Snail and E47 repressors of *E-cadherin* induce distinct invasive and angiogenic properties in vivo

Hector Peinado[1,3], Faustino Marin[2], Eva Cubillo[1], Hans-Juergen Stark[3], Norbert Fusenig[3], M. Angela Nieto[2] and Amparo Cano[1,*]

[1]Departamento de Bioquímica, UAM. Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC-UAM), Arturo Duperier 4, 28029 Madrid, Spain
[2]Instituto Cajal CSIC, Doctor Arce, 37, 28002 Madrid, Spain
[3]German Cancer Research Center, DKFZ, Im Neuenheimer Feld 280, Heidelberg 69120, Germany
*Author for correspondence (e-mail: acano@iib.uam.es)

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Summary

The transcription factors Snail and E47 are direct repressors of *E-cadherin*, with both inducing a full epithelial-mesenchymal transition and invasive behaviour in vitro when expressed in the prototypic epithelial MDCK cell line. The role of these repressors in the invasive process and in other tumorigenic properties is, nevertheless, still poorly understood. However, organotypic cultures and in vivo transplantation assays indicate that cells expressing MDCK-Snail and MDCK-E47 exhibit significant differences. MDCK-Snail cells have a higher infiltrative potential than MDCK-E47 cells. Interestingly, both cell types induce angiogenesis of the host stromal tissue in transplantation assays, but this property is greatly enhanced in transplants of MDCK-E47 cells. Xenografted tumours induced in nude mice also show signs of strong angiogenic potential, again markedly increased in tumours induced by MDCK-E47 which exhibit a higher vessel density and proliferation rate than those induced by MDCK-Snail cells. These results suggest differential roles for Snail and E47 *E-cadherin* repressors in tumour progression where Snail is implicated in promoting the initial invasion and E47 plays an active role in tumour cell growth by promoting angiogenesis.

Key words: Snail, E47, E-cadherin, Invasion, Angiogenesis, Transplantation assays

Introduction

Carcinoma progression is a multi-step process that involves the accumulation of mutations in several oncogenes and tumour-suppressor genes leading to the acquisition of common altered cellular mechanisms that provide the hallmarks of malignant transformation (Hanahan and Weinberg, 2000). Among the acquired cellular mechanisms, local invasion and metastasis provide tumour cells with the ability to disseminate to distant organs and tissues and represent the most threatening event for the survival of cancer patients. New blood vessel formation (angiogenesis) is also required for the growth of tumours above a few millimetres in both the primary and secondary tumour foci and can provide an additional pathway for the dissemination of tumour cells (Hanahan and Weinberg, 2000; Bergers and Benjamin, 2003). Loss of expression or function of the E-cadherin cell-cell adhesion molecule has emerged as an important event for local invasion and metastasis, supporting a role for *E-cadherin* as an invasion-suppressor gene (Birchmeier and Behrens, 1994; Berx et al., 1995; Perl et al., 1998; Christofori and Semb, 1999). The process of invasion is frequently associated with the loss of other epithelial markers and with the acquisition of mesenchymal markers and a migratory and in vitro invasive behaviour, collectively known as epithelial-mesenchymal transitions (EMTs) (for a review, see Thiery, 2002). EMT processes also occur during normal embryonic development under strict spatio-temporal control and are always accompanied by the loss of functional E-cadherin-mediated cell-cell adhesion (Hay et al., 1995; Boyer et al., 2000; Nieto, 2002; Thiery, 2002). Furthermore, repression of *E-cadherin* must be maintained in highly migratory cells both during embryonic development and in tumour progression (Takeichi, 1993; Behrens et al., 1989; Burdsal et al., 1993; Perl et al., 1998).

Several molecular mechanisms underlying *E-cadherin* downregulation have been unravelled in the past few years. Among them, hypermethylation of the *E-cadherin* promoter and transcriptional alterations have emerged as the main mechanisms responsible for *E-cadherin* downregulation in most carcinomas (Yoshiura et al., 1995; Hennig et al., 1995; Hajra et al., 1999; Rodrigo et al., 1999; Tamura et al., 2000; Cheng et al., 2001). Several transcriptional repressors of *E-cadherin* have been isolated, including the zinc-finger factors Snail (Battle et al., 2000; Cano et al., 2000) and Slug (Hajra et al., 2002; Bolós et al., 2003), the two-handed zinc factors δEF1 (ZEB-1) and SIP-1 (ZEB-2) (Grooteclaes and Frisch, 2000; Comijn et al., 2001) and the bHLH factor E12/E47 (Pérez-Moreno et al., 2001). Snail, Slug and E47 repressors apparently induce a similar phenotype when overexpressed in epithelial MDCK cells, as they elicit a full EMT with all the hallmarks of the process: loss of E-cadherin and other epithelial markers, increased expression and organization of mesenchymal markers, and a motile behaviour (Cano et al., 2000; Pérez-Moreno et al., 2001; Bolós et al., 2003). Furthermore, MDCK cells expressing either Snail or E47 are able to migrate through collagen gels in Boyden-chamber assays and exhibit tumorigenic properties when injected into nude mice (Cano et

al., 2000; Pérez-Moreno et al., 2001). The involvement of Snail and E47 in tumour progression is also supported by the expression of both genes in carcinoma cell lines with invasive and metastatic properties (Cano et al., 2000; Pérez-Moreno et al., 2001; Cheng et al., 2001; Poser et al., 2001) and by the expression of Snail in dedifferentiated breast carcinomas and invasive hepatocarcinomas (Blanco et al., 2002; Sugimachi et al., 2003). Despite these apparent similarities, the implication of Snail and E47 in specific stages of tumour progression is not yet fully understood. Indeed, several observations suggest that Snail and E47 repressors might play distinct functions. Significant differences in the interaction of both factors with the *E-cadherin* promoter have been recently detected, with Snail showing a much higher binding affinity (2×10^{-10} M) than E47 (6×10^{-9} M) for specific E-boxes (Bolós et al., 2003). In addition, *Snail* and *E2A* (encoding E12/E47) exhibit distinct expression patterns in early embryonic development of mammals; *Snail* is expressed at EMT areas (Cano et al., 2000; Carver et al., 2001; Locascio et al., 2002) whereas *E2A* is expressed in the already migratory mesodermal cells (Pérez-Moreno et al., 2001).

To gain further insight into the role of Snail and E47 in tumour progression, we used previously characterized MDCK-Snail and MDCK-E47 cell lines (Cano et al., 2000; Pérez-Moreno et al., 2001; Bolós et al., 2003) and performed an in-depth analysis of their properties when grown in vitro in organotypic cultures, as well as in vivo in transplantation assays and xenografted tumours.

Materials and Methods

Cell lines and organotypic cultures

The generation and characterization of two-dimensional cultures of MDCK-CMV (control), MDCK-Snail and MDCK-E47 cell lines has been previously reported (Cano et al., 2000; Pérez-Moreno et al., 2001). All experiments with organotypic cultures and transplantation assays were performed with single clones of each transfectant cell type. Cells were grown in DMEM in the presence of 10% FBS, 10 mM glutamine (Gibco BRL) and 100 μg/ml ampicillin, 32 μg/ml gentamicin (Sigma Chemical Co.) at 37°C in a humidified CO_2 atmosphere. For organotypic cultures 1×10^6 cells were seeded onto collagen gels (Type I, rat tail tendon) in filter-culture inserts (pore size 3.0 μm, polycarbonate; Becton Dickinson, Heidelberg, Germany). The gel was prepared in the presence (co-culture) or absence of postmitotic fibroblasts, as previously described (Maas-Szabowski et al., 2000; Stark et al., 1999). The organotypic cultures were immersed in DMEM and grown for the indicated time periods. Medium was completely replaced every 3 days. At the end of the culture period, membranes with the organotypic cultures were dissected from the filter inserts and either immediately frozen in liquid nitrogen embedded in Tissue Tek OCT Compound (Medim, Gießen, Germany), or fixed in formaldehyde and embedded in paraffin. At least four organotypic cultures of each cell clone were analysed at the indicated time points of the experiment (1, 2 and 3 weeks after seeding); for each experimental setting, half of the cultures were analysed using immunofluorescence or in situ hybridization and half by histology.

Transplantation assays

MDCK cells were precultured on collagen I gels in DMEM. They were covered with a silicon transplantation chamber (Renner, Gmbh) and transplanted onto the dorsal muscle fascia of 6-week-old male BALB/c nude mice, following the published protocol (Boukamp et al., 1990; Skobe et al., 1997). Eight mice were used for each MDCK cell line. Animals were maintained under sterile conditions and observed every 2 days. Four animals from each group were sacrificed after 10 and 20 days of transplantation. Transplants were resected and half were embedded in OCT and frozen in liquid nitrogen for cryostat sectioning and immunofluorescence with the remainder fixed in formaldehyde and embedded in paraffin as described above.

