Neural Stem/Progenitor Cells Express 20 Tenascin C Isoforms That Are Differentially Regulated by Pax6*

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Tenascin C (Tnc) is an alternatively spliced, multimodular extracellular matrix glycoprotein present in the ventricular zone of the developing brain. Pax6-deficient small eye (sey) mouse mutants show an altered Tnc expression pattern. Here, we investigated the expression of Tnc isoforms in neural stem/progenitor cells and their regulation by the paired-box transcription factor Pax6. Neural stem/progenitor cells cultured as neurospheres strongly expressed Tnc on the protein level. The Tnc isoform expression in neural stem/progenitor cells was analyzed by reverse transcriptase-PCR and dot blot hybridization. In total, 20 different Tnc isoforms were detected in neurospheres derived from embryonic forebrain cell suspensions. The Tnc isoform containing the fibronectin type III domains A1A4BD is novel and might be a neural stem/progenitor cell-specific. Transient overexpression of Pax6 in neurospheres of the medial ganglionic eminence did not alter the total Tnc mRNA expression level but showed a pronounced regulative effect on different Tnc isoforms. The larger Tnc isoforms containing four, five, and six additional alternatively spliced fibronectin type III domains were up-regulated, whereas the small Tnc isoforms without any or with one additional domain were down-regulated. Thus, Pax6 is a homeodomain protein that also modulates the splicing machinery. We conclude that the combinatorial code of Tnc isoform expression in the neural stem/progenitor cell is complex and regulated by Pax6. These findings suggest a functional significance for individual Tnc isoforms in neural stem/progenitor cells.

The multimodular glycoprotein Tenasin C (Tnc)4 is a prominent constituent of the extracellular matrix during CNS development and repair (1). In the literature one does find many different abbreviations for Tenasin C (e.g. TN-C, TN, Ten, Hxb, and TNC). Therefore, we have decided to use the official gene symbol Tnc (2) as abbreviation for Tenasin C and will accordingly to international standards use Tnc for the mouse protein, TNC for the human protein, and the italicized versions Tnc and TNC for the murine and human gene/mRNA, respectively.

Structurally, Tnc consists of several serially arranged protein domains. At the amino terminus it has a cysteine-rich region where six Tnc molecules assemble to the so called hexabrachion (1). In the mouse, 14.5 EGF-like repeats and in the smallest variant eight constitutive fibronectin type III (FnIII) domains follow. The carboxyl-terminal domain is a fibrinogen-like glob. Tnc is encoded by a single gene, which encodes for several alternatively spliced exons (1). The complexity of murine Tnc is dramatically increased by the use of up to six alternatively spliced FnIII domains named A1, A2, A4, B, C, and D, which can be independently inserted between the 5th and 6th constitutive FnIII domain and leads to a large variety of Tnc isoforms (3). Theoretically, up to 64 different Tnc isoforms could be generated and 27 of them have been detected during embryonic and early postnatal mouse CNS development (3). However, the cell types responsible for the generation of this Tnc isoform complexity in vivo have not been elucidated. In culture, oligodendrocytes have been shown to preferentially express larger Tnc isoforms, whereas astrocytes are biased toward smaller Tnc isoforms (4). However, a large complexity of Tnc isoforms exists already before astrocytes and oligodendrocytes are generated during CNS development. Functionally, the alternatively spliced region of Tnc harbors at least two active domains, which stimulate neurite outgrowth of central neurons. The activity of the FnIIIID domain alone was reported to depend on interaction with the a7β1 integrin receptor (5), whereas the activity of FnIIIIBD selectively required interaction with the cell adhesion molecule F3/contactin (6).

Tnc is prominently expressed during forebrain development (7, 8) in the ventricular and subventricular zone (9, 10) where neural stem cells reside (11). Early on, Tnc is expressed by radial glia cells (12, 13) and functions as a modulator of growth factor responsiveness controlling the transition from early to late neural stem cells (14). Furthermore, Tnc is required for normal neural progenitor proliferation as well as for proper differentiation of neurons and oligodendrocytes (10, 14). At later develop-

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4 The abbreviations used are: Tnc, Tenasin C; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; CNS, central nervous system; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; FnIII, fibronectin type III; GLAST, glutamate and aspartate transporter; MEF, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; RT, reverse transcription; sey, small eye; MGE, medial ganglionic eminence; MOPS, 4-morpholinopropanesulfonic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
opmental stages Tnc expression is largely confined to astrocytes (8) before it becomes down-regulated in the CNS, with the exception of the adult neurogenic stem cell niche (9). The regulation of Tnc expression by growth factors and cytokines has been extensively studied in various cell lines (15) and also to some extent in primary glial cultures (4). However, it has not been investigated whether individual Tnc isoforms are differentially regulated. Furthermore, the relevance of the individual growth factors and cytokines for the expression of Tnc in vivo remains unclear so far. On the other hand, studies of the promoter region of Tnc revealed that transcription factors Evx-1, Brn-2, and Otx2 interact with defined response elements and were shown to regulate Tnc expression (16–19). Again, it has not been investigated whether these transcription factors control the expression of Tnc in vivo. The best evidence for regulation of Tnc has been obtained with the paired-box transcription factor Pax6 that, comparable with Tnc, is expressed by radial glia cells in the ventricular zone of the developing cortex (20), becomes down-regulated postnatally, and is maintained in the adult neurogenic stem cell niche (21). In the natural Pax6 mouse mutant small eye (sey) Tnc expression is selectively lost in the cortical ventricular zone and reduced in the ventricular zone of the ganglionic eminence during early forebrain development, whereas Tnc expression in cortical astrocytes at later developmental stages appears normal (20). Apparently, the expression of Tnc in neurogenic radial glia depends on Pax6, but how Pax6 regulates Tnc expression has not been studied.

Here, we investigated the expression of Tnc isoforms in free-floating neurospheres that serve as a widespread model for neural stem/progenitor cells in vitro (22). We found that 20 different Tnc isoforms are present in neurospheres. Transient overexpression of Pax6 resulted in a shift from smaller to larger levels. Thus, Pax6 acts as a modulator of alternative splicing and growth factors and cytokines for the expression of Tnc potentially regulated postnatally, and is maintained in the adult neurogenic stem cell niche (9). The regulation of Tnc expression by growth factors and cytokines has been extensively studied in various cell lines (15) and also to some extent in primary glial cultures (4). However, it has not been investigated whether individual Tnc isoforms are differentially regulated. Furthermore, the relevance of the individual growth factors and cytokines for the expression of Tnc in vivo remains unclear so far. On the other hand, studies of the promoter region of Tnc revealed that transcription factors Evx-1, Brn-2, and Otx2 interact with defined response elements and were shown to regulate Tnc expression (16–19). Again, it has not been investigated whether these transcription factors control the expression of Tnc in vivo. The best evidence for regulation of Tnc has been obtained with the paired-box transcription factor Pax6 that, comparable with Tnc, is expressed by radial glia cells in the ventricular zone of the developing cortex (20), becomes down-regulated postnatally, and is maintained in the adult neurogenic stem cell niche (21). In the natural Pax6 mouse mutant small eye (sey) Tnc expression is selectively lost in the cortical ventricular zone and reduced in the ventricular zone of the ganglionic eminence during early forebrain development, whereas Tnc expression in cortical astrocytes at later developmental stages appears normal (20). Apparently, the expression of Tnc in neurogenic radial glia depends on Pax6, but how Pax6 regulates Tnc expression has not been studied.

Here, we investigated the expression of Tnc isoforms in free-floating neurospheres that serve as a widespread model for neural stem/progenitor cells in vitro (22). We found that 20 different Tnc isoforms are present in neurospheres. Transient overexpression of Pax6 resulted in a shift from smaller to larger Tnc isoforms, without changing overall Tnc mRNA expression levels. Thus, Pax6 acts as a modulator of alternative splicing and differentially regulates the expression of the various Tnc isoforms in neural precursors implicating that functional differences are encoded in the alternatively spliced Tnc isoforms.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture**—Neurosphere cultures were performed as described previously (14). Briefly, timed pregnancy NMRI mice were sacrificed and staged according to Theiler’s criteria (23). E13 (Theiler stage 21) animals were used for the dissection of the cortex, the lateral ganglionic eminence, and medial ganglionic eminence (MGE). The tissue was digested using 30 mg/ml papain (Worthington) in minimal essential medium (DMEM:F-12, 1:1 (Sigma)) for 20 min by mechanical trituration. The single cell suspension was cultured in a humidified CO2 incubator at 37 °C, 6.0% CO2 with 105 cells/ml in neurosphere medium, which was changed after 24 h and again after 3 days. Neurospheres were resuspended in neurosphere medium, which was changed after 24 h and again after 3 days. Neurospheres showing EGFP fluorescence after 6 days were used for RNA isolation (RNeasy mini kit, Qia-gen). MEF cultures and the A7 cell line were transfected using the Rotifect kit according to the manufacturer’s instructions (Roth, Germany). Briefly, 20 μl of transfection solution was mixed with 2.5 μg of plasmid DNA and incubated for 20 min at room temperature before addition to 70–90% confluent cultures for 4.5 h in DMEM. After washing, the medium was replaced by DMEM, 5% FCS for 24 h. The transfected cultures where then kept in DMEM, 10% FCS, 1% penicillin/streptomycin until confluent cultures were harvested for RNA isolation as described above for neurosphere cultures.

**RT-PCR Analysis**—1 μg of total RNA obtained from defined CNS areas of E13.5 sye mice, their wild type littermates, or from the cell cultures detailed above, was reverse transcribed using random hexamer primers (first strand cDNA synthesis kit, Fermentas). 1 μl of these cDNAs was used in a 25-μl PCR reaction containing 1.5 mM MgCl2, 5 nmol of each dNTP, 1.25 units of Taq polymerase, and 5 pmol of the appropriate forward and reverse primers. The following oligonucleotide primer pairs were used at the indicated annealing temperatures: Tenascin C (Tnc), 5′-GCTCTAGAGGACTCTGTACCCCATTTCC-3′, 5′-CGGGATCCCTCCCCAGATTTCGGAAGTTGCT-3′ at 59 °C; Tnc isoforms, 5s, 5′-CGGGATCCGAAATTGATGCACCC-AAGGAC-3′, 6as, 5′-CGAATTCTTATGTTAGATTAGTCCCGAGA-3′ at 57 °C; 5f, 5′-CGCTCGAGACGTGTGAAGGACCATCACG-3′ at 56 °C; 5r, 5′-CGCAAGCTTTATCTCCTTGGAGAACCCATGGC-3′ at 59 °C (3); paired box gene 6 (Pax6), 5′-CGCCGAGAAGATCGTGAAG-3′, 5′-TCTCGGATTTCCTCCAAGAGATG-3′ at 56 °C; Integrin α7 (Itga7), 5′-CGTCCGGAGCCAACTCAGAC-3′, 5′-CTCTGGATGGTCGCTGCTT-3′ at 60 °C; Integrin β1 (Itgb1), 5′-AAAACTGTTGTGCACATTGTA-3′, 5′-GCACTGTGGGTGATTTGTGG-3′ at 60 °C; F3/contactin (Cntn1), 5′-CCACAGAGTGA-GGGTCAA-3′, 5′-CATTGGAGATGTCACACAGA-3′ at 60 °C; Ds-1-Proteoglycan/Phosphacan (Ptprz1), 5′-TATGC-TACCSCCGAGACAC-3′, 5′-CCTGCTGCTGTAAGCCAGATATT-3′ at 55 °C; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ACCTCCTACGGGAAATTCTC-3′, 5′-CCTTCCATCAGTTGGAATG-3′ at 60 °C; and β-actin (Actb) 5′-TATGCAACACATGCTGTCTGTTG-3′, 5′-AGAAGCCACTTGCAGTCAGATGG-3′ at 60 °C (27). All reactions were carried out in a Mastercycler gradient (Eppendorf) starting with a 160-s denaturation step at 94 °C followed by 35–38
cycles of 20 s at 94 °C, 30 s at the respective annealing temperature and 30 s elongation at 72 °C. The reaction was followed by a 5-min final elongation step at 72 °C and cooled down to 4 °C. The PCR products were separated on a 1.5% agarose gel and the bands cut out of the gel and extracted using the Qiaex II gel extraction kit (Qiagen). The isolated PCR products were ligated into the vector pCRII-TOPO (TOPO-TA cloning kit, Invitrogen) using the A overhangs that were produced by the Taq polymerase during the PCR. The resulting transformants were checked for Tnc fragments of the expected size by direct lysis of the bacteria with 25 μl of 70% ethanol and PCRRed using primers flanking the alternatively spliced region of Tnc.

Dot Blot Hybridization Analysis—The positively identified clones were cultured and their plasmids isolated (Qiaprep Spin Miniprep Kit, Qiagen) and dot blotted onto Hybond N+ membranes (Amersham Biosciences). For this procedure the membrane was prewetted in 10× SSC and up to 50 ng of target sequence in a volume of up to 6 μl was spotted on the membrane. The loaded DNA was denatured in 500 mM NaOH, 1.5 M NaCl and renatured in 500 mM Tris/HCl, 1.5 M NaCl, pH 7.5. The membranes were baked for 2 h at 80 °C for covalent binding of the DNA. Seven equivalent blots were produced with the same pattern of plasmids. The membrane-bound DNA was hybridized overnight at 72 °C with probes that are specific for the different alternatively spliced FnIII domains of Tnc. The probes were the same as in Ref. 3. A probe against FnIII domain 6 was used as a negative control because the PCR-amplified fragments should not contain this part of the Tnc molecule. For detection the membranes were blocked in 100 mM Tris/HCl, 300 mM NaCl, 10% