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Isoform-Specific Role of Transforming Growth Factor-β2 in the Regulation of Proliferation and Differentiation of Murine Adrenal Chromaffin Cells In Vivo

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Chromaffin cells, the neuroendocrine cells of the adrenal medulla, play an important role in molecular, cellular, and developmental neurobiology. Unlike the closely related sympathetic neurons, chromaffin cells are able to proliferate throughout their whole life span. Proliferation of chromaffin cells in vivo is thought to be regulated by the interaction of neurogenic and hormonal signals. Previous studies have shown that chromaffin cells synthesize and release transforming growth factor-βs (TGF-βs). In the present study, effects of TGF-βs on proliferation and differentiation of chromaffin cells in mouse adrenal chromaffin cells were investigated in a genetic mouse model. We observed a significant increase in the total number of tyrosine hydroxylase-positive (TH^+) cells in *Tgf*β*2*$^{-/-}$ knockout mouse embryos at embryonic day (E) 18.5 compared with wild-type animals (*Tgf*β*2*$^{+/+}$), but no changes in the number of TH^+ cells were observed in *Tgf*β*3*$^{-/-}$ mouse mutants. At E15.5, but not at E18.5, there was a marked increase in the number of proliferative cell nuclear antigen-positive chromaffin cells in *Tgf*β*2*$^{-/-}$ knockout embryos compared with the wild-type group. On the other hand, there was a clear decrease in the ratio of total number of phenylethanolamine-N-methyltransferase-positive cells to the total TH^+ in *Tgf*β*2*$^{-/-}$ mice embryos at E18.5 compared with wild-type animals. This is the first documentation of the physiological significance of the TGF-β2, an isoform that has been suggested to play a role in the regulation of chromaffin cells proliferation and differentiation based on in vitro experiments.

Key words: transforming growth factor-β; tyrosine hydroxylase; phenylethanolamine-N-methyltransferase; proliferative cell nuclear antigen; chromaffin cells

Transforming growth factor-βs (TGF-βs) are multifunctional cytokines with a widespread distribution (Dünker and Krieglstein, 2000). Three highly homologous isoforms of TGF-β are known from several species, including mammals and birds: TGF-β1, TGF-β2, and TGF-β3 (Roberts and Sporn, 1990). All TGF-β isoforms signal via the same heteromeric receptor complex, consisting of a ligand binding TGF-β receptor type II (TβR-II) and a TGF-β receptor type I (TβR-I; Wrana et al., 1992). Signal transduction from the receptor to the nucleus is mediated via memberes of the intracellular effector molecules called *SMADs* (Baker and Harlan, 1997; Heldin et al., 1997). TGF-βs exert a wide range of biological responses on a variety of cells belonging to different organ types. They are involved in the regulation of proliferation; differentiation; survival; extracellular matrix formation; bone, cartilage, and skeletal development; wound healing; haematopoiesis; immune and inflammatory cell responses; and nervous system development (Roberts and Sporn, 1990; Unsicker and Krieglstein, 2000; Böttner et al., 2000). With regard to cell proliferation, it has been shown that TGF-β can function depending on the context, e.g., dependently either promote or inhibit mitosis (Roberts and Sporn, 1990).

Chromaffin cells are neuroendocrine cells of neural crest origin that were termed *chromaffin* by Kohn (1902) because of their characteristic staining property with chrome salts. Unlike the closely related sympathetic neurons, which also originate from the neural crest and probably from a common progenitor cell, chromaffin cells are able to proliferate throughout the life span (Unsicker et al., 1978; Anderson, 1989; Unsicker, 1993a). Even in adult animals, a small fraction of cells is undergoing cell division. The capacity of the cells to divide declines rapidly from birth to adulthood (Malvaldi et al., 1968; Tischler et al., 1989; Verhofstad, 1993). In vivo, proliferation of chromaffin cells is thought to be regulated by interaction of neurogenic and hormonal signals (Tischler et al., 1989). In vitro studies suggested that adrenal cortex-derived glucocorticoid hormones are essential

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inhibitors of chromaffin cell mitosis (Lillien and Claude, 1985; Frödin et al., 1995). Recent studies have shown that TGF-β—synthesized and secreted from developing chromaffin cells—inhibits DNA synthesis of adrenal chromaffin cells (Krieglstein and Unsicker, 1995; Wolf et al., 1999). Reduction of endogenous TGF-β increases proliferation of developing chick adrenal chromaffin cells in vivo (Combs et al., 2000). In the present study, we investigated whether chromaffin cell development in mice is regulated and controlled by TGF-βs and whether TGF-βs may act isoform specifically when regulating chromaffin cell proliferation and differentiation in the murine adrenal gland.