Induction of xenografted tumours

Cells grown in two-dimensional cultures were trypsinized, washed and resuspended at a density of 1×10^7 cells/ml in PBS. 1×10^6 cells of the indicated cell lines were injected into the flanks of 8-week old male BALB/c nude mice (Charles River). Mice were maintained under sterile conditions and observed every two days. The size and growth of the tumours were estimated from the external diameter measured with a calibre, and the mice sacrificed when the tumours reached a size of 1.5 cm of larger external diameter. Control mice (injected with MDCK-CMV cells) were observed for up two months. Tumours were excised and immediately frozen in isopentane-cooled liquid nitrogen embedded in OCT. All animal experiments were performed according to institutional guidelines for animal care. A total of ten tumours from MDCK-E47 transfectants were generated from a single clone and 12 tumours were generated from two independent clones of MDCK-Snail transfectants (six tumours each). At least four different tumours derived from each cell line were analysed by histology, immunostaining and in situ hybridization.

Immunofluorescence analysis

Sections (5-10 μm) of the OCT-embedded samples from either the organotypic cultures, in vivo transplants or xenografted tumours were fixed in methanol (–20°C) and acetone (–20°C) and then incubated with the primary antibodies (Navarro et al., 1993; Cano et al., 2000). The primary antibodies included: rat monoclonal anti-mouse E-cadherin (1:100) (ECCD2) (a gift of M. Takeichi, Kyoto University, Japan), mouse monoclonal anti-vimentin (1:100) (Dako), anti-cytokeratin 8 (1:20) (Progen, Heidelberg, Germany), anti-endoglin (1:100) (South. Biotech. Associates) and rat monoclonal anti-mouse CD31 (1:100) (BD Pharmingen). Secondary antibodies included goat anti-rat and goat anti-mouse Ig coupled to Alexa 594 and Alexa 488 respectively (1:1000) (Molecular Probes). Samples from the organotypic cultures and transplants were also stained with DAPI to detect nuclei.

In situ hybridization analysis

Vibratome sections at 40-100 μm were obtained from the OCT specimens. After elimination of the OCT compound by washing in 4% paraformaldehyde, sections were subjected to in situ hybridization as previously described (Blanco et al., 2002). The riboprobes labelled with digoxygenine nucleotides used were: full length mouse E-cadherin, Snail and E47 cDNA, as previously described (Cano et al., 2000; Pérez-Moreno et al., 2001); and full length mouse VEGF-A and TGFβ-1 cDNA, kindly provided by F. Larcher (CIEMAT, Madrid, Spain) and M. Quintanilla (IIB. Madrid, Spain), respectively. Some sections were processed for immunohistochemistry with a monoclonal antibody for α-smooth muscle actin (Sigma), following treatment with peroxidase-coupled secondary antibody and developing with DAB-H_2O_2.

Proliferation assays

To monitor proliferation, cells were seeded in triplicate onto 96-well plates at a density of 5000 cells/well. After 24 hours of growth in normal medium, radiolabelled [^{3}H]thymidine (0.5 μCi) was added and the cells were grown for an additional 24 hours. Cells were harvested and [^{3}H] radioactivity was measured in a solid scintillation counter.

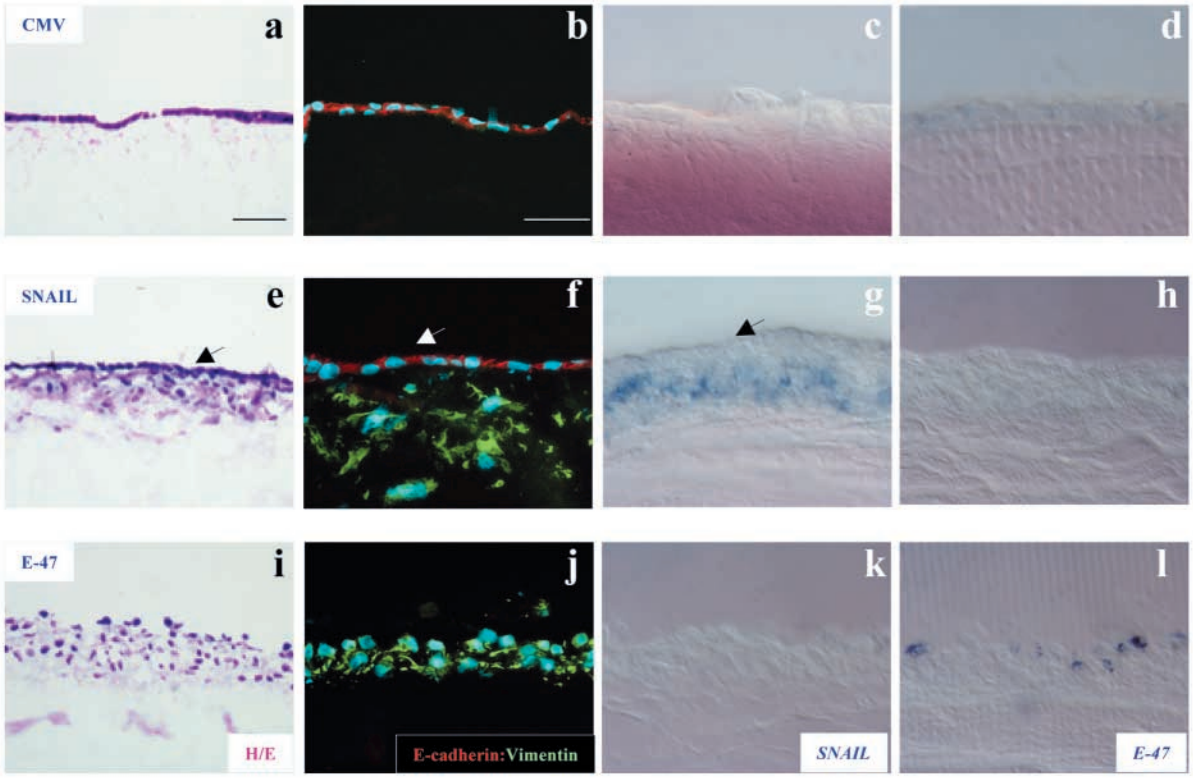


Fig. 1. Analysis of organotypic cultures. Organotypic cultures of MDCK-CMV (CMV) (a-d), MDCK-Snail (Snail) (e-h) and MDCK-E47 (E47) cells (i-l) were prepared as described in Materials and Methods and grown for 3 weeks. Histological (a,e,i) and double immunofluorescence analyses for E-cadherin (red) and vimentin (green) (b,f,j) were performed on cryostat sections; nuclei were stained with DAPI (blue). In situ hybridization analysis for *Snail* (c,g,k) and *E47* transcripts (d,h,l) were performed on vibratome sections. Note the infiltration of *Snail*-expressing cells into the collagen gel and the transdifferentiation to an epithelial non-invading phenotype of the upper layer of MDCK-Snail cultures which re-express E-cadherin and have lost vimentin and *Snail* expression (arrows in e, f and g). Bars, 50 μm.

Quantification of migrated cells in collagen gels and markers in xenografted tumours

Sections prepared for immunofluorescence analysis obtained from 10-day organotypic cultures and transplantation assays were analysed to score the number of cells invading the collagen gel both from the upper part (MDCK infiltrated cells) and lower part of the cultures (emigrated host stromal cells). Quantification was performed by calculating the percentage of the distance migrated into the collagen type I gel by either cell type in relation to the total gel thickness. At least four sections of each culture and cell type were analysed and the data presented as the average. Internal variation among the different samples of each cell type was less than 10%. Quantification of *E-cadherin/Snail/E47* expression in xenografted tumours was estimated on in situ hybridization sections by calculating the number of positive cells for each of the marker on the overall surface section. Quantification of CD31 positive cells in MDCK-Snail and MDCK-E47 induced tumours was also performed on immunofluorescence sections.

Results

Organotypic cultures reveal a distinct invasive behaviour of MDCK-Snail and MDCK-E47 cells

Expression of Snail and E47 in MDCK cells induces EMT and a highly motile behaviour on collagen type IV gels (Cano et al., 2000; Pérez-Moreno et al., 2001), suggesting that both cell types might exhibit invasive properties. To analyse the influence of both factors further in a more physiological context, we performed organotypic cultures of either cell type, as well as control MDCK-CMV cells. Histological analysis of the cultures showed that control MDCK-CMV cells form an epithelial cell monolayer after 1 week (data not shown) which remained unstratified after 3 weeks of culture without infiltration into the collagen gel (Fig. 1a). Immunofluorescence staining indicated that the monolayer expresses the typical simple epithelial markers cytokeratin 8 (CK-8) (data not shown) and E-cadherin at cell-cell contacts of the basolateral membranes, and do not express the mesenchymal marker vimentin (Fig. 1b). As expected, in situ hybridization (ISH) analysis showed the absence of *Snail* and *E47* transcripts in the MDCK-CMV cultures (Fig. 1c,d). Cultures of MDCK-Snail cells form a disorganized multilayer after 1 week of culture in which some cells from the deeper layers were infiltrating the collagen gel (data not shown). All cells in the 1-week MDCK-Snail cultures maintain the mesenchymal-like phenotype characterized by the expression of vimentin and the absence of E-cadherin and CK-8 (data not shown). After 3 weeks of culture, MDCK-Snail cells were infiltrating about 20% of the total collagen type I gel thickness (Fig. 1e). Infiltrating MDCK-Snail cells exhibit a mesenchymal phenotype characterized by high expression of vimentin and complete absence of E-cadherin (Fig. 1f) and CK-8 (data not shown). Surprisingly, after 3 weeks in culture, the uppermost layer of the MDCK-

