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Original Article

Bone Morphogenetic Proteins (BMPs) induce epithelial differentiation of NT2D1 human embryonal carcinoma cells

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ABSTRACT Human embryonal carcinoma (EC) cells represent the stem cells of testicular germ cell tumours (TGCTs) and are morphologically, antigenically and functionally related to the stem cells of early mammalian embryos. Despite the large capacity for differentiation displayed by TGCT stem cells, little is known of the factors controlling their developmental potency. We have analyzed the differentiation elicited in NT2D1 human embryonal carcinoma (EC) cells by Bone Morphogenetic Proteins (BMPs) and compared it with that elicited by retinoic acid (RA). We have found that while RA induced expression of neuronal, endodermal and epithelial markers in NT2D1 human EC cells, treatment with BMPs resulted in a predominantly epithelial phenotype. We also provide evidence to suggest that at least some of the effects elicited by RA in human EC cells might be mediated through RA-induced expression of BMP-7. Thus BMPs may play an important role in specifying the type of differentiation arising from human multipotent stem cells. The manipulation of BMP signalling in human embryonic multipotent stem cells may therefore prove a useful approach in attempts to generate specific differentiated cell types *in vitro,* and loss of the malignant and/or transformed phenotype.

KEY WORDS: *BMP-7, embryonal carcinoma, stem cells, epithelial differentiation.*

Introduction

Testicular germ cell tumours of adolescents and adults (TGCTs) are neoplasms thought to arise as a result of abnormal germ cell development. Their disparate morphology (reviewed in Andrews *et al.*, 1987) has resulted in classification into two main groups, the seminomas (SE) and non-seminomas (NS). The latter class comprises a heterogeneous set of tumours displaying variable degrees of differentiation into embryonic and extraembryonic lineages. Non-seminomatous TGCTs range from the undifferentiated embryonal carcinomas (EC) to the teratomas (TE), where the stem cell compartment is absent and the tumour mass is comprised of fully differentiated somatic tissue. Tumours where the differentiated component belongs to extra-embryonic lineages are also observed, namely the yolk sac carcinomas (YS) and the choriocarcinomas (CH). Mixed tumours, where undifferentiated and differentiated components co-exist, are also found (teratocarcinomas, TC). For the pathologist, the differentiation capacity and type of differentiation in TGCTs has important implications for the prognosis and treatment of these tumours. For the developmental biologist, the proliferative capacity and developmental potency of EC cells has made essential contributions to the study of early mammalian developmental processes. In spite of this, the factors controlling potency, the extent and the direction of differentiation in these tumours are still largely unknown. Numerous cell lines of variable potency have been isolated in the past from the stem cells of NS (see Andrews *et al.*, 1987). Of these, the T2cl13 and the NT2D1 have been extensively used as *in vitro* models of TGCT stem cells. Human EC cell lines are functionally and morphologically related to murine embryonic (ES) stem cell lines and to the more recently isolated human ES cell lines (Thompson, 1998; Shamblott *et al.*, 1998; Reubinoff *et al.*, 2000), and thus represent models of early human development. Pluripotent human EC cell lines can be cultured *in vitro* as stem cells and differentiate in response to treatment with a number of chemical agents, the best

Abbreviations used in this paper: EC, embryonal carcinoma; TGCT, testicular germ cell tumours; SE, seminomatous TGCT; NS, non-seminomatous TGCT; TE, Teratoma; TC, teratocarcinoma; BMP, bone morphogenetic proteins; OP, osteogenic protein; RA, retinoic acid; ES, embryonic stem; TGFβ, transforming growth factor beta; SMAD, similar to Mothers against decapentaplegic and Sma; PCNA, proliferating cell nuclear antigen; RAR, retinoic acid receptor; hGDF3, human growth differentiation factor-3.

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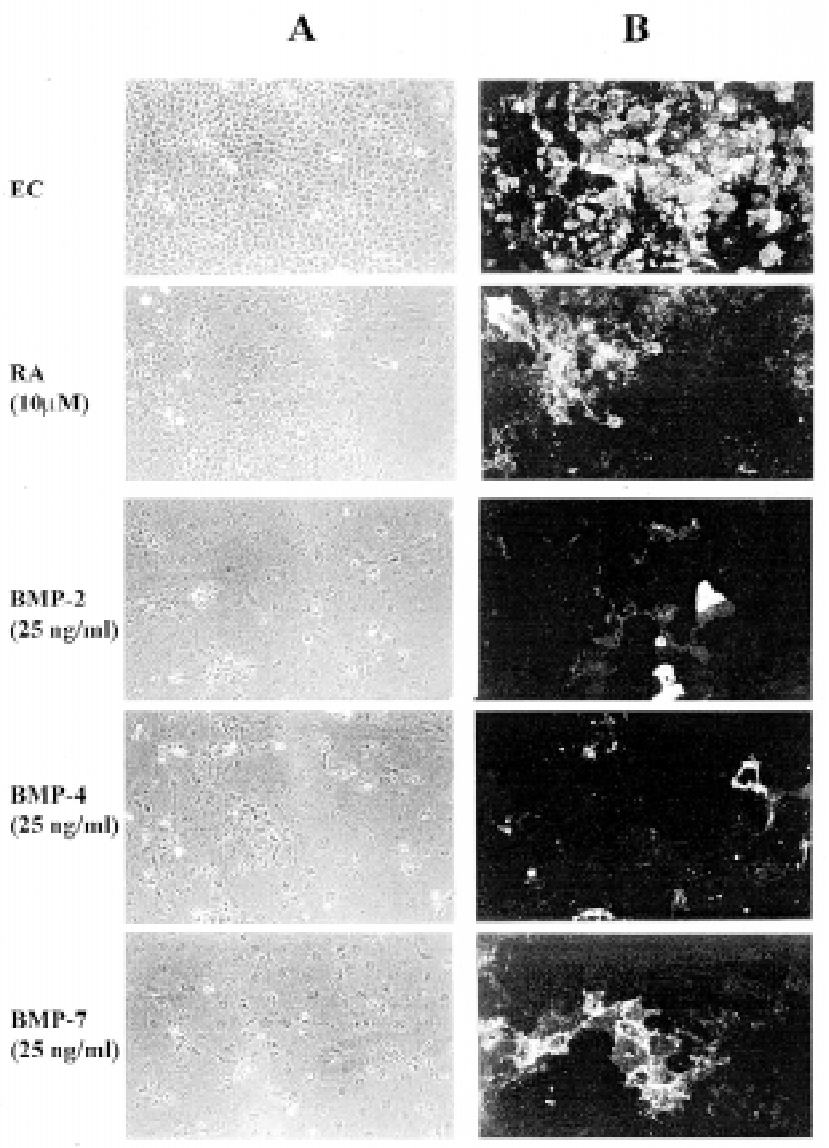

