TGF- β cooperates with TGF- α to induce the self-renewal of normal erythrocytic progenitors: evidence for an autocrine mechanism

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Simultaneous addition of both TGF- α and TGF- β induces the sustained, long-term outgrowth of chicken erythrocytic progenitor cells, referred to as T2ECs from both chick bone marrow and 2-day-old chicken embryos. By analysis for differentiation antigens and gene expression, these cells were shown to represent very immature haematopoietic progenitors committed to the erythrocytic lineage. T2ECs differentiate into almost pure populations of fully mature erythrocytes within 6 days, when TGF- α and TGF- β are withdrawn and the cells exposed to anaemic chicken serum plus insulin. Outgrowth of these cells from various sources invariably required both TGF- α and TGF- β , as well as glucocorticoids. Proliferating, established T2ECs still require TGF- α , but are independent of exogenous TGF- β . Using a TGF- β -neutralizing antibody or expressing a dominant-negative TGF- β receptor II, we demonstrate that T2ECs generate an autocrine loop involving TGF- β during their establishment, which is required for sustained proliferation. Using specific inhibitors, we also show that signalling via Mek-1 is specifically required for induction and maintenance of cell proliferation driven by cooperation between the TGF- α and - β receptors. These results establish a novel mechanism by which self-renewal of erythrocytic progenitors is induced and establish avian T2ECs as a new, quasi-optimal model system to study erythrocytic progenitors.

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Introduction

Despite much progress in the last few years, the elucidation of the network of growth and differentiation factors controlling the individual fate of erythrocytic progenitor cells remains a major challenge. Besides increasing our understanding of the normal mechanisms involved, the pursuit of this question should also provide us with much-needed tools to dissect the alterations resulting in the leukemogenic transformation process.

We have been addressing those questions in the chicken

erythrocytic progenitor model. Numerous molecules have been described that influence the decision-making of these cells between proliferation, differentiation and apoptosis. Their ability to self-renew (i.e. proliferate without detectable differentiation) can be controlled by cooperation between various ligands of membrane receptors and of steroid receptors. These are: TGF- α (Pain *et al.*, 1991), stem cell factor (SCF) plus TGF- α (Hayman *et al.*, 1993; Steinlein *et al.*, 1995), SCF plus Epo (Wessely *et al.*, 1999), estradiol (Schroeder *et al.*, 1993) and glucocorticoids (Wessely *et al.*, 1997a). The small lipophilic molecules retinoic acid (RA) and triiodothyronine (T3), acting through their respective nuclear receptors, have been shown to represent key players in the control of the commitment process that leads from a self-renewing cell to a committed cell (Gandrillon *et al.*, 1994; Bauer *et al.*, 1998; Gandrillon and Samarut, 1998). Once committed, these cells either die by apoptosis or terminally differentiate, depending upon the absence or presence of the hormonal factors erythropoietin and insulin in the culture medium (Gandrillon *et al.*, 1994). Furthermore, those cells can easily be transformed by the avian erythroblastosis virus (AEV), a retrovirus carrying both a growth-promoting component (the *v-erbB* oncogene) and a differentiation-inhibiting component (the *v-erbA* oncogene; for a review see Rascole *et al.*, 1997).

Using the avian erythrocytic progenitor model system, we explored the possibility that other, previously uncharacterized growth factors could participate in inducing the self-renewal of erythrocytic progenitor cells. We decided to test the activity of TGF- β in this system for two main reasons: (i) TGF- β was shown to induce apoptosis in normal chicken macrophages (Solari, 1996). As the growth of contaminating macrophages negatively affects the growth of erythrocytic progenitor cells (Wang *et al.*, 1992), we reasoned that adding TGF- β to the cultures should indirectly boost erythrocytic progenitor cell growth. (ii) Both TGF- α and - β have initially been described as one, unique activity transforming various cell types (De Larco and Todaro, 1978; Roberts *et al.*, 1980). We reasoned that by adding these two molecules together, we might be able to reconstitute such an activity, thereby causing an acceleration of erythrocytic progenitor cell growth.

TGF- β belongs to a very large family of secreted signalling molecules that appear to mediate an incredibly vast array of biological functions ranging from normal growth to developmental activities (for a review see Kingsley, 1994). Although much attention has been focused on the growth-repressing activities of TGF- β , numerous studies have shown that these molecules can also promote growth, depending on the cell type studied and/or on the growth factor combination that is applied to a given cell type. This has been demonstrated for, among others, haematopoietic cells (Keller *et al.*, 1992).

In vitro, conflicting data have been generated concerning the role of TGF- β molecules on erythropoiesis with the description of both an inhibition and a stimulation of erythropoiesis (Mizuguchi *et al.*, 1993; Krystal *et al.*, 1994; Dybedal and Jacobsen, 1995). The phenotype of knockout mice, exhibiting defects in vasculogenesis and haematopoiesis that result in reduced numbers of erythrocytic cells, favour TGF- β being a positive growth regulator *in vivo* (Dickson *et al.*, 1995).

TGF- β is thought to mediate its action through binding to surface receptors consisting of a characteristic combination of type I and type II receptors (Heldin *et al.*, 1997). Other surface molecules do bind TGF- β but their biological role is still obscure (Massague, 1992). The signal is then transduced within the cells via phosphorylation of serine/threonine residues of proteins of the SMAD family. Both activating and inhibitory SMAD proteins have been described with opposite effect upon the final signalling output. The activating SMAD proteins are thought to act via the generation of a heterodimeric complex that translocates into the nucleus to activate transcription of a set of specific genes, many of which are still to be defined (for a review see Heldin *et al.*, 1997) but which could involve AP-1-responsive genes (Zhang *et al.*, 1998). Interestingly, signal transduction via the Mek-MapK pathway interferes with the signalling via Smad 1 by phosphorylation inhibitory sites in the linker region of this molecule, establishing a link between receptor tyrosine kinase- and TGF- β receptor signalling (Kretschmar and Massague, 1998).

In this paper, we demonstrate that the concomitant addition of TGF- α and TGF- β induces an impressive, long-term outgrowth of chicken erythrocytic progenitor cells in suspension. These cells were referred to as T2ECs (for TGF- α /TGF- β -induced erythrocytic cells). T2ECs can be grown in very large quantities resulting from >30 generations in culture, and can be induced to differentiate into mature erythrocytes by the concomitant withdrawal of TGF- α and TGF- β and the addition of anaemic chicken serum plus insulin. These cells require TGF- α , the c-ErbB/EGFR ligand, TGF- β and glucocorticoids for sustained outgrowth and can be established from chick bone marrow from various chicken flocks as well as from 2-day-old chicken embryos. Finally, we present evidence that the establishment of T2ECs requires both TGF- α and TGF- β , but that the cells generate a functional TGF- β autocrine loop during their establishment, most probably induced by the initial addition of TGF- β . This TGF- β autocrine loop was also functional in the establishment of cells previously unrecognized as being TGF- β dependent (TECs and SCF/TGF- α cells). Our results identify the TGF- α plus TGF- β combination as a novel, potent growth-promoting activity acting on normal progenitor cells.

Results

TGF- α and TGF- β cooperate in the outgrowth of immature haematopoietic progenitor cells from chicken bone marrow

Normal bone marrow cells prepared from the tibias of 19-day-old chicken embryos were seeded in a liquid medium containing both TGF- α and TGF- β (LM1; see Materials and methods for details). Small clumps of cells were

already apparent 48 h after the start of the cultures. These cells continued to grow steadily at a very high growth rate for ~1 month (Figure 1A), at which time they stopped proliferating and died, most likely due to cellular senescence.

The observed growth curves differ significantly from those observed with previously used factor combinations (Hayman *et al.*, 1993; Schroeder *et al.*, 1993; Wessely *et al.*, 1997a). First, there is no observable lag time before a measurable growth could be observed, in contrast to cells grown in TGF- α , E2, Dex and chicken serum (Schroeder *et al.*, 1993). Secondly, the cells showed no transient reduction of cell growth as those established in SCF, TGF- α , E2 and Dex (Hayman *et al.*, 1993; Wessely *et al.*, 1997a). Rather, the cells grew at a very high rate once they entered the exponential growth phase, displaying a population doubling time of 18 h. Both of these characteristics translate into a number of cells that can theoretically be obtained (10^{15} – 10^{18} starting from 5×10^5 bone marrow cells) equivalent to >30 cell generations. This *in vitro* lifespan of the TGF- α /TGF- β induced cells is significantly higher than those obtained using previously published protocols (Hayman *et al.*, 1993; Schroeder *et al.*, 1993; Wessely *et al.*, 1997a).

During their exponential growth phase, the TGF- α /TGF- β induced cells grew as extremely large, tightly packed clumps of non-adherent, round and very refractile cells (Figure 1B). Hardly any adherent cells were visible at the time of the first passage and thereafter, in contrast to control cultures in which non-erythrocytic cell types survived in large amounts. This validates our strategy of using TGF- β in order to kill or inhibit adherent macrophagic or fibroblastic cells. In addition, the early absence of non-adherent cells distinguishes this culture system from the previously published ones where adherent cells had to be eliminated through numerous passages of the non-adherent cells.