Snail cultures showed an epithelial-like organization (Fig. 1e, arrow), confirmed by the homogenous expression of E-cadherin at basolateral contact areas and the absence of vimentin (Fig. 1f, arrow). Interestingly, ISH of the 3-week MDCK-Snail cultures showed an intense and homogeneous expression of *Snail* transcripts in the lower infiltrating mesenchymal cells and its complete absence in the uppermost non-invading epithelial cell layer (Fig. 1g). Absence of *E47* mRNA was observed in the MDCK-Snail cultures (Fig. 1h). In contrast to this behaviour, cultures of MCDK-E47 cells form disorganized multilayers without any apparent organization of the uppermost layers but with fewer cells infiltrating the collagen gel after 1 week (data not shown) and invading about 5-10% of the overall gel thickness after 3 weeks of culture (Fig. 1i). All cell layers of the MCDK-E47 cultures maintain the mesenchymal phenotype with expression of vimentin and absence of E-cadherin (Fig. 1j) and CK-8 (not shown). However, ISH analysis of MDCK-E47 cultures showed that *E47* transcripts are detected in all cell layers, but with a mixed 'salt and pepper' pattern, (Fig. 1l) and do not express *Snail* transcripts (Fig. 1k). Similar results were obtained for the different cell types when organotypic cultures were performed in the presence of fibroblasts (data not shown), indicating that extracellular factors secreted by fibroblasts are not involved in the behaviour of the different MDCK transfectants.

These results indicate that MDCK-Snail cells have a 2- to 3-fold higher infiltrative potential in organotypic cultures than MDCK-E47 cells, and this behaviour seems to be an intrinsic property of the cells. They also suggest that sustained expression of *Snail* is required for induction of the invasive behaviour of mesenchymal cells under these experimental conditions, since its loss in the uppermost layer is associated with an epithelial non-invading phenotype.

MDCK-Snail and MDCK-E47 cells have distinct invasive behaviour in transplantation assays

The in vivo invasive potential of Snail- and E47-expressing cells was analysed in surface transplantation assays. Cells were seeded on the top of collagen type I matrices that were then transplanted onto the back muscle fascia of nude mice, as previously described for keratinocyte cultures (Boukamp et al., 1990; Skobe et al., 1997). The transplants were allowed to grow for up to 10 and 20 days when mice were sacrificed and analysis performed. After 10 days of culture, transplants from control MDCK-CMV cells grew as an organized epithelial multilayer (Fig. 2Aa,Ba) with apparently differentiated cyst-like structures (Fig. 2Aa, black arrowheads). MDCK-CMV cells were unable to infiltrate the adjacent collagen type I gel (Fig. 2Aa,Ba). In agreement with this phenotype, E-cadherin expression was detected at cell-cell contacts in most cells of the 10-day control MDCK transplants (Fig. 2Ad,Bd). Co-expression of vimentin was detected in some isolated cells of the deepest layer of 10-day control transplants (Fig. 2Bd). Transplants of MDCK-Snail cells grown for 10 days form an undifferentiated multilayer with infiltration of the deeper cells up to around 45% of the total collagen type I gel thickness (Fig. 2Ab,Bb, black arrowheads). No expression of E-cadherin was noted but homogeneous expression of vimentin was detected in all cells of the 10-day MDCK-Snail transplants (Fig. 2Ae,Be). The 10-day transplants of MDCK-E47 cells grew as undifferentiated multilayers (Fig. 2Ac,Bc) which maintain the repression of E-cadherin and homogenous expression of vimentin (Fig. 2Af,Bf). However, only 15% of the collagen type I gel was infiltrated in the superficial part by MDCK-E47 cells (Fig. 2Ac,Bc, black arrowheads). In contrast, the lower part of the collagen type I gel (in direct contact with the host) of 10-day MDCK-E47 transplants shows clear accumulation of vimentin-positive cells (Fig. 2Ac,f, red arrows). These cells have apparently migrated from the host stromal tissue and infiltrated around 55% of the gel's thickness. A similar situation was detected in the lower part of the collagen gel in the 10-day MDCK-Snail transplants, although the gel was only invaded from the bottom to about 10-15% of its thickness (Fig. 2Ab,e, red arrows). By contrast, no migrated cells were observed in that region of the 10-day MDCK control transplants (Fig. 2Aa,d, red arrows).

Analysis after 20 days of in vivo culture showed that control MDCK transplants were overgrown as a highly organized multilayer of differentiated epithelial cells with an abundance of cyst-like structures that were unable to infiltrate the remaining collagen type I gel (Fig. 3Aa,d). Indeed MDCK-CMV cells clearly display a delimitation of the seeded cells from the collagen gel, suggesting that they have organized a basal membrane (Fig. 3Ad and data not shown; yellow arrow in panel d indicates basal membrane delimitation). Immunofluorescence analysis of parallel transplants with a less hyperplastic epithelium showed E-cadherin expression at cell-cell contacts of all cell layers of 20-day control MDCK transplants (Fig. 3Ba), which also showed complete absence of vimentin expression (Fig. 3Bd). This indicates that MDCK-CMV cells fully differentiate under these experimental conditions, losing the residual expression of mesenchymal markers observed in the 10-day transplants. The 20-day transplants of MDCK-Snail and MDCK-E47 cells showed an overgrowth of highly undifferentiated cells that completely infiltrated the whole collagen type I gel and the stromal host tissues up to the muscle and adipose tissues (Fig. 3Ab,c,e,f and data not shown). As previously observed in the organotypic cultures, the 20-day cultures of MDCK-Snail transplants showed a transdifferentiation to an epithelial-like phenotype in the uppermost layers, characterized by expression of CK-8 (data not shown) and E-cadherin at cell-cell contacts and the absence of vimentin (Fig. 3Bb,e, white arrows). Co-expression of E-cadherin and vimentin was observed in some cells at the interface between the epithelial and mesenchymal layers (Fig. 3Be, white arrowheads), indicative of an intermediate phenotype. As observed in the organotypic cultures, 20-day transplants of MDCK-E47 cells maintained the undifferentiated phenotype in all cell layers (Fig. 3Ac,f), as confirmed by the complete absence of E-cadherin and homogenous expression of vimentin even in the uppermost cell layers (Fig. 3Bc,f).

MDCK-Snail and MDCK-E47 cells exhibit a distinct in vivo angiogenic potential

The detection of vimentin-positive cells in the lower part of the collagen gel in 10-day transplants of MDCK-E47 and to a lesser extent in those of MDCK-Snail cells (see Fig. 2A), together with the absence of a clear boundary between the collagen gel and the host tissue (Fig. 2A, dotted lines), suggest

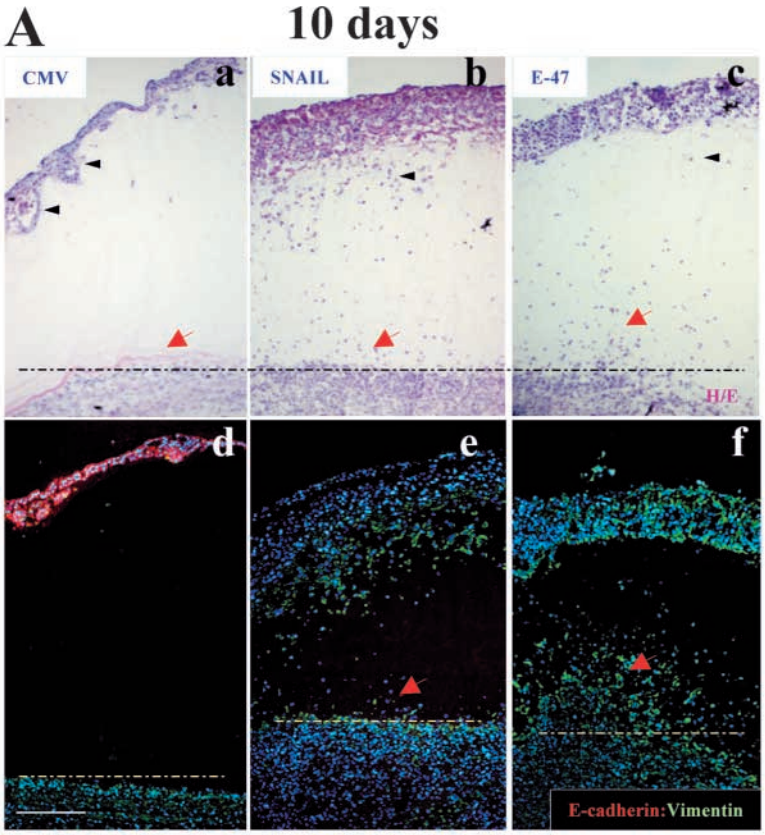
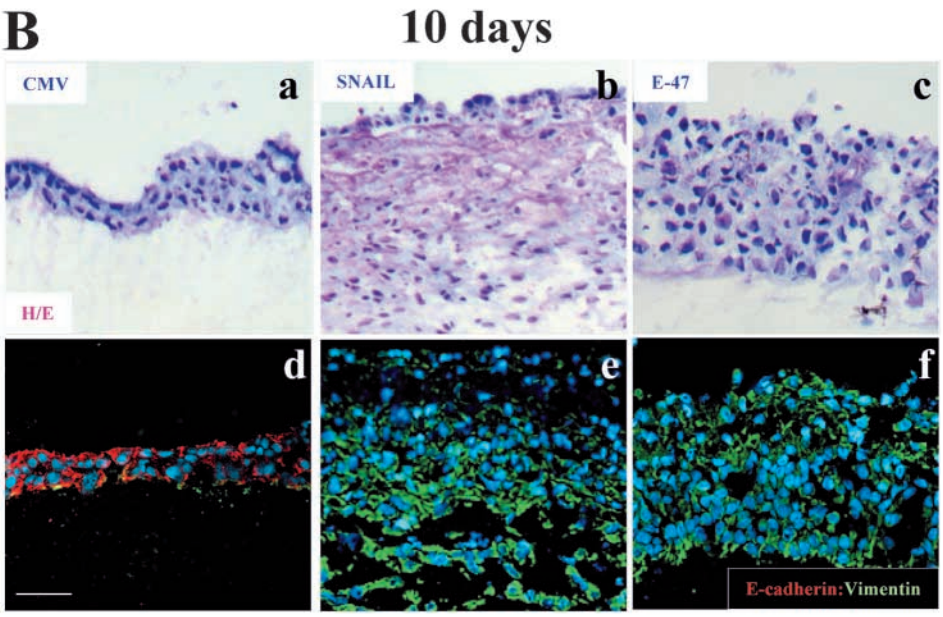