Fig. 1. Immunocytochemistry of human NT2D1 EC cell monolayers cultured in the absence or presence of RA or BMP-2, -4 or -7 for 5 days. **(A)** *phase contrast; EC: NT2D1 cells cultured in the absence of a differentiation inducer. Note the characteristic EC cell morphology. RA: NT2D1 cells cultured in the presence of retinoic acid, revealing a heterogenous cell population including morphologically differentiated cells. BMP-2, -4, -7: NT2D1 cells cultured in the presence of the indicated recombinant BMP. Note the relatively homogeneous population of morphologically differentiated cell types.* **(B)** *Tra-1-60 staining of the fields displayed in A. Note the decreased Tra-1-60 staining in cultures treated with RA or with BMP protein, indicative of differentiation.*

known of which is retinoic acid (RA). High concentrations of RA are required however, and these are unlikely to occur naturally *in vivo* or to be attainable in therapy. A similar consideration can be made for the other chemical agents known to induce differentiation of human EC cell lines. Other factors, produced by TGCT components, by the gonadal environment or by embryos must therefore be able to influence the extent and direction of stem cell differentiation in tumours and during embryonic development. Members of the TGFβ-superfamily, such as Mullerian inhibiting substance, activin and inhibin, are known to be expressed in the gonadal environment, in TGCTs themselves (reviewed in Hogan, 1996; van Schaik *et al.*, 1997) and in embryos (Hogan, 1996). The finding that BMP-7/OP-1 can induce differentiation of NT2D1 human EC cell monolayers (Andrews *et al.*, 1994) provides further evidence for the existence of peptide growth factors capable of modulating differentiation choices in human TGCT stem cells.

In the present study, we provide evidence that BMPs can induce epithelial differentiation in NT2D1 cultures, as opposed to the predominantly neuronal differentiation elicited by RA (Andrews *et al.,* 1984a). Finally, evidence is presented to show that BMPs may be responsible for at least some aspects of RA-mediated differentiation of NT2D1 cells. We conclude that BMPs represent candidates for the factors regulating stem cell differentiation in TGCTs and may play a role in early human development.

Results

BMP-2, -4 and -7 induce differentiation in NT2D1 monolayer cultures

The effects of BMP-2, BMP-4, BMP-7 (25 ng/ml) and RA (10μM) on the morphology and differentiation of NT2D1 cultures was examined over a 5 days of treatment (Fig.1). Differentiation was monitored by morphological criteria (loss of characteristic EC cell morphology, increase in the cytoplasmic/nuclear volume ratio, flattening of the cells) and by staining with antibody TRA-1-60, which recognizes a stem cell-specific surface antigen (Andrews *et al.*, 1984a). Loss of TRA-1-60 staining is evident in both RA and BMP-treated cultures, with BMP-treated cultures presenting a more uniform, epithelial-like morphology than the corresponding RA-treated cultures (Fig.1). Cultures treated with a combination of BMP and RA were morphologically and antigenically indistinguishable from NT2D1 cultures treated with BMP alone (not shown). In order to compare RA-mediated differentiation with the effects elicited by BMPs an analysis was carried out with markers specific for the neuronal, endodermal and osteogenic/chondrocytic pathways on NT2D1 monolayer cultures treated with RA or BMP over a 14 day period. While an extensive neurofilament network was evident in NT2D1 cultures treated with RA for 14 days, no evidence of neurofilament staining (NF-165) was present is parallel NT2D1 cultures treated with BMP-7 (Fig. 2), indicating that the neuronal phenotype is not a feature of BMP-mediated differentiation, as previously reported (Andrews *et al.*, 1994). It has been reported that certain human EC cell lines differentiate into endodermal cell lineages upon RA treatment (Roach *et al.*, 1994). Northern blot analysis of the expression of the endodermal markers HNF-3α and -β in NT2D1 cells treated with BMP-7 or RA over a prolonged period was therefore carried out. An induction of these endodermal markers during the late (from 6 days onwards) stage of RA-mediated differentiation was evident, but no induction was observed in parallel BMP-treated cultures (Fig. 3). An analysis of the expression of markers associated with the chondrogenic (matrix GLA protein) and osteogenic (osteocalcin) lineages by Northern

