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Malignant progression of invasive tumour cells seen in hypoxia present an accumulation of β-catenin in the nucleus at the tumour front

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ABSTRACT

Of all processes involved in tumour progression, local invasion and formation of metastases are the clinically most relevant but the scientifically least well understood at their molecular level. The loss of cell adhesion, then tumour cell migration with changes in the cytoskeleton, invasion and metastatic dissemination are the steps of the "metastatic cascade". The E-cadherin–catenin complex plays a key role in cell adhesion thus building the first step in malignant progression. In many epithelial cancers, E-cadherin is lost concomitantly with tumour progression. Thus β-catenin dissociates in the cytoplasm and accumulates in the nucleus as a transcription factor. Recent experimental progress has identified that tumour hypoxia not only induces tumour angiogenesis, but also modulates malignant progression to initiate tumour invasion and metastasis. It was hypothesised that hypoxia within tumours causes dysfunction of the E-cadherin–catenin complex with an accumulation of β-catenin in the nucleus and produces an invasive phenotype of tumour cells. For this purpose fertilized chicken eggs were incubated for ten days in normoxic conditions. Subsequently colon carcinoma cells (SW-480) were placed on the chorioallantoic membrane. During the following six days the eggs were incubated either in normoxic conditions or in stepwise decreasing hypoxic conditions. SW-480 colon carcinoma cells did not invade the epithelial layer in normoxic conditions. β-catenin was membrane bound or in the cytoplasm. The nuclei were regularly omitted. In contrast, an invasion through the epithelial layer into the mesoderm was already seen after three days when incubated in hypoxic conditions. β-catenin was membrane bound in non-invasive regions of the tumour nodule but there was an accumulation of β-catenin in the nucleus in the invasive tumour front. Hypoxia seems to be responsible for accumulation of β-catenin in the nucleus which is accompanied by a more invasive phenotype of tumour cells at the tumour front.

Introduction

The ability of tumour cells to invade adjacent tissues and disseminate into distant organs has for a long time been considered the biological hallmarks of malignancy (Hart and Saini, 1992; Woodhouse et al., 1997). The process of metastasis appears to be regulated by a variety of gene products. These include cell-to-cell and cell-to-extra cellular matrix receptors (Bernstein and Liotta, 1994; Juliano and Varner, 1993) proteolytic enzymes that facilitate breakdown and invasion of the basement membrane, vascular channels and organs (Ellis et al., 1992; Liotta, 1992; Matrisian, 1992) motility factors which allow migration through tissues (Stoker and Gherardi, 1991), receptors mediating organ-specific invasion (Nicolson, 1991); growth factors necessary for the maintenance of the tumour micro-colonies in the secondary organ (Baserga, 1994) and angiogenic factors that result in vascularisation of the metastasis, allowing the supply of nutrients, removal of metabolites and haematogenous spread of metastatic cells (Folkman, 1995; Liotta et al., 1991). Weakening of cell-to-cell adhesion is obviously imperative for tumour cells to metastasize. The first step in the metastasis cascade is the loss of cell adhesion. The E-cadherin–catenin complex plays a key role in cell adhesion (Beavon, 1999). The role of E-cadherin in metastasis has become highly topical in the past few years due to its apparent promise as a prognostic indicator, with loss or reduction of expression correlating with enhanced aggressiveness and dedifferentiation of many carcinomas (Birchmeier and Behrens, 1994; Hunt et al., 1997; Mareel et al., 1994; Shino et al., 1995; Takeichi, 1993; Tamura et al., 1996). Some tumours

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also display the ability to regulate E-cadherin expression during the process of metastasis, which raises questions about the role of the tumour microenvironment (Bukholm et al., 2000). Moreover recent experimental findings identified tumour hypoxia not only to induce tumour angiogenesis, but also to modulate the expression of several genes that have been implicated in tumour invasion, metastasis and poor prognosis (Brizel et al., 1996; Hockel et al., 1996; Sullivan and Graham, 2007; Vaupel, 2008; Vaupel et al., 2004).

It has been hypothesised that hypoxia within tumours, resulting in tumour necrosis, causes downregulation of E-cadherin, and ultimately sets the metastatic cascade in motion (Beavon, 1999). To investigate this hypothesis, an *in vivo* model is required to meet the following properties: tumour cells should be able to invade the epithelial layer through changes in cell-to-cell receptors, cell-to-extra cellular matrix receptors, secreting proteolytic enzymes, growth factors, induction of angiogenesis etc. It is of importance that all these steps of "metastatic cascade" can be investigated in different oxygen supply conditions to evaluate the effect of hypoxia. For this purpose, we established the modified chorioallantoic membrane (CAM) model to a tumour invasion model to investigate the first step – contact inhibition – of the "metastatic cascade" under hypoxic conditions (Demir et al., in press).

