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Expression of the TGF-β coreceptor endoglin in epidermal keratinocytes and its dual role in multistage mouse skin carcinogenesis

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Endoglin is an integral membrane glycoprotein primarily expressed in the vascular endothelium, but also found on macrophages and stromal cells. It binds several members of the transforming growth factor (TGF)-β family of growth factors and modulates TGF-β_1-dependent cellular responses. However, it lacks cytoplasmic signaling motifs and is considered as an auxiliary receptor for TGF-β. We show here that endoglin is expressed in mouse and human epidermis and in skin appendages, such as hair follicles and sweat glands, as determined by immunohistochemistry. In normal interfollicular epidermis, endoglin was restricted to basal keratinocytes and absent in differentiating cells of suprabasal layers. Follicular expression of endoglin was high in hair bulb keratinocytes, but decreased in parts distal from the bulb. To address the role of endoglin in skin carcinogenesis *in vivo*, *Endoglin* heterozygous mice were subjected to long-term chemical carcinogenesis treatment. Reduction in endoglin had a dual effect during multistage carcinogenesis, by inhibiting the early appearance of benign papillomas, but increasing malignant progression to highly undifferentiated carcinomas. Our results are strikingly similar to those previously reported for transgenic mice overexpressing TGF-β_1 in the epidermis. These data suggest that endoglin might attenuate TGF-β_1 signaling in normal epidermis and interfere with progression of skin carcinogenesis.
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Keywords: endoglin; TGF-β; keratinocyte; tumor progression

Introduction

The transforming growth factor-β (TGF-β) plays an important role in epithelial homeostasis, as it modulates the cellular phenotype and is a powerful inhibitor of cell growth (Derynck *et al*., 2001; Piek and Roberts, 2001). Members of the TGF-β superfamily exert their biological effects by binding to a heteromeric complex containing two different transmembrane serine/threonine kinases known as type I and type II signaling receptors (Letterio and Roberts, 1998; Massagué, 1998). The TGF-β receptor complex also contains two auxiliary receptors named endoglin and betaglycan (Massagué, 1998). These are transmembrane proteins with large extracellular domains and serine/threonine-rich cytoplasmic regions without consensus signaling motifs. Endoglin binds TGF-β_1, TGF-β_3, activin-A, BMP-2, and BMP-7 in the presence of the signaling receptors types I and II (Cheifetz *et al*., 1992; Bellón *et al*., 1993; Letamendia *et al*., 1998a; Barbara *et al*., 1999), and modulates TGF-β-dependent cellular responses (Lastres *et al*., 1996; Letamendia *et al*., 1998a; Li *et al*., 2000; Diez-Marques *et al*., 2002). Endoglin plays an important role in mammalian physiology as demonstrated by genetic inactivation, since *Endoglin* null (*Eng*$^{-/-}$) murine embryos die of vascular defects at 10–10.5 days post coitum (Bourdeau *et al*., 1999; Li *et al*., 1999; Arthur *et al*., 2000). Endoglin is a disulfide-linked homodimer of 180 kDa expressed in endothelial cells (Gougos and Letarte, 1988, 1990; Li *et al*., 2000), macrophages (Lastres *et al*., 1992), stromal cells (St-Jacques *et al*., 1994; Robledo *et al*., 1996), and other cellular lineages within the vascular system and connective tissues (Fonsatti *et al*., 2001). Several tumor cells also express endoglin, including pre-B-cell and monocytic leukemia (Quackenbush and Letarte, 1985; Haruta and Seon, 1986; Lastres *et al*., 1992; Zhang *et al*., 1996), melanoma (Altomonte *et al*., 1996), choriocarcinoma (Letamendia *et al*., 1998b), and ovarian carcinoma (Jindal *et al*., 1995). However, little is known about the possible expression and function of endoglin in epithelial cells.

Approximately 90% of all human tumors are of epithelial origin. TGF-β has both positive and negative effects on carcinogenesis. It behaves as a tumor suppressor, early in tumorigenesis, but can also drive malignant progression, invasion, and metastasis (see

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Akhurst and Balmain, 1999; Massagué *et al*., 2000; Derynck *et al*., 2001, for reviews). In spite of the large number of studies addressing the role of TGF-β in epithelial carcinogenesis, the specific role played by the TGF-β coreceptor endoglin has not been evaluated *in vivo*.

The mouse skin model of multistage carcinogenesis has been widely studied and is well characterized with respect to phenotypic and genetic changes (Yuspa, 1994; Akhurst and Balmain, 1999). Tumor initiation is induced by a single topical application of the carcinogen 7,12-dimethylbenz(*a*)anthracene (DMBA), and involves a specific oncogenic mutation in the H-Ras gene (Quintanilla *et al*., 1986). Thereafter, repeated application of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) results in the outgrowth of highly differentiated benign papillomas. A small subset of papillomas eventually progresses to malignant squamous cell carcinomas, some of which undergo an epithelial–mesenchymal transition to spindle cell carcinomas (Klein-Szanto *et al*., 1989; Buchmann *et al*., 1991). The spindle cell, the most invasive and motile carcinoma cell type has altered the expression of keratinocyte-specific markers, such as cytokeratins, and show loss of E-cadherin expression (Navarro *et al*., 1991; Díaz-Guerra *et al*., 1992; Frontelo *et al*., 1998).

Here, we describe the presence of endoglin in keratinocytes of basal epidermis and hair follicles, the two major cell compartments for tumor initiation in skin carcinogenesis (Akhurst and Balmain, 1999; Taylor *et al*., 2000). To investigate the role of endoglin in carcinogenesis, *Endoglin* heterozygous ($Eng^{+/-}$) mice were subjected to long-term chemical carcinogenesis treatment with DMBA and TPA. We found that endoglin haploinsufficiency had a dual effect during multistage skin carcinogenesis, inhibiting the early appearance of benign tumors, but increasing the late malignant phenotype.

Results

Endoglin is expressed in keratinocytes of normal skin and cutaneous lesions

The presence of endoglin was analysed in human normal skin sections by immunohistochemistry using the specific monoclonal antibody (mAb) SN6h. Endoglin was expressed in the epidermis, restricted exclusively to basal keratinocytes (Figure 1a, b). The immunostaining pattern was discontinuous in the basal layer with some cells staining weakly or not at all, and in patches in certain regions of the plasma membrane and/or cyto-

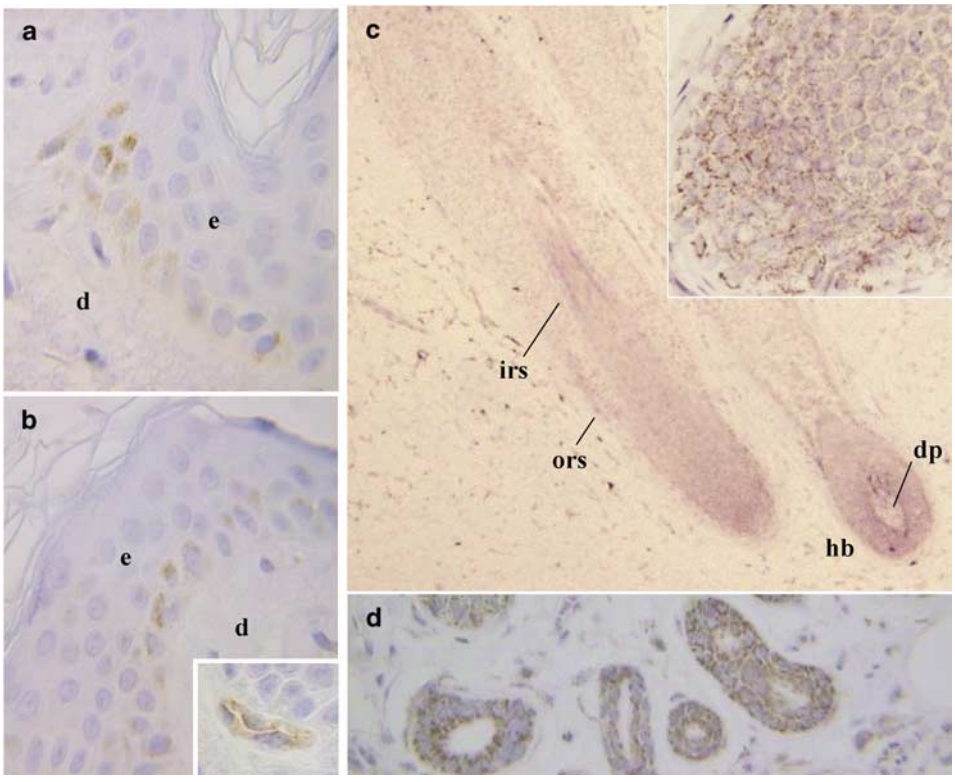

Figure 1 Immunohistochemical detection of endoglin in human skin and skin appendages. (**a, b**) Interfollicular skin. Endoglin is expressed in basal keratinocytes of the epidermis (e) and in dermal (d) blood vessels (inset in panel b). (**c**) Hair follicles. Note staining in keratinocytes of the hair bulb (hb) and outer (ors) and inner (irs) root sheaths. No staining is found in the dermal papilla (dp) and in parts distal from the hair bulb. The inset in this panel shows a higher magnification of the hair bulb. (**d**) Sweat glands showing positive staining in basal and ductal cells. Original magnifications: $\times 100$ (**c**), $\times 400$ (**a**, **b**, **d**, insets in **b** and **c**)

plasm. This is in contrast to the continuous and cell-surface pattern of endoglin expression seen in endothelial cells of dermal blood vessels (Figure 1b, inset), used as a positive control. The antibody did not stain suprabasal keratinocytes and dermal fibroblastic cells. Endoglin was also present in keratinocytes of hair follicles (Figure 1c). Intense staining was observed in the hair matrix and in the proximal regions of the outer and inner root sheaths, and decreased in parts distal from the bulb. Mesenchyme-derived dermal papilla fibroblasts were endoglin negative. As seen for interfollicular epidermal cells, hair follicle keratinocytes expressed endoglin in a polarized fashion, with the protein located in certain regions of the plasma membrane and/or cytoplasm in the form of granules or aggregates (Figure 1c, inset). Basal and ductal cells of the sweat gland were found to express endoglin strongly (Figure 1d), while high nonspecific staining background was observed in cells of the sebaceous gland (not shown). Immunostaining of normal mouse skin sections with the rat mAb MJ7/18 specific for murine endoglin revealed the same pattern of protein expression in follicular and interfollicular epidermis as in human skin (see below in Figure 6).

Since endoglin was present in cells of the epidermis and skin appendages, we analysed its expression in several human cutaneous disorders. To this aim, sections of verruca vulgaris ($n=3$), seborrheic keratosis ($n=3$), nodular basal cell carcinomas ($n=7$), and squamous cell carcinomas ($n=10$) were stained with the mAb SN6h. A pattern of staining similar to that of normal epidermis was found in all cases of verruca vulgaris (not shown). In seborrheic keratosis, endoglin staining was extended to proliferating keratinocytes above the basal layer, while absent from terminally differentiating keratinocytes (Figure 2a). All specimens of nodular basal cell carcinomas were stained with the specific mAb, revealing that endoglin was expressed mainly at the periphery of tumor nests, as dots, on the cell surface in contact with the basement membrane zone. Reduced staining in the inner cell layers was seen in all cases (Figure 2b). Immunohistochemical analysis of squamous cell carcinomas showed that all tumors expressed endoglin at variable levels regardless of the differentation grade (Figure 2c, d). Positive staining was observed in the periphery of tumor nests, but not in the central keratinized areas of well-differentiated tumors from which endoglin was excluded (Figure 2c).