MATERIALS AND METHODS

Animals

*Tgf*β*2*$^{+/-}$ and *Tgf*β*3*$^{+/-}$ heterozygous mice were offspring from breeding pairs kindly provided by T. Doetschman, University of Cincinnati. The generation of these strains and the characterization of the respective knockout phenotypes were previously described (Proetzel et al., 1995; Sanford et al., 1997). Mice heterozygous for TGFβ2 null allele and TGFβ3 null allele present no apparent defects, and no significant embryonic lethality was observed (Proetzel et al., 1995; Sanford et al., 1997). Mice homozygous for TGFβ2 null mutants die from congenital cyanosis shortly before or during birth. Homozygous TGF-β3 mouse mutants die within 24 hr of birth. *Tgf*β*3*$^{-/-}$ pups exhibit a cleft palate and, thus, are unable to suckle, gasp for air, and become cyanotic just before death (Proetzel et al., 1995; Kaartinen et al., 1995). Experiments were performed according to the National Health and ethical regulations. Animals were kept in a regulated environment (23°C ± 1°C, 50% ± 5% humidity) on a 12-hr light:12-hr dark cycle, with food and water ad libitum. At embryonic day (E) 18.5, embryos were transcardially perfused with 4% paraformaldehyde (PFA). Whole adrenal glands were fixed in Bouin's fixative (picrid acid, formaldehyde, and glacial acetic acid) for about 6 hr, dehydrated in a graded series of ethanol, and embedded in paraffin. Serial sections (10 μm) were mounted on glycerin-coated slides, dried at 38°C, and kept at room temperature. E15.5 embryos were fixed by immersion, opening the specimen at the ventral midline to allow the Bouin's fixative to penetrate more easily.