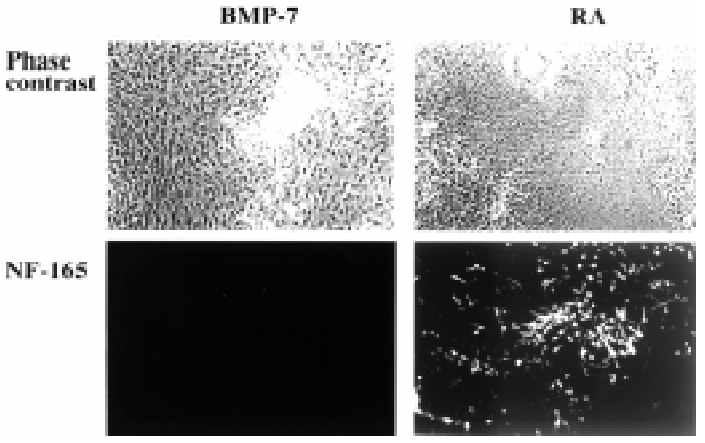

Fig. 2. Immunocytochemistry of human NT2D1 cell aggregates cultured in the presence of RA (10 μM) or BMP-7 (25 ng/ml) for 14 days. *Phase contrast and NF-165 staining, revealing the presence of neuronal cell types in RA-treated but not in BMP-treated cultures.*

blotting also failed to demonstrate expression of these markers in BMP or RA treated cultures (not shown). Thus the type of differentiation elicited by BMPs in NT2D1 cultures differs from that elicited by retinoic acid, as reported previously (Andrews *et al.*, 1994) and the identity of the differentiated cells resulting from BMP treatment is unlikely to fall within the neuronal, endodermal or chondrogenic/osteogenic classes.

BMP-7 activation of SMAD-specific transcription in NT2D1 cells

TGFβ superfamily members are thought to act via the SMAD class of intracellular signal transduction mediators, a family of related proteins similar to *D. melanogaster* Mothers against Decapentaplegic and *C. elegans* Sma (reviewed by Massague, 1998). RT-PCR analysis revealed that the common SMAD-mediator *SMAD*4 and the BMP-specific SMADs (*SMAD*1 and 5) are expressed in NT2D1 cells (data not shown). As evidence for SMAD involvement in BMP-effects in NT2D1 cells, we investigated the responses of a SMAD-dependent reporter (Jonk *et al.*, 1998) in NT2D1 cultures subjected to transient transfection and subsequent treatment with 50 ng/ml BMP-7, 50 ng/ml Activin A, 20 ng/ml TGFβ or 10 μM retinoic acid for 48 h. This reporter can respond to signalling by BMPs, activin and TGFβ. All three members of the TGFβ superfamily, but not RA, induced reporter expression significantly, indicating that the signal transduction pathways for BMPs, activin and TGFβ are functional in NT2D1 monolayer cells (Fig. 4). In spite of this, BMPs but not activin can induce differentiation as judged by loss of stem-cell specific morphology and stage-specific antigen expression (Caricasole *et al.*, 1997). Although the differentiation-inducing potential of TGFβ in NT2D1 cells is not known, Weima *et al.* (1989) failed to detect differentiation in monolayer cultures of the related T2cl13 line treated with TGFβ.

Differentiation of NT2D1 cells mediated by BMP-7 or RA: a comparison

In order to identify differences in the profile of gene expression between BMP- and RA-induced derivatives we carried out a comparative analysis of expression of a panel of markers. BMP-7 was selected as a representative BMP (of the three BMPs tested). NT2D1 monolayer cultures were treated in parallel with 25 ng/ml BMP-7 or 10 μM RA and total cellular protein was isolated and analyzed by Western blotting, employing a panel of specific antibodies (Fig. 5). Differentiation induced by both BMP-7 and RA is characterized by a decrease in protein levels of the activin binding protein follistatin, a stem cell marker in the human EC cell lines (de Jong *et al.*, 1993). A pronounced decrease in proliferating cell nuclear antigen (PCNA; a proliferation-related antigen) protein levels in BMP-treated cultures (and to a much lesser extent in RA-treated cultures) is indicative of decreased proliferation rates, consistent with previous findings (Andrews *et al.*, 1994). The rapid and strong increase in c-JUN protein levels associated with both BMP and RA mediated differentiation of NT2D1 cells is consistent with findings in the mouse EC and ES cell systems (e.g. de Groot *et al.*, 1990 and 1991). Production of extracellular matrix components seems to be differentially modulated by BMP or RA in NT2D1 cells, with laminin and fibronectin levels increasing in BMP-treated cultures but not in RA treated cultures. Finally, the expression of the retinoic acid receptors (RAR) α, β and γ was analysed. All RARs were induced by RA in NT2D1 cells (as previously reported, e.g. Moasser *et al.*, 1994). Interestingly, induction of RARβ protein levels was observed in BMP-treated cultures, albeit at later time points. Thus induction of differentiation by both BMP and RA is associated with a decrease in cellular proliferation and in follistatin protein levels, and increased c-JUN protein levels. Differences are observed with regard to the expression of the extracellular matrix proteins laminin and fibronectin, and of the RARs (with the exception of RARα). The observed increase in RARβ protein level in BMP-treated cultures could indicate a role for RARβ in BMP-mediated differentiation of NT2D1 cells. This prompted us to investigate the expression of the RARβ gene during the early stages of NT2D1 differentiation. Northern blot analysis was carried out to investigate the expression of the stem cell-specific marker hGDF3 (human Growth-Differentiation Factor-3; Caricasole *et al.*, 1998), RARα, RARβ and SMAD-7, an inhibitory SMAD whose expression is known to be rapidly induced by TGFβ family members (Nakao *et al.*, 1997). As shown in Fig. 6, expression of the stem cell-specific marker hGDF3 falls below the detection limit by 4 days of differentiation in both BMP and RA-treated cultures. Induction of RARβ expression in BMP-treated cultures is con-