Endoglin expression in murine keratinocyte cell lines

To characterize endoglin expression in cultured keratinocytes at the mRNA and protein levels, we used a panel of premalignant and malignant mouse epidermal cell lines: PB (papilloma-derived cells), Pam212, HaCa4, and PDV (squamous carcinoma cell lines), and the spindle carcinoma cell lines Car C and Car B. Northern blot experiments with a cDNA probe corresponding to the extracellular domain of the molecule (see Materials and methods) revealed a transcript of 3.1 kb in all the cell lines (Figure 3a), but detection required a long exposure of the autoradiography indicating a low level

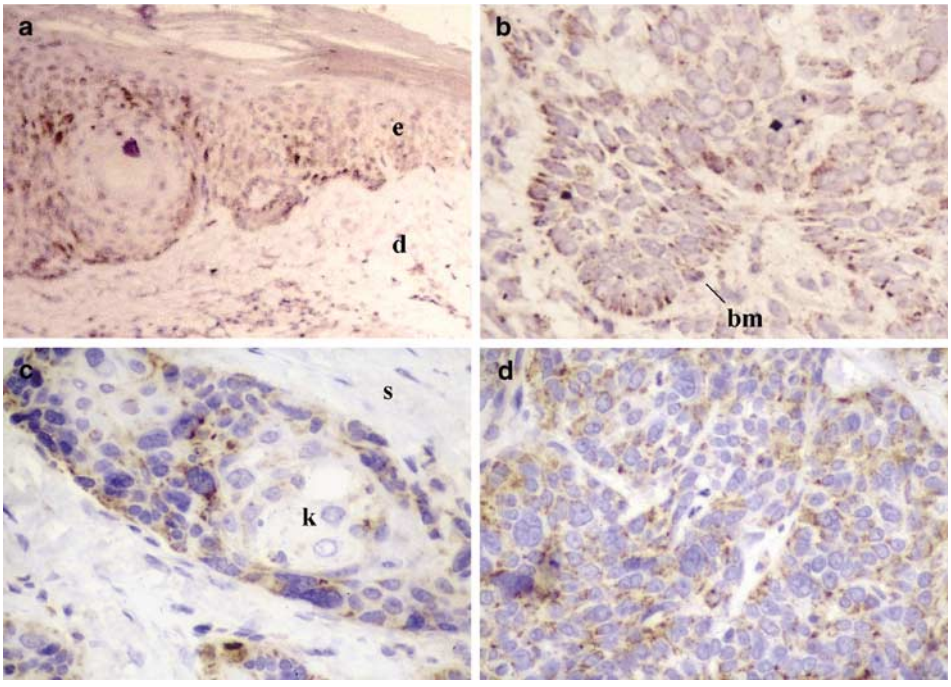

Figure 2 Immunohistochemical detection of endoglin in human cutaneous disorders. (**a**) Seborrheic keratosis; e, epidermis; d, dermis. (**b**) Nodular basal cell carcinoma showing polarized positive staining at the periphery of tumor nests in contact with the basement membrane (bm). (**c**) Well-differentiated squamous cell carcinoma. Note positive staining in the periphery of the tumor while endoglin is absent from the keratinized regions (k); s, stroma. (**d**) Poorly differentiated squamous cell carcinoma showing endoglin staining in tumor cells. Original magnifications: $\times 100$ (**a**), $\times 200$ (**d**), $\times 400$ (**b**, **c**)

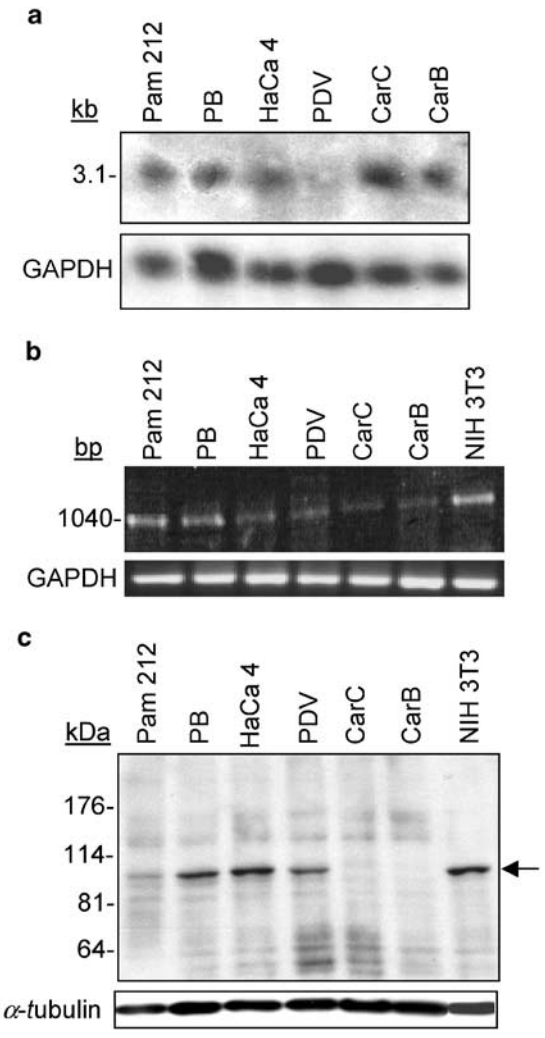

Figure 3 Analysis of endoglin expression in mouse epidermal cell lines. (**a**) Northern blot hybridization reveals a transcript of 3.1-kb expressed in all cell lines. A GAPDH probe was used as control for RNA quality and amount. Times of exposure of the autoradiographies were about 3 days for GAPDH and 20 days for endoglin mRNA. (**b**) Detection of endoglin transcripts by PCR amplification. Samples of total RNA from the indicated cell lines were incubated with the reverse transcriptase and the generated cDNAs were used for PCR amplification in the presence of specific primers. A single 1040-bp band of the predicted size was detected in all the cell lines. (**c**) Western blot analysis with the mAb MJ7/18 specific for murine endoglin. The arrow indicates the position of the specific band detected in keratinocyte and NIH3T3 cell lines. In the bottom panel, the filter was reblotted with an anti-α-tubulin mAb as a control for protein loading