Material and methods

Eggs and preparation

Fertilized chicken eggs (White Leghorn, 50–54 g, Charles River Wiga, Sulzfeld, Germany) were disinfected with a sterile gauze saturated with 70% ethanol and incubated for 9 days at 37 °C, 80% humidity and normoxia (20.5% O_2, 78.9% N_2, 0.04% CO_2, 0.56% Others) in an upright position with the blunt pole on top (Fig. 1). After nine days, the eggs were prepared as follows: first a 2 mm hole was milled carefully into the egg shell on the tip of the blunt pole using a graving instrument (Proxon Micromat System®, Niersbach, Germany). The dust generated during milling was removed using moist sterile gauze. The outer shell membrane was not ruptured. Then an anatomical forceps was used for perforation of the outer shell membrane to open up the air cell. Subsequently, the egg shell was broken according to the topography of the air cell. Thus a circled window of 2–3 cm could be opened up (Fig. 1b). Using a stereomicroscope, the inner shell membrane was moistened with 0.5 ml of Ringer solution. This procedure allows removing the inner shell membrane with micro-tweezers without destroying the epithelial layer and the vascular architecture of the CAM (Fig. 1c). The window was closed with a slip, which was fixed with scotch tape. The eggs were then incubated for another day so that possible microlesions could recover. On the 10th breeding day (BD) the eggs were inoculated with 10^6 tumour cells (SW480) dropped as a suspension in isotonic NaCl solution on the CAM (Fig. 1d). Afterwards, the window was again closed with the slip and tape. Now samples were divided into two groups of different breeding conditions. The normoxic group (nor-group, $n = 10$) was incubated in normoxic conditions. The hypoxic group (hyp-group, $n = 10$) was incubated in an atmosphere of decreasing oxygen levels atmosphere from 18% O_2 (on 10th BD) via 15% O_2 (on 11th BD) to 13% O_2 (12th BD). This procedure allows the chick embryo, and also the tumour cells, to adjust to the changing breeding conditions down to levels of hypoxia similar to that in the microenvironment of tumour cells spreading *in vivo*. The tumour nodules on the CAM were harvested either on the 13th BD ($n = 5$) or on the 16th BD ($n = 5$). In total we looked at 20 samples divided into four groups of five samples each: first of all two groups of breeding conditions (nor-group and hyp-group) subdividing into two different groups of incubation time [3 or 6 days (D): nor-group-3D ($n = 5$) or nor-group 6D ($n = 5$), hyp-group-3D ($n = 5$), hyp-group-6D ($n = 5$)]. After the planned incubation intervals, individual tumour growth and spread was documented by photographs (Fig. 1e) (0.6×, 1.2× and 2.5×) (Photo-camera, Cannon® Tokyo, Japan) and subsequently the CAM was harvested and fixed in formaldehyde (4 vol.%, 24 h).

Tumour cells

The SW480 human colorectal adenocarcinoma cell line (ATCC® number: CCL-228™) was cultured in Dulbecco's modified Eagle's medium (DMEM®, PAA Laboratories, Pasching, Austria) supplemented with 10% fetal bovine serum. Tumour cells were cultivated in an environment of 21.5% O_2, 8% CO_2 and 70.9 N_2 up to a confluence rate of 80%. For inoculation on the CAM, tumour cells were trypsinized (PAA Laboratories, Pasching, Austria) and washed with phosphate buffered saline (PBS) (2× centrifuge: 20 °C, 5 min, 500 RCF). A suspension of 13–15 µl representing 10^6 cells was inoculated on the CAM.

Paraffin embedding

Each tumour nodule was fixed in 4% formaldehyde for 24 h. For the paraffin embedding, the tissue samples were dehydrated in increasing

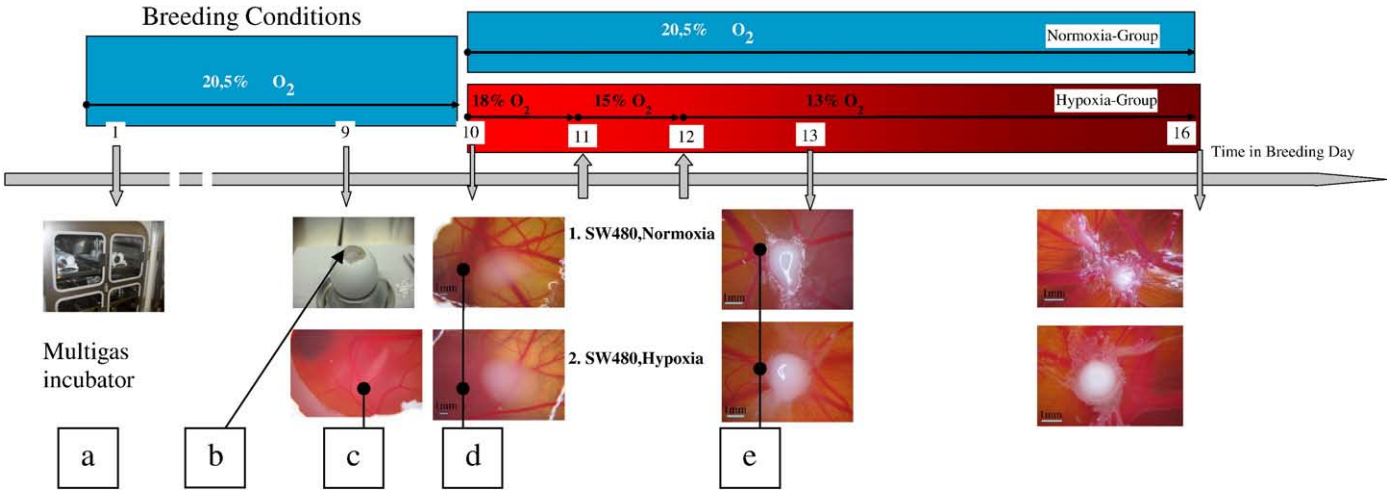

Fig. 1. Chorioallantoic model assay for investigating tumour invasion in hypoxia. Work flow in breeding days (BD) (a). During the first 10 BD the eggs were incubated in an upright position in normoxic conditions (20.5% O_2) and were opened on the blunt pole on 9th BD (b). To get the chorioallantoic membrane out the inner shell membrane was removed (c). 10^6 tumour cells (SW-480) were seeded as a suspension on 10th BD (d). Next, the eggs were incubated either in normoxic or in stepwise hypoxic conditions. Tumour nodules occurred on 13th BD (e). Histomorphological and immunohistochemical evaluation were performed after paraffin embedding and slices vertical to the CAM surface (f).