We then tried to establish the influence of TGF- β mediated inhibition of adherent cell growth on the observed outgrowth of haematopoietic cells. The possible involvement of cell-cell interactions was addressed in a limiting dilution cloning experiment. In this assay, cell-cell interactions are minimized at low cell density, thus involvement of cell-cell interactions would lead to non-linear clonal outgrowth of the cells with respect to cell density (Wessely *et al.*, 1997a). Normal, 19-day embryo bone marrow cells were plated at various densities in semi-solid medium containing both TGF- α and TGF- β , and colonies were counted 1 week later (Figure 1C). The frequency of observed colonies was strictly proportional to the number of seeded cells, even for the lowest cell densities tested. These results strongly argue that the TGF- α + TGF- β combination acts directly on the growing progenitor cells. Our assay also permits estimation of the frequency of cells responding to the TGF- α + TGF- β combination in bone marrow as 1:300, a frequency significantly higher than the one estimated for SCF/TGF- α cells present in the bone marrow of 7-day-old chickens (1:15 000, Hayman *et al.*, 1993).

We also tried to eliminate the influence of myelomonocytic cells through cell sorting. We decided to sort the MEP26-positive fraction of bone marrow cells since this antigen is not expressed on mature cells from the myeloid

Fig. 1. Growth and morphological characteristics of T2ECs. **(A)** Growth curve of bone marrow cells seeded at 5×10^5 cells per ml in LM1 medium. Shown is a curve drawn from raw data obtained in six independent experiments. Each time point corresponds to the mean $\pm$ SD observed in at least two independent determinations. The linear regression method permitted to determine the following equation for the adjusted curve: cell number = $0.299 \times 10^{0.344t}$. Statistical analysis permitted determination of a correlation coefficient $r^2 = 0.97$. **(B)** Phase-contrast microphotography of living T2ECs. **(C)** Limiting dilution analysis. Bone marrow cells were seeded at 33, 330, 3300 and 33 000 cells/well in 346 mm² wells in LM1 medium containing 1.3% methylcellulose. Cells were grown for 1 week and the number of developing colonies were counted. Shown is the mean $\pm$ SD of one experiment performed in duplicate. Error bars values are contained within the symbols used. We calculated a correlation coefficient value $r^2 = 1.00$. **(D)** and **(E)** Micrographs of T2ECs cultures **(D)** and the same obtained after sorting the MEP26-positive fraction of bone marrow cells **(E)** are shown, obtained by staining with Wright and Giemsa stains after cytocentrifugation. Bar = 5 μ m.

and the eosinophilic lineages (McNagny *et al.*, 1992). Furthermore, MEP26 is highly expressed at the surface of the cells responding to the TGF- α + TGF- β combination (see below, Figure 2A). We therefore isolated the MEP26-positive fraction from bone marrow using magnetic beads. The sorted cells, corresponding to $\sim 3\%$ of the initial cell number used, were seeded in LM1. They behaved identically to the non-sorted bone marrow cells, immediately starting to grow as large clumps of round refractile cells. The morphology of the cells was indistinguishable from the morphology of cells growing from non-fractionated cells (Figure 1E), as well as their antigen expression pattern (see below).

Taken together, these data indicate that the TGF- α + TGF- β combination acts directly at the progenitor level.

Light microscopic examination after histological staining of both sorted and non-sorted cells growing in LM1 showed that they were composed of a very homogeneous population of immature haematopoietic cells (Figure 1D and E). Numerous mitotic figures were detected throughout the cultures. No overt sign of differentiation could be detected, even after screening large numbers of cells, except for very rare ($<0.1\%$) fully mature erythrocytes.

Because of their association with the erythrocytic lineage (shown below by their expression of erythrocytic-specific molecules as well as their ability to differentiate into mature erythrocytes), the cells will subsequently be referred to as T2ECs.

The T2EC population contains very early erythrocytic progenitors

In order to determine to which haematopoietic lineage T2ECs belong, we first analysed the surface expression of a number of molecules that have been shown to be specific or characteristic for various haematopoietic lineages and differentiation stages.

As control cells two cell lines were employed: (i) the HD57-E cell line, which is derived from the multipotent HD57 cell line (Metz and Graf, 1991b); and (ii) the HD4 cell line, which is a 100% erythrocytic cell line composed of cells arrested in their differentiation programme at the CFU-E stage by the AEV retroviral oncogenes (Beug *et al.*, 1982).

As expected, no expression of eosinophilic (EOS) or myelomonocytic (Myl 51/2) markers was detected on any of these cell lines (Figure 2A). Both lines, however,

Fig. 2. Surface markers and gene expression in T2ECs. (A) FACS analysis of the expression of specific antigens at the surface of three cell types: two control cell lines (HD4 and HD57E) and a culture of T2ECs. A minus sign denotes the absence of expression of the considered antigen as compared with a negative control (PBS + secondary antibody). One star denotes expression of the considered antigen at the surface of 5–20% of the cells, two stars: 20–85% of positive cells, three stars: 85–100% of positive cells. For the raw FACS data for certain antigens at the surface of T2ECs, see Figures 3 and 5. (B) RT-PCR analysis of gene expression in three cell types: two control cell lines (HD4 and BM2) and a culture of T2ECs. We checked for the expression of a ubiquitously expressed gene (GAPDH) and of three genes known to be expressed in erythrocytic cells (GATA-1 and histone H5) or to be restricted exclusively to the erythrocytic lineage (globin). As a marker for non-erythroid cells, we also analysed the expression of the Spi-1/PU.1 gene, known to be expressed in myeloid and lymphoid cells. By analysing the signal generated by a 1:100 dilution of BM2 cDNA co-amplified with a non-diluted T2EC cDNA, we estimate contaminating myeloid cells to be present at <1%.

behaved like typical erythrocytic progenitors in that they expressed high levels of the transferrin receptor, recognized by the JS8 antibody. Both cell lines are also recognized by the 4.5.A.5 and MC-5J5-B2 antibodies, which stain immature cells from several lineages. Analysis by four other monoclonal antibodies proved to be more informative. The erythrocytic-specific JS4 antibody strongly stained HD4 cells, while it failed to interact with the HD57-E line, suggesting that the latter cells are much more immature than HD4 cells. This immature nature of HD57-E cells was also confirmed by the fact that only

the latter cells expressed high levels of all three MEP antigens tested (specific for E26 transformed multipotent cells), including MEP21 which is most indicative of very immature avian haematopoietic cells (Frampton *et al.*, 1995).

The T2EC cultures displayed a somewhat intermediate phenotype between HD4 and HD57-E cells. T2ECs did not express the most immature MEP21 antigen and very low levels of the JS4 antigen (typical for more mature cells) but did express the two other MEP antigens. This suggests that T2EC cultures contained cells somewhere in between multipotent progenitors and mature CFU-Es. This pattern of antigen expression is very reminiscent of haematopoietic chicken cells transformed by retroviruses expressing PML-RAR α or Δ RAR α plus v-erbB (Altan *et al.*, 1996, 1997).

Furthermore, T2ECs were shown to express high levels of the immature-specific antigens detected by the BEN, MC22.3 and MC-4M12-26 antibodies. None of these three antigens were detected at the surface of the reference cell lines. They could therefore represent antigens specific for very immature yet erythroid-restricted progenitors, besides their reactivity with certain myeloid cell types (Kornfeld *et al.*, 1983).

This pattern of antigen expression is very similar to the one we observed at the surface of erythrocytic progenitors grown in the previously described TGF- α /SCF/E2/Dex combination (Wessely *et al.*, 1997a). Nevertheless, the T2ECs displayed a somewhat more immature phenotype than the SCF/TGF- α cells as evidenced by lower JS4 and higher MEP17 and MEP26 expression levels (data not shown).

Finally, T2ECs were analysed for gene expression by RT-PCR (Figure 2B). Both an erythrocytic (HD4) and a myeloid (BM2) cell line were used as controls. The observed qualitative gene expression pattern was clearly similar between HD4 and T2ECs: both of these cell types displayed the expression of the erythrocytic-specific gene α -globin, and of the histone H5 and GATA-1 genes that are both known to be expressed in erythrocytic cells, among other cell types. This demonstrated that at least some of the T2ECs were erythrocytic in nature. The absence of mature myeloid or lymphoid cells in the cultures was ascertained by the absence of expression of the transcription factor Spi-1/PU.1 (Figure 2B) known to be expressed in various non-erythrocytic lineages, including megakaryocytic, B-lymphoid and myeloid cells (Fisher and Scott, 1998). The Spi-1/PU.1 signal was clearly detected in a mixture of 1% BM2-cDNA mixed with 99% T2EC-cDNA (Figure 2B). Thus, T2ECs contain less than 1% of PU-1 expressing myeloid or lymphoid cells.

We also investigated the possibility that our cultures could contain very immature, multipotent progenitors that could be induced to generate non-erythrocytic haematopoietic lineages. For this, T2ECs were exposed to 100 nM TPA for 1 week, a treatment previously shown to induce the myeloblastic differentiation of Myb-Ets transformed multipotent progenitors (Graf *et al.*, 1992). No differences in cell cycle progression, morphology or differentiation antigen expression (using antibodies to EOS, MYL 51/2, MEP17 and MEP26 antigens) were detected when comparing TPA-treated versus untreated T2EC cultures (data not shown). Similarly, treatment with the myeloid avian

cytokine cMGF (myelomonocytic growth factor; Leutz *et al.*, 1984) did not induce any detectable morphological or antigenic changes (data not shown).

Taken together our data indicate that T2EC cultures contained very immature progenitors apparently restricted to the erythrocytic lineage.

Differentiation ability of T2ECs

We further confirmed the erythrocytic nature of T2ECs by analysing their differentiation potential. T2EC cultures were induced to differentiate by replacing growth-promoting factors (TGF- α , TGF- β and Dex) with a differentiation-inducing cocktail [anaemic chicken serum (ACS) plus insulin]. Cell differentiation was then characterized for various parameters.

Morphological analysis. The final stages of erythrocytic differentiation are characterized by a strong reduction in cell volume and a narrower size distribution (Dolzign *et al.*, 1995). Both of these phenomena were observed in our differentiating T2EC culture (Figure 3A). Starting from ~3 days in culture, we began to observe the appearance of very characteristic oval-shaped normal erythrocytes. After 6 days in the presence of ACS, we consistently observed almost pure populations of morphologically normal erythrocytes (Figure 3B).

Cell cycle parameters. It has been described previously that the initial stages of erythrocytic differentiation results in an increase of the proliferation rate due to a shortening of the G₁ period (Mullner *et al.*, 1996). We verified that these events also occurred in our T2EC cultures: the percentage of cells in G₁ dropped from ~60% to ~50% 24 h after differentiation induction (Figure 3C).

Haemoglobin content. Differentiating T2EC culture showed a very rapid increase in the percentage of benzidine-positive cells reaching close to 100% of positive cells 6 days after the induction of differentiation (Figure 3D).

Changes in differentiation antigens. As expected, we detected a strong reduction in the expression of the immature-specific MEP17 and MEP26 antigens (Figure 3E and F) as well as a dramatic increase in the frequency of cells expressing the mature-specific JS4 antigen at their surface (Figure 3G).

Taken together, these results confirm the erythrocytic nature of T2ECs and establishes its usefulness as a differentiation model.

We then investigated the possibility of analysing the differentiation process at a clonal level. For this, single T2ECs were sorted by FACS in a 96-well plate under 100 μ l of differentiation medium. Colonies showed logarithmic growth at least during the first 3 days (Figure 3H), but the size of the resulting colonies was found to be highly variable (see error bars in Figure 3H). This was still true when the colonies consisted of fully mature erythrocytes at day 6 (data not shown). In order to determine whether the antigenically most immature fraction of T2ECs would have a tendency to form larger colonies, we sorted the 2% of the T2ECs that most strongly expressed the immature-specific MEP17 antigen. In that case, we observed the generation of very large

colonies, containing 500 erythrocytes or more in ~4% of the wells. Assuming symmetrical division, this would represent nine divisions of the cell giving rise to the colony. This demonstrates that T2EC cultures do contain cells that are clearly much more immature than CFU-Es which give rise to colonies of 32 cells or less, i.e. that lie five divisions antecedent to mature erythrocytes.

Growth factor requirements and sources for T2ECs

Having characterized T2ECs as very immature erythrocytic progenitors, we next analysed their growth factor requirements. Normal bone marrow cells were plated in LM1 medium or in LM1 lacking either TGF- α or TGF- β . The outgrowth of T2ECs from bone marrow was strictly dependent upon the presence of both TGF- α (Figure 4A) and TGF- β (Figure 4B). The dependence on TGF- α during both cell establishment and T2EC growth was also shown by the fact that a specific inhibitor of c-ErbB/EGFR (PD153.035; Fry *et al.*, 1994) rapidly inhibited proliferation of T2ECs (data not shown).

We then tested the necessity for the addition of either estradiol or glucocorticoids. Neither were found to be necessary for the initial outgrowth of T2ECs (Figure 4C). Nevertheless, exogenous dexamethasone addition was found to be mandatory for the long-term (>2 weeks) growth of these cells. No estradiol dependence could be detected even on long-term growth. These conclusions were strengthened by the use of specific inhibitors. The addition of an estrogen antagonist (ICI 182.780; Wakeling, 1989; Schroeder *et al.*, 1993) caused no growth retardation until day 15, and a slight proliferation inhibition thereafter (Figure 4D), thereby demonstrating that growth of T2ECs is essentially estradiol independent. In contrast, the addition of a glucocorticoid antagonist (ZK 112.993; Wessely *et al.*, 1997a) suppressed both the outgrowth of T2ECs from bone marrow cells (Figure 4E) and the growth from established T2ECs (not shown). This demonstrates that the initially observed outgrowth of T2ECs in the absence of exogenous dexamethasone addition relied on the presence of sufficient endogenous glucocorticoids within the culture serum and establishes endogenous glucocorticoids as an essential growth factor in that system, similarly to the previously published culture systems (Wessely *et al.*, 1997a).

Normal chicken serum (NCS) has been described as containing an unknown but mandatory activity that was confined to certain serum batches (Schroeder *et al.*, 1993; Steinlein *et al.*, 1995). We therefore tested whether T2ECs were still dependent upon NCS addition. This proved not to be the case and we even detected a slight inhibitory effect of NCS addition on long-term growth (Figure 4F). This suggests that T2ECs are fully independent of chicken-specific factors (like avian Epo; Wessely *et al.*, 1999) for their growth.

TGF- β 1 is one member of a large and very diverse family of secreted signalling molecules active in various aspects of growth control and development. We therefore addressed the question as to whether the observed self-renewal-promoting effect of TGF- β 1 was specific to a particular subgroup within this family or not. The distantly related molecules erythroid differentiation factor (EDF) and bone morphogenetic protein 4 (BMP4; Kingsley, 1994) failed to elicit any outgrowth (Figure 4G), suggesting that

Fig. 3. Differentiation parameters of T2ECs. (A) Cell size distribution. T2ECs grown for 4 days in LM1 (–) or in a differentiation-inducing medium (+) containing ACS plus insulin (see Materials and methods for details) were analysed by FACS for their forward scatter (FSC) parameter that measures the cell size. (B) Morphological analysis. T2ECs grown for 6 days in a differentiation-inducing medium were stained with Wright and Giemsa stains after cytocentrifugation. Bar = 5 μ m. (C) Cell cycle analysis. T2ECs grown for 24 h in LM1 (–) or in a differentiation-inducing medium (+) were analysed by flow cytometry after propidium iodide staining. (D) Benzidine staining. T2ECs grown for 6 days in LM1 (–) or in a differentiation-inducing medium (+) were analysed at different time points for their percentage of benzidine-positive cells. Shown is the mean $\pm$ SD of two independent determinations. (E), (F) and (G) Expression of differentiation antigens. T2ECs grown for 4 days in LM1 (–) or in a differentiation-inducing medium (+) were analysed by FACS for the expression of MEP17 (E), MEP26 (F) and JS4 (G) antigens. The blue line indicates the negative FACS profile of cells incubated with the FITC-labelled goat anti-mouse antibody only. (H) Clonal differentiation analysis. Individual T2ECs were seeded by FACS sorting in 96-well plates in 100 μ l of differentiation medium. The progeny of individual clones was counted at various time intervals after seeding. Shown is the mean $\pm$ SD of the size of 48 clones giving rise to a detectable progeny upon seeding of 186 wells.

the induction of T2EC self-renewal was a TGF- β 1-specific effect. This was confirmed by the observation that both TGF- β 2 and TGF- β 3 failed to induce T2EC outgrowth (Figure 4G).

T2ECs can be established from very early chicken embryos as well as from genetically diverse chickens

Earlier results showed that SCF/TGF- α progenitors could easily be obtained from young SPAFAS chicks, but were more difficult to establish from other chicken flocks. In addition, it was impossible to induce outgrowth of ery-

throid progenitors from 48 h chicken embryos (Schroeder *et al.*, 1993; P.Steinlein and H.Beug, unpublished). Therefore, we investigated various sources of haematopoietic cells for the possibility of establishing T2EC cultures from them. First, we tested the age of the donor and found that T2ECs can be generated from adult chickens up to 6 months old, albeit with a prolonged lag phase (probably due to a lower frequency of T2ECs in the bone marrow cell preparation seeded (Figure 4H). More surprisingly, we could grow T2ECs from 48-h-old chicken blastodermal cells. Outgrowth required a clear lag phase, but then resulted in cells showing a similar, exponential outgrowth to that of T2ECs derived from the bone marrow of chicks (Figure 4I). No response of these early embryonic cells to SCF was detected, as far as proliferation or increased red cell production is concerned (H.Beug, unpublished). Epo-R progenitors could not be grown from the same embryos under a variety of conditions. Like T2ECs from chicks, the 48 h embryo-derived T2ECs expressed various immature antigens, including the MEP26 and MC22/3, two immature-specific antigens not expressed by Epo-R progenitors (Wessely *et al.*, 1999). Thus, the ability of T2ECs to grow out from embryonic blood islands clearly distinguishes them from the erythrocytic progenitor types described previously.

Secondly, we investigated whether the genetic background of the chicken strain used would have a similar influence on T2EC outgrowth to that observed in SCF/TGF- α progenitors. Bone marrow cells of a commercially available, outbred chicken flock were treated similarly to those from SPAFAS chicks and behaved indistinguishably from the latter with respect to outgrowth in the TGF- α + TGF- β combination (Figure 4J). Thus, erythrocytic progenitor self-renewal under the influence of TGF- α + TGF- β seems to be much less dependent upon the genetic background than progenitor outgrowth in media lacking TGF β .