of endoglin mRNA expression in cultured keratinocytes. This transcript has already been described in other murine tissues such as the ovary, thymus, placenta, and kidney (Ge and Butcher, 1994; St-Jacques *et al*., 1994; Rodriguez-Peña *et al*., 2002). The relative abundance of the endoglin mRNAs among the cell lines was roughly similar, except for PDV carcinoma cells that showed lower levels in this experiment. The presence of endoglin transcripts was confirmed by RT–PCR (Figure 3b). Using specific primers to mouse endoglin, a single 1040-bp PCR product of the predicted size was obtained in all the cell lines tested, including NIH3T3 fibroblasts used as a control. To confirm that this band represented endoglin, it was purified and sequenced, demonstrating its identity with the published murine sequence (Ge and Butcher, 1994; St-Jacques *et al*., 1994). Western blots (under reducing conditions) clearly detected a polypeptide of about 95 kDa in PB, HaCa4, and PDV keratinocytes (Figure 3c). A weaker band was present in Pam212, whereas no specific signal could be detected in Car C and Car B cell lines. Overall, the intensities of the bands detected in the keratinocyte cell lines were low, although comparable to that of NIH3T3 fibroblasts. Parallel blots challenged with preimmune rat IgG, as a negative control, confirmed the specificity of the mAb MJ7/18 (data not shown).

Reduced incidence of chemically induced benign papillomas and enhanced progression to malignant carcinomas in Endoglin *heterozygous mice*

To analyse the role of endoglin in carcinogenesis, we took advantage of the $Eng^{+/-}$ mouse that expresses a single endoglin allele on a 129/Ola and C57Bl/6 mixed background (backcrossed onto the C57Bl/6 background for four generations, see Bourdeau *et al*., 1999). Two independent skin carcinogenesis experiments were performed using groups of seven (experiment 1) and eight to 10 (experiment 2) male and female mice. $Eng^{+/-}$ mice and the homozygous littermates ($Eng^{+/+}$) were treated with an initial dose of DMBA followed twice weekly with TPA for about 20 weeks, as described in Materials and methods. A significant reduction in the number of tumors per mouse and the total yield of tumors was observed in the $Eng^{+/-}$ versus $Eng^{+/+}$ mice throughout the observation period (Figure 4). The first appearance of papillomas was noticed on weeks 12 (experiment 1) or 8 (experiment 2) in $Eng^{+/+}$ mice and 1–2 weeks later in the corresponding $Eng^{+/-}$ mice. Tumor induction occurred in 87% of $Eng^{+/+}$ mice and 44% of $Eng^{+/-}$ mice. No significant differences in tumor development were observed between males and females.

The histopathological examination of the tumors at the end of the experiments (25 and 23 weeks post-DMBA initiation in experiments 1 and 2, respectively) revealed that all tumors in the $Eng^{+/+}$ mice were benign papillomas. However, in the $Eng^{+/-}$ mice, despite a threefold reduction in the total number of tumors versus the controls, more than half of the developed tumors were malignant carcinomas (Table 1). Papillomas in the $Eng^{+/-}$ and $Eng^{+/+}$ mice were histologically very similar with abundant keratin and a high level of differentiation (Figure 5a). $Eng^{+/-}$ carcinomas showed a variable pattern of differentiation ranging from well-differentiated squamous carcinomas grades I–II (Figure 5b) to highly undifferentiated carcinomas grades III–IV that showed very little evidence of cellular organization, differentiation, or keratin production (Figure 5d, see also Table 1). Carcinomas in the $Eng^{+/-}$ mice were, in general, very invasive and infiltration into the muscle

