of migratory behaviour in vertebrates, providing us with clues about the evolutionary origin of the neural crest (see Manzanares and Nieto, 2003). Again, as in the case of cleft palate formation, it would be interesting to know why EMT does not occur in these cells. It could be that certain Snail downstream targets have been recruited only in the vertebrate lineage. Alternatively, if amphioxus *snail*-expressing cells could migrate, it is possible that the environment does not provide the necessary clues to trigger this process. Nevertheless, there is no evidence that snail is fully active in the neural tube of pre-vertebrates. In the absence of good anti-snail antibodies, the possibility that the snail protein is not translated or is maintained in an inactive state by post-translational mechanisms (see below) in these organisms cannot be excluded. However, we know that Snail genes are active in the cleft palate situation at least to induce cell survival, making it an excellent model in which to study Snail-dependent/EMT-independent processes

Snail genes as cell-adhesion regulators

In addition to finding processes that are governed by Snail independently of the induction of EMT, different experimental models have revealed that Snail functions in some cell movements that do not require a full EMT. Interestingly, recent evidence shows that Snail genes also participate in these processes by regulating cell adhesion and migration (Yamashita et al., 2004; Savagner et al., 2005). One example of this is mesoderm formation in anamniote vertebrate embryos (such as amphibia and fish). During mesoderm formation in amniotes, cells delaminate and migrate individually. However, in anamniotes, a complex interplay of different morphogenetic movements causes a mass, sheet-like migration of cells, in which cells maintain contact with each other while moving (Keller et al., 2000). Ironically, it is the lack of a bona fide EMT during mesoderm formation in *Xenopus* and zebrafish that might have made some investigators reluctant to consider the Snail genes as being important players in convergence and extension movements (reviewed by Locascio and Nieto, 2001). However, very recently, *snail1a* has been implicated in the anterior movement of the axial mesendoderm in the zebrafish embryo (Yamashita et al., 2004). Although the mechanism underlying its function in this process is not yet understood, it seems clear that LIV1 is necessary for the nuclear localisation of Snail1a and that its downregulation induces defects in anterior mesoderm migration in zebrafish (Yamashita et al., 2004). Curiously enough, and possibly due to the implication of snail, the authors mention that EMT is impaired by both *liv1* and *snail1a* misexpression. As already discussed, mesendoderm migration does not occur through a bona fide EMT, but still, Snail1a is important for the extension movement. Our own data on the other *snail1* gene in zebrafish (*snail1b*) extend and reinforce this idea (M. J. Blanco, A. B.-G., A. E. Reyes, M. Tada, M. Allende, S. W. Wilson, R. Mayor and M. A. N., unpublished).

The idea that Snail is involved in the movement of cells that maintain contact with each other as they move is consistent with its role during gastrulation in *Drosophila*. In the fly, *snail* is expressed in the cells of the ventral furrow that invaginate

and give rise to the mesoderm (Leptin, 1991). In this context, again, the changes in cell shape that accompany the morphogenetic movements do not require a full EMT. Cells move together even though Snail still functions as an E-Cadherin repressor. Cell-cell adhesion is reduced but maintained due to a switch in expression from E- to N-Cadherin (Oda et al., 1998). At the cellular level, a similar process occurs during mesoderm formation in ascidians (Wada and Saiga, 1999) and hair bud formation in mice (Jamora et al., 2005). As already mentioned, *Snail1* is expressed during the invagination of the hair bud precursor cells, which occurs concomitantly with E-Cadherin downregulation.

Another example in which Snail genes participate in cell movements that do not require a full EMT has been recently published (Savagner et al., 2005). This study found that Snail2 is involved in the re-epithelialisation of cutaneous wounds in mice, a process that requires migration and reduced cell-cell adhesion, but in which the cells involved retain intercellular junctions and remain associated with each other in a cohesive sheet (Savagner et al., 2005). During this process, there is no upregulation of *Snail1* expression, which is reflected in the maintained expression of E-cadherin in the margins of the wound and which supports the idea that Snail2 is a much weaker repressor of E-cadherin than Snail1 (Bolós et al., 2003). It will be interesting to identify the adhesion molecules that Snail2 regulates in this process. Future work should also aim to identify all the targets that are directly or indirectly regulated by Snail genes and which are in common or specific to each family member. Taking into account that similar roles are carried out by Snail1 and Snail2 in different species (Nieto, 2002), the search for these targets and their specificity is going to be a difficult task, although undoubtedly extremely interesting. With respect to targets, Fig. 4 summarises those that are involved in processes in which Snail genes: repress epithelial markers (Cano et al., 2000; Batlle et al., 2000; Guaita et al., 2002; Ikenouchi et al., 2003; Tripathi et al., 2005a); upregulate mesenchymal markers (Cano et al., 2000; Guaita et al., 2002); or participate in the change of cell shape and invasive properties (del Barrio and Nieto, 2002; Yokoyama et al., 2003), cell proliferation (Vega et al., 2004) or cell survival (Kajita et al., 2004; Tribulo et al., 2004; Vega et al., 2004).