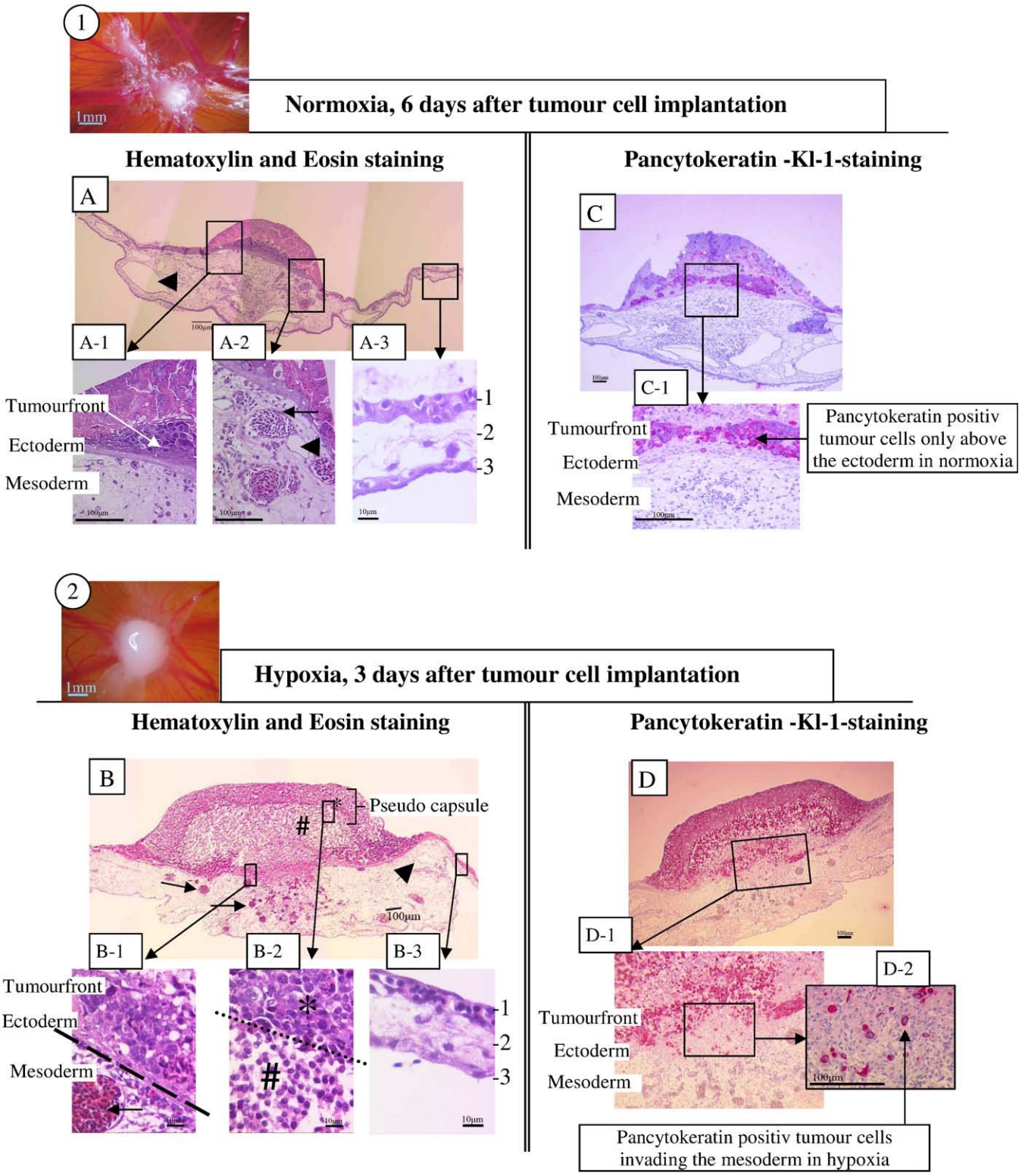

Fig. 2. Hematoxylin and Eosin staining (A, B) and pancytokeratin-Kl-1-staining (C, D) of a tumour nodule grown in the chorioallantoic membrane (CAM). The normal CAM (A-3 and B-3) consists of three layers (1) ectoderm, (2) mesoderm and (3) endoderm in both breeding conditions (normoxia or hypoxia) without apparent differences. This is seen next to the tumour nodules. In both breeding conditions a thickening of the CAM up to 500 µm, especially of the mesoderm underneath the tumour nodule (A, A-1, B, B-1)) could be seen [blood vessels (arrows), lymph vessels (arrows head)]. Three different phenotypes (B, B-2) of tumour cells could be distinguished in hypoxia. First: in the apex cell-to-cell contacts seem to be pronounced (*). The tumour cells form a "pseudocapsule" (B-2, interrupted line). The cells are small and the core plasma relation is balanced. Second: in the ventral part the cells are loosely configured (#). Cell-to-cell contact is sparse. Third: in the tumour front, especially in the centre of progression, necrosis with cell detritus was regularly seen next to vital tumour cells infiltrating through the ectoderm (D-2). In normoxic breeding conditions, even after 6 days of tumour cell implantation no tumour cells could be detected in the mesoderm (C, C-1).