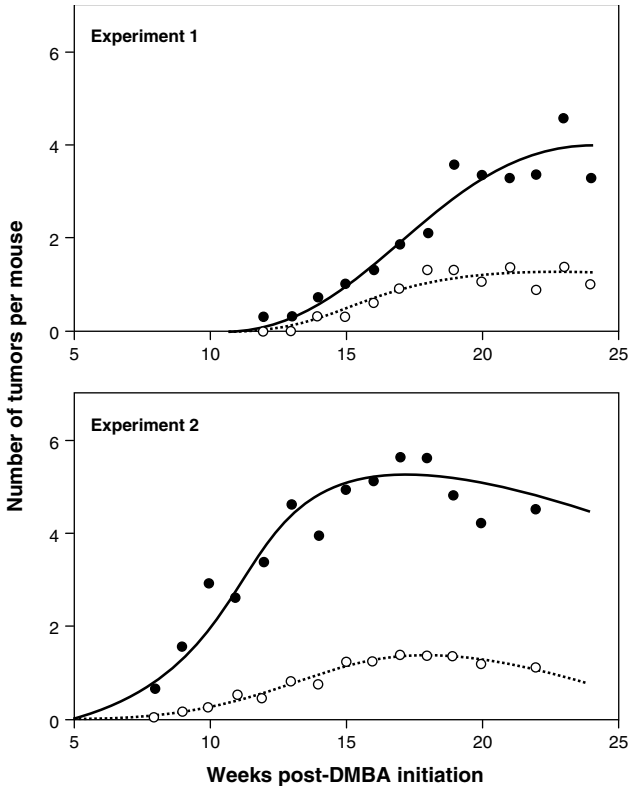

Figure 4 Rate of appearance of tumors in $Eng^{+/+}$ (•) and $Eng^{+/-}$ (○) mice. The average number of tumors (over 2 mm in diameter) per mouse is plotted versus the number of weeks after initiation with DMBA. In both experiments, reduction in the average tumor number per mouse in $Eng^{+/-}$ versus $Eng^{+/+}$ mice is statistically significant when the tumor numbers reach the plateau. Thus, for example, at weeks 22 and 23 in experiment 1 (P-values <0.05 and <0.01, respectively, using a Student t-test) and at weeks 17 and 18 in experiment 2 ($P<0.05$ and $P=0.01$, respectively)

layer was often observed (Figure 5c). No obvious differences in the inflammatory response were observed between $Eng^{+/-}$ and $Eng^{+/+}$ tumors.

Endoglin expression was vastly increased in $Eng^{+/+}$ papillomas relative to normal skin, as shown by immunohistochemistry. Strong immunoreactivity was observed in basal and suprabasal keratinocytes (Figure 6c), but not in differentiating keratinocytes of the upper epidermal layers. Staining was mainly located at the plasma membrane, although some cells also showed an intense intracellular staining. Endoglin expression in suprabasal keratinocytes was preserved in hyperplastic epidermis adjacent to the tumors (not shown). However, in skin distant from the lesions, staining shifted to a pattern similar to that found in the normal epidermis (Figure 6a), endoglin being restricted to basal-like cells and preferentially intracellular (Figure 6b). Tumors induced in $Eng^{+/-}$ mice showed poor immunoreactivity with mAb MJ7/18, although a weak plasma membrane and cytoplasmic staining could be observed in basal and suprabasal keratinocytes of papillomas (Figure 6d).

These results show that a reduction of endoglin gene dosage inhibits the formation of benign skin tumors, but highly increases the frequency of malignant conversion.

Discussion

So far, most of the research studies on endoglin have focused on its role in angiogenesis and vascular remodeling. This is due to the fact that endoglin is preferentially and strongly expressed in endothelial cells (Gougos and Letarte, 1988; Li *et al*., 2000). Furthermore, mutations in the endoglin gene lead to haploinsufficiency and are responsible for the hereditary hemorrhagic telangiectasia type 1 (HHT1), an autosomal dominant disorder characterized by multisystemic vascular dysplasia, and recurrent hemorrhage (Guttmacher *et al*., 1995; Shovlin and Letarte, 1999; Marchuk and Lux, 2001). In addition, endoglin expression is regulated during heart development in human and chicken (Qu *et al*., 1998; Vincent *et al*., 1998); and endoglin-null mice obtained by targeted disruption of the endoglin gene die at 10–10.5 days post coitum due to vascular and cardiac abnormalities (Bourdeau *et al*., 1999; Li *et al*., 1999; Arthur *et al*., 2000).

Aside from the endothelial expression of endoglin, several laboratories have reported the presence of endoglin in other cell types, mostly on cellular lineages within the vascular system and connective tissues (see Fonsatti *et al*., 2001, for a review). In this work, we show for the first time that endoglin is expressed in the epidermis and skin appendages, such as hair follicles and sweat glands. The presence of endoglin in normal interfollicular epidermis is restricted to keratinocytes of the basal, proliferative, layer, whereas none is detected on suprabasal keratinocytes. This pattern of expression is disrupted in human cutaneous hyperproliferative lesions, such as seborrheic keratosis, and in tumors, such as human basal and squamous cell carcinomas and mouse DMBA/TPA-induced papillomas, where aberrant endoglin expression in suprabasal layers occurs.

To analyse the potential role of endoglin in tumor formation and malignant progression, we used a skin multistage carcinogenesis model in heterozygous C57Bl/6 $Eng^{+/-}$ mice. These mice develop normally, and clinical signs of HHT were present in the 129/Ola strain, but almost absent in the C57Bl/6 strain (Bourdeau *et al*., 1999, 2001). None of the $Eng^{+/-}$ C57Bl/6 mice used in our experiments showed nosebleeds or telangiectases. Our results show that a reduction of *Endoglin* gene dosage has profound effects on skin neoplasia, affecting both early and late stages of tumorigenesis. Decreased endoglin expression suppresses benign tumor outgrowth, but accelerates malignant conversion and development of highly aggressive poorly differentiated carcinomas, a phenotype first observed in transgenic mice overexpressing TGF-β_1 in the epidermis (Cui *et al*., 1996). This study as well as those from other laboratories have led to postulation of a dual role for TGF-β_1