Together, these findings show that Snail genes should be considered in a more general sense as being regulators of cell adhesion and movement, rather than being regarded just as regulators of EMT. In this context, we believe that the triggering of the EMT by Snail genes would be one of the mechanisms these genes use to promote cell movement.

Controlling Snail activity by subcellular localisation

It has been recently shown in vitro that the activity of the Snail1 protein is regulated by phosphorylation, which, in turn, regulates its subcellular localisation (Domínguez et al., 2003; Zhou et al., 2004; Yook et al., 2005). Current data suggest that exportins (such as CRM1), which control the translocation of proteins from the nucleus to the cytoplasm, are involved in exporting phosphorylated Snail1 and, thus, in its inactivation as a transcription factor (Domínguez et al., 2003). Interestingly, one of the kinases that phosphorylates Snail1 is GSK3, which not only promotes the nuclear export of Snail1 but also its rapid degradation via the proteasome (Zhou et al., 2004; Yook et al., 2005) (Fig. 3). Thus, the phosphorylation of Snail1 exquisitely controls its activity. These data are also compatible with the finding that active GSK3, which maintains Snail1 in an inactive state, is required to prevent an EMT from occurring in breast and skin epithelial cell lines. In these cells, inhibition of GSK3 activity also induces *Snail* transcription, adding a new mechanism by which GSK3 regulates Snail1 (Bachelder et al., 2005). In addition to GSK3, the p21-activated kinase (PAK1) is also able to phosphorylate Snail at a different residue and to control its subcellular localisation. Interestingly, PAK1-induced phosphorylation favours the nuclear localisation of Snail and, thus, its activity as a transcription factor (Yang et al., 2005).

The relationship of Snail1 with GSK3 connects Snail to the WNT signalling pathway. Indeed, in the presence of WNT signalling, GSK3 is unable to phosphorylate its targets and thus, both β-catenin and Snail1 are stabilised and ready to act as transcription factors. β-catenin itself, acting as a transcription factor through its interaction with TCF/LEF, is required for EMT both in epithelial cells (Kim et al., 2002) and during heart cushion development (Liebner et al.,