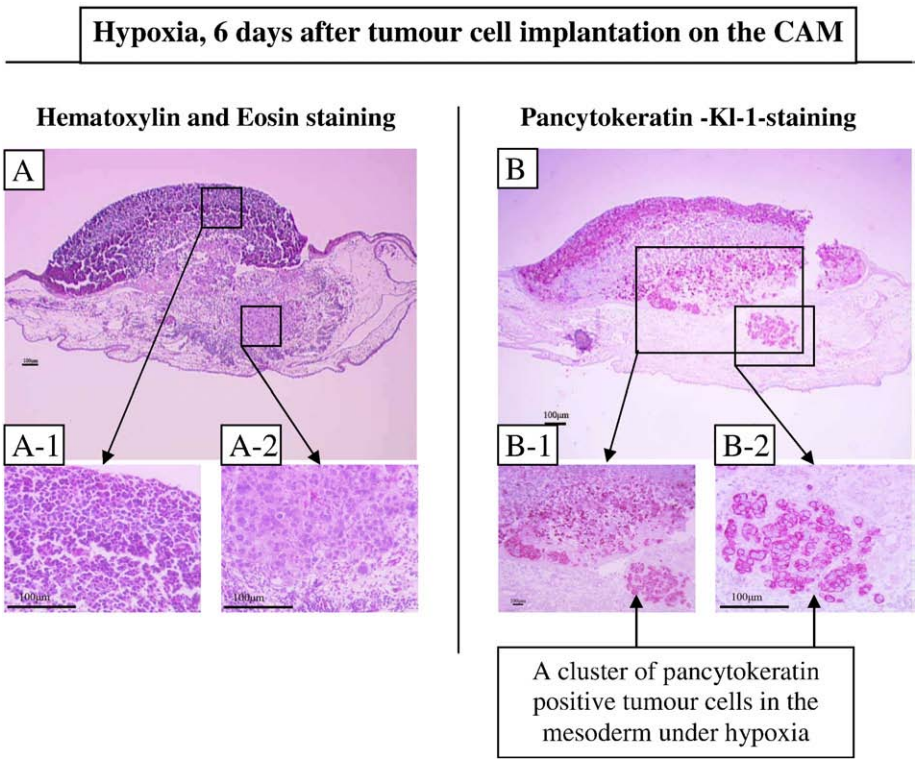

Fig. 3. "Satellite metastasis" in the mesoderm occurred after 6 days of tumour cell implantation on the CAM. CAM is negative for immunostaining of pancytokeratin-Kl-1. Only human tumour cells (SW-480) are positive. The depth and the kind of invasion in the mesoderm by the tumour cells are easily detectable (B, B-1, B-2). Cluster of tumour cells in the mesoderm (A-2, B-2) showing an increased nucleus–plasma relationship. Cell-to-cell contacts seem to work again (A-1 vs. A-2).

amounts of alcohol. Then the planar portion of the CAM with the tumour nodule was embedded in paraffin in an upright position for later sections vertical to the CAM's surface. All further investigations were performed on 5 µm tissue slices.

Immunohistochemistry

Immunostaining was performed on 5 µm sections of paraffin embedded tissues. To detect the tumour cells, we utilized pancytokeratin-Kl-1-staining, performed as described in the protocol (1:200, IgG1 (mouse), Immunotech®, Marseille, France). Hematoxylin and Eosin staining was used to analyze classic histological criteria such as invasion and penetration through the epithelial layer or nucleus–plasma relation of the cells. The viability of the cells was demonstrated by the proliferation marker, monoclonal mouse anti-human Ki-67 antigen (1:100 IgG1, kappa, Clone MIB-1, DAKO M 7240, DAKO Cytomation, DK-2600, Glostrup Denmark). Expression of β-catenin was investigated by a mouse anti-β-catenin monoclonal antibody (1:100, clone 14; Transduction Labs., Lexington, KY, USA).

Results

Gradual lowering of oxygen levels, produces more accurate results with regard to the least possible deranging of the physiology of the CAM. Tumour nodules were formed in all groups (diameter: 1.5 ± 0.5 mm (Fig. 2: 1 and 2). The CAM consists of three layers (Fig. 2: A-3, B-3): (1) the ectoderm forms an epithelial layer without fenestration. (2) The mesoderm consists of the extracellular matrix and the blood vessels (Fig. 2: A-2, ◄) and lymph vessels (Fig. 2: A-2, ◄). (3) The endoderm builds the barrier to the albumen and towards the chick embryo. In fact, there were no morphological differences seen in the CAM in greater distance to the tumour nodule under both breeding conditions [(Fig. 2: A-3, B-3), thickness 65 ± 5 µm]. A thickening of the CAM could be observed, especially of the mesoderm (vessels, lymph vessels, extracellular matrix), underneath the tumour nodule as induced by the tumour cells (up to 500 ± 50 µm, Fig. 2: A, B) under both breeding conditions. CAM is negative for immunostaining pancytokeratin-Kl-1. Only human tumour cells SW-480 are positive (Fig. 2: C, D). The depth and the type of invasion in the mesoderm by the tumour cells were thus easily detectable. In normoxia, even after 6 days of incubation, the tumour cells were vital and clearly positive