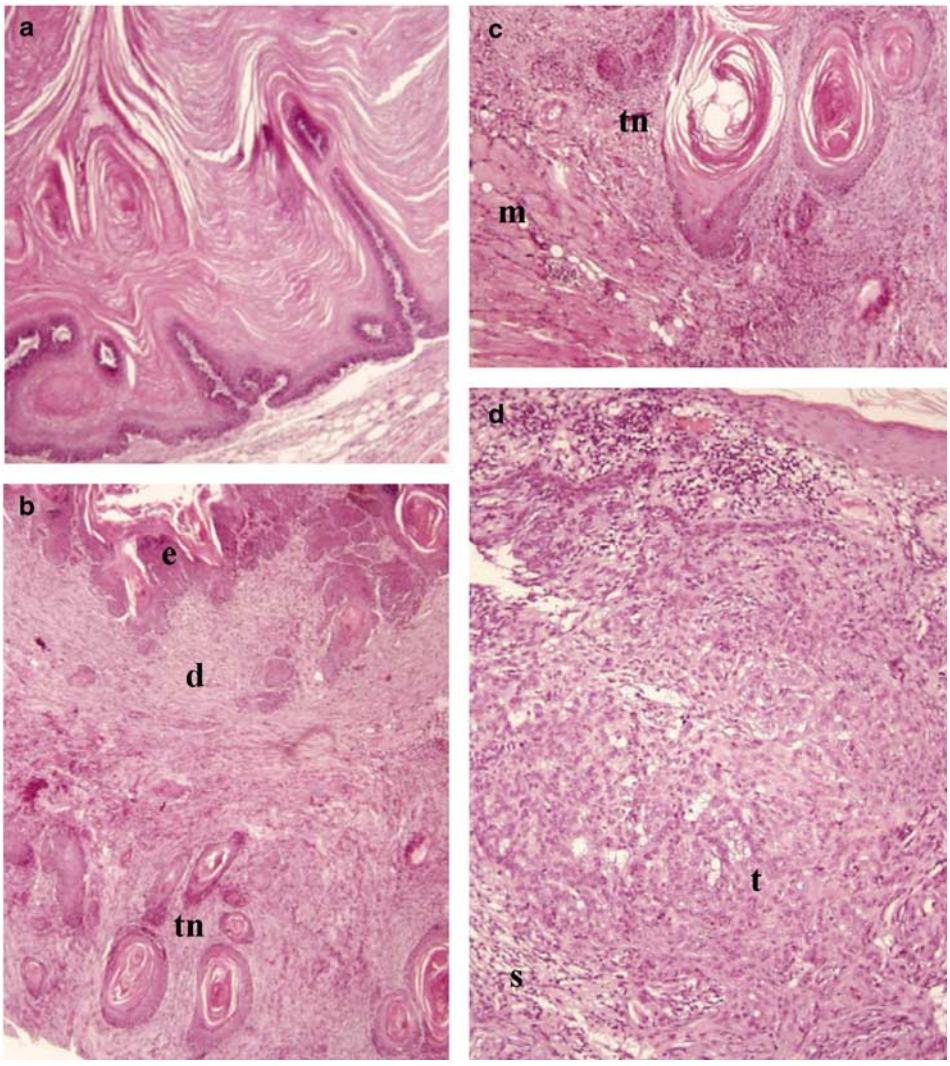

Figure 5 Histology of tumors induced in $Eng^{+/-}$ mice. (**a**) Well-differentiated papilloma showing abundant keratin. (**b**) Squamous cell carcinoma grades I–II. Note nests of tumor cells (tn) and keratin invading deep into the dermis (d); e, epidermis. (**c**) Detail of a well-differentiated squamous cell carcinoma infiltrating muscle tissue (m). (**d**) Poorly differentiated carcinoma grades III–IV; t, tumor; s, stroma. Original magnifications: $\times 100$ (**b**), $\times 200$ (**a**, **d**), $\times 400$ (**c**)

Table 1 Evaluation of tumor yield and histological grade in $Eng^{+/-}$ with respect to $Eng^{+/+}$ mice after DMBA/TPA skin carcinogenesis[a]

		Cumulative number of tumors			
Mouse line	*Number of animals with tumors*	*Papilloma*	*SCC I–II*	*SCC III–IV*	*Total*
$Eng^{+/+}$	13/15	45	—	—	45
$Eng^{+/-}$	7/16	5	5	4	14

[a]At the termination of the carcinogenesis experiment (25 weeks post DMBA in experiment 1 and 23 weeks post DMBA in experiment 2), all tumors were enumerated and histologically typed. SCC, squamous cell carcinoma.

in carcinogenesis (Akhurst and Balmain, 1999; Massagué *et al*., 2000; Derynck *et al*., 2001; Rich *et al*., 2001). TGF-β_1 acts as a suppressor of tumor formation, early in carcinogenesis – by virtue of its well-established antimitogenic function in epithelial cells (Roberts and Sporn, 1990; Alexandrov and Moses, 1995). At later stages, TGF-β_1 stimulates malignant progression by inducing an epithelial–mesenchymal transition toward an invasive and metastatic phenotype (Caulín *et al*., 1995; Cui *et al*., 1996; Oft *et al*., 1996; Frontelo *et al*., 1998; Portella *et al*., 1998), likely in cooperation with oncogenic Ras (Oft *et al*., 1996, 2002). These TGF-β