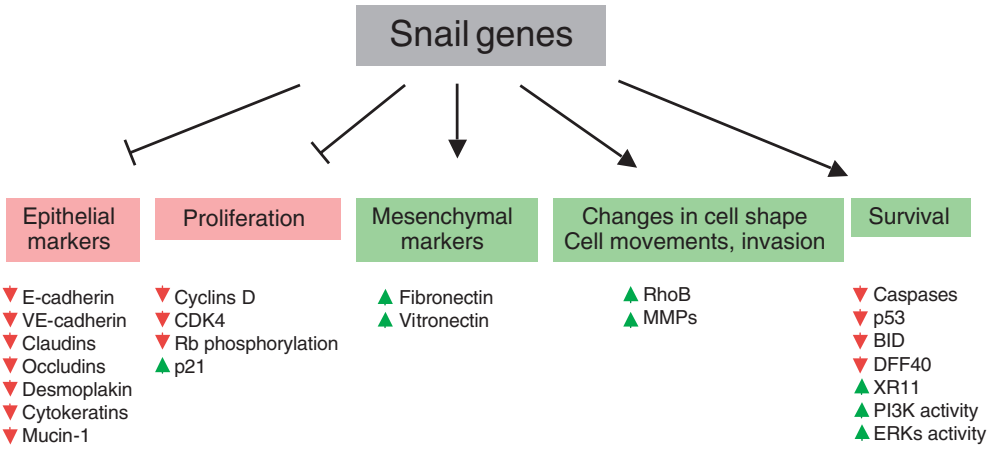

Fig. 4. Downstream targets of Snail. Snail gene expression induces the loss of epithelial markers and the gain of mesenchymal markers, as well as inducing changes in cell shape, and changes related to morphology and to the acquisition of motility and invasive properties. The Snail genes also regulate cell proliferation and cell death. Not all of these targets are directly regulated by Snail genes: because Snail genes function as repressors, from *Drosophila* to humans (reviewed by Nieto, 2002), target upregulation might be due to the Snail-mediated repression of a repressor. However, their role as activators cannot be excluded. The molecules and processes shown in red are downregulated or impaired by Snail, and those in green are upregulated or promoted by Snail. BID, Bcl-interacting death agonist; CDK, cyclin-dependent kinase; DFF, DNA fragmentation factor; ERKs, extracellular signal-regulated kinases; MMPs, metalloproteinases; PI3K, phosphoinositide 3-kinase; p21, cyclin-dependent kinase inhibitor; p53, tumour suppressor; Rb, retinoblastoma; XR11, *Xenopus* Bcl-xL homologue.

2004). These data suggest that cooperation occurs between WNT signalling and other Snail-induced signalling pathways, such as FGF, in the triggering of the EMT. This cooperation has been already highlighted in several developmental systems, in particular, in the mesoderm and the neural crest (Ciruna et al., 2001; Bastidas et al., 2004; Meulemans and Bronner-Fraser, 2004). For example, when Snail1 activity is maintained, E-cadherin is repressed and is therefore not available to bind β-catenin and form adherens junctions. As a result, β-catenin is available to bind to TCF/LEF and to act as a transcription factor, promoting WNT signalling. Although this situation will only occur concomitantly with an inactive β-catenin degradation system, WNT signalling can increase Snail1 function by preventing its nuclear export and degradation, and Snail1 can promote WNT signalling by keeping E-cadherin downregulated.

LIV1 also appears to regulate Snail function by controlling it by an as-yet unknown mechanism (Yamashita et al., 2004). Thus, GSK3 and LIV1 might play opposite roles, downregulating and activating the function of Snail, respectively (Fig. 3).

In addition to being regulated at the transcriptional level, the data discussed above indicate that Snail function is also regulated by its subcellular localisation. Indeed, Snail is a pleiotropic protein that needs to be tightly regulated, as its misexpression is detrimental in many ways, as discussed below.

Snail functions: development versus pathology

One conclusion that can be drawn from the numerous recent studies of the Snail proteins is that they mainly function to regulate cell movement and to provide cells with survival properties. While these functions are crucial for embryonic development, they become fatal in pathological situations in the adult. The Snail-mediated induction of cell movements is translated in the embryo into the ability to generate different tissues and organs that are located far from where their precursors originate. In cancer, however, they facilitate the delamination of cells from the primary tumour and their metastasis to other parts of the body (Fig. 5).

Snail: tumour progression marker and anti-invasive drug target

In addition to their important roles during embryonic development, the loss of cell adhesion and the induction of EMT has a more sinister role during tumour progression, as previously discussed (Thiery, 2002). Indeed, the induction of EMT constitutes the first step in the metastatic cascade, allowing cells to delaminate from the primary tumour and to intravasate into lymph or blood vessels. Thus, the pathological activation of Snail1 leads to the acquisition of invasive properties by epithelial tumours, as suggested after the first indication of the involvement of Snail genes in the triggering of EMT (Nieto et al., 1994). For example, Snail1 is activated at the invasive front of tumours induced in mouse skin (Cano et al., 2000). Because the loss of E-cadherin from tumours is associated with a poor prognosis (Perl et al., 1998), Snail genes, as E-cadherin repressors, could be regarded as early markers of tumour malignancy. In addition to downregulating E-cadherin, *Snail*-expressing cells acquire migratory and invasive properties through changes in their cytoskeleton and through the induction of metalloproteinases (Fig. 4). The analyses of biopsies obtained from breast tumours (Blanco et al., 2002; Elloul et al., 2005); gastric cancers (Rosivatz et al., 2002); hepatocellular carcinomas (Sugimachi et al., 2003; Miyoshi et al., 2005); colon cancers (Palmer et al., 2004) and synovial sarcomas (Saito et al., 2004) have confirmed that *Snail* expression correlates with decreased E-cadherin expression, and with dedifferentiation and invasiveness. In colon cancer, SNAIL1 has been found both to downregulate E-cadherin and to repress the expression of the vitamin D receptor gene (*VDR*) (Palmer et al., 2004). Interestingly, the vitamin D analogue seocalcitol has been used in clinical trials against some tumours (Dalhoff et al., 2003) and *VDR* expression, which is associated with a good clinical outcome, is lost during tumour progression (Kallay et al., 2002).