Immunohistochemistry

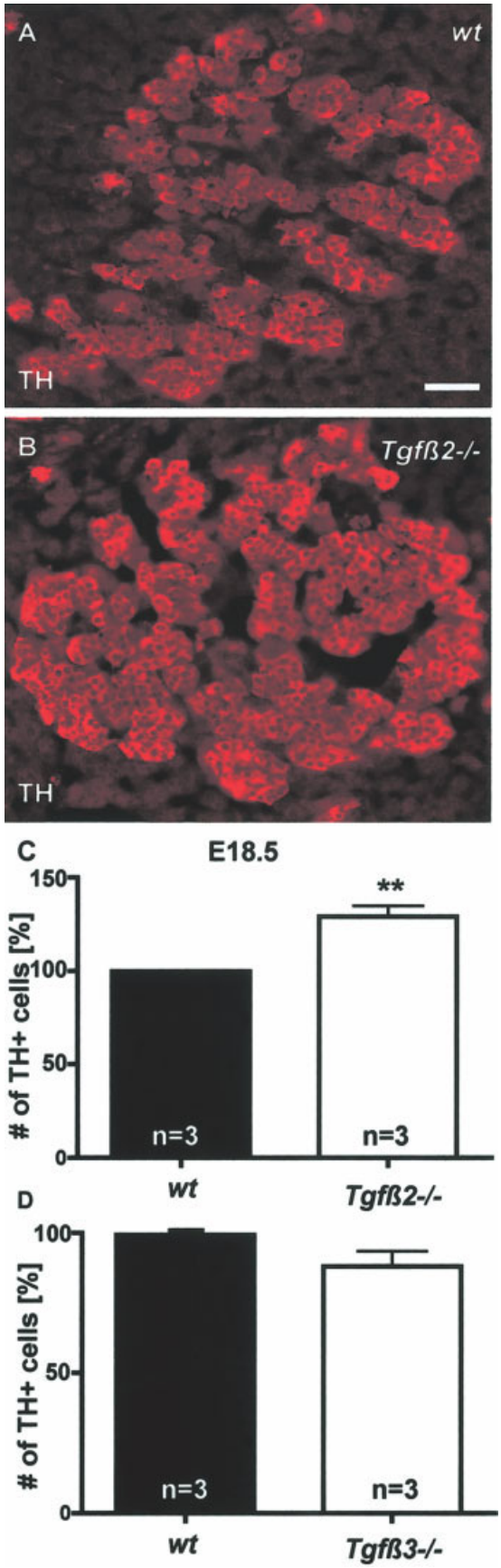
For specific staining of chromaffin cells, paraffin sections of adrenal gland were immunostained with a rabbit antityrosine hydroxylase (anti-TH) antibody (Chemicon, Temecula, CA). Immunostaing for PNMT was performed with a polyclonal rabbit anti-PNMT antibody (Chemicon) specifically staining differentiated chromaffin cells. To detect chromaffin cells that are still proliferating, a mouse monoclonal antibody directed against the proliferative cell nuclear antigen (PCNA) was used (Novokastra). Paraffin sections were deparaffinized and heated in citrate buffer (0.1 M, pH 6) in a microwave oven at 600 W for 5 min to improve antigen retrieval. Endogenous peroxidase activity was quenched by preincubation in 3% H_2O_2 (Merck). Nonspecific binding was blocked by preincubation in 10% normal goat serum (NGS; Sigma, St. Louis, MO) containing 0.1% Triton for 2 hr. Sections were incubated with an anti-TH antibody (1:200) at 4°C overnight and rinsed in phosphate-buffered saline (PBS). The antibody was visualized by incubation with a goat anti-rabbit Cy3-labeled (GaR-Cy3) secondary antibody (Molecular Probes, Eugene, OR) for 1 hr at room temperature. The section were mounted with prolong antifade kit (Molecular Probes). For phenylethanolamine-N-methyltransferase (PNMT) and PCNA staining, sections were incubated with anti-PNMT antibody (1:500) or anti-PCNA antibody (1:100), respectively, at 4°C overnight. After rinsing with PBS, sections were incubated with GaR (1:100), goat anti-mouse (GaM; 1:100; Nordic), respectively, for 1 hr at room temperature, rinsed in PBS, and incubated with rabbit peroxidase-antiperoxidase (PaP-R; 1:1,000; Nordic) or mouse peroxidase-antiperoxidase (PaP-M; 1:800; Nordic), respectively, for 1 hr at room temperature. Visualization of the immune complex was achieved by incubation with diaminobenzidine (DAB; Sigma) according to Adam's method (1977). The sections were dehydrated in ethanol and mounted.

To evaluate the total number of TH^+ and $PNMT^+$ chromaffin cells, every third serial section of adrenal gland was counted to avoid double counting. For $PCNA^+$ cells, the ratio for $PCNA^+/TH^+$ cells in a total of 300 TH^+ cells was calculated. Data represent means of $n = 3 \pm$ SEM. The significance of intergroup differences was detected by applying Student's *t*-test for unpaired samples. Differences were considered significance at $P < 0.05$.

RESULTS

A previous study by our group showed that in ovo neutralization of all TGF-β isoforms results in a significant increase in the number of chromaffin cells in the developing quail adrenal gland (Combs et al., 2000). In the present study, we investigated whether this increase in the number of chromaffin cells is mediated by an individual TGF-β isoform by analyzing *Tgf*β*2*$^{-/-}$ and *Tgf*β*3*$^{-/-}$ null mouse mutants with a specific gene deletion for the TGF-β isoform -β2 or -β3. Figure 1 shows that total numbers of TH^+ chromaffin cells in mice deficient for TGF-β2 (*Tgf*β*2*$^{-/-}$) were significantly increased compared with wild-type mice embryos (*Tgf*β*2*$^{+/+}$), whereas the data for *Tgf*β*3*$^{-/-}$ null mouse mutants reflect wild-type numbers, indicating that the deletion of the TGF-β3 gene seems to have no or only a marginal effect on the development of chromaffin cells. These data suggest that the effect of TGF-β on total numbers of chromaffin cells is isoform specific and regulated by TGF-β2 rather than by TGF-β3.

Chromaffin cells retain the capacity to proliferate throughout their whole life span. To elucidate the mechanisms leading to increased numbers of chromaffin cells, we tested whether the increase in numbers of chromaffin cells may be a consequence of increased proliferation. Proliferating cells were identified by PCNA staining and chromaffin cells by costaining for TH. Figure 2D shows that at E18.5 the ratio of PCNA-positive cells is equivalent in *Tgf*β*2*$^{-/-}$ null mice compared with wild-type littermates. Moreover, analysis at E15.5 (Fig. 2), an earlier time point in adrenal development, reveals a significant increase in the ratio of PCNA-positive chromaffin cells, suggesting



that an increase in proliferation of chromaffin cells leads to an overall increase in chromaffin cell numbers when development proceeds. During organogenesis, proliferating cells may be delayed in their state of differentiation. Chromaffin cells differentiate from noradrenaline- to adrenaline-producing cells. To test for their status of differentiation, tissue sections were stained for PNMT, a differentiation marker, and counterstained for TH. At E18.5, about half of the cells express PNMT. As shown in Figure 3 fewer PNMT-positive cells are detectable in adrenals from *Tgf*β*2*$^{-/-}$ mice than in organs from wild-type littermates. Quantification of this morphological data shows that the ratio of PNMT-positive chromaffin cells is reduced from about 60% to about 35%. Together, these data provide clear evidence that TGF-β2 has an isoform-specific role in the regulation of proliferation as well as differentiation of chromaffin cell in the developing murine adrenal gland.

DISCUSSION

The present study suggests a specific role for the TGF-β2 isoform rather than the TGF-β3 isoform in the regulation and control of chromaffin cell proliferation and differentiation. Analysis of *Tgf*β*2*$^{-/-}$ and *Tgf*β*3*$^{-/-}$ double-deficient mice revealed a more important role for TGF-β2 mediating processes in the development of the eye (Dünker and Krieglstein, 2003) as well as in the development of ribs and midline fusion (Dünker and Krieglstein, 2002). TGF-β, insulin like growth factor-II IGF-II), and nerve growth factor (NGF) are potent promoters of chromaffin cell proliferation in vitro (Lillien and Claude, 1985; Tischler et al., 1989; Frödin and Gammeltoft, 1994; Wolf et al., 1999). PACAP has been identified as a negative regulator of chromaffin cell proliferation in vitro (Frödin et al., 1995). TGF-βs play a role in stimulation and inhibition of astroglial cell proliferation and regulation of astroglial cell phenotypic expressions (Flanders et al., 1993). In vivo, proliferation of chromaffin cells is thought to be regulated by interaction of neurogenic and hormonal signals (Tischler et al., 1989). Wolf et al. (1999) have shown that TGF-β did not interfere with the spontaneous proliferation of chromaffin cells dissociated from postnatal day 6 rat adrenal gland in culture. However, when DNA synthesis and proliferation of

Fig. 1. Paraffin cross-sections of E18.5 mouse adrenal medulla stained with antityrosine hydroxylase (anti-TH) antibody. **A,B:** *Tgf*β*2*$^{-/-}$ null mouse mutants (B) show an increase in the total number of TH-positive chromaffin cells compared with wild-type *Tgf*β*2*$^{+/+}$ littermates (A). **C:** Quantification of the total number of TH-positive chromaffin cells in *Tgf*β*2*$^{-/-}$ null mouse mutants compared with wild-type *Tgf*β*2*$^{+/+}$ littermates at embryonic day (E) 18.5. **D:** Quantification of the total number of TH-positive chromaffin cells of *Tgf*β*3*$^{-/-}$ null mouse mutants compared with wild-type *Tgf*β*3*$^{+/+}$ littermates at E18.5. Results are means ± SEM of three embryos per group. $^{**}P < 0.01$. Scale bar = 50 μm. Figure can be viewed in color online via www.interscience.wiley.com.