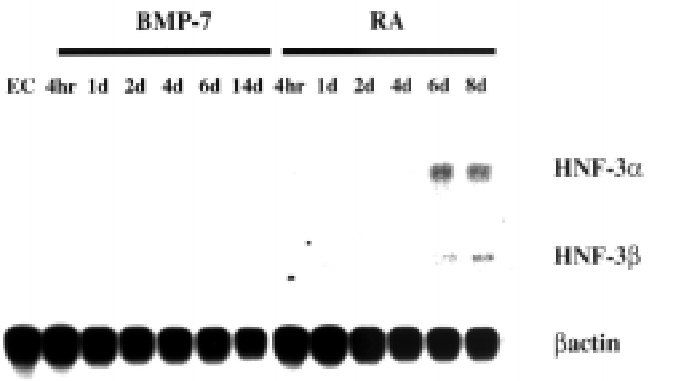

Fig. 3. Northern blot of polyadenylated RNA (2 μg) from NT2D1 cell monolayers cultured in the presence of BMP-7 (25 ng/ml) or RA (10 μM) for the indicated time. *Blots were analyzed with HNF-3α and -3β probes. A β-actin probing provided a loading control.*

firmed by RNA expression analysis, but this induction occurs after 4 days of treatment and is therefore unlikely to represent an early effect of BMP stimulation. By contrast, increased steady-state levels of SMAD-7 transcripts are observed by 4 h of treatment with BMP. Expression of both RARα and RARβ is rapidly increased upon RA treatment, consistent with previous findings (e.g. Moasser *et al.*, 1994).

BMP-7 induces epithelial differentiation in NT2D1 cultures

Several reports have pointed to BMPs as factors capable of promoting the differentiation of embryonal stem cells towards the epithelial phenotype (Hoodless and Hemmati-Brivanlou, 1997, and references therein). Immunocytochemistry with a pan-keratin-specific antibody revealed that BMP treatment of NT2D1 cultures for 6 days results in enhanced expression and organization of keratins, indicative of epithelial differentiation (Fig. 7). This is in contrast to treatment with RA for the same time period, which results in little change in expression of keratins relative to control (EC) NT2D1 cultures (Fig. 7). Enhanced expression of keratins in BMP-treated NT2D1 suggests an epithelial phenotype as the end-point of BMP-mediated differentiation in this cell system. To confirm the immunocytochemical data over a more comprehensive time period, a Western blotting study was performed employing the anti-pan cytokeratin antibody on total cell lysates of control NT2D1 monolayer cultures and monolayer cultures treated with BMP-7 or RA for up to 14 days. Similarly, lysates from control NT2D1 cell aggregates or aggregates cultured in the presence of BMP-7 or RA were also tested with the same antibody. The results are shown in Fig. 8A and B, respectively. In monolayer cultures, cytokeratin levels increased rapidly in cultures treated with BMP-7, reaching high levels of expression by day 14 of differentiation. RA treatment of NT2D1 monolayers also resulted in high levels of cytokeratin expression by day 14 of treatment, but a difference in the kinetics of cytokeratin expression was noted. In aggregated NT2D1 cultures, a clear difference in cytokeratin expression was noted between BMP- and RA-treated cultures, with high levels of keratin expression being reached in BMP-7 treated cells only.

In order to investigate the observed effects of RA on cytokeratin expression in NT2D1 cells, the effects of RA and BMP treatment on the expression of endogenous BMP genes (BMP -2, -4 and -7) in NT2D1 cells was analysed. It is in fact known that RA can induce expression of BMP-2 in F9 mouse EC cells (Rogers *et al.*, 1992), and induction of cytokeratins by RA during the late phases of differentiation might conceivably be mediated by a surge in BMP expression. BMP-2, -4 and -7 expression was therefore investigated by Northern blotting of polyadenylated RNA from NT2D1 control monolayers and monolayers treated with BMP-7 or RA over a comprehensive time course (Fig. 8C). No BMP-2 expression was detected in any of the samples (data not shown). BMP-4 expression in NT2D1 EC cells is barely detectable under the conditions employed, but increases rapidly and to high levels as a response to BMP-7 treatment. No significant induction of BMP-4 expression is observed in RA-treated cultures. While BMP-4 expression is induced by BMP-7 in NT2D1 cells, expression of BMP-7 (detectable in EC cells) is rapidly downregulated by culture in the presence of BMP-7. However, RA treatment results in increased levels of BMP-7 transcripts from day 4 of treatment onwards. This surge in BMP-7 expression, coupled with a downregulation in follistatin expression (Fig. 5), might result in the availability of bioactive BMP-7 in the medium and therefore provide an explanation for the observed increased levels of cytokeratin expression at later time points.