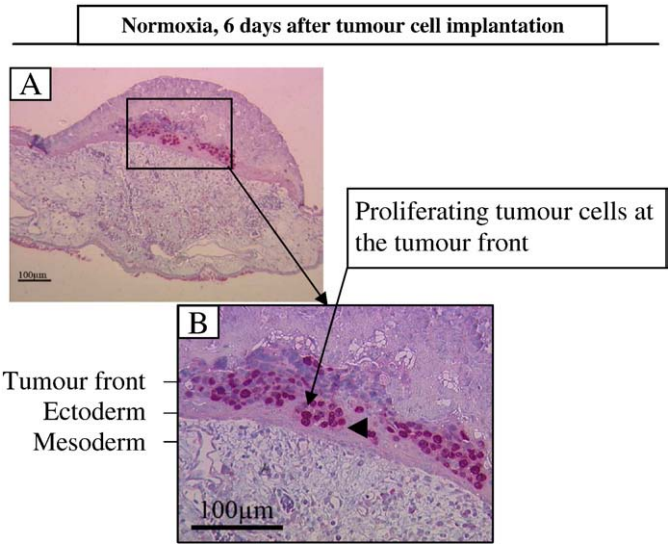

Fig. 4. Ki-67 immunostain of the tumour nodule on the chorioallantoic membrane six days after breeding in normoxic conditions (A). Especially at the tumour front, the nuclei (◄) of the tumour cells are clearly positive for Ki-67 immunostaining. The tumour cells are viable but they do not invade the mesoderm even after six days of incubation (B).

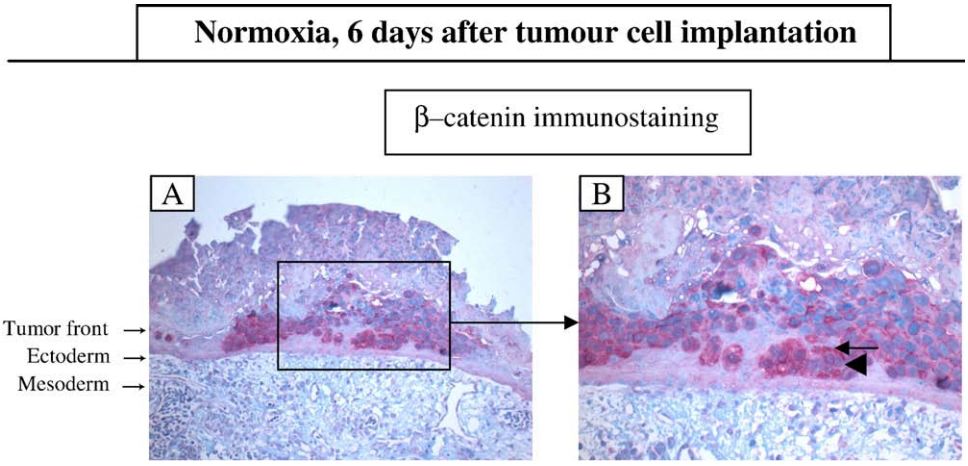

Fig. 5. β-catenin immunostain of the tumour nodule on the chorioallantoic membrane six days after breeding under normoxic conditions (A). Tumour cell membrane and cytoplasm are clearly positive for β-catenin (B, ←). The nucleus is generally negative for β-catenin (B, ◄). Tumour cells are viable and proliferate but they are non-invasive and are negative for β-catenin in the nucleus.

for Ki-67 immunostaining (Fig. 4: A, B) as a sign of active proliferation but they did not invade the mesoderm (Fig. 2: A-1; C-1).

Tumour cells incubated in hypoxia initially demonstrated two different phenotypes adjusted to the physiological stress they had to cope with (Fig. 2: B). At the outer zone (towards to air) the tumour cells are close together and form a "pseudocapsule". This zone is characterized by poor proliferation (Fig. 6: B) and a membranous β-catenin formation (Fig. 7: C). In the ventral zone and especially at the tumour front, the tumour cells are shedded. This part of the tumour nodule is characterized by a high proliferation rate (Fig. 6: C), β-catenin formation in the nucleus (Fig. 7: D) and invasiveness through the ectodermal layer (Fig. 2: D-1, D-2). After 6 days of incubation in hypoxia (Fig. 3 and Fig. 9) the tumour cells showed the same phenotype at the non-invasive phenotype area of the outer zone (Fig. 9: A-3) and at the invasive phenotype area of the tumour front (Fig. 9: A-1). The third phenotype of tumour cells formed a "satellite metastasis" in the mesoderm (Fig. 3, A-2, B-2). Here the tumour cells are again close together and β-catenin is again membranous (Fig. 9: A-2).