Fig. 4. Growth analysis of T2ECs. (A) Bone marrow cells were seeded in LM1 (+TGF- α) or in LM1 from which TGF- α was omitted (-TGF- α) and counted at various time intervals. (B) Bone marrow cells were seeded in LM1 (+TGF- β) or in LM1 from which TGF- β was omitted (-TGF- β) and counted at various time intervals. (C) Bone marrow cells were seeded in LM1 (+Dex + E2) or in LM1 from which E2 (+Dex -E2) or Dex (-Dex +E2) or both (-Dex -E2) were omitted and counted at various time intervals. (D) Bone marrow cells were seeded in LM1 or in LM1 plus 10^{-6} M ICI 182,780 and counted at various time intervals. (E) Bone marrow cells were seeded in LM1 or in LM1 from which Dex was omitted (-Dex), or in LM1 minus Dex plus 3×10^{-6} M ZK 112,993 (+ZK) and counted at various time intervals. (F) Bone marrow cells were seeded in LM1 (1% NCS) or in LM1 from which NCS was omitted (0% NCS) or in LM1 containing 5% NCS (5% NCS) and counted at various time intervals. (G) Bone marrow cells were seeded in LM1 (+ TGF- β 1) or in LM1 where TGF- β 1 was replaced with TGF- β 2, TGF- β 3, BMP4 or EDF and counted at various time intervals. (H) Bone marrow cells from a 6-month-old male chicken were enriched for progenitors through centrifugation on top of a Lymphocyte Separation Medium layer, seeded in LM1 and counted at various time intervals. (I) Cells prepared from 48-h embryos (blastoderm stage) were seeded in LM1 medium plus SCF, E2 and IGF-1 (open squares) and counted at various time intervals. Similar results were obtained in the absence of SCF, E2 and IGF-1 (data not shown). (J) Bone marrow cells from a commercially available outbred chicken strain were seeded in LM1 and counted at various time intervals.

Continuous proliferation of T2ECs is dependent on the induction and maintenance of a growth-stimulatory TGF- β autocrine loop

Expression of the v-ErbA oncoprotein was previously reported to induce the outgrowth of differentiation-arrested erythrocytic progenitors in the absence of exogenous oncoproteins or receptor tyrosine kinases (Gandrillon *et al.*, 1989; Casini and Graf, 1995). However, this outgrowth still required the cooperation of v-ErbA with endogenously expressed receptor tyrosine kinases such as avian c-ErbB or c-Kit (Schroeder *et al.*, 1990; Pain *et al.*, 1991; Beug *et al.*, 1996). Therefore, we determined whether or not the expression of v-ErbA might alter the TGF- β growth response. For this, fresh bone marrow cells were infected with a retrovirus expressing v-ErbA together with a *Neo*^R selectable gene or, as a control, with a respective empty retrovirus vector. G418-resistant cells were cultivated in LM1 medium, in both the presence and absence of TGF- β . To our surprise, both v-ErbA- and control-vector-infected cells no longer required TGF- β for sustained proliferation (data not shown), suggesting that they had either lost or never developed a dependence on TGF- β for growth.

These findings prompted us to analyse whether or not uninfected bone marrow cells would also require TGF- β for initial outgrowth only, becoming TGF- β independent once established as a mass culture. T2ECs were grown for 1 week in the presence of TGF- α and TGF- β . One aliquot was then shifted to medium lacking TGF- β while the other was cultivated in complete medium. In the absence of TGF- β , T2ECs continued to exhibit sustained proliferation ability, showing only a slight, hardly significant reduction in their growth rate (Figure 5A). One possible explanation for this behaviour was that the cells had started to produce sufficient amounts of their own bioactive TGF- β , which then stimulated cell proliferation via an autocrine loop (see below).

TGF- β was then withdrawn at different time points during the first week of outgrowth, to delineate the time point at which such an autocrine loop was starting to become functional. Cells had to be grown for ~5 days in the presence of exogenous TGF- β before they were able to develop some factor independence (Figure 5B).

We then asked whether this period of exogenous TGF- β requirement was needed for the cells producing bioactive TGF- β to reach a cell density sufficient to establish an autocrine loop or whether autocrine TGF- β production was initially absent and only developed during the first week of cultivation. We therefore tried to enrich the cell populations seeded for T2ECs. For this, we used the previously described MEP26-positive fraction of bone marrow cells, as this fraction gave rise to cells that were morphologically (Figure 1E) and antigenically (Figure 5C) indistinguishable from T2ECs. MEP26-positive cells were sorted from total bone marrow using magnetic beads and incubated at 10^6 cells/ml in either LM1 medium or in the same medium minus TGF- β 1. No significant growth could be detected in the absence of added TGF- β (not shown), therefore suggesting that no autocrine loop is initially active in this MEP26-positive fraction, even when grown at high cell density.

Three types of experiment were then performed to determine whether T2ECs indeed utilized an autocrine loop involving TGF- β , i.e. expressed TGF- β receptors,

synthesized and secreted TGF- β and actually required the produced, autocrine TGF- β for growth. First, we determined whether T2ECs expressed both TGF- β -receptor subunits as well as TGF- β . The chicken homologue of human TGF- β 1 is TGF- β 4, endogenously produced by many chicken cell types (Burt and Paton, 1992). We therefore analysed the transcript levels for TGF- β 4 in T2ECs withdrawn from exogenous TGF- β 1 and restimulated or not stimulated with the same factor, using relative quantification by RT-PCR. All cells invariably produced TGF- β 4 and no significant differences could be detected between stimulated and non-stimulated cells (Figure 6A). Furthermore, both cell types produced similar amounts of TGF- β receptor (TGF- β R) subunit I or II transcripts, again determined using relative quantification by RT-PCR (Figure 6A). T2ECs could also be shown to secrete TGF- β into serum-free medium using an ELISA assay that detects ~80 pg TGF- β per ml of medium (Figure 5D). Finally, the bioactivity of the secreted TGF- β was demonstrated by the fact that the supernatant of T2EC cultures induced a significant growth reduction of the TGF- β -sensitive CCL64 cells (Figure 5E). The inhibition we observed was equivalent to that induced by a TGF- β 1 concentration of ~0.5 ng/ml. This is somewhat higher than that shown by ELISA and therefore suggest that the CCL64 bioassay is more prone to detect chicken TGF- β 4 molecules than ELISA.

The second step to establish that a TGF- β autocrine loop was indeed involved in sustained proliferation of T2EC cells was to demonstrate that interruption of such a hypothetical autocrine loop by a TGF- β neutralizing antibody would indeed interfere with T2EC proliferation. For this, established T2ECs growing in a medium lacking exogenous TGF- β were supplemented with a neutralizing anti-TGF- β antibody. In comparison with cells without antibody, the neutralizing antibody caused a very efficient, although somewhat delayed, reduction in the growth of T2ECs (Figure 5F). We demonstrated that this reduction was not due to some toxic effect of the neutralizing antibody by adding a 100 times excess of TGF- β to the culture medium containing the antibody. This completely reversed the growth inhibition induced by the anti-TGF- β antibody (Figure 5F). These results show that T2ECs indeed require a TGF- β autocrine loop for their sustained proliferation.

In a third series of experiments, we tried to determine whether the development of T2ECs itself required signalling through TGF- β receptors, in addition to their dependence on ligand-activated c-ErbB. The early stages of T2EC cultures were not suitable for biochemical experiments due to insufficient cell numbers. Therefore we made use of the observation that Epo-R progenitors (undergoing self-renewal in the presence of Epo, SCF, Dex and IGF-1; see Wessely *et al.*, 1999, and above) were induced to premature differentiation, cell cycle arrest and apoptosis by the addition of exogenous TGF- β , on top of all previous factors. This suggests that those cells are not dependent upon an autocrine loop of TGF- β for their growth. We nevertheless showed that Epo-R progenitors can be induced to develop, after a lag period, into T2EC-like cells when exposed to TGF- α , TGF- β , estradiol, dexamethasone and IGF-1 (see below).

To study the possible role of TGF- β -dependent signal

Fig. 5. Evidence for an autocrine growth mechanism in T2ECs. (A) Bone marrow cells were seeded in LM1 for 1 week, at which time (arrow) cells were rinsed and replated in LM1 (+ → +) or in LM1 from which TGF- β was omitted (+ → -) and counted at various time intervals. (B) Bone marrow cells were seeded in LM1 for various periods of time (4 days, blue triangles; 5 days, red squares; 6 days, green circles), rinsed and replated in LM1 from which TGF- β was omitted (+ → -) and counted at various time intervals. As a positive control, the growth curve obtained in the continuous presence of TGF- β (LM1) is shown. (C) FACS analysis of the expression of specific antigens at the surface of T2ECs grown from either non-sorted bone marrow cells (red lines) or from the MEP26-positive fraction of bone marrow cells after magnetic sorting (green lines). All cells were grown for >1 week in LM1 before being analysed for their antigen expression pattern. (D) ELISA detection of TGF- β . ELISA detection was performed on the 20-fold concentrated supernatants from both Epo-R and T2EC cultures. The values obtained with Epo-R cells are borderline with respect to the background. Shown is the mean $\pm$ SE of a duplicate determination. (E) Detection of bioactive TGF- β . CCL64 cells were grown for 3 days in the presence of LM1 minus TGF- β (Co), LM1 (+ T β) or LM1 minus TGF- β conditioned by T2ECs (T2EC-CM; see Materials and methods). Shown is the mean $\pm$ SD of two independent experiments performed in triplicate. (F) Bone marrow cells were seeded in LM1 for 1 week, at which time ($t = 0$) cells were rinsed and replated in LM1 (squares), in LM1 from which TGF- β was omitted plus neutralizing anti-TGF- β antibody (circles) or in LM1 plus neutralizing anti-TGF- β antibody plus 100 ng/ml of TGF- β (triangles) and counted at various time intervals. (G) Epo-R progenitors were infected with a retrovirus expressing a dominant-negative form of TGF- β RII (clone 1, filled circles; clone 2, open circles) or left uninfected (open squares), grown in the presence of avian Epo plus SCF and Dex, and counted at various time intervals. (H) Aliquots from the same cell populations as in (G) were switched to a medium where Epo and SCF were replaced by a TGF- α + TGF- β combination and counted at various time intervals.

Fig. 6. Relative quantification by RT-PCR analysis of various genes in cells grown in the absence (–) or presence (+) of TGF- β . (A) Established T2ECs were deprived of TGF- β by a 24 h incubation in the presence of the neutralizing anti-TGF- β antibody. Cells were then incubated for an additional 24 h either in the absence of TGF- β (LM1 from which TGF- β was omitted plus the neutralizing anti-TGF- β antibody) or in the presence of TGF- β (LM1 medium). (B) Unfractionated bone marrow cells were incubated for 24 h either in the absence of TGF- β 1 (LM1 from which TGF- β was omitted) or in the presence of TGF- β 1 (LM1 medium). (C) MEP26-positive bone marrow cells were sorted using magnetic beads and incubated for 24 h either in the absence of TGF- β 1 (LM1 from which TGF- β was omitted) or in the presence of TGF- β 1 (LM1 medium). In all cases, the RNAs were extracted and reverse-transcribed. For relative quantification purposes, dilutions of the RT mixture were used in pilot experiments in order to estimate the region of template amounts that would produce a plateau effect. All further experiments were carried out outside the plateau region, using such conditions that a decrease in template quantities would result in a decrease in the PCR-generated signal (Freeman *et al.*, 1999). In that linear region, three successive dilutions (initial, five times diluted and 25 times diluted) were analysed by PCR. Besides the GAPDH control, three genes were analysed: the TGF- β 4 gene (Burt and Paton, 1992), one class I TGF- β receptor (RPK-2; Nohno *et al.*, 1993) and one class II TGF- β receptor (Barnett *et al.*, 1994). (D) Thrombospondin expression analysis. Chicken embryo fibroblasts (lane 1), MEP26-positive bone marrow cells incubated for 24 h either in the absence of TGF- β 1 (LM1 from which TGF- β was omitted; lane 2) or in the presence of TGF- β 1 (LM1 medium; lane 3) or established T2ECs (lane 4) were analysed for thrombospondin (Lawler *et al.*, 1991) expression levels.

transduction in this development of T2ECs from Epo-R progenitors, we expressed a dominant-negative variant of the TGF- β R subunit II (T β RIIdn) in Epo-R progenitors, using suitable retrovirus vectors. After drug selection, the cells were grown continuously as Epo-R progenitors, supplied with the respective factors. As those cells were not dependent upon an autocrine loop of TGF- β for their growth, the blocking of TGF- β signalling by expressing T β RIIdn did not result in an adverse effect on cell proliferation (Figure 5G). Indeed the cells even increased in growth rate relative to empty vector-infected control cells (not shown) possibly due to protection of the Epo-R cells from the growth-inhibitory effects of TGF- β present in ACS.

A completely different result was obtained when T β RIIdn-expressing and control cells were switched to a medium containing TGF- α and TGF- β , but no SCF or Epo, and compared for proliferation behaviour. Here, the control cells resumed exponential growth after a lag phase, during which the majority of cells died, while outgrowth occurred from persisting clumps of healthy, immature-looking cells. In contrast, the T β RIIdn-expressing cells died rapidly, being completely disintegrated after 7 days, when the control culture was again actively growing (Figure 5H).

These results suggest that signal transduction through the TGF- β R II is required for T2EC establishment from erythrocytic progenitors. Our data demonstrate that during

the first 7–10 days of culture, a TGF- β autocrine loop is turned on, sending via a type II receptor a signal that ultimately results in TGF- β -independent growth of T2ECs.

The growth-stimulatory TGF- β autocrine loop does not rely upon massive induction of TGF- β or TGF- β receptor gene expression

Next, we investigated possible mechanisms that could generate and maintain the described autocrine loop involving TGF- β . The simplest explanation would be that the exogenous TGF- β 1 would generate a signal eventually inducing transcriptional activation of the endogenous TGF- β 4 gene. Such an auto-induction has previously been described for the TGF- β 1 gene in mammalian cells (Kim *et al.*, 1990). We therefore analysed the transcript levels for TGF- β 4 in T2ECs first deprived of TGF- β by addition of a neutralizing antibody and then grown for 24 h in the absence or presence of TGF- β 1. No significant difference could be detected in the amount of TGF- β 4 transcripts using relative quantification by RT-PCR in such cells (Figure 6A). We then reasoned that the up-regulation of one of the two subunits needed to transduce the TGF- β signal could participate in the generation of an autocrine loop. Nevertheless, no significant difference could be detected in the amount of TGF- β receptor I or II transcripts using relative quantification by RT-PCR (Figure 6A).

We then reasoned that the establishment of the autocrine loop might somehow differ from its maintenance. We therefore repeated the above experiments on 'naïve' bone marrow cells. Those cells were grown for 24 h in the absence or presence of TGF- β 1 and analysed for the expression levels of TGF- β 4 and TGF- β receptor I or II transcripts. Again, no significant differences could be detected using relative quantification by RT-PCR (Figure 6B).

It was nevertheless possible that the overall transcript level we detected in bone marrow was indeed due to the overwhelming presence of mature cells of various lineages. We therefore addressed the same question, employing the MEP26-positive fraction described above, which was shown to give rise to cells that were morphologically (Figure 1E) and antigenically (Figure 5C) indistinguishable from T2ECs. MEP26-positive cells were sorted from total bone marrow using magnetic beads and incubated for 24 h in the absence or presence of TGF- β 1. Cells were then analysed for the expression levels of TGF- β 4 and TGF- β receptor I or II transcripts. Again, no significant differences could be detected using relative quantification by RT-PCR (Figure 6C).

The mechanism responsible for the generation of an autocrine loop therefore could be post-transcriptional, or might involve other molecules besides TGF- β and its receptors I and II. For instance, addition of exogenous TGF- β 1 at the start of the cultures could induce an activity converting the secreted, latent and inactive TGF- β 4 form into a bioactive form, as suggested in other model systems (Zeng *et al.*, 1996). One obvious candidate is thrombospondin, a molecule recently identified as a major activator of TGF- β 1 (Crawford *et al.*, 1998) that had been identified in the establishment of an autocrine loop in a different model system (Majack *et al.*, 1986). Although thrombospondin mRNA could be easily detected by PCR in chicken embryo fibroblasts, no such expression could be

Fig. 7. Evidence for an autocrine growth mechanism in TECs and SCF/TGF- α cells. (A) Bone marrow cells were seeded in semi-solid medium for 1 week in the absence (–) or presence (+) of a neutralizing anti-TGF- β antibody and the number of viable TEC colonies were counted. The results are expressed as the percentage of the untreated control (minus the neutralizing antibody). Shown is the mean $\pm$ SE of two independent experiments performed in duplicate. (B) Bone marrow cells were seeded in SCF/TGF- α medium in the absence (–) or presence (+) of a neutralizing anti-TGF- β antibody and counted at various time intervals.

detected in established T2ECs or in the MEP26-positive fraction of bone marrow cells treated for 24 h with TGF- β 1 or not (Figure 6D).

Continuous proliferation of TECs and SCF/TGF- α cells requires induction and maintenance of a growth-stimulatory TGF- β autocrine loop

We then assessed whether or not a growth-stimulatory TGF- β autocrine loop was involved in the proliferation of other, previously characterized immature erythrocytic progenitors. For this TECs (TGF- α -induced erythrocytic cells; Pain *et al.*, 1991) were seeded in semi-solid medium in the absence or presence of a neutralizing anti-TGF- β antibody to determine whether the outgrowth of immature erythrocytic cell colonies was inhibited. Relative to the untreated control cells, the neutralizing antibody caused a dramatic reduction in the number of TEC colonies (Figure 7A).

Very similar results were obtained with SCF/TGF- α cells growing in suspension in chicken serum-containing media (Hayman *et al.*, 1993; Figure 7B). Similar to T2ECs, the outgrowth of these cells was clearly inhibited when

the neutralizing anti-TGF- β antibody was added from the start of the cultures. Taken together, those data show that the induction and maintenance of a growth-stimulatory TGF- β autocrine loop is a characteristic shared by various c-ErbB-expressing progenitors, some of which have never been exposed to exogenous TGF- β .

Establishment and sustained proliferation of T2ECs require cooperation between the TGF- α R and the TGF- β R via the Mek-MapK pathway

Above, we demonstrated that establishment of T2ECs required both TGF- α and TGF- β (Figure 4A and B). In addition, removal of TGF- α from established T2ECs or inhibition of the avian EGF/TGF- α receptor with PD 153.035 caused their rapid growth arrest followed by apoptosis (data not shown). This suggested that a cooperation between the receptors for TGF- α and TGF- β is required for both development and continuous proliferation of T2ECs. It has been shown earlier that SCF/TGF- α progenitors up-regulate expression of the avian EGF/TGF- α receptor (c-ErbB) during their establishment (Steinlein *et al.*, 1995). In addition, recent results by others suggest that signalling via the Ras-MapK pathway inhibits the growth-inhibitory signalling of the TGF- β receptor via Smads (Kretzschmar *et al.*, 1997). We therefore addressed the question of whether or not inhibition of signalling via the Ras-MapK pathway would specifically affect establishment and/or maintenance of T2ECs.