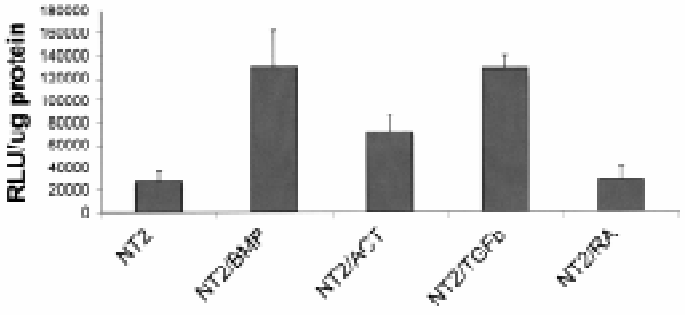

Fig. 4. SMAD-dependent transcriptional response in NT2D1 cells. *Cells were cultured in the absence or presence of BMP-7 (50 ng/ml), activin A (50 ng/ml), TGFβ (20 ng/ml) or RA (10 μM), as judged by transient transfection with the* SMAD*-response reporter 3x(CAGACA)pGL3-Ti (Jonk et al., 1998). Shown are the averages of duplicate samples from 4 independent experiments.*

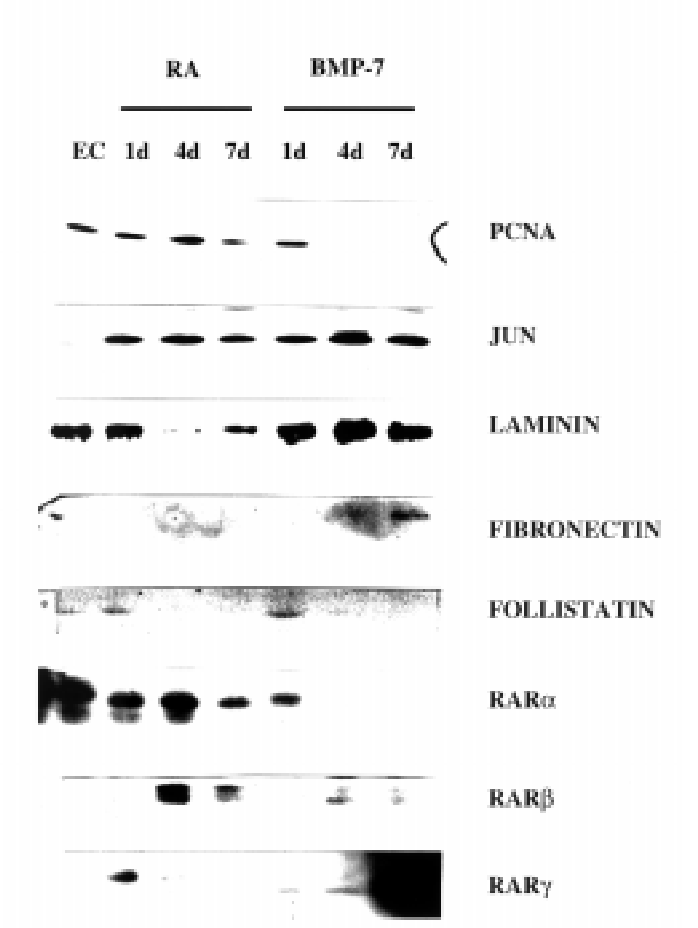

Fig. 5. Profile of marker protein expression during differentiation of NT2D1 cells. *Western blot of total cellular protein (60 μg) from NT2D1 cell monolayers cultured in the presence of BMP-7 (25 ng/ml) or RA (10 μM) for the indicated time, and probed with a panel of antibodies specific for PCNA, c-JUN, Laminin, Fibronectin, Follistatin and the RARs (see Materials and Methods for details).*

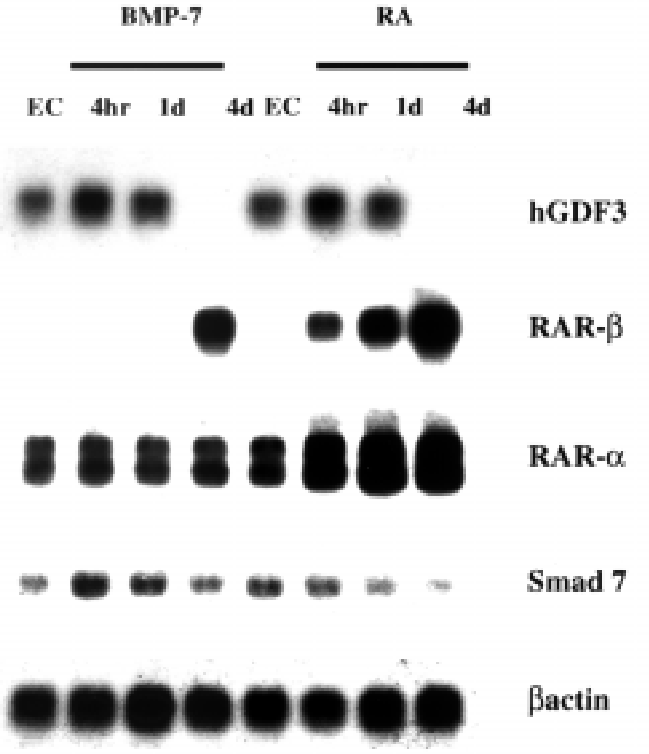

Fig. 6. Profile of marker gene expression during differentiation of NT2D1 cells. *Northern blot of total RNA (40μg) from NT2D1 cell monolayers treated with BMP-7 (25 ng/ml) or RA (10 μM) for the indicated time, and analyzed with probes specific for hGDF3, RARβ, RARα and* SMAD-7. *A β-actin probing provided a loading control.*

Discussion

Human EC cells serve as a model system for the stem cells of both early human embryos and human testicular germ cell tumours and as such they constitute common ground between developmental biology and oncology. An understanding of the factors controlling the balance between proliferation and differentiation in these cell systems is likely to provide clues as to the processes controlling early human embryogenesis and germ cell oncogenesis. In *Xenopus laevis* embryos, BMPs have been characterized as inhibitors of neurogenesis and inducers of epidermal differentiation (e.g. Wilson and Hemmati-Brivanlou, 1995). More recently, evidence obtained in mouse P19 EC cells demonstrated that BMP-4 can interfere with RA-mediated neural differentiation and induce expression of keratins, indicative of epithelial differentiation (Hoodless and Hemmati-Brivanlou, 1997). Thus, ectodermal patterning mediated by BMPs may be conserved between amphibians and mammals. The finding that BMPs can induce differentiation of the NT2D1 human EC cell line (Andrews *et al*., 1994), a human EC cell line displaying neuronal differentiation upon RA treatment (Andrews *et al*., 1984), indicates that BMPs may also regulate ectodermal differentiation in models of early human embryogenesis and TGCTs. Little is known of the changes in gene expression occurring during BMP-mediated differentiation of human EC cells and how these compare with RA-mediated differentiation. In particular, no clues exist as to the end-point reached in BMP-treated cultures. In the present study we aimed at defining the type of differentiation elicited by BMPs in NT2D1 cells. All BMPs tested (BMP-2, -4, -7) were found capable of inducing differentiation in NT2D1 cells at ng/ml amounts. Despite the presence of an apparently functional SMAD response to BMPs, TGFβ and activin in NT2D1 cells, a clear differentiation phenotype in monolayer cultures has only been observed in the presence of BMPs (this report and Caricasole *et al*., 1997). This differentiation was analysed through the employment of a panel of molecular markers specific for stem cells or their differentiated derivatives. BMP-7 elicits downregulation of Tra-1-60, hGDF3 and follistatin expression (stem cell markers; Andrews *et al*., 1994; Caricasole *et al*., 1998; de Jong *et al*., 1993), indicative of differentiation. Further evidence in support of an induction of differentiation comes from the observation that expression of extracellular matrix components (laminin, fibronectin), of c-JUN and at least one of the RARs (RARβ) is induced in BMP-treated NT2D1 monolayers. A substantial decrease in PCNA expression is consistent with the reported BMP-mediated inhibition of NT2D1 cell proliferation (Andrews *et al*., 1994). Downregulation of stem cell markers and induction of c-JUN and RARβ expression are features common to both RA- and BMP-mediated differentiation of NT2D1 cells. However, increased levels of expression of SMAD-7, of BMP-4 and of extracellular matrix proteins are a feature of BMP-mediated differentiation, while expression of RARα, RARγ , HNF-3α, HNF-3β and neurofilament protein are a characteristic of RA-mediated differentiation. Through the use of an anti-pan cytokeratin antibody (recognizing cytokeratins 1-19), we characterized the type of differentia-

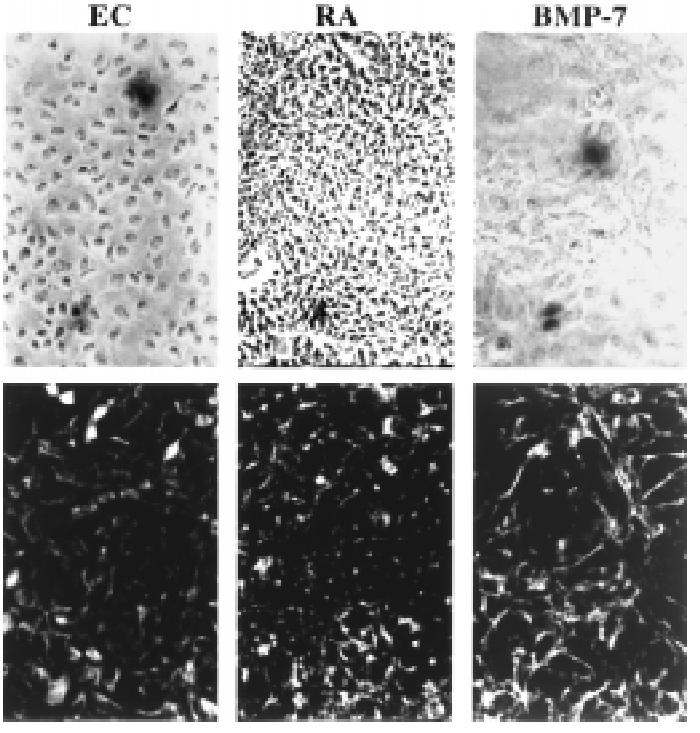

Fig. 7. Immunocytochemistry of human NT2D1 EC cell monolayers cultured in the absence or presence of RA (10 μM) or BMP-7 (25 ng/ml) for 6 days. *Phase contrast (top row) and staining with a pan-cytokeratin antibody (bottom row), showing increased and organised cytokeratin staining in BMP-treated cultures.*