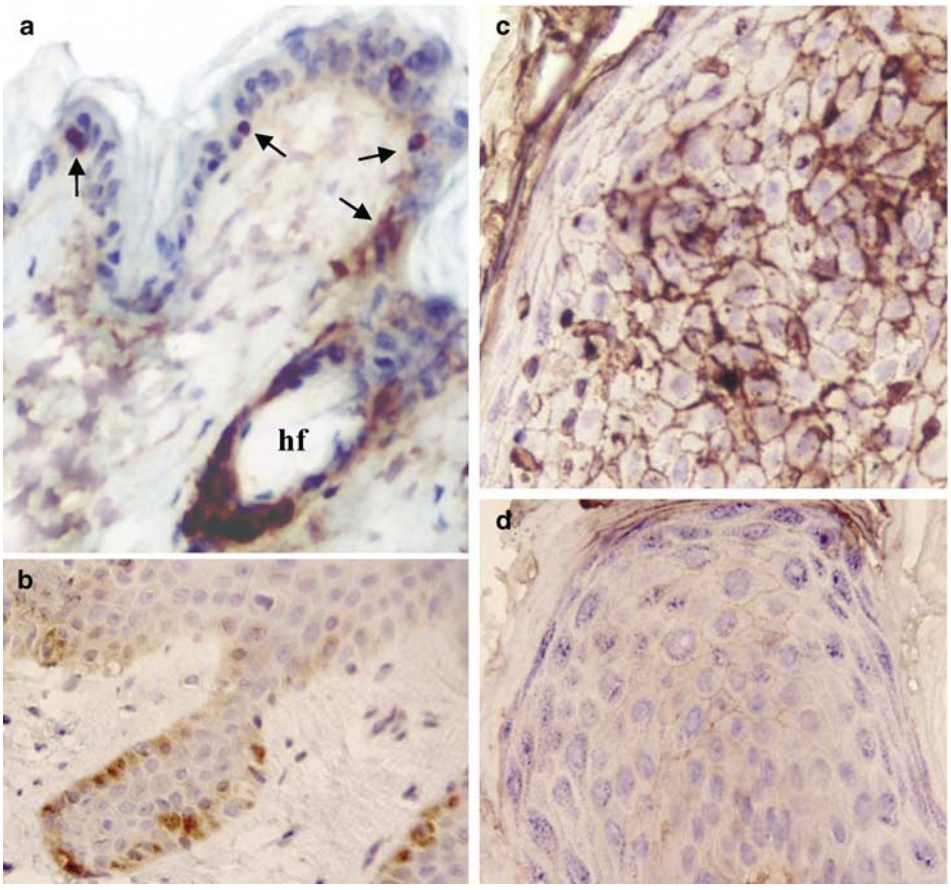

Figure 6 Immunohistochemical detection of endoglin in tumors induced in $Eng^{+/+}$ and $Eng^{+/-}$ mice. (**a**) $Eng^{+/+}$ normal skin. Note positive staining in basal keratinocytes (arrows) and hair follicles (hf). (**b**) Normal skin distant from a papilloma induced in an $Eng^{+/+}$ mouse. Note that the epidermis is still slightly hyperplastic. (**c**) $Eng^{+/+}$ papilloma. (**d**) $Eng^{+/-}$ papilloma. Original magnifications: $\times 200$ (**a**, **b**, **d**), $\times 400$ (**c**)

effects occur within the tumor cells themselves and require signaling through serine/threonine kinase receptors (Oft *et al*., 1998; Portella *et al*., 1998). Considering this mechanism of action of TGF-β_1 our results strongly suggest that the endoglin level on epithelial keratinocytes is critical for the mediation of TGF-β_1 inhibitory effect since the generation of benign tumors is inhibited in $Eng^{+/-}$ mice. However, transformation to a more malignant phenotype is observed in these mice, suggesting that normal levels of endoglin would inhibit the effect of TGF-β_1 on malignant progression.

It can be argued, however, that defects in neovascularization could play an additional role in the phenotype observed in our $Eng^{+/-}$ mice, since endoglin is highly expressed on the tumor-associated angiogenic vascular endothelium (Burrows *et al*., 1995; Bodey *et al*., 1998; Kumar *et al*., 1999; Li *et al*., 2001; Takahashi *et al*., 2001). The importance of angiogenesis for the growth of solid tumors is well recognized (Hanahan and Folkman, 1996). It has been shown that angiogenesis is a rate-limiting step in the genesis of premalignant lesions such as mouse skin papillomas (Bolontrade *et al*., 1998; Larcher *et al*., 1998), decreased in $Eng^{+/-}$ mice in this study. Angiogenesis is also decisive for tumor progression, and halting angiogenesis inhibits development of invasive carcinomas and restores the benign phenotype of malignant keratinocytes (Skobe *et al*., 1997). Therefore, it is unlikely that defective angiogenesis would account for the dual phenotype exhibited by $Eng^{+/-}$ mice during carcinogenesis: decreased tumor formation and accelerated malignant progression. Interestingly, a recent report shows that decreased endoglin expression in cultured human prostate cancer cells enhances cell migration and invasion, while increased expression has opposite effects (Liu *et al*., 2002). These findings suggest that the level of endoglin expression can modulate adhesion and motility in transformed epithelial cells, and are in line with the results presented here that reduced endoglin expression in keratinocytes favors progression to invasive carcinomas.

A function for endoglin as an attenuator of TGF-β_1 signaling is supported by our previous studies showing that endoglin inhibits the TGF-β_1 antiproliferative and certain TGF-β_1-dependent cellular responses (Lastres *et al*., 1996; Letamendia *et al*., 1998a; Diez-Marques *et al*., 2002). Although the molecular mechanism by which endoglin modulates TGF-β responses remains to be determined, a recent report suggests that by directly

interacting with the signaling receptors types I and II, endoglin regulates their phosphorylation status and subsequently their signaling activity (Guerrero-Esteo *et al*., 2002). TGF-β_1 is an endogenous regulator of epidermal homeostasis that is preferentially expressed in suprabasal layers, where it is thought to block proliferation of differentiating keratinocytes (Akhurst *et al*., 1988; Fowlis *et al*., 1992; Patamalai *et al*., 1994). Interestingly, endoglin is synthesized in basal proliferative keratinocytes, the appropriate cell type to counteract the negative regulation of growth exerted by this factor. Anomalous endoglin expression in suprabasal keratinocytes, as observed in papillomas and carcinomas and other cutaneous hyperplastic disorders, is likely associated with expansion of the proliferative keratinocyte compartment in these lesions (Yuspa, 1994). Thus, we postulate that the relative levels of endoglin and TGF-β_1 production can mediate the strength of the cellular responses to the growth factor, pointing to endoglin as a potential new target to explore therapeutic strategies to control TGF-β-mediated tumor progression.