Snail2 has also been recently added to the list of Snail family members that are involved in tumour progression (Uchikado et al., 2005). This study's finding that SNAIL2 is downregulated in human esophageal squamous carcinomas suggests that Snail2, in addition to Snail1, can also induce pathological EMT to occur in particular cell types or to cooperate with Snail1 in this process. An example of this co-operation might occur in human breast tumour cells, where SNAIL1 expression has been correlated with dedifferentiation and metastasis (Cheng et al., 2001; Blanco et al., 2002; Elloul et al., 2005), and SNAIL2 expression with the repression of the tumour suppressor gene *BRCA2* (Tripathi et al., 2005b). Snail2 is also activated in malignant mesotheliomas, where it is induced by stem cell factor (SCF) (Catalano et al., 2004), which is in agreement with its previous description as a target of the SCF/c-Kit pathway (Pérez-Losada et al., 2002). In these tumours, *SNAIL2*

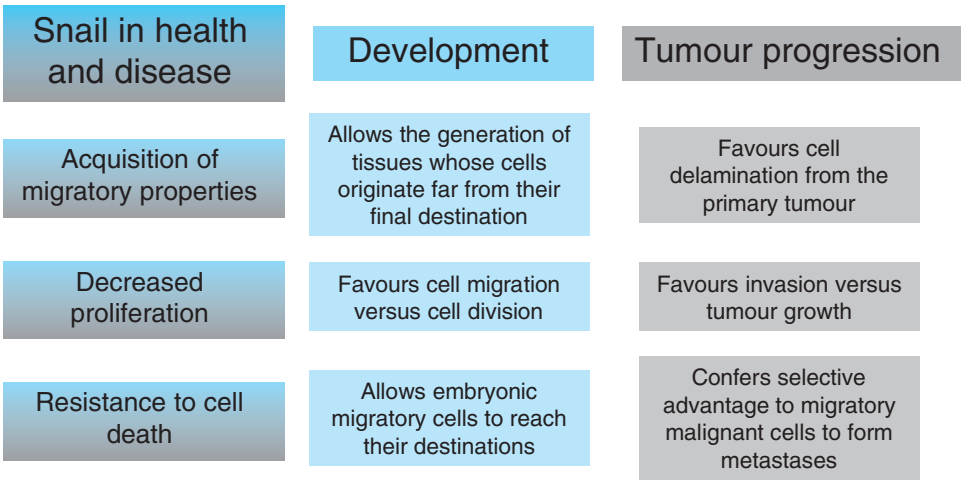

Fig. 5. Snail functions in development and disease. The cellular properties conferred by Snail expression are beneficial under normal circumstances, but can be fatal in pathological situations.

expression seems to be associated with a patient's resistance to chemotherapeutic agents, in keeping with the described role of Snail2 in protecting cells from cell death (Inoue et al., 2002; Pérez-Losada et al., 2003; Kajita et al., 2004).

There are circumstances in which the dissemination of a primary tumour involves its exposure to low levels of oxygen, which helps it to acquire malignant properties. A recent study has shown that hypoxia indeed induces SNAIL1 expression and invasiveness in ovarian carcinoma cells (Imai et al., 2003). Although the mechanism for Snail induction is not yet understood, a hint may come from pancreatic cancer cells, in which hypoxia induces the transcription of the autocrine motility factor (AMF; Fig. 3), a protein that can act both as a cytoplasmic enzyme and as an extracellular cytokine. AMF, in turn, induces Snail and can generate liver metastasis when overexpressed in cells orthotopically administered to nude mice (Tsutsumi et al., 2004).

In summary, advances in the last few years have lead to our understanding that *Snail1* in particular, and the Snail genes in general, are new potential targets of anti-invasive drugs, owing to their association with dedifferentiated metastatic tumours of different origins.