Two inhibitors of the Ras-MapK pathway were selected that were both able to specifically revert Ras/TGF- β -induced epithelial-mesenchymal transition (EMT) in Ha-Ras-expressing mammary epithelial cells, without revealing any toxicity in this system (Oft *et al.*, 1996, S.Gruenert, E.Janda and H.Beug, unpublished). These were: L-739.749, a specific inhibitor of Ha-Ras farnesylation (Kohl *et al.*, 1994) and PD98059, a widely used inhibitor of Mek-1 (Dudley *et al.*, 1995). First, we determined whether these inhibitors would affect proliferation of erythroblasts which do not require cooperation between TGF- α and TGF- β , i.e. mEpoR progenitors (Wessely *et al.*, 1999). These cells express the murine Epo receptor (mEpoR) and require human recombinant Epo, SCF and Dex for proliferation (Wessely *et al.*, 1999). In contrast, TGF- β R signalling is not required for EpoR progenitor growth (Figure 5G). Indeed, TGF- β inhibits their proliferation and induces differentiation or apoptosis (H.Beug, unpublished). L-739.749 inhibited proliferation of mEpoR progenitors even at low concentrations (2 μ M, data not shown) and was therefore unsuitable for our studies. In contrast, even high concentrations of PD98059 (40 μ M) completely failed to affect proliferation of the mEpoR progenitors (Figure 8A, EpoR). However, proliferation of T2ECs was completely inhibited by PD98059 after 4–6 days (Figure 8A, T2ECs). These results suggest that the cooperation between TGF- α and TGF- β in T2ECs requires signalling via Mek-1.

Finally we asked whether the development of T2ECs from EpoR progenitors, shown above to require signalling via the TGF- β R (Figure 5H), is also dependent on signalling via Mek-1. mEpoR progenitors were switched to a medium containing TGF- α and TGF- β , but no SCF or Epo, and cultivated in the presence or absence of PD98059 (Figure 8B). While the cells developed into

Fig. 8. Evidence for the requirement of Mek-1 signalling for proliferation of T2ECs but not mEpoR progenitors. (A) mEpoR progenitors (circles, grown in hrEpo, SCF, E2, Dex and IGF-1) and T2ECs, [squares, cultivated in TGF- α , TGF- β , E2, Dex, IGF-1, as well as a specific inhibitor for c-Kit (Ex50; Wessely *et al.*, 1999)] were cultivated in the presence (filled symbols; +PD) or absence (open symbols; -PD) of 40 μ M PD98059 and counted daily. Cumulative cell numbers have been plotted. (B) mEpoR progenitors were grown either in hrEpo, SCF, E2, Dex and IGF-1 (open squares, EpoR) or switched to medium containing TGF- α , TGF- β , E2, Dex, IGF-1 (TTEDI), either in the presence (filled circles, +PD) or absence (open circles) of 40 μ M PD98059 and subjected to daily counting as above.

T2ECs in the absence of PD98059, as indicated by exponential growth in TGF- α /TGF- β -containing medium after a lag phase of 5 days, outgrowth in the same medium was completely inhibited in the presence of PD98059. These results clearly demonstrate that establishment and continuous proliferation of T2ECs not only requires bioactive receptors for TGF- α and TGF- β , but also a functional Mek-1 kinase, which may be important for cross-signalling between the two receptors (Kretzschmar *et al.*, 1997).

Discussion

T2ECs: a new model system to study decision-making in erythrocytic progenitor cells

Since the initial description that TGF- α induced the self-renewal of chicken erythrocytic progenitor cells (Pain *et al.*, 1991), considerable progress has been made in identifying molecules that could promote, either alone or in combination, such an effect (for a review see Beug

et al., 1996). The T2ECs exhibit two main features that distinguish them from previously described culture systems. First, the cells exhibit a prolonged *in vitro* lifespan, which permits very large numbers of cells to be obtained. Secondly, T2ECs are committed to the erythrocytic lineage, but display a very immature erythrocytic phenotype, characterized by the expression of cell surface markers also expressed on multipotent cells. Despite this immature phenotype, the cells differentiate into almost pure populations of fully mature erythrocytes. These characteristics render the T2EC culture system a particularly valuable model for studying erythropoiesis, permitting access to the most immature stages of erythrocytic differentiation known so far.

Another interesting aspect of T2ECs is that similar progenitors could be grown from very young, 48-h chicken embryos, using the same factor combination. At this stage, primitive, erythrocytic progenitors start to differentiate from haemangioblasts, but terminally differentiated red cells are not yet apparent (Flamme and Risau, 1992). Other factor combinations lacking TGF- β completely failed to yield any outgrowth of primitive erythrocytic progenitors from blastoderms in liquid cultures (H.Beug and O.Gandrillon, unpublished). Pilot experiments suggest that T2ECs from chicken blastoderms may retain the ability to produce embryonic-type globin chains throughout long culture periods, which would allow analysis and manipulation of primitive-definitive haemoglobin switching in a cell culture model.

Are T2ECs transformed or non-transformed cells?

The possibility of obtaining highly immature, fast-growing progenitors raises the intriguing question as to whether those cells should be considered as transformed cells or not. Although we do reconstitute a transforming activity, by adding TGF- α and TGF- β together (De Larco and Todaro, 1978; Roberts *et al.*, 1980; note that this activity was characterized at that time as 'reversible'), we nevertheless feel that T2ECs should be considered 'normal' based on the following arguments: (i) no growth was ever noticed beyond 4 weeks in culture corresponding roughly to 30–35 generations. This is a period of growth only slightly larger than those previously described for 'normal' progenitors (Pain *et al.*, 1991; Wessely *et al.*, 1997a) and corresponds to the 'Hayflick limit' of normal chicken embryo fibroblasts. However, oncogene-transformed chicken bone marrow cells also display a limited *in vitro* lifespan (Ulrich *et al.*, 1992). (ii) T2ECs can be induced to differentiate to a fully mature phenotype at any time in culture. Again, this does not fully qualify as evidence of non-transformation. For example, acute promyelocytic leukaemia cells can also be induced to differentiate by retinoid acid addition (Chomienne *et al.*, 1996). (iii) No exogenous oncogene has been introduced into T2ECs during their establishment and the activation of endogenous proto-oncogenes is highly unlikely. First, T2ECs are mortal, non-immortalized chicken cells. In contrast to immortalized mouse fibroblasts, spontaneous activation of oncogenes has never been reported in normal chicken cells during *in vitro* culture. More importantly, the outgrowth of T2ECs from 18 day embryo bone marrow cells is instantaneous, thus further excluding the remote possibility of selection for oncogene mutations during establishment

of the cultures. (iv) T2ECs remain fully dependent on several factors and hormones during their entire lifespan. Withdrawal of the c-ErbB ligand TGF- α , interruption of the autocrine TGF- β loop by neutralizing antibody or inactivation of the glucocorticoid receptor by addition of a Dex antagonist caused them to stop proliferating. Although factor-dependent leukaemic cell lines do exist, their factor dependence usually differs from that of the respective normal haematopoietic progenitors.

The non-transformed nature of T2ECs therefore is confirmed by the simultaneous fulfilment of all the above 'soft' criteria rather than by one isolated parameter strictly defining normal or transformed cells.

Self-renewal of erythrocytic progenitors: signalling pathways required and *in vivo* significance?

We demonstrate here that at least two different combinations (SCF/TGF- α /E2/Dex or TGF- α /TGF- β /Dex) can induce proliferation without any detectable differentiation (self-renewal) of erythrocytic progenitor cells. A third, totally different factor combination (Epo, SCF and Dex) likewise induces self-renewal, although spontaneous differentiation of a fraction of the progenitors is clearly enhanced in these cells (Wessely *et al.*, 1999).

TGF- β mostly signals through pathways (Smads) that are unrelated to those utilized by the former receptors (Linnekin *et al.*, 1997 and references therein; Massague *et al.*, 1997). However, evidence for alterations of Smad signalling via the Ras–MapK pathway does exist (Kretzschmar *et al.*, 1997), and we have presented evidence (Figure 8A and B), that establishment and maintenance of T2ECs requires Mek-1, likely to function in this cross signalling between receptor tyrosine kinases and the TGF- β receptor.

Another group of signal transduction intermediates possibly relevant for erythroblast self-renewal are the signal transducers and activators of transcription (STATs). The ability of c-ErbB (plus TGF- β R) and Epo-R/c-Kit to induce self-renewal is correlated with a similar ability of both receptor combinations to activate STAT5-dependent gene transcription (Mellitzer *et al.*, 1996; Wessely *et al.*, 1998, Wessely *et al.*, 1999). STAT5 was also reported to interact and cosignal with the GR (Stoecklin *et al.*, 1996, 1997). This correlative evidence has to await confirmation by more direct data (e.g. use of dominant-negative STAT5 or analysis of STAT5-deficient mice).

Is there any *in vivo* relevance of erythrocytic progenitor self-renewal? A positive answer may be given with respect to Epo-R progenitors. Both c-Kit and the Epo-R are required for erythropoiesis (Huang *et al.*, 1990; Wu *et al.*, 1995). In addition, recent evidence using adult mice expressing a defective GR (Reichardt *et al.*, 1998) suggests that the GR may be required for the expansion of the erythrocytic progenitor compartment during stress erythropoiesis (A.Bauer, F.Tronche, O.Wessely, C.Kellendonk, H.M.Reichardt, P.Steinlein, G.Schutz and H.Beug, in preparation).