Discussion

Hypoxia leads to malignant progression of tumour cells. This hypothesis is supported by many clinical studies (Hockel et al., 1996; Vaupel, 2008) and *in vitro* experimental set ups (Baserga, 1994; Krishnamachary et al., 2003; Sullivan and Graham, 2007). As to the authors' knowledge, this is the first description of an experimental set up to investigate defined tumour hypoxia and malignant progression on an *in vivo* model at the molecular level. The stepwise daily

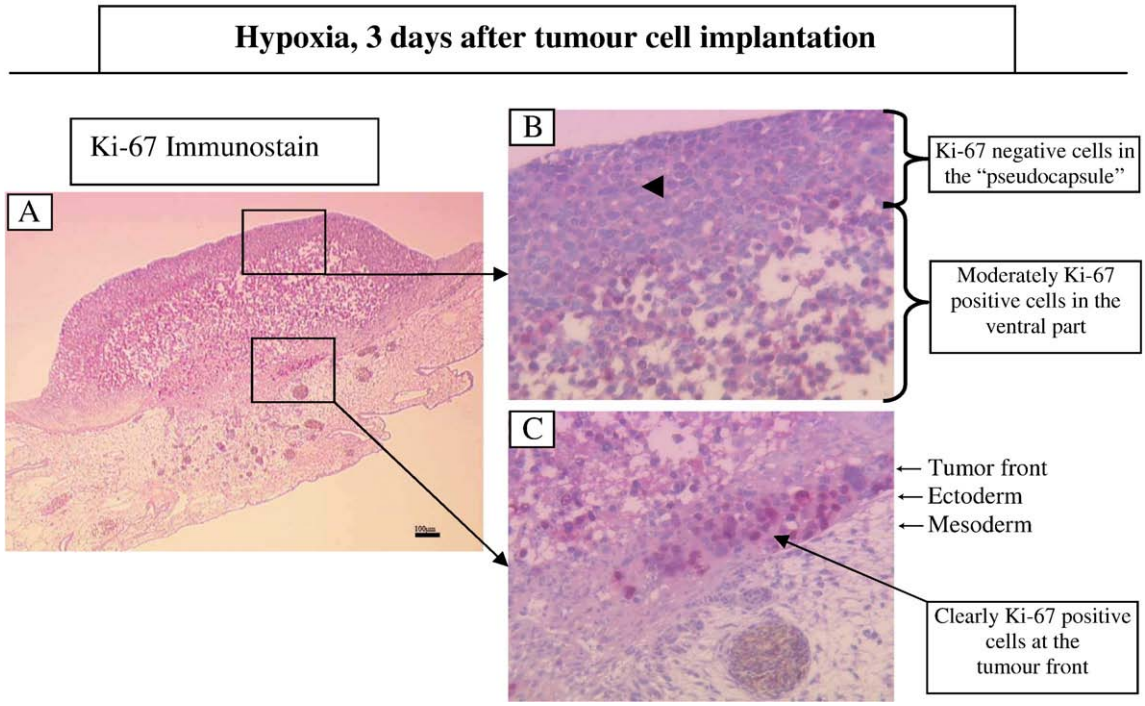

Fig. 6. Ki-67 immunostain of the tumour nodule on the chorioallantoic membrane three days after breeding under hypoxic conditions (A). Two different phenotypes of tumour cells are demonstrated. In the outer zone, the tumour cells form a "pseudocapsule", they are densely packed and the nuclei are generally negative for Ki-67 (B, ◄). In the ventral part, especially at the tumour front, the nuclei of the tumour cells are clearly positive for Ki-67 immunostaining. Obviously, the tumour cells proliferate at the tumour front but not in the zone of the "pseudocapsule".