Fig. 2. MDCK-Snail and MDCK-E47 cells exhibit invasive and migratory abilities in in vivo transplantation assays. (A) low power and (B) high power images of MDCK-CMV (CMV) (a,d), MDCK-Snail (Snail) (b,e) and MDCK-E47 (E47) cells (c,f) grown on collagen type I gel, transplanted onto the backs of nude mice and allowed to grow for 10 days. Cryostat sections of each type of transplant were analysed by histology (a-c) and double immunofluorescence for E-cadherin (red) and vimentin (green) (d-f); nuclei were stained with DAPI (blue). (A) The lower limit of the collagen gel in contact with the host stroma is indicated by dashed black (a-c) and yellow lines (d-f). MDCK-CMV cells form an organized epithelial multilayer with cyst like structures without infiltration into the collagen gel (black arrowheads in a), whereas MDCK-Snail cells are clearly infiltrating (black arrowheads in b). Note also vimentin-positive cells apparently migrating from the host stroma into the collagen gel in MDCK-Snail and MDCK-E47 transplants (red arrows in b, c, e and f), in contrast to the complete absence of cells in the lower part of the collagen gel in MDCK-CMV transplants (a,d). Bar, 50 μm. (B) High power images of the upper part of the indicated 10-day transplants showing the histology (a-c) and immunofluorescence analyses for E-cadherin (red) and vimentin (green) (d-f); nuclei were stained with DAPI (blue). Note the absence of E-cadherin and expression of vimentin in all cell layers of MDCK-Snail (e) and MDCK-E47 (f) transplants. Bar, 50 μm.



that these vimentin-expressing cells may represent mesenchymal cells that have migrated from the host stroma. However, it is also possible that other cell types from the host tissue, such as endothelial cells, are also migrating into the collagen gel. To examine this aspect further, double immunofluorescence staining for the CD31 endothelial marker and vimentin were performed on the 10-day transplants. As can be observed in Fig. 4, adjacent sections of the MDCK-Snail and MDCK-E47 transplants shown in Fig. 2 exhibited CD31 staining in all the host stroma in close contact with the collagen gel edge (Fig. 4e,f, yellow arrows). In contrast, only a few CD31-positive cells (around 10-20% of the total host volume tissue) were detected in the stroma of control MDCK transplants, and these cells were localized far away from the lower collagen gel edge. Furthermore, hardly any vimentin or CD31-positive cells were observed inside the upper zone of the collagen gel in control transplants (Fig. 4a,d). Interestingly, although few CD31-positive cells appeared to be migrating into the collagen gel of the MDCK-Snail transplants (Fig. 4e) (colonizing 5-10% of the collagen gel in its lower portion), a large number of CD31-positive cells were detected infiltrating (from the bottom up) about 50% of the collagen gel in MDCK-E47 transplants (Fig. 4f, top level of CD31 positive cells indicated by red arrows). In addition, some blood vessel-like structures were detected in the host stromal compartment of MDCK-Snail and MDCK-E47 transplants even expanding into the colonized region of the collagen gel in MDCK-E47 transplants (Fig. 4h,i, white arrows). The blood vessel-like structures detected in much deeper regions of the stromal compartment of control MDCK transplants (Fig. 4d,g) are likely to be the normal blood vessels of the host tissue. Similar results were obtained in other

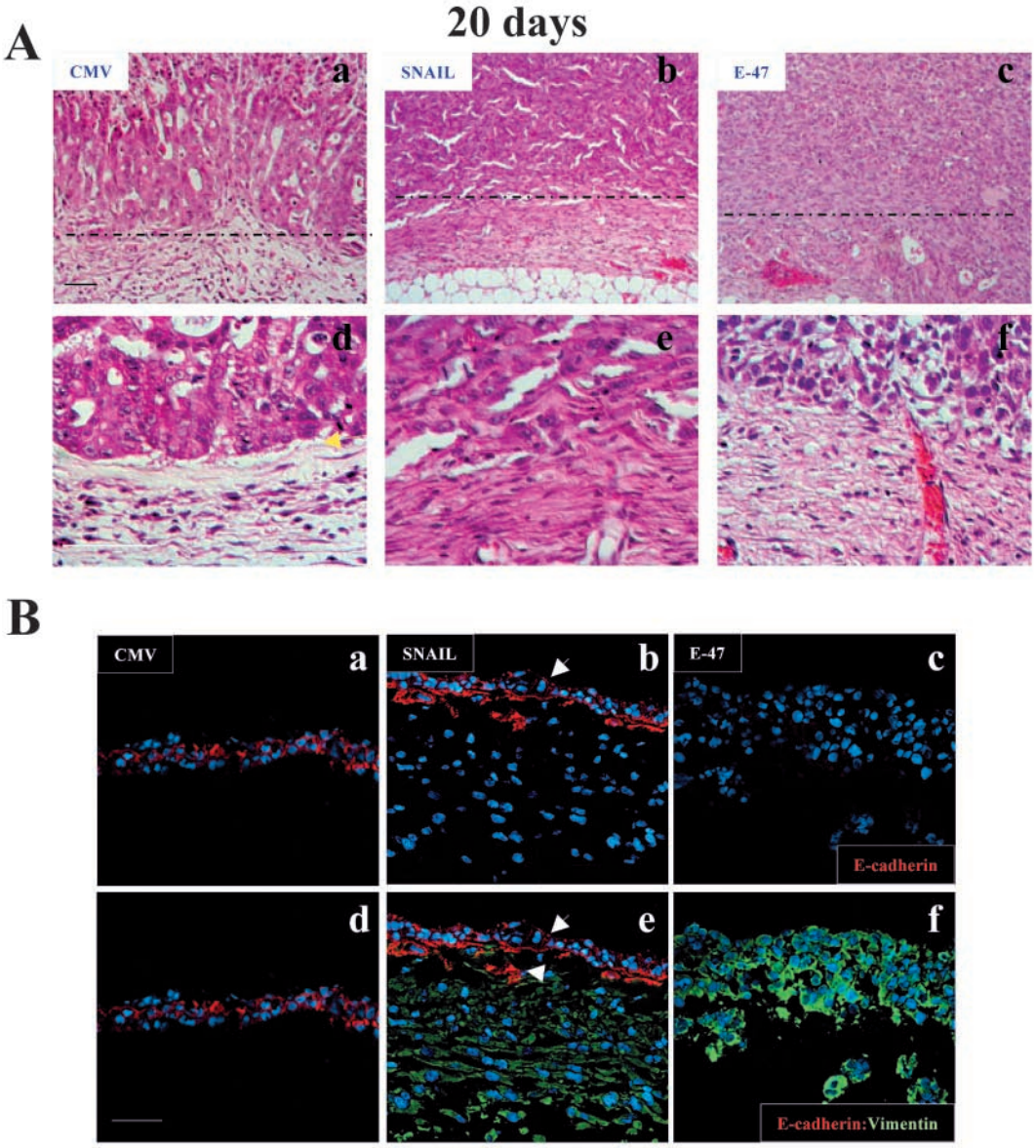


Fig. 3. Twenty day-transplants of MDCK-Snail and MDCK-E47 cells exhibit a high infiltration potential. Transplants of the indicated cells lines were allowed to grow on the backs of nude mice for 20 days and subsequently either (A) fixed in formaldehyde and processed for histology or (B) frozen in OCT and subjected to immunofluorescence staining after cryostat sectioning. (A) MDCK-CMV (CMV) (a,d) cells form a multilayer of highly differentiated epithelial cells with a clear basal membrane delimitation from the remaining collagen gel (dashed line in a, and yellow arrow in d). MDCK-Snail (b,e) and MDCK-E47 (c,f) cells fully infiltrate the collagen gel and adipose and muscle stromal tissues; the dashed lines in b and c indicate the hypothetical limit of the collagen gel. Panels d to f show magnified images of the lower part of the transplants. See also blood vessels in the lower part of MDCK-E47 transplants (c,f). Bar, 50 μm. (B) MDCK-CMV transplants maintain E-cadherin expression and absence of vimentin (a,d) in all cell layers, while no expression of E-cadherin and homogeneous expression of vimentin is observed in all cells of MDCK-E47 transplants (c,f). Invading cells of MDCK-Snail transplants do not express E-cadherin and are vimentin positive, while the upper non invading layers re-express E-cadherin and have lost vimentin (b,e). Nuclei were stained with DAPI (blue). Bar, 50 μm.

samples from independent transplants of each cell type. The above results strongly suggest that MDCK-Snail and particularly MDCK-E47 cells are inducing an angiogenic response of the host stromal tissue under these experimental conditions.