Materials and methods

Cell lines and culture

The origin of the mouse epidermal cell lines: Pam212, PB, PDV, HaCa4, Car C, and Car B used in this work has been described (Yuspa *et al*., 1986; Díaz-Guerra *et al*., 1992). NIH3T3 mouse fibroblast cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in Ham's F12 medium supplemented with amino acids and vitamins (Pam212, PB, HaCa4, PDV) or Dulbecco's modified Eagle's medium (NIH3T3, Car C, and Car B), with 10% fetal calf serum (Gibco Invitrogen Corp., Barcelona, Spain) and antibiotics (2.5 μg/ml amphotericin B, 100 μg/ml ampicillin, and 32 μg/ml gentamicin; Sigma-Aldrich, Madrid, Spain). Cultures were maintained on plastic at 37°C in a 5% CO_2-humidified atmosphere.

Endoglin heterozygous mice

Endoglin heterozygous ($Eng^{+/-}$) mice were generated by homologous recombination using embryonic stem cells of 129/Ola (129) origin and by backcrossing onto the C57BL/6 (B6) background, as previously described (Bourdeau *et al*., 1999). B6-$Eng^{+/-}$ mice of N4 generation were used in the present studies, as well as their B6-$Eng^{+/+}$ littermate controls. Genotypes were determined by PCR analysis of tail DNA isolated using two sets of primers amplifying a 470 bp product in the mutated allele, and a 300 bp product in the wild-type allele (Bourdeau *et al*., 1999). Mice were bred and kept in ventilated rooms, in a germ-free facility, under controlled conditions of light, temperature, humidity, food, and water (Rodriguez-Peña *et al*., 2002). $Eng^{+/-}$ mice were fertile, showing a normal ratio of male to female progeny, had a normal lifespan, and did not show any visible vascular abnormality. In all procedures, mice were treated in accordance with the international and national institution's guidelines for the care and use of laboratory animals: Conseil de l'Europe (published in the Official Daily No. L358/1-358/6, 18th December 1986), Spanish Government (published in Boletín Oficial del Estado No. 67, pp. 8509–8512, 18th March 1988, and Boletín Oficial del Estado No. 256, pp. 31349–31362, 28th October 1990), and the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85–23).

Chemical carcinogenesis

Tumors were induced on the shaved dorsal skin of 10-week-old mice by a single topical application of 32 μg of DMBA in 200 μl acetone, followed by treatment twice a week with 12.5 μg. of TPA in 200 μl acetone (10^{-4} M). The number of tumors (>2 mm in diameter) on each mouse was recorded every week. Mice were anesthetized and killed at the end of the experiments, and tumors were fixed in formalin and embedded in paraffin. The tumors were histologically typed by hematoxylin and eosin staining of paraffin sections. Although the pure B6 genetic background is refractory to DMBA/TPA carcinogenesis (Reiners *et al*., 1984), the N4 generation of 129 × B6 mice used in this study yielded four to five tumors per mouse.

Antibodies and immunohistochemical analysis

The human endoglin-specific mAb SN6h (Dako Diagnosticos, Barcelona, Spain) was used for staining formalin-fixed tissues. The mAb MJ7/18 recognizes mouse endoglin (Ge and Butcher, 1994), and was purchased from BD Biosciences (Heidelberg, Germany). Immunohistochemical detection of endoglin was performed in deparaffinized human and mouse tissue sections by the Envision plus peroxidase method (Dako A/S, Glostrup, Denmark), as previously reported (Velasco *et al*., 2001). The reaction product was developed with diaminobenzidine tetrahydrochloride and H_2O_2. The sections were dehydrated in graded ethanols, cleared in xylene, and mounted in Permount after counterstaining with hematoxylin.

Northern, RT—PCR, and Western blot analyses

Northern blot hybridization experiments were performed with total RNA extracted from the cell lines by the guanidinium thiocyanate procedure. RNA samples (25 μg) were fractionated on 1% agarose–formaldehyde gels and transferred to Nylon membranes (Schleicher and Schuell, Dassel, Germany). The probe for endoglin mRNA detection was a fragment of 1040 bp generated by RT–PCR from mouse keratinocytes (PB cell line), using oligonucleotides flanking the coding sequence corresponding to the extracellular domain: 5′-GTGTGTCTGGTCACAAGGAGGC-3′ (nucleotides 1001–1022) and 5′-GGACAAGGTGCTAGGGCGCAGA-3′ (nucleotides 2019–2040). PCR conditions were 35 cycles: 95°C for 1 min, 63°C for 1 min 30 s, 72°C for 1 min. For RT–PCR analysis, total RNA samples were incubated with the Moloney murine leukemia virus reverse transcriptase (Promega Corporation, Madison, WI, USA) and the generated cDNAs were used for PCR amplification as described above. PCR products were fractionated in 1% agarose–ethidium bromide gels. The 1040-bp band was isolated using a gel extraction kit (QIAquick; QiagenGmbH, Hilden, Germany) and sequenced in an ABI PRISM 377 machine (PE Applied Biosystems, Foster City, CA, USA).

For detection of endoglin in Western blots, cells were lysed at 4°C in buffer RIPA and a cocktail of proteinase inhibitors as previously described (Frontelo *et al*., 1998). Aliquots of total cell lysates containing equivalent amounts of protein (30 μg) were separated by 7.5% SDS–PAGE under reducing conditions and electrotransferred to Immobilon P membranes (Millipore Corporation, Bedford,

MA, USA) for immunodetection with the rat anti-mouse endoglin mAb MJ7/18 or preimmune rat IgG. As secondary Ab, goat anti-rat IgG coupled to horseradish peroxidase (Pierce, Perbio Science, Bonn, Germany) was used. Filters were reblotted with the anti-α-tubulin mAb DM1A (Sigma Aldrich). The peroxidase activity was developed using an enhanced chemiluminescence kit (Amersham Biosciences).

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