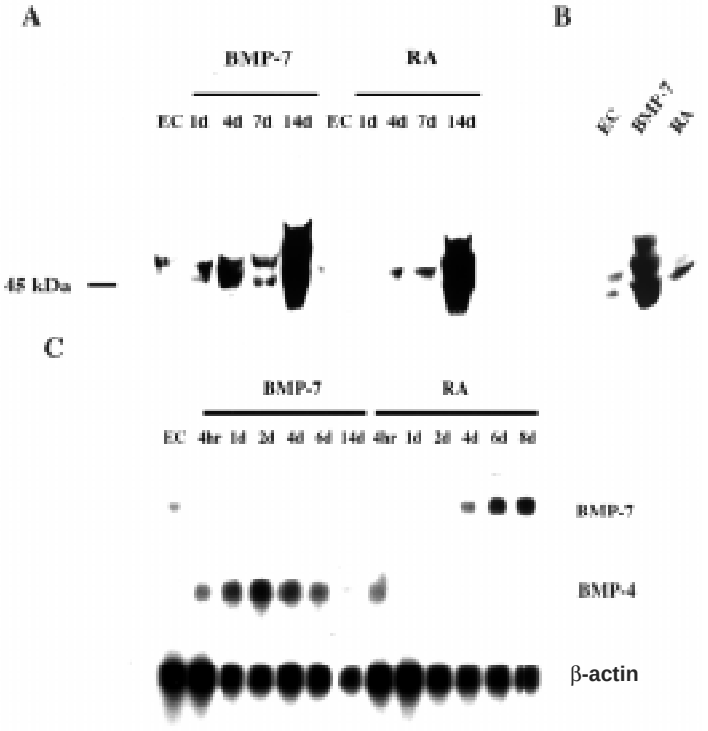

Fig. 8. Expression of cytokeratins and of BMP genes in NT2D1 cells cultured in the presence of BMP-7 or RA. (A) *Western blot of total cellular protein (120 μg) from NT2D1 cell monolayers cultured in the presence or absence of BMP-7 (25 ng/ml) or RA (10 μM) for the indicated times, and analysed for cytokeratin expression employing a pan-cytokeratin antibody, demonstrating increased expression of cytokeratins in BMP-7 and RA treated cultures.* **(B)** *Western blot of total cellular protein (120 μg) from NT2D1 cell aggregates cultured in the presence or absence of BMP-7 (25 ng/ml) or RA (10 μM) for 14 days, and analysed for cytokeratin expression employing a pan-cytokeratin antibody. Note increased expression of cytokeratins in BMP-7 but not RA treated cultures.* **(C)** *Northern blot of polyadenylated RNA (2 μg) from NT2D1 cell monolayers cultured in the presence of BMP-7 (25 ng/ml) or RA (10 μM) for the indicated time, and analysed for BMP-4 and BMP-7 expression by employing specific probes. A β-actin probing provided a loading control.*

tion induced in NT2D1 by BMPs as predominantly epithelial, with no evidence of neuronal or endodermal differentiation as judged by the absence of expression of markers specific for these lineages. While expression of cytokeratins is not a predominant feature of the early phases of RA-mediated differentiation, significant levels of cytokeratins are evident at later differentiation time points. This might be explained by the surge in BMP-7 expression observed during later stages of RA-induced differentiation, which is coincident with the decreased expression of the activin/ BMP-binding protein, follistatin (e.g. see Iemura *et al.*, 1998). Thus, although the conclusions of Andrews *et al.* (1994) regarding the different end-point reached in BMP-treated as opposed to RA-treated NT2D1 cultures are essentially correct, it is clear that the two end-points share an epithelial component, which in the case of RA-induced differentiation might be induced by the observed surge in BMP-7 expression. As to the type(s) of epithelial differentiation elicited in NT2D1 cells by BMPs, it will be of interest to determine the specific type of keratin(s) expressed in fully differentiated cells with the aid of keratin-specific antibodies.

The mechanisms controlling the potency, proliferation and differentiation of human TGCT stem cells are still largely unknown. The known factors which can induce differentiation of pluripotent human EC cells *in vitro* (e.g. RA) are active at concentrations which are unlikely to be present *in vivo*. In the light of the present findings and of those of Andrews *et al.* (1994) it would be of great interest to determine whether BMPs are expressed in TGCTs and their environment, and to compare expression of BMP receptors with tumour histology. The creation of NT2D1 lines stably expressing a SMAD-responsive reporter would be extremely valuable to determine sites of BMP signalling in tumours generated in xenografts, and would allow an analysis of the correlation between SMAD responses and specific tumour components.

In conclusion, while efforts have been made to understand the molecular and cellular basis underlying the differentiation of murine EC and ES cells, little is known of the genetic events occurring during human EC cell differentiation. An understanding of these events should elucidate the mechanisms underlying the choice between proliferation and differentiation in germ cell tumours as well as in early human development. With the availability of human ES cell lines (Thompson *et al.*, 1998; Shamblott *et al.*, 1998), an important potential application of these lines will be the *in vitro* generation of tissues for transplantation therapy. This requires a knowledge of factors capable of maintaining or differentiating human stem cells, of molecular markers necessary to follow differentiation in culture and of the type of differentiation elicited by individual factors. It is likely that knowledge gathered through the analysis of pluripotent human EC cell lines will form the foundations for the study of human ES cell lines.