The angiogenic response was more obvious after 20 days of culture when clear infiltration of blood vessels into the implanted MDCK-Snail and MDCK-E47 cells was observed (Fig. 5b,c, blue arrows). In marked contrast were MDCK control transplants in which the vessels are restricted to the host stroma (Fig. 5a,d, blue arrows). A detailed observation of the histological sections revealed that the angiogenic response of MDCK-Snail and MDCK-E47 cells extends all over the transplants, since sprouting blood vessels reached the uppermost external regions of the implants (Fig. 5e,f, blue arrows). However, the angiogenic response of the control MDCK cells was restricted to the stromal tissue (Fig. 5d, blue arrow). The in vivo malignant behaviour of MDCK-Snail and MDCK-E47 cells was also correlated in the 20-day transplants with a total invasion of the host stroma and reabsorption of the implanted collagen gel detected (Fig. 5b,c, yellow arrows indicate the hypothetical edge of collagen gel). In contrast, in control MDCK transplants, the collagen gel largely persists and the implanted cells maintain the epithelial phenotype and a clear basal membrane-like delimitation without invading the gel (Fig. 5a, yellow arrow indicates the edge of collagen gel).

The results presented so far indicate that MDCK-Snail and MDCK-E47 cells exhibit distinct in vitro and in vivo behaviour when grown as cultures. Snail-expressing cells are more likely to invade the collagen gel at short incubation periods than E47 expressing cells, exhibiting a 2- to 3-fold increase in their invasion capacity. Although both cell types induce an

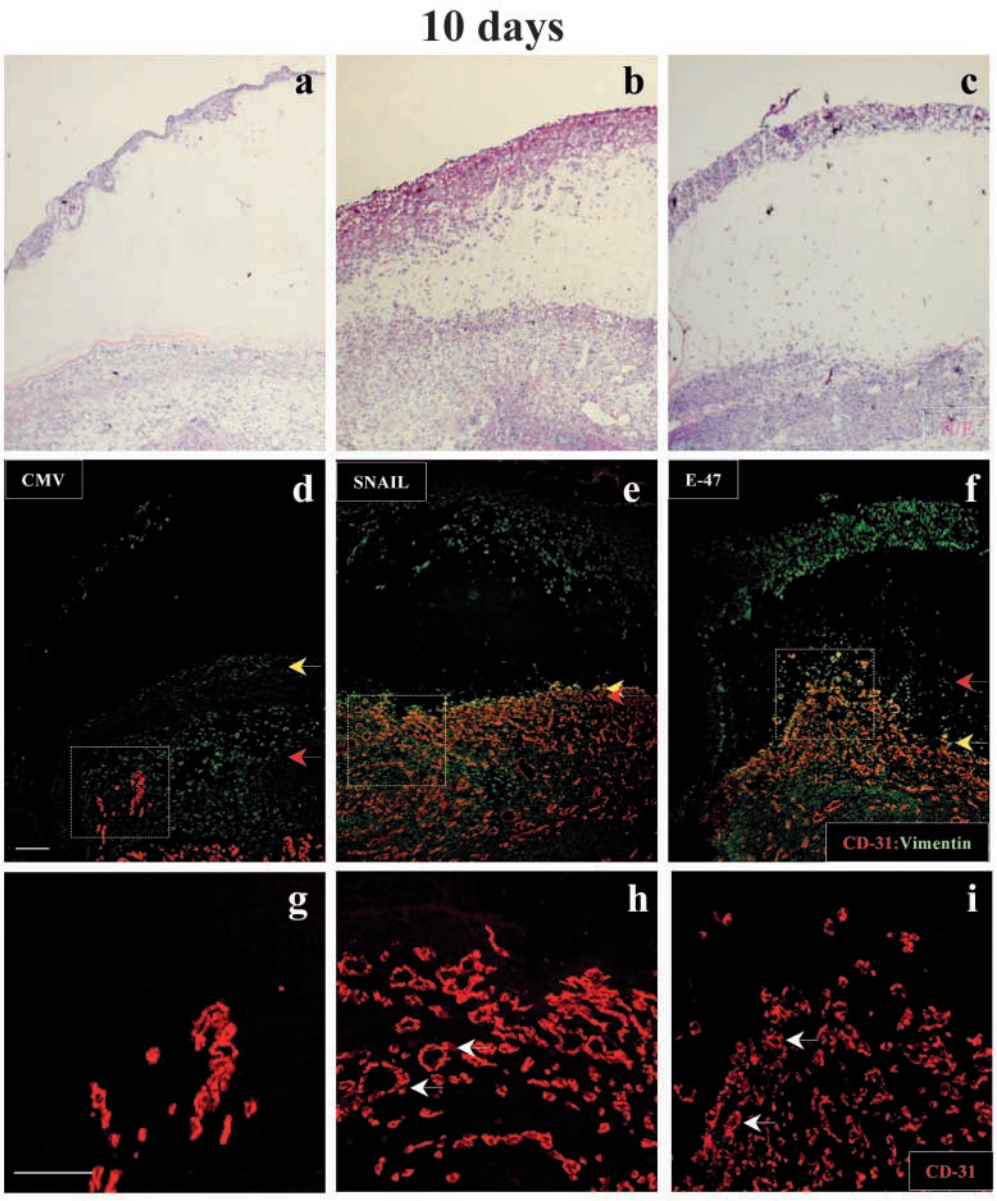


Fig. 4. MDCK-Snail and MDCK-E47 cells induce an angiogenic response in the host stromal tissue. Cryostat sections of 10-day transplants of MDCK-CMV (a,d,g), MDCK-Snail (b,e,h) and MDCK-E47 (c,f,i) cells were subjected to histological (a-c) and immunofluorescence analysis (d-i) for CD31 (red) and vimentin (green). Yellow arrows indicate the border between the collagen gel and host stromal tissue, red arrows indicate the upper limit of CD31-positive cells. (g-i) Magnified images of CD31 positive cells (indicated as insets in d-f) are shown; white arrows indicate blood vessels. Bars, 50 μm.

angiogenic response of the host tissue, this property is approximately 3- to 4-fold higher in MDCK-E47 cells.

Tumorigenic properties of MDCK-Snail and MDCK-E47 cells.

MDCK-Snail and MDCK-E47 cells are tumorigenic when injected into nude mice (Cano et al., 2000; Pérez-Moreno et al., 2001). The different behaviour exhibited by each cell type in organotypic cultures and transplantation assays, led us to carry out a closer examination of their in vivo properties when growing in nude mice. Additional injection studies into nude mice confirmed previous results, with both cell types inducing tumours at all injection sites. However, the tumours induced by MDCK-E47 cells grew much faster than those induced by MDCK-Snail cells (Fig. 6A). Analysis of the tumour growth dynamics demonstrated that MDCK-E47 transfectants show a 2-fold higher proliferation potential than MDCK-Snail transfectants (Fig. 6A). Proliferation assays performed in two-dimensional cultures also showed that MDCK-E47 cells grow between 1.6- and 2-fold faster than MDCK-Snail cells in in vitro culture conditions (Fig. 6B).