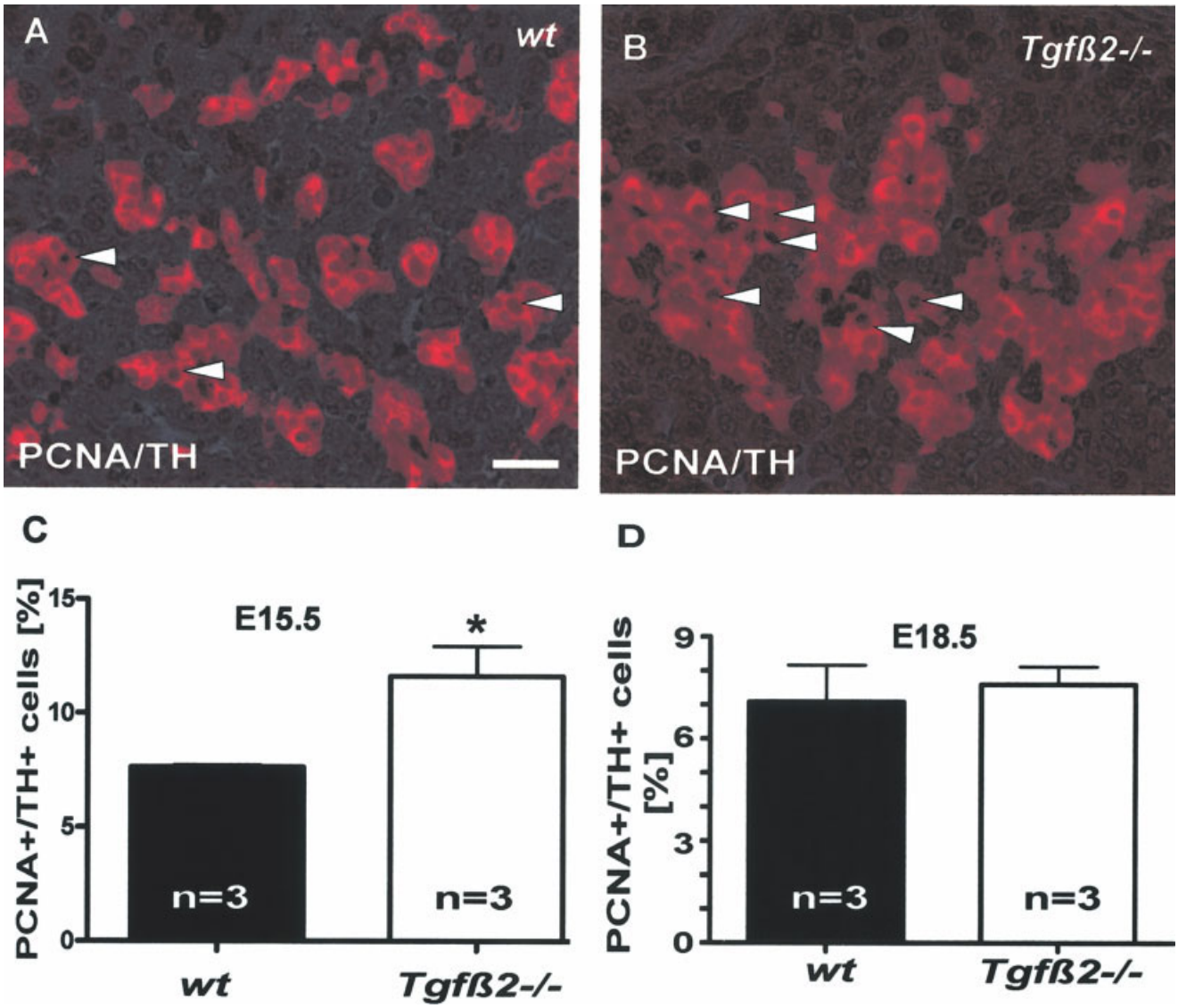
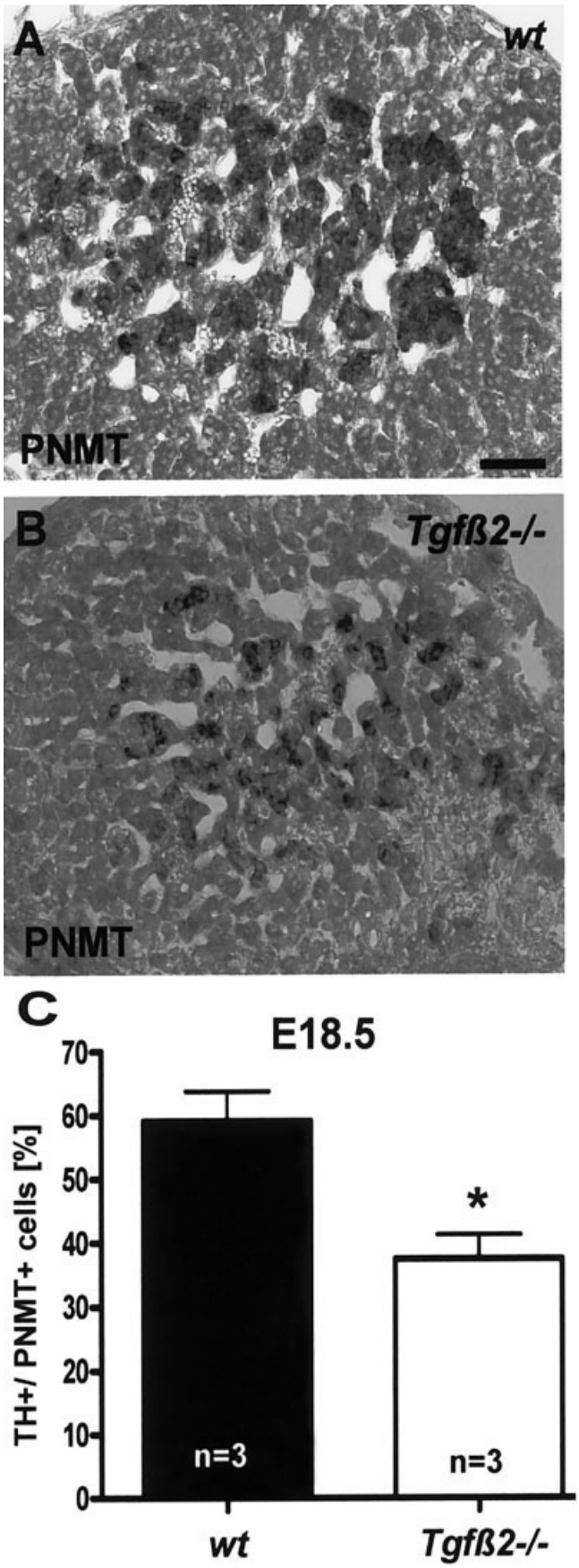