Materials and Methods

Tissue culture

NT2D1 human embryonal carcinoma cells (Andrews *et al.*, 1984b) were cultured on gelatinized plastics in a 1:1 ratio of Dulbecco's minimal essential medium and Ham's F12 medium, buffered with $NaCO_3$ (44 nM) in a humidified incubator (7.5% CO_2). The medium was supplemented with 10% fetal calf serum as described before (Caricasole *et al.*, 1998). Differentiation was induced by culture in the presence of recombinant BMP (25 ng/ml; Genetics Institute, USA), RA (10 μM) or a combination of the two

TABLE 1

DETAILS OF PRIMARY AND SECONDARY ANTIBODIES EMPLOYED FOR IMMUNOBLOTTING

Antibody	Reference	Antibody	Reference
PCNA	Santa Cruz cat.n.SC-56	c-JUN	Santa Cruz cat.n.SC-U5
Laminin	Sigma cat.n.L-9393	Follistatin	van den Eijnden-van Raaij, unpublished.
Fibronectin	Sigma cat.n.F-3648	pan-cytokeratin	SIGMA cat.n.C-2562
RARα	Fisher *et al.*, 1994	Neurofilament	anti-NF-165 Developmental Studies Hybridoma Bank. Dept. of Pharmacology and Mol. Sciences, John Hopkins Univ. School of Medicine, Baltimore, MD.
RARβ	Fisher *et al.*, 1994		
RARγ	Fisher *et al.*, 1994		

as indicated. For prolonged culture (longer than 3 days), medium (control or containing BMP, RA or a combination of the two) was refreshed every 3 days. For aggregation experiments, cells were cultured in bacteriological Petri dishes in the absence or presence of differentiation inducers for 3 days, plated out and cultured further for the indicated times. Differentiation was monitored through morphological criteria (e.g. see Andrews *et al.*, 1987), by immunocytochemistry with the Tra-1-60 antibody, recognizing an EC cell-specific antigen (Andrews *et al.*, 1984a, 1994), and by analyzing expression of the stem cell marker hGDF3 (Caricasole *et al.*, 1998). Activin A was a kind gift from Dr. de Waele, Innogenetics, Ghent, Belgium. TGFβ was obtained as described (van den Eijnden-van Raaij *et al.*, 1988).

Transient transfection assays

Cells were transfected according to the calcium phosphate protocol (Sambrook *et al.*, 1989). Briefly, NT2D1 cells were grown to subconfluence on 12 well tissue culture plastics and each well transfected with 2 μg *SMAD*-response promoter pGL3TI-4x(CAGACA) (Jonk *et al.*, 1998) and 1 μg pgk-*LacZ* (as an internal control for transfection efficiency; deWinter *et al.*, 1996) in 1 ml culture medium. Agents (BMP, TGFβ, activin, RA, as indicated) were then added 4 h following transfection, bringing the culture volume to 2ml. Cells were harvested 24 h later, and processed as described (Sambrook *et al.*, 1989) for luciferase, LacZ and protein measurements.

Immunocytochemistry and Western Blotting

Immunocytochemistry was performed essentially as described (Goumans *et al.*, 1998). NT2D1 cells were grown to subconfluence on gelatinized plastics and fixed by incubation in 4% paraformaldehyde in phosphate buffered saline (PBS), followed by incubation with primary antibody at the appropriate concentrations (Tra-1-60, Andrews *et al.*, 1984; pan-cytokeratin, mixture of mouse monoclonals specific for keratins 1-19 from Sigma Chemical Co., cat. n. C-2562). Fluorescent conjugate-coupled anti-mouse secondary antibody was then applied, followed by immunofluorescence analysis. Western blotting was performed as described (Sambrook *et al.*, 1989), employing total cellular protein (60 μg for Fig. 5 and 120 μg for Fig. 8 A,B) primary antibody and horseradish peroxidase-coupled secondary antibody as detailed in Table 1. For detection, the ECL system (Amersham Inc., UK) was employed., followed by exposure of X-ray film.

RNA isolation and Northern blotting

RNA isolation and Northern blotting was performed as described (Caricasole *et al.*, 1998). Two μg of polyadenylated RNA or 40 μg total RNA (as indicated) were loaded onto the gel. For hGDF3, the probe used was that described in Caricasole *et al.* (1998). RAR probes were gifts from Dr. P. van der Saag (Hubrecht laboratory, Utrecht) and were derived from the human RARα cDNA (a 1.8 kb EcoRI fragment) and from the human RARβ cDNA (a 1.4 kb SstI-BamHI fragment) . For SMAD-7, the full length mouse cDNA was employed (Ishisaki *et al.*, 1998). The β-actin probe was described previously (Mummery *et al.*, 1990). For BMP-2, BMP-4 and BMP-7, probes comprising portions of the respective cDNAs were generated from mouse cDNA by PCR using primers as described (Roelen *et al.*, 1997); product specificity was tested by restriction fragment analysis. For Hepatocyte Nuclear factors (HNF-3α and HNF-3β), osteocalcin and Matrix Gla Protein (MGLA), probes were generated from mouse cDNA employing PCR primers specific for the mouse genes as detailed below; product specificity was tested by restriction fragment analysis.

Target	Primer (5' or 3')	Primer sequence (number indicates position of first base in published sequence)	Accession n.
HNF-3α	5'	699-ccaagccgccttactcctaca	X74936
	3'	1252-cgcagatgaagacgctggaga	
HNF-3β	5'	555-caagacataccgacgcagcta	X74937
	3'	1233-gttgaaggcgtaatggtgctc	
Osteocalcin	5'	421-ttctccacagccttcatgtcc	L24429
	3'	940-gcgctgctgtgacatccatac	
MGLA protein	5'	69-cctgtgctacgaatctcacga	D00613
	3'	450-cagccagctaggagatcatgt	

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