Xenografted tumours induced by both MDCK-Snail and MDCK-E47 cells were subjected to histological, immunofluorescence and ISH analysis. At least four different tumours generated by each cell line were studied, and representative examples are presented in Fig. 6C. Tumours induced by MDCK-Snail and MDCK-E47 cells are highly undifferentiated spindle cell tumours (Fig. 6Ca,f), although one of the MDCK-Snail-induced tumours showed a 20-30% of the total area with apparent differentiation (data not shown). The degree of differentiation of tumours was confirmed by immunostaining for E-cadherin. No E-cadherin expression was observed in the undifferentiated tumours induced by either cell type (Fig. 6Cb,g), in contrast to the normal expression of E-cadherin at cell-cell contacts of the epidermis adjacent to the

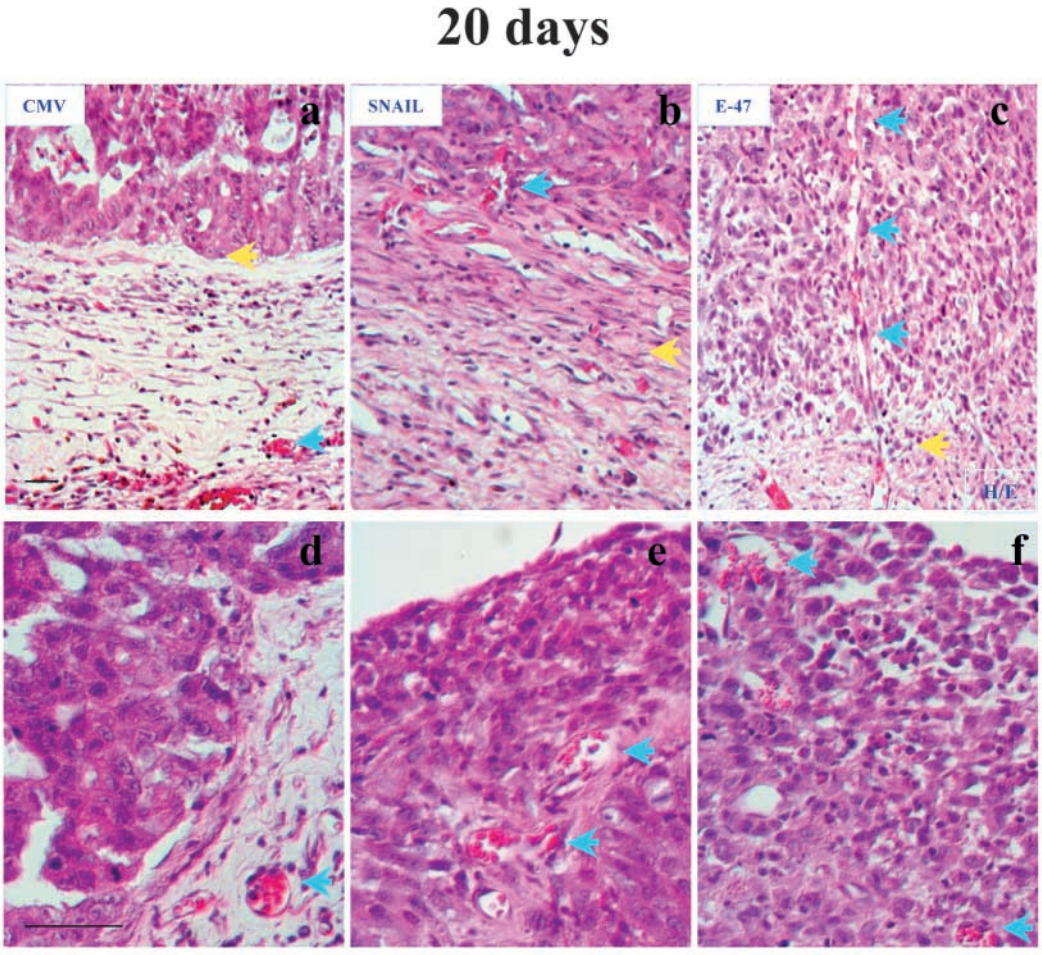


Fig. 5. Angiogenic response of 20-day transplants. MDCK-CMV (CMV) (a,d), MDCK-Snail (b,e) and MDCK-E47 (c,f) transplants grown for 20 days on the backs of nude mice were fixed in formaldehyde and processed for histological analysis. (a-d) Images corresponding to the lower part of the transplants; yellow arrows indicate the limit within the basal membrane and the remaining collagen gel (a) or the hypothetical edge of the gel (b,c). (d-f) Magnified images of the lower (d) and upper part (e,f) of the transplants. Blood vessels (indicated by blue arrows) were detected in the stromal region of CMV transplants (a,d) while they extend all over the surface of Snail (b,e) and E47 (c,f) transplants. Bars, 50 μm.

tumours (Fig. 6Cb, inset). ISH analysis showed complete absence of *E-cadherin* mRNA in the undifferentiated tumours induced by MDCK-Snail cells (Fig. 6Cc) which showed strong expression of *Snail* transcripts in 80% of all tumour cells (see detail of an inner area of the tumour in Fig. 6Cd). Surprisingly, a diffuse but consistent expression of *E-cadherin* mRNA was observed in undifferentiated tumours induced by MDCK-E47 cells (Fig. 6Ch); these showed a 'salt and pepper' pattern with expression of *E47* mRNA in around 30-40% of tumour cells (see detail of an inner area of the tumour in Fig. 6Cj). As expected, the areas of MDCK-E47 tumours with high levels of *E47* transcripts seem to correspond to those with complete absence of *E-cadherin* mRNA, indicating an effective repression of *E-cadherin* transcription in the cells with active expression of the E47 repressor. Interestingly, ISH of MDCK-Snail tumours also showed expression of *E47* mRNA in some scattered cells (Fig. 6Ce), which might coincide with those showing stronger *Snail* expression (Fig. 6C, compare panels d and e). No expression of *Snail* mRNA was detected in the MDCK-E47 induced tumours (Fig. 6Ci). These results suggest that Snail can induce *E47* in restricted areas of tumours, whereas the reverse situation does not occur.

The angiogenic capacity of the tumours induced by MDCK-Snail and MDCK-E47 cells was also analysed by staining with the endothelial markers CD31 and endoglin. As shown in Fig. 7A, both kinds of tumour contained abundant endothelial cells co-expressing both markers that organized into blood vessel-like structures. However, tumours induced by MDCK-E47 cells contain more blood vessels (Fig. 7Ae,f) of smaller size compared to those induced by MDCK-Snail cells (Fig. 7Aa,b). The differences in blood vessel organization could also be detected by immunohistochemical staining of α-smooth muscle actin (αSMA) in both kinds of tumour (Fig. 7Ac,d,g,h). Quantitative estimation of the vessel density of the different tumours was performed by confocal analysis of serial sections stained with the CD31 marker. This study showed that tumours induced by MCDK-E47 cells have a higher CD31 staining density (3500 CD31 units/mm^2 surface) than those induced by MDCK-Snail cells (1400 CD31 units/mm^2 surface). Two-dimensional reconstitution of the images obtained from the confocal serial analyses showed that MDCK-E47-induced tumours did contain a very high density of small CD31-positive vessels covering most of the tumour surface. Tumours induced by MCDK-Snail cells have a lower density of larger CD31 vessels that are restricted to the inner area of the tumours (data not shown). These results indicate that tumours induced by both MDCK-Snail and MDCK-E47 cells have a high angiogenic potential, although they have different organization and/or size of blood vessels. Analysis of pro-angiogenic factors VEGF-A and TGFβ1 was performed on both types of tumours by ISH. MCDK-Snail and MDCK-E47 induced tumours showed expression of *TGFβ1* transcripts in a rather scattered pattern across the extent of the tumour (Fig. 7Ba,c). MDCK-Snail-induced tumours also showed expression of *VEGF-A*