The potential *in vivo* relevance of T2ECs is much less clear. The phenotype of TGF- β 1–/– mice does show defects in haematopoiesis resulting in reduced numbers of erythrocytic cells (Dickson *et al.*, 1995). In contrast, mice lacking EGFR/c-ErbB have no obvious defect in erythropoiesis. However, the EGFR could be expressed

in normal murine Epo-R progenitors and was able to drive the proliferation of these cells in cooperation with Dex, in the absence of a functional c-Kit and Epo-R (E.Deiner, H.Dolznig, J.Hofmann, E.Mullner and H.Beug, in preparation). The question as to whether c-ErbB or related receptor tyrosine kinases cooperate with TGF- β R family signalling or not in murine erythrocytic progenitors is currently under intense investigation.

How do T2EC cells relate to other types of avian erythroid progenitors?

T2ECs share c-ErbB expression and dependence on a c-ErbB ligand (TGF- α) and a GR ligand (Dex) with the so-called SCF/TGF- α progenitors (Hayman *et al.*, 1993). However, SCF/TGF- α progenitors seem to differ from T2ECs in that they also require estradiol for long-term self-renewal (Hayman *et al.*, 1993; Schroeder *et al.*, 1993; Wessely *et al.*, 1999). SCF/TGF- α progenitors express some multipotent cell antigens, but to a lesser extent than T2ECs (this paper; P.Steinlein and H.Beug, unpublished). Interestingly, SCF/TGF- α progenitors require chicken serum (containing relatively high TGF- β levels; H.Beug, unpublished) for their outgrowth from bone marrow. Their initial requirement for TGF- β during outgrowth may have been met by the chicken serum, while they would no longer require exogenous TGF- β after the establishment of a TGF- β autocrine loop (see Figure 7). This assumption would explain several confusing observations. First, the variable outgrowth of SCF/TGF- α progenitors from chicken flocks with different genetic background may be explained by a different response to the limiting amounts of TGF- β present in the sera. If TGF- β is provided in excess, as in the T2ECs, outgrowth occurs in an identical fashion in all chicken flocks tested to date (see Figure 4J). Secondly, colonies of SCF/TGF- α progenitors in limiting dilution assays grew very slowly during the first 5–7 days, but then increased rapidly in growth rate (Steinlein *et al.*, 1995). Again, the initial slow outgrowth may have been caused by the limiting amounts of TGF- β in chicken serum, but cells grew rapidly once the TGF- β autocrine loop had developed. Thus, SCF/TGF- α progenitors may just represent T2ECs established under limiting TGF- β levels and therefore differing in their properties. This idea is supported by our finding that the proliferation of bona fide SCF/TGF- α progenitors was inhibited by TGF- β -neutralizing antibodies (see Figure 7).

Similarly, our finding that the proliferation of TECs is also inhibited by the addition of a TGF- β -neutralizing antibody strongly suggests that TECs might very well also represent T2ECs established under limiting TGF- β levels. TEC outgrowth would therefore rely upon the generation of a cooperative TGF- β autocrine loop (Dawson and Wynford-Thomas, 1995). This would explain why TECs could not be grown in liquid cultures, where the cellular dispersion would make a cooperative TGF- β autocrine loop especially inefficient, by preventing accumulation or trapping of minute amounts of TGF- β .

How do T2ECs relate to the various progenitors that require the c-Kit ligand SCF for sustained proliferation? Recently, we found that common erythrocytic BFU-E/CFU-E progenitors from mice, humans and chickens can be induced to self-renew by the joint action of erythropoietin, SCF and the GR ligand Dex (Beug *et al.*,

1996; Reichardt *et al.*, 1998; Wessely *et al.*, 1999; von Lindern *et al.*, 1999). In the chicken, this progenitor type has been referred to as Epo-R progenitors. These cells do not require TGF- β R signalling for proliferation (as shown by the expression of a dominant-negative mutant of the TGF- β -receptor II, T β RIIdn; see Figure 5). Rather, TGF- β inhibits the growth of Epo-R progenitors and induces differentiation or apoptosis in the presence or absence of Epo/Insulin, respectively (H.Beug, unpublished).

If Epo-R progenitors are grown in the presence of SCF and Dex (lacking Epo), they only transiently proliferate (Wessely *et al.*, submitted). In earlier communications, bone marrow erythroblasts grown in SCF plus chicken serum (containing low levels of Epo and/or GR ligand) showed only transient outgrowth and were referred to as SCF progenitors (Hayman *et al.*, 1993; Wessely *et al.*, 1997b). A similar transient outgrowth caused by SCF alone (again not ruling out the contribution of limiting Epo and GR ligand levels present in serum) was reported for human BFU-E (Sawada *et al.*, 1991; Muta *et al.*, 1995). SCF progenitors might thus represent Epo-R progenitors grown in limiting levels of Epo and/or GR ligand.

A similarly enigmatic issue has been the development of SCF/TGF- α progenitors from SCF progenitors (Steinlein *et al.*, 1995) and the influence of Dex on this process (Wessely *et al.*, 1997a). In TGF- α , estradiol and chicken serum, SCF/TGF- α progenitors grow out from chick bone marrow at very low frequency (1 in 15 000). Addition of SCF increases this frequency to values similar to those obtained with the transiently growing progenitors obtained in SCF alone (1 in 500). We show here that T2ECs can develop from Epo-R progenitors and that this development is inhibited by T β RIIdn (see Figure 5). We also have evidence that SCF/TGF- α progenitors and T2ECs can develop back into Epo-R progenitors, if we inhibit c-ErbB signalling by a specific inhibitor (PD 153.035; Fry *et al.*, 1994) and provide the cells with Epo, SCF and Dex (H.Beug, unpublished). These cells no longer require TGF- α or TGF- β for proliferation.

Taken together, these findings suggest that the compartment of erythrocytic progenitors is highly plastic, consisting of interconvertible populations of cells displaying different stages of maturity and a highly variable pattern of plasma membrane receptor expression. It will be interesting to determine if and how this erythrocytic progenitor plasticity can be reconciled with the prevailing linear model of erythrocytic differentiation, leading from BFU-Es via CFU-Es to erythrocytes.

A growth-promoting, autocrine loop involving TGF- β in normal erythrocytic progenitors

In most cell types, TGF- β induces growth inhibition and apoptosis. This is true also in situations where TGF- β autocrine loops mediate negative regulation of cell growth (Falk *et al.*, 1991; Fiorelli *et al.*, 1994; Pratsinis *et al.*, 1997). To our knowledge, T2ECs are the first haematopoietic cell system in which a TGF- β autocrine loop is involved in positive growth control. It is possible that signalling from TGF- α -activated c-ErbB via the Ras-Mek-MapK pathway inhibits growth inhibitory signalling of the TGF- β R via Smads and that this process is involved in inducing a positive proliferation signal by the TGF- β R. A similar positive effect of an autocrine loop involving

TGF- β has been described recently in mammary epithelial cells transformed by receptor tyrosine kinases or downstream signal transducers (Oft *et al.*, 1996; A.Pacher, M.Oft and H.Beug, in preparation). Here, the cooperation of TGF- β R signalling with the Ras–MapK pathway results in establishment of a TGF- β autocrine loop, causing complete protection from the growth-inhibiting effects of TGF- β and dedifferentiation of the epithelial cells into proliferating cells with a mesenchymal phenotype (Oft *et al.*, 1996). This corresponds closely to our finding here, that the cooperation between EGFR and TGF- β R signalling in T2ECs causes a similar protection from growth inhibition and generation of a more immature erythrocytic phenotype.

In conclusion, TGF- α + TGF- β , the ‘transforming’ activity initially described to transform rat fibroblastic cells (De Larco and Todaro, 1978), has a totally unexpected, proliferation-stimulating and differentiation-inhibiting effect in very different cell types, namely erythrocytic progenitor cells and epithelial cells. This raises the intriguing possibility that induction of proliferation as well as regression to more immature cell types by cooperation of RTKs with members of the TGF- β R family may well be a general phenomenon, to be observed in a multitude of cell types.

Materials and methods

If needed, more detailed protocols are available on request by e-mail from ogandrill@ens-lyon.fr.

Chemicals

TGF- α was purchased from R&D as a lyophilized human recombinant protein. It was resuspended in 0.01 M acetic acid/1% fetal bovine serum (FBS) at a 100 μ g/ml stock solution. TGF- β 1, TGF- β 2 and TGF- β 3 were purchased from R&D as lyophilized human recombinant proteins. They were resuspended in 0.05 M HCl/1% FBS at a 1 μ g/ml stock solution.

Dexamethasone (Dex; Sigma) and β -estradiol (E2; Sigma) were prepared as 10^{-2} M solution in ethanol and subsequently diluted in α -MEM. The ER and GR antagonists ICI 182.780 and ZK 112.993 were kind gifts of ICI (Dr Wakeling) and Schering (Professor M.Schneider), respectively. Stock solutions were made up in ethanol at 10^{-3} M and 3×10^{-3} M, respectively. Phorbol, 20-oxo-20-deoxy-12,13-dibutyrate (TPA; Sigma) was prepared as a 1 mM stock dilution in DMSO and subsequently diluted in α -MEM. EDF (Tsuji *et al.*, 1988) was a gift from Dr Y.Eto (Ajinomoto Co., Inc., Japan) and was provided as a purified human recombinant protein in an acetate buffer solution. cMGF was a gift from Chantal Domenget (ENS de Lyon) and was provided as a crude supernatant from LPS-treated HD11 cells (Leutz *et al.*, 1984). BMP4 was a gift from Dr Pierre Savatier (ENS de Lyon) and was provided as a crude supernatant from COS cells expressing the BMP4 cDNA. Insulin-like growth factor (IGF)-1 was purchased from R&D and used at a final concentration of 40 ng/ml.

Antibodies

The MEP17, MEP21, MEP26, EOS47 and MYL 51/2 antibodies (McNagney *et al.*, 1992) were a gift of Dr T.Graf (EMBL, Heidelberg). The JS4, JS8, MC-4M12-26, 4.5.A.5 and MC-5J5-B2 antibodies have been previously described (Schmidt *et al.*, 1986a).

The MEP antigens are expressed mainly on immature progenitors, whereas the EOS, MYL 51/2 and JS4 antigens are expressed respectively on more mature eosinophilic, myelomonocytic and erythrocytic progenitors (Frampton *et al.*, 1995). The JS8 antibody recognizes the 90 kDa avian transferrin receptor and is quantitatively expressed at higher levels on erythrocytic (mature and immature) cells (Schmidt *et al.*, 1986b).

The BEN antibody has been previously described (Corbel *et al.*, 1992). The MC-4M12-26, 4.5.A.5 and MC-5J5-B2 antibodies recognize immature erythrocytic progenitors but are not specific for the erythrocytic lineage (Schmidt *et al.*, 1986a; this study). The MC 22.3 antibody

recognizes a marker present on multipotent and very immature erythrocytic progenitors (Steinlein *et al.*, unpublished). The neutralizing anti-TGF- β antibody was purchased as monoclonal mouse anti-TGF- β 1, - β 2, - β 3 antibodies from Genzyme (ref. 80-1835-03) and was used at a final concentration of 20 μ g/ml.

Cells

Unless stated otherwise, normal bone marrow cells were prepared from 19-day-old SPAFAS embryos. In most assays, a medium called LM1 (liquid medium 1) was used. This consisted of α -modification of Eagle's medium (α -MEM) containing 10% FBS (Gibco-BRL), 1% normal chicken serum (NCS; Gibco BRL), 10^{-4} M β -mercaptoethanol (Sigma), 5 ng/ml TGF- α , 1 ng/ml TGF- β 1, 10^{-6} M dexamethasone and 1% of a 100 $\times$ antibiotics solution (penicillin plus streptomycin; Flow Laboratories). Any departure from this medium will be referred to in the figure legends.

For standard T2EC cultures, 10^7 bone marrow cells from 19-day SPAFAS embryos were plated in 10 ml of LM1, split 1:10 2 days later, at which time clumps of cells were apparent. The cultures were then kept at a density of $\sim 2 \times 10^5$ cells/ml, by passing them every other day.

The limiting dilution cloning analysis was performed in LM1 containing 1.3% methylcellulose. Bone marrow cells were seeded at 33, 330, 3300 and 33 000 cells/well in 346 mm² wells, grown for 1 week and the number of colonies counted.

The HD4 cell line is a chicken erythroblast cell line composed of cells which are blocked in their differentiation program at the CFU-E stage by the AEV (Beug *et al.*, 1982) and were grown in α -MEM containing 10% FBS, 1% NCS, 10^{-4} M β -mercaptoethanol and 1% of a 100 $\times$ antibiotics solution. The HD57-E cell line was derived from the haematopoietic multipotent HD57 cell line, a gift from Dr T.Graf (EMBL, Heidelberg). These cells were grown in a modified ‘blastoderm medium’ (Metz and Graf, 1991a) in the absence of insulin and with replacement of conalbumin by bovine serum albumin (BSA; Boehringer Mannheim). The BM2 cell line consists of monoblasts transformed by the avian myeloblastosis virus and was grown as previously described (Bagnis *et al.*, 1993). The culture conditions for TECs and for SCF/TGF- α cells have been described in Pain *et al.* (1991) and Hayman *et al.* (1993), respectively.

The selection for MEP26-positive bone marrow cells was performed by incubating 4×10^7 bone marrow cells with a 1:100 dilution of MEP26 antibody in PBS + 1% BSA for 45 min. The cells were rinsed twice and incubated for 2 h in the presence of 8×10^6 rinsed magnetic dynabeads (Dynal) coated with an anti-mouse antibody. The target cells were then positively isolated using a Dynal MPC magnet, rinsed in PBS + BSA, numbered (usually $\sim 10^6$ cells were obtained) and seeded.

Chicken embryo fibroblasts were grown as described previously (Gandrillon *et al.*, 1987).

CCL64 inhibition assay and TGF- β ELISA

CCL64 cells, kindly provided by Dr C.Mummery and Dr P.Van Arum-Swanink, were grown in Dulbecco's modification of Eagle's medium containing 7.5% FBS, 2 mM glutamine and 1% of a 100 $\times$ antibiotics solution. For assaying TGF- β bioactivity, 2500 CCL64 cells were seeded in 500 μ l per well of a 24-well plate. Two hours later, the supernatant was replaced by the T2EC-conditioned medium (see below) and cells were grown for an additional 3 days, at which point the relative number of cells was determined using a WST-1 test (Boehringer Mannheim) according to manufacturer's instructions. T2EC-conditioned medium was prepared by incubating 2×10^7 cells in 4 ml of LM1 devoid of added TGF- β for 24 h. As a positive control, we used LM1 containing 1 ng/ml TGF- β 1.

The TGF- β 1 ELISA was performed using 20-fold concentrated supernatants of T2ECs grown for 24 h in serum-free medium. For this, cells were grown to $\sim 5 \times 10^7$ cells in 20 ml of medium, washed, suspended in serum-free medium [Stem-Pro 34TM (Life Technologies) plus Dex and IGF-1] and incubated at the same concentration overnight. The supernatant medium was concentrated 20-fold, acid-treated to activate the latent form of TGF- β , and dilutions were analysed by ELISA as described (Oft *et al.*, 1996).

Retroviral infection of avian erythrocytic progenitors

A cDNA encoding a dominant-negative mutant of the human transforming growth factor β receptor subunit II (T β RII-K277R c-DNA; Wrana *et al.*, 1992) was inserted into the pOGI avian retroviral construct. The construction and properties of this retrovirus will be described elsewhere (M.Kieslinger, I.Woldmann, R.Moriggl, J.Hofmann, J.Ihle, H.Beug and T.Decker, manuscript in preparation). Chicken embryo

fibroblasts producing this virus (pOGITbRIIdn) as well as the empty retroviral vector were then used to infect bone marrow cells from 2- to 10-day-old chicks cultivated in DMEM plus 2% high titre anaemic serum (providing avian Epo), 100 ng/ml avian SCF, 5×10^{-7} M estradiol and dexamethasone and 40 ng/ml human IGF-1. The outgrowing Epo-R progenitors (Wessely *et al.*, submitted) were selected in 700 μ g/ml hygromycin for 6 days. The cells were then expanded to $>2 \times 10^7$ cells and frozen.

Assay for TGF β Rdn function during T2EC establishment

The above mentioned Epo-R progenitors expressing either empty vector or TbRIIdn were cultivated in Epo/SCF-containing medium as described in the previous section. Aliquots were transferred to medium containing 10 ng/ml TGF- α , 2 ng/ml TGF- β 1, estradiol, dexamethasone and IGF-1. Cells were maintained at densities between 2×10^6 and 4×10^6 cells/ml by daily medium changes or passaging, using the respective factor-containing media. Cell proliferation was monitored by daily counting in an electronic cell counter (CASY-I, Scharfe Systems, Germany) and cumulative cell numbers determined.

Flow cytometric analysis

Cell surface antigens were detected after incubation for 20 min at 4°C with the primary antibody diluted 1:100 to 1:10 in DPBS plus 1% BSA. FITC-labelled goat anti-mouse antibody (BioSys) diluted 1:50 was used as the second antibody for 20 min at 4°C.

Cell cycle analysis was performed on cells rinsed in PBS, fixed in cold ethanol (66% final concentration) on ice for 30 min, treated with RNase at 1 mg/ml for 30 min, and stained with propidium iodide at a final concentration of 50 μ g/ml.

In both cases, the fluorescence of individual cells was measured using a FACStar PLUS flow cytometer (Becton-Dickinson). Data acquisition and analysis were performed using the CELLQuest program (Becton-Dickinson).

Cell sorting was performed using the CELLQuest plus CLONE-CYT programs (Becton-Dickinson).

RT-PCR analysis

RT-PCR was performed essentially as described previously (Gandrillon and Samarut, 1998). The primers used, the size of the expected fragments and the PCR conditions are available on request from ogandrill@ens-lyon.fr.

Differentiation assay

T2ECs were induced to differentiate by the concomitant removal of TGF- α , TGF- β and Dex from the medium and the addition of 10 ng/ml insulin and of 5% ACS for the indicated period of time. The preparation of ACS as well as the estimation of the level of differentiation by acidic benzidine staining have been described previously (Gandrillon and Samarut, 1998).

Inhibitor experiments

Two specific inhibitors of Ha-Ras and Mek-1, i.e. L-739,749 (a kind gift from Dr F.Himmelsbach, Boehringer Ingelheim, Biberach, Germany; Kohl *et al.*, 1994), and PD98059 (Calbiochem; Dudley *et al.*, 1995), respectively, were employed to inhibit signal transduction in mEpoR progenitors (Wessely *et al.*, 1999) and T2ECs. The inhibitors were dissolved in DMSO at 20 mM (PD98059) and 10 mM (L-739,749) and, after titration for toxicity (see Results) used at working dilutions of 4 μ M (PD98059) and 2 μ M (L-739,749). Measurement of inhibitor action during T2EC proliferation and T2EC establishment was performed as described above (see assay for TGF β Rdn function during T2EC establishment).

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