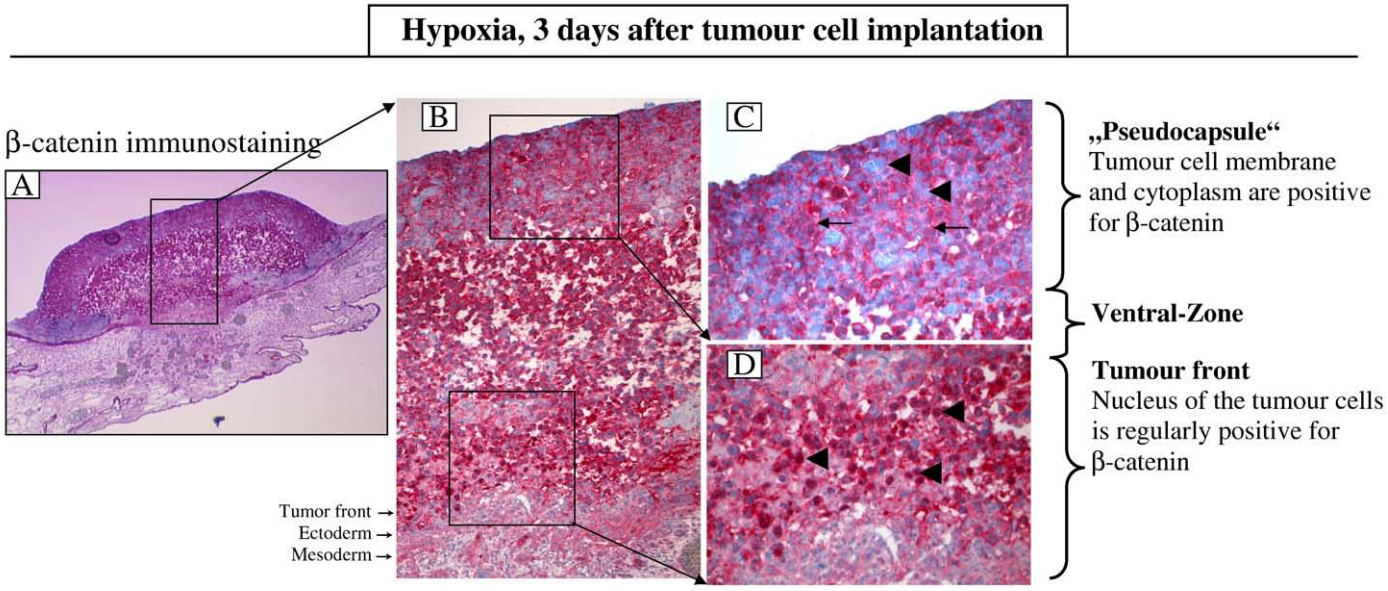

Fig. 7. β-catenin immunostaining of the tumour nodule on the chorioallantoic membrane three days after breeding in hypoxic conditions (A). Two different phenotypes of tumour cells are demonstrated in hypoxia. In the outer zone, the tumour cells are densely packed and form a "pseudocapsule". Tumour cell membranes are clearly positive for β-catenin (C, ←). The nuclei are regularly negative for β-catenin (C, ◄). In the ventral zone, especially at the tumour front, the nuclei of the tumour cells are clearly positive for β-catenin immunostaining (D, ◄). Obviously, in hypoxia, the tumour cells at the tumour front are positive for β-catenin in the nucleus, which is accompanied by higher proliferation rate and invasiveness.

worsening of the oxygen supply (20.5%, 18%, 15% and 13% O_2) allows tumour cells and chick embryos to adapt to hypoxia. No morphological changes were seen in greater distance from the tumour nodule as demonstrated in Fig. 2: A-3 and B-3. The thickness of the CAM in this region (as described in the literature (Reizis et al., 2005)) was 69 ± 28.7 μm. In both breeding conditions underneath the tumour nodule especially the mesoderm gets thicker. An increase of blood and lymph vessels and extracellular matrix could be observed (Fig. 2: A, B). There is a paracrine interaction of human tumour cells with avian CAM. It has already been shown, that human cytokines like vascular growth factor VEGF (Wilting et al., 1993), interferon (IFN)-α (Ribatti et al., 1996) and IFN-β (Demir and Hoper, 1997) interact with the CAM locally and increase or decrease vascular density, respectively. The next goal of our work will be to find out which of the secreted proteins are responsible for the thickening and invasion of the mesoderm (VEGF, metalloproteinases etc.). In

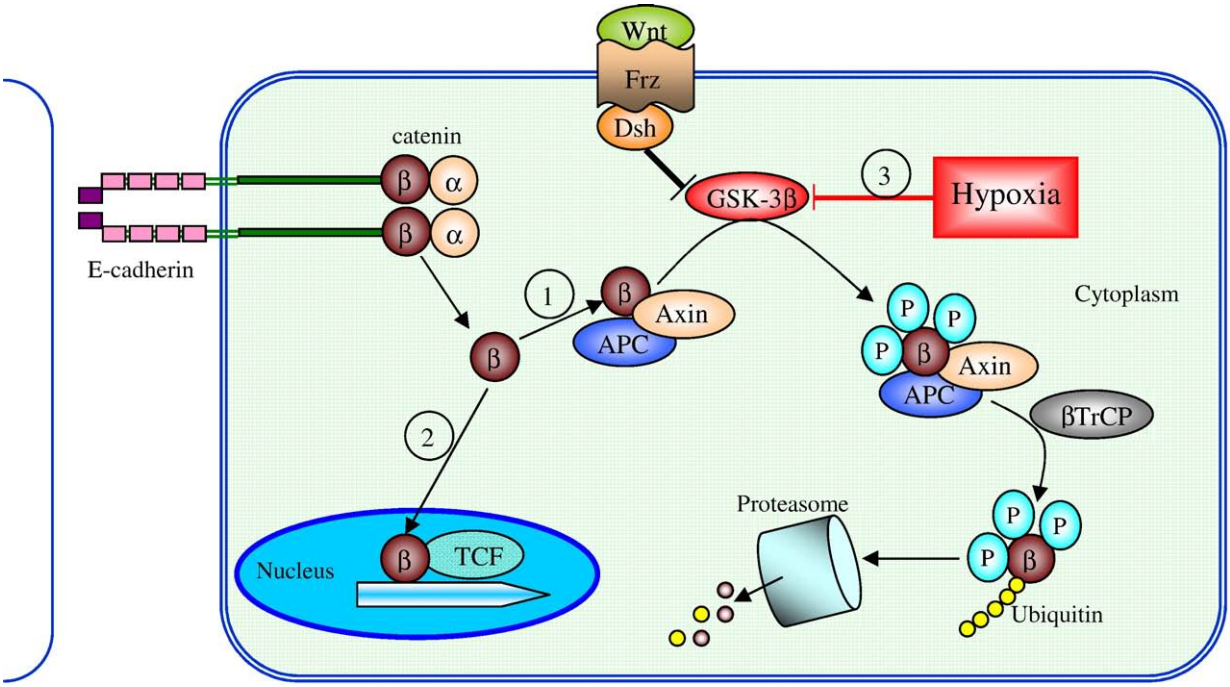

Fig. 8. Potential signalling pathways downstream of the loss of E-cadherin function. Following the loss of E-cadherin function, β-catenin (1) is sequestered by the adenomatous polyposis coli (APC)–axin–GSK-3β complex and phosphorylated by GSK-3β. This phosphorylated β-catenin is specifically bound and ubiquitylated by βTrCP, a subunit of the E3 ubiquitin ligase complex. Ubiquitylation earmarks β-catenin for proteasomal degradation. GSK-3β is repressed and does not phosphorylate β-catenin, when the Wnt signalling pathway is activated or when in hypoxia (3). β-catenin then translocates to the nucleus. Together with TCF/Lef-1 transcription factors, it modulates the expression of several target genes involved in cell proliferation and tumour progression (modification to Christofori (2006)). α, α-catenin; Dsh, dishevelled; Frz, frizzled.

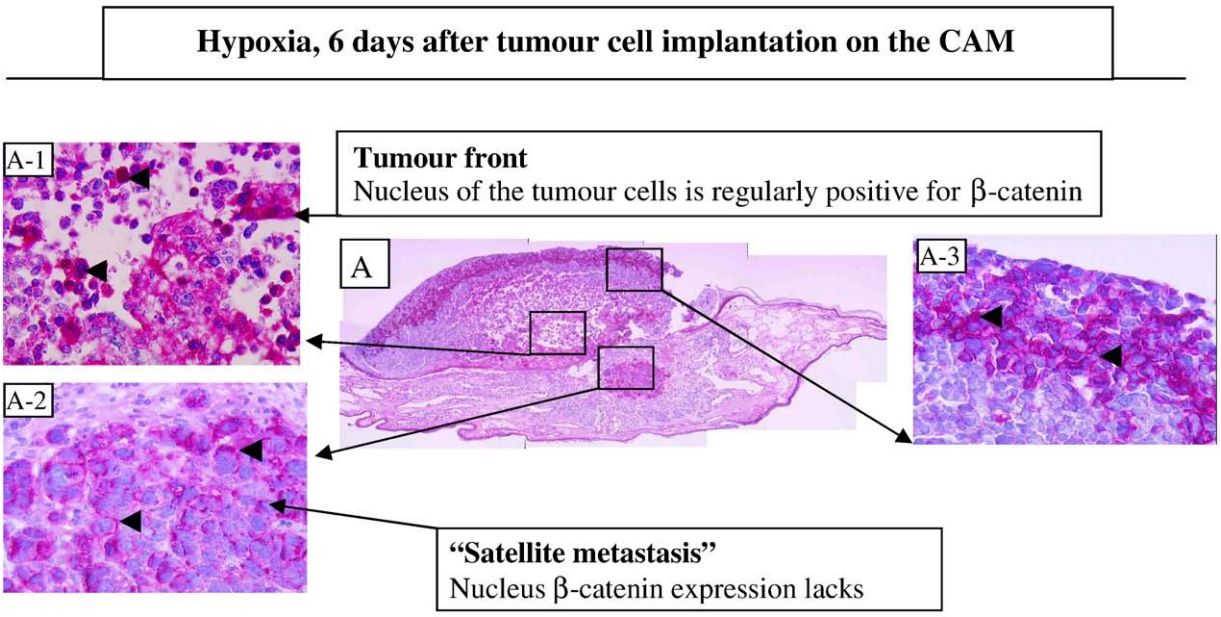

Fig. 9. β-catenin staining of a tumour nodule six days after incubation on the CAM. Three different phenotypes of tumour cells can be observed. (1) In the outer zone the tumour cells form a pseudocapsule, cell-to-cell contact is functioning which is demonstrated by membranous β-catenin (A-3, ◄). (2) At the tumour front, β-catenin is in the nucleus and tumour cells are invading the ectodermal layer (A-1, ◄). (3) In the "satellite metastasis", tumour cells are densely packed and β-catenin is again membranous (A-2, ◄). This means that tumour cells can show different adaptive behaviour at different stages of hypoxic stress, which can be demonstrated by β-catenin localization.

normoxia, the tumour cells are alive but do not invade the ectoderm even after 6 days of incubation (Figs. 2 and 4). This is accompanied by expression of membranous β-catenin (Fig. 5). This means that the first step of the metastatic cascade (contact inhibition) is not initiated. In hypoxia, different phenotypes of tumour cells were observed in one tumour nodule. In the outer zone (towards the air) they form a "pseudocapsule" for protection in which proliferation is downregulated (Fig. 6: B) and cell-to-cell contact is dominant (Fig. 7: B). In the ventral zone and especially at the tumour front, the tumour cells are shedded and proliferate, which is accompanied by invasion of the mesoderm. β-catenin is membranous in the outer zone. At the tumour front β-catenin is translocated into the nucleus which is accompanied by tumour cells infiltration into the mesoderm. Intact E-cadherin–catenin complex in normal physiological conditions shows a membrane bound or cytoplasmatic location of β-catenin (Fig. 8, (Christofori, 2006)). It has been shown (Behrens and Lustig, 2004) that glycogen-synthetase-kinase-3β (3GSK-3β) is repressed when the Wnt signalling pathway is activated and instead of being phosphorylated, β-catenin translocates to the nucleus. Together with TCF/Lef-1 transcription factors, β-catenin modulates the expression of several target genes involved in cell proliferation and tumour progression. Moreover there are data showing that GSK-3β (Beitner-Johnson et al., 2001) is phosphorylated by hypoxia, which results in its enzymatic inactivation (Cross et al., 1995). There is now evidence that inactivation of GSK-3β by hypoxia leads to accumulation of β-catenin (Fig. 9) which is accompanied by a more invasive phenotype of tumour cells (Figs. 7 and 9).

In conclusion, hypoxia seems to generate a more invasive phenotype of tumour cells.

β-catenin seems to work as a transcription factor in the nucleus modulating tumour progression in dependence of hypoxia.

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