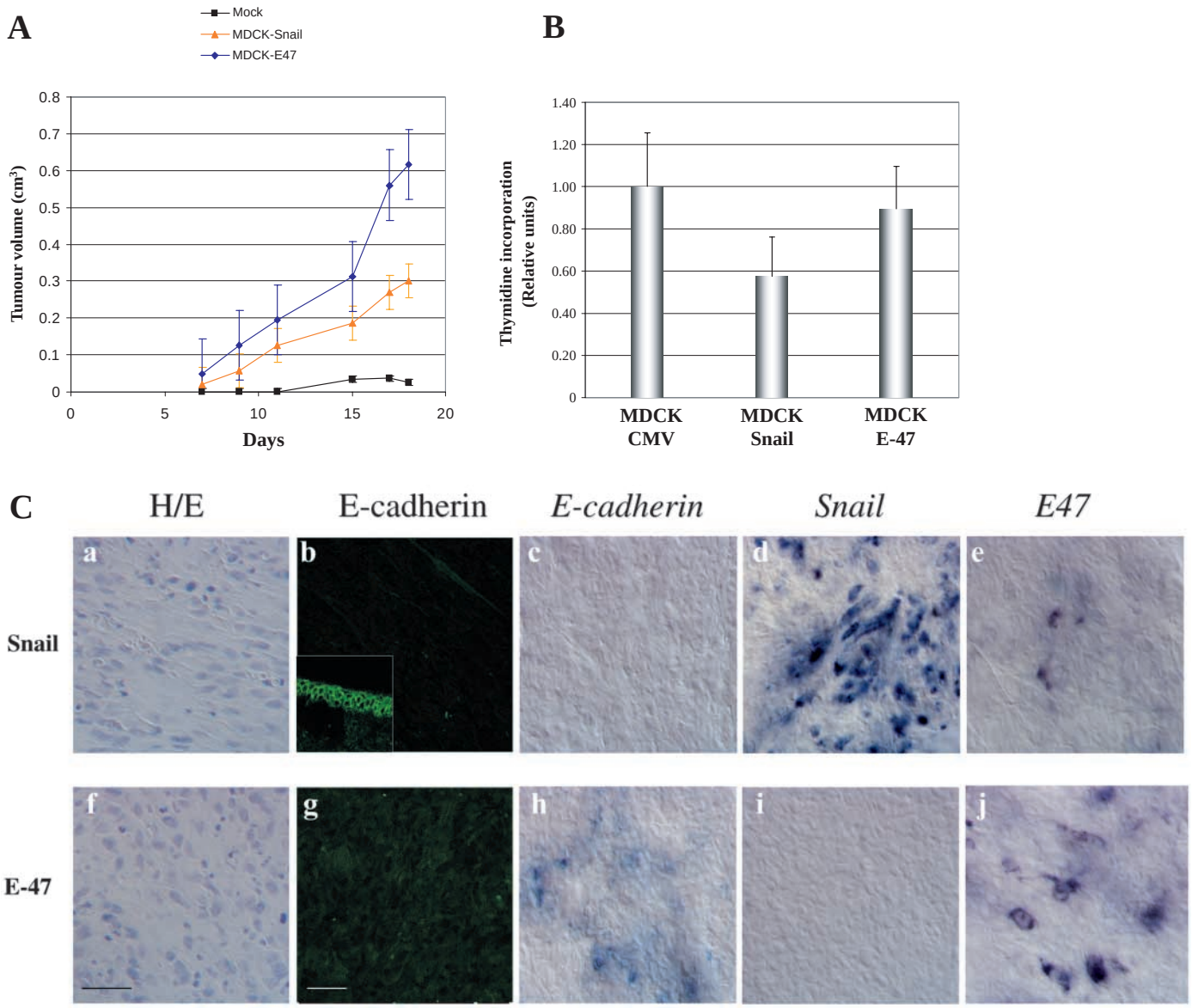


Fig. 6. Analyses of xenografted tumours induced by MDCK-Snail and MCDK-E47 cells. (A) Growth behaviour of MDCK-CMV, MDCK-Snail and MDCK-E47 cells after injection in nude mice. (B) Proliferation of the three cell types in two-dimensional cultures. (C) Tumours induced into nude mice by MDCK-Snail (Snail) (a-e) and MDCK-E47 (E47) (f-j) cells were analysed by histology (a,f); immunostaining for E-cadherin (b,g); and in situ hybridization for *E-cadherin* (c,h), *Snail* (d,i) and *E47* (e,j) transcripts. Note the absence of *E-cadherin* (c) and focal expression of *E47* (e) transcripts in MDCK-Snail induced tumours; and the more heterogeneous expression of *E-cadherin* (h) and *E47* (j) transcripts, but complete absence of E-cadherin protein (g) in MDCK-E47 induced tumours. Inset in b shows E-cadherin staining in the epidermis adjacent to the tumour. Cryostat sections were used for the histological and immunofluorescence analyses; vibratome sections were used in the ISH analyses. Bars, 50 μm.

transcripts in isolated patches that were also positive for αSMA, although this ectopic expression did not correspond to morphologically visible blood vessels (Fig. 7Bb). These results indicate that proangiogenic factors are produced by tumours induced by MDCK-Snail and MCDK-E47 cells.

Discussion

The zinc finger transcription factor Snail and the bHLH factor E47 have been described as strong repressors of *E-cadherin* expression. Both factors induce a process of EMT when expressed in prototypic epithelial cells, such as MDCK cells, conferring a highly motile and migratory behaviour in in vitro culture systems (Cano et al., 2000; Pérez-Moreno et al., 2001). The strong association between the expression of *Snail* and *E12/E47* and invasive and metastatic properties of different carcinoma cell lines and tumours (in the case of Snail), support the active participation of both repressors in the acquisition of invasiveness (Cano et al., 2000; Pérez-Moreno et al., 2001; Poser et al., 2001; Cheng et al., 2001; Yokoyama et al., 2001; Blanco et al., 2002; Sugimachi et al., 2003). However, the capacity to induce invasion by either factor in vivo or any

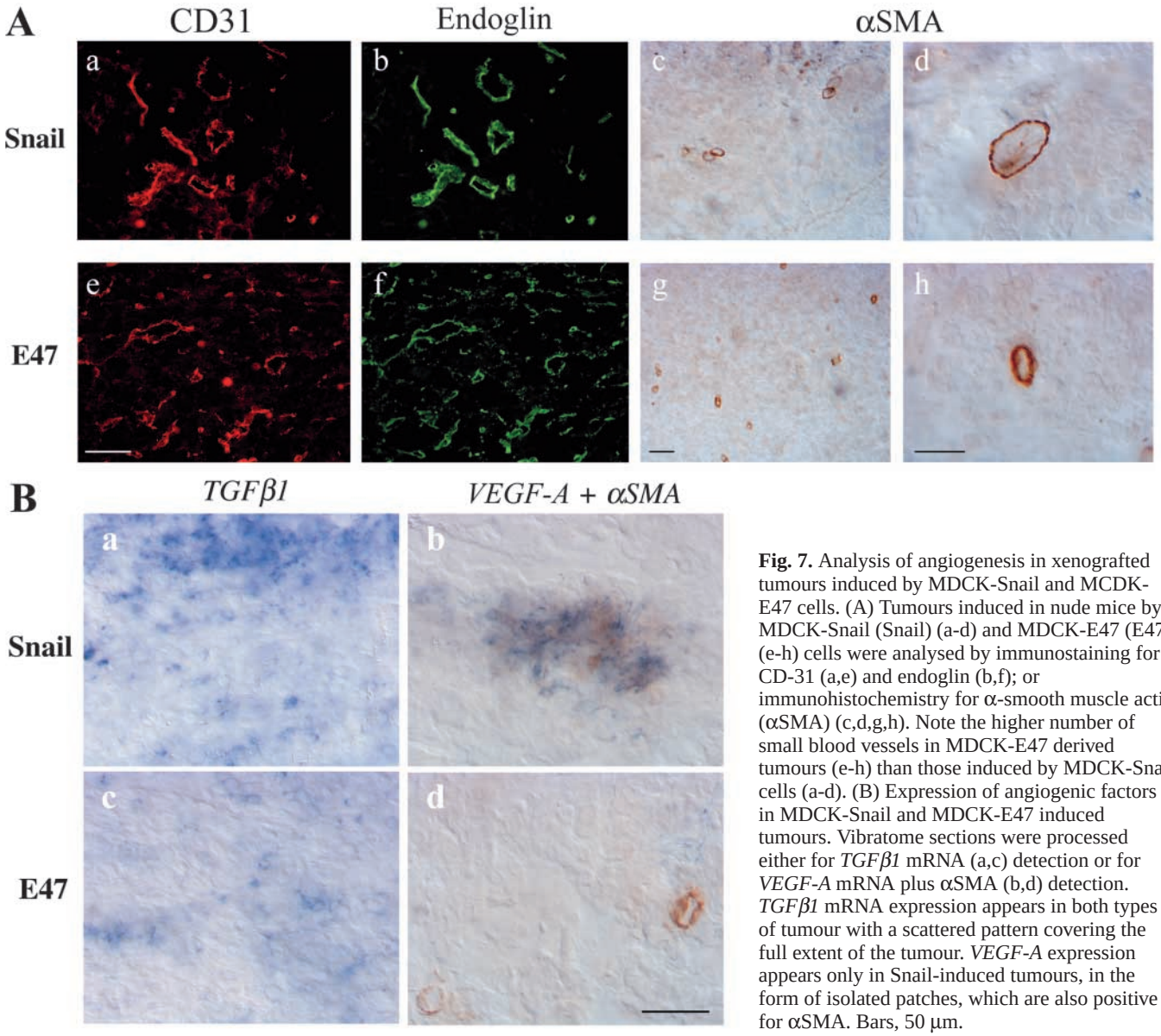


Fig. 7. Analysis of angiogenesis in xenografted tumours induced by MDCK-Snail and MCDK-E47 cells. (A) Tumours induced in nude mice by MDCK-Snail (Snail) (a-d) and MDCK-E47 (E47) (e-h) cells were analysed by immunostaining for CD-31 (a,e) and endoglin (b,f); or immunohistochemistry for α-smooth muscle actin (αSMA) (c,d,g,h). Note the higher number of small blood vessels in MDCK-E47 derived tumours (e-h) than those induced by MDCK-Snail cells (a-d). (B) Expression of angiogenic factors in MDCK-Snail and MDCK-E47 induced tumours. Vibratome sections were processed either for *TGFβ1* mRNA (a,c) detection or for *VEGF-A* mRNA plus αSMA (b,d) detection. *TGFβ1* mRNA expression appears in both types of tumour with a scattered pattern covering the full extent of the tumour. *VEGF-A* expression appears only in Snail-induced tumours, in the form of isolated patches, which are also positive for αSMA. Bars, 50 μm.

differences in the action of these repressors has not yet been established. We have analysed this important issue and present evidence supporting the ability of Snail and E47 to induce in vivo invasion and angiogenesis. Furthermore the two repressors were shown to have different modes of action.

In vitro organotypic cultures and in vivo transplantation assays of MDCK cells stably expressing Snail or E47 repressors clearly indicate that MCDK-Snail cells have stronger infiltration capacity than MDCK-E47 cells over short time periods. Organotypic cultures showed 20% and transplantation assays 45% invasion of the gel layer by MDCK-Snail cells. In contrast, only 5-10% of the gel surface was invaded by MDCK-E47 cells in either experimental setting after 1 week or 10 days of culture. After a longer period in culture, MDCK-Snail cells continued to infiltrate into the collagen gel, but MCDK-E47 cells seem to be more restrained from invasion, particularly in the organotypic cultures. MCDK-Snail cells that infiltrate the collagen gel maintain the mesenchymal-like phenotype, as characterized by the loss of epithelial markers E-cadherin and CK-8 and high expression of vimentin. However, MDCK-Snail cells in the uppermost non-invading layers showed a clear transdifferentiation to an epithelial-like phenotype with re-expression of E-cadherin organized in cell-cell contacts, in both organotypic cultures and transplantation assays (Fig. 1 and Fig. 3B). The transdifferentiation behaviour of MDCK-Snail cells has never been observed in two-dimensional culture conditions, where the cells remain as a homogenous population of mesenchymal-like cells after prolonged passage. This suggests that either the host environment or the tri-dimensional organization of the cells is responsible for the transdifferentiation observed.

Interestingly, the mesenchymal-like cells which maintain *Snail* expression are those able to invade the collagen gel in both organotypic cultures and transplantation assays, whereas the epithelial-like cells that have lost *Snail* expression are non-invasive. These results strongly suggest that sustained expression of *Snail* is required for induction of invasiveness, and are in full agreement with the phenotype of *Snail* null mice,

which are unable to undergo EMT (Carver et al., 2001). This situation is also reminiscent of the transient expression of *Snail* observed in specific developmental contexts in mammals. Expression is associated with the regions undergoing EMT but silenced in some developmental stages once cells are already migrating or when they suffer the reverse mesenchymal to epithelial transition (Sefton et al., 1998; Nieto, 2002). In the context of development, *Snail* expression seems to require both inductive and maintenance signals provided by different growth factors, such as TGFβ and FGFs, respectively (Thiery, 2002; Nieto, 2002; Ciruna and Rossant, 2001). The loss of *Snail* expression observed in the uppermost layer of the organotypic cultures and transplants of MDCK-Snail cells after longer times of culture suggest the absence or weakness of maintenance signals in those regions. Additional mechanisms affecting to the stability of *Snail* mRNA in those experimental conditions could also explain the observed expression pattern.

Since *Snail* expression is driven by the CMV promoter, mechanisms such as those affecting the stability of *Snail* mRNA may account for the loss of *Snail* expression observed in the uppermost layer of the organotypic cultures and transplants of MDCK-Snail cells after longer times of culture. This hypothesis is supported by the fact that the *Snail* cDNA expressed in stable transfections carries the original 3′UTR region of *Snail* mRNA (Cano et al., 2000). Therefore, *Snail* mRNA degradation can be promoted by an as yet unidentified mechanism in the upper levels of organotypic culture under a cell compaction environment. Intra-clonal variations of *Snail* expression may also explain the observed *Snail* downregulation in the upper layers of the cultures. However, the fact that MDCK-E-47 cells have more intra-clonal *E-47* expression variability with a clear 'salt and pepper' pattern, whereas transdifferentiation does not occur in MDCK-E47 cells at any time of culture under either experimental situation, makes this possibility highly unlikely.

Although further studies are needed to clarify this specific aspect, the present results suggest that EMT induced by Snail might be reversible, at least in some specific cell or tissue contexts. Alteration of mRNA stability could also explain the heterogeneous expression of *E47* transcripts observed in both kinds of culture of MCDK-E47 cells. Nevertheless, and in contrast to MDCK-Snail cells, MDCK-E47 cells maintain the mesenchymal phenotype and the absence of E-cadherin protein in all culture conditions either in in vitro three-dimensional organotypic cultures, in in vivo transplant assays and in the xenografted tumours. In spite of this, MDCK-E47 cells have a lower invasive capacity than MDCK-Snail cells at short incubation periods, suggesting that the EMT mediated by E47 is not sufficient to induce a highly invasive potential and may require a proper environment to develop the invasion process. Indeed, after long culture periods, sustained expression of E47 is able to confer invasiveness, since the 3-week transplants of MDCK-E47 cells exhibit an in vivo infiltrating potential similar to that induced by MDCK-Snail transplants (see Fig. 3A and Fig. 5). These observations support the idea that besides *E-cadherin* repression, Snail and E47 must regulate additional genes (as primary or secondary targets) whose expression or repression might be required to provide cells with a high invasion potential. In this context, it is worthy of note that local or transient expression of *Snail* in a few cells might be sufficient to produce local invasion, an event that will initiate the escape of tumour cells and the metastatic cascade. The local and restricted expression of *Snail* at EMT regions in the mouse embryo (Sefton et al., 1998; Cano et al., 2000; Carver et al., 2001) and, more importantly in tumours induced in the mouse skin, as well as in human breast carcinomas and hepatocarcinomas (Cano et al., 2000; Blanco et al., 2002; Sugimachi et al., 2003) fully supports this idea. Interestingly, the MDCK-Snail-induced tumours showed a focal expression of *E47* apparently coincident with regions of stronger *Snail* expression, indicating a potential cooperation of both factors in specific in vivo contexts. On the other hand, focal E-*cadherin* mRNA expression could be detected in MDCK-E47 induced tumours that, nevertheless exhibited a complete absence of E-cadherin protein, indicating additional levels of regulation of E-cadherin in this specific tumoral context. Post-transcriptional regulation of E-cadherin affecting protein stability might be one of the mechanisms involved. Indeed, our previous studies on ectopic expression of E-cadherin in dedifferentiated spindle carcinoma cells showed that E-cadherin turnover was highly increased, rendering the cells unable to express high levels of the ectopic protein or to organize it in defined cell-cell contacts, despite the high levels of exogenous *E-cadherin* mRNA expression (Navarro et al., 1993; Lozano and Cano, 1998). Additional studies are required to clarify this specific aspect.

An interesting and unexpected observation was made in the transplantation assays: the induction of a migratory response of host cells into the collagen gel. At short time periods (1 week of culture) this response was much stronger in the transplants of MDCK-E47 than in those of MDCK-Snail cells. Interestingly, a significant proportion of the migrated cells from the host stroma are endothelial cells, determined by staining with the CD31 marker, and have the ability to organize themselves into blood vessel-like structures in the host tissue in proximity to the transplant and within the collagen gel. Angiogenesis is defined as the formation of new capillaries from existing vessels. Although this process occurs in physiological conditions during embryonic development and wound healing, it is crucial in tumour progression. Indeed, tumour cells release angiogenic factors or repress the synthesis of inhibitors. Blood vessels were particularly evident in the MDCK-E47 transplants (Fig. 4). However, after prolonged culture (3 weeks) a strong and widespread angiogenic response is observed in the transplants of both cell types. These observations indicate that a strong angiogenic response of the host tissue is induced by MDCK-E47 cells and to a lesser extent by MDCK-Snail cells under the transplant assay conditions. Indeed, analysis of xenografted tumours induced by both kinds of cell indicates that they are highly angiogenic, with a higher density of endothelial vessels being detected in the MDCK-E47 than in MDCK-Snail-induced tumours. These differential angiogenic properties might be, at least in part, responsible for the distinct proliferation capacity of the tumours. Thus, the higher in vivo angiogenic capacity of MDCK-E47 cells compared to MDCK-Snail cells might contribute to the higher proliferation potential of the tumours induced by the first cell type. However, other effects exerted by both factors on cell proliferation and/or cell survival, can not be discounted at present.

In this context, elucidation of the angiogenic and migratory factors induced by either cell type or the host stroma will be

required to fully understand the role of Snail and E47 in tumour progression. Candidates for the induction of angiogenesis and cell migration during development and tumour progression include members of the TGFβ, FGF and VEGF families. Indeed, TGFβ-1 and FGFs are involved in the regulation of *Snail* expression (Spagnolli et al., 2000; Ciruna and Rossant, 2001; Valdés et al., 2002; Yañez-Mo et al., 2003; Peinado et al., 2003) and are strong candidates for roles in tumour progression and migration. In agreement with this, *TGFβ-1* and *VEGF-A* expression has been detected in the MDCK-Snail tumours, while MDCK-E47 tumours seem to preferentially express *TGFβ-1* transcripts at the analysed time points (Fig. 7B). These observations do not discount expression of other members of the VEGF family and/or additional angiogenic factors by MDCK-E47 cells at different time periods of in vivo growth. In fact, our ongoing studies on expression profiling in MDCK-Snail and MDCK-E47 cells indicate differential induction of several pro-angiogenic factors in both cell types such as VEGF, jagged1 and nocth2 (H.P., G. Moreno-Bueno, E.C., D. Sarrio, S. Villa, V. Bolós, J. Palacios and A.C., unpublished). Interestingly, jagged1 has been implicated in angiogenesis during development through activation of Notch signalling (Shimizu et al., 1999; Shimizu et al., 2000). In this context, a recent study (Morel et. al., 2003) proposes that Snail can downregulate negative regulators of Notch signalling during development, providing a possible link between those molecules in the control of gene expression. These observations suggest that Snail and E47 are directly or indirectly involved in the regulation of several genes implicated in tumour progression and angiogenesis.

The present results suggest differential and distinct roles of Snail and E47 in in vivo invasiveness and tumour progression. Snail has a prominent role in the promotion of local invasion and E47 acts to maintain a dedifferentiated and migratory phenotype contributing to a strong angiogenic response from the host stromal tissue. These results, therefore, provide new information of interest to the design of therapeutic strategies for blocking specific stages of tumour progression, such as local invasion and angiogenesis.

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