Fibrosis and wound healing

Pathological EMT is not only observed during tumour progression, but also in other circumstances, such as fibrosis. Thus, it not surprising to find that SNAIL1 is activated in the mesothelial cells of patients during the secondary fibrosis that is associated with prolonged peritoneal dialysis (Yáñez-Mo et al., 2003). TGFβ1 induces the same effects both in cultured human mesothelial cells (Yáñez-Mo et al., 2003) and in rats that receive an intraperitoneal injection of an adenovirus vector that transfers active TGFβ1 (Margetts et al., 2005). EMT and Snail1 induction is also observed after the incubation of mesothelial cells with menstrual effluent (Demir et al., 2004).

Fibrosis also appears in the kidney concomitant with Snail1 expression after injury, and TGFβ can also mimic this effect both in vitro and in vivo (Sato et al., 2003). Snail expression may be a general response to injury in epithelial cells, as Snail2 is activated during skin wound healing, as already mentioned (Savagner et al., 2005), and Snail1 appears during cataract extraction in lens epithelial cells that concomitantly undergo EMT (Saika et al., 2004).

Snail effects on cell proliferation and survival in development and disease

There is one more theme to consider regarding the conversion of cells from an epithelial to a mesenchymal phenotype. This conversion gives rise to cells that resemble fibroblasts, which, intuitively, one would expect to have an increased proliferation rate. However, cell division is impaired in Snail-expressing epithelial cells that have undergone EMT (Valdés et al., 2002; Peinado et al., 2003; Vega et al., 2004). For example, changes in cell shape concur with low proliferation and the expression of Snail in several systems, such as in the premigratory neural crest in chick and mouse (Burstyn-Cohen and Kalcheim, 2002; Vega et al., 2004), in the ventral furrow during *Drosophila* gastrulation (Foe, 1989) and in the invasive front of carcinomas (Jung et al., 2001). This observation is striking because tumour development is usually associated with increased cell proliferation. However, it is worth considering that the change to an invasive phenotype is related to a tumour's acquisition of malignant properties, not with its formation or growth. Thus, Snail would favour invasion versus tumour growth (Fig. 5). Invasion is also favoured by the angiogenic properties of Snail (Peinado et al., 2004).

During hair bud formation, Snail-expression correlates with E-cadherin downregulation and increased proliferation (Jamora et al., 2005). Interestingly, in this system, cells maintain the epithelial phenotype. Thus, decreased cell proliferation could be linked to the profound reorganization of the cytoskeleton that occurs concomitantly with the EMT, and which may be incompatible with a highly proliferative state. Alternatively, it could depend on cellular context and might be related to the absence of some Snail targets or co-factors. The latter is consistent with the finding that particular *Drosophila snail* mutant alleles show an intermediate phenotype in the cells that normally express the gene. These cells express both mesodermal and ectodermal markers, suggesting that the regulation of different targets is independently affected in these mutants (Hemavathy et al., 1997).

As discussed above, during embryonic development, Snail gene expression protects certain cell populations from cell death, such as the neural crest (Vega et al., 2004; Tribulo et al., 2004), and the epithelial cells at the medial edge of the palate when the palate fails to fuse (Martinez-Alvarez, 2004). In a mouse model of colonic neoplasia, the *min* mouse, Snail downregulation by antisense oligonucleotides has been shown to increase cell death in colon tumours (Roy et al., 2004), confirming its role in cell survival in cancer. In this model, tumour incidence also decreased with Snail downregulation and the tumours showed decreased proliferation rates.

These findings thus show that the acquisition of movement, survival and invasive properties by Snail-expressing cells that delaminate from the mesoderm or the neural tube in developing embryos, or from a primary tumour in an adult, gives these cells a selective advantage and ability to travel considerable distances. Such cells might stop being exposed to survival factors that are present in their tissue of origin as they move away from this tissue, and might also encounter apoptotic factors during their migration through hostile territories. If Snail proteins indeed protect migrating cells from death, they would thus allow embryonic migratory cells to reach their final destinations, while unfortunately also disseminating malignant cells through an adult to give rise to metastasis (Fig. 5).

Conclusion

It is clear that future research will provide much more information on the functions of this gene family both in development and in disease. It will be important to identify endogenous repressors to understand how embryonic cells stop migrating when they reach their target tissues and also synthetic inhibitors to specifically interfere with the delamination of cells from primary tumours. It is possible that Snail inactivation could help to prevent invasiveness and help in making invasive cells more susceptible to destruction. Thus, hopefully, future advances in our understanding of the Snail gene family's mechanisms of action and regulation will help us to gain insights into essential developmental pathways and into one of the worst sides of cancer, the onset of the metastatic process.

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