Fig. 2. Micrograph from paraffin cross-sections of E15.5 mouse adrenal medulla stained with antityrosine hydroxylase (anti-TH) and antiproliferative cell nuclear antigen-positive cells (anti-PCNA) antibodies. **A,B:** At E15.5, an increase in the ratio of $PCNA^+/TH^+$ chromaffin cells was observed in *Tgf*$\beta 2^{-/-}$ null mouse mutants (B) compared with wild-type *Tgf*$\beta 2^{+/+}$ littermates (A). **C:** Quantification of the ratio of $PCNA^+/TH^+$ cells in *Tgf*$\beta 2^{-/-}$ null mouse mutants compared with wild-type *Tgf*$\beta 2^{+/+}$ littermates at E15.5. **D:** Quantification of the ratio of $PCNA^+/TH^+$ cells in *Tgf*$\beta 2^{-/-}$ null mouse mutants compared with wild-type *Tgf*$\beta 2^{+/+}$ littermates at E18.5. Results are represented as means ± SEM of three embryos per group. $^*P < 0.05$. Scale bar = 50 μm. Figure can be viewed in color online via www.interscience.wiley.com.

chromaffin cells were stimulated by fibroblast growth factor-2 (FGF-2) and IGF-II, TGF-β had a pronounced inhibitory effect.

TGF-β immunoreactivity occurs in developing mouse chromaffin cells (Flanders et al., 1990). TGF-β is also synthesized and secreted by chromaffin cells (Wolf et al., 1999) via exocytosis (Krieglstein and Unsicker, 1995; Krieglstein et al., 1998). Chromaffin cells express TGF-β receptors, which may be activated upon release of chromaffin cell-derived TGF-β (Wolf et al., 1999). However, the function of TGF-β2 within the mouse adrenal medulla is engimatic. Culturing of mouse neural crest cells in the presence of glial cell line-derived neurotrophic factor (GDNF) has been shown to result in a dramatic increase in the number of TH-positive cells with neuronal morphologies (Maxwell et al., 1996). Mice-deficient for the NGF receptor trkA show apparent losses in chromaffin cells numbers until day 12 postnatally (Schober et al., 1999). Mice lacking FGF-2 and PACAP and TGF-β2 have not been analyzed with regard to their adrenal phenotype. Thus, TGF-β2 is the first identified peptide growth factor shown to be a negative regulator of chromaffin cell pro-



liferation and a stimulator for differentiation of chromaffin cells from norepinephrine-producing cells to epinephrine-producing cells. TGF-β is known as a contextually acting molecule, in that its action often depends on environmental cues, i.e., the cell type, of state of differentiation, and the presence or absence of other growth factors, best exemplified by its capacity either to stimulate or to inhibit proliferation (Roberts and Sporn, 1990; Nathan and Sporn, 1991; Skoff et al., 1998; Ashley et al., 1998). Several mechanisms have been suggested by which TGF-β suppresses cell growth (Wolf et al., 1999). In epithelial cells, TGF-β inhibits mitosis by blocking the transition from G1 to S phase (Alexandrow et al., 1995). TGF-β prevents the hyperphosphorylation of the retinoblastoma gene product (pRB), which is necessary for the progression from G1 to S phase, via interaction with cyclins and cyclin-dependent kinases (Howe et al., 1991; Laiho et al., 1990; Koff et al., 1993; Alexandrow et al., 1995). Previous studies showed that a reduction in the endogenous TGF-β increased proliferation of developing adrenal chromaffin cells in vivo in quail embryos, and these result are consistent with our result on mouse embryos (Combs et al., 2000).

The present study adds murine neuroendcrine cells (chromaffin cells) to the list of documented cell types responding to TGF-β by inhibition of proliferation and stimulation of differentiation. These data also extend the list of neural crest-derived cells whose development is specifically impaired by the lack of TGF-β2 (Sanford et al., 1997). In vitro TGF-β isoform treatment may result in reductive biological effects; in vivo, however, TGF-β isoforms seem to serve unique roles. Future studies will reveal whether TGF-β2 acts alone or in cooperative synergy with other growth factors. In conclusion, this is the first demonstration of a physiological and biological role of TGF-β2 in the regulation of chromaffin cell proliferation and, furthermore, the first documented role of this factor in the regulation of differentiation of chromaffin cells in mouse adrenal glands.

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Fig. 3. Micrograph from paraffin cross-sections of mouse adrenal medulla stained with an antiphenylethanolamine-N-methyltransferase antibody (anti-PNMT). **A,B:** A significant decrease in the ratio of total number of phenylethanolamine-N-methyltransferase-positive ($PNMT^+$) cells compared with the total number of tyrosine hydroxylase-positive (TH^+) cells was found in *Tgf*$\beta 2^{-/-}$ null mouse mutants (B) compared with wild-type animals (A) at E18.5. **C:** Quantification of the ratio of $PNMT^+$/ TH^+ in *Tgf*$\beta 2^{-/-}$ null mouse mutants compared with wild-type *Tgf*$\beta 2^{+/+}$ littermates at E15.5. Results are represented as means ± SEM of three embryos per group. $\star P < 0.05$. Scale bar = 50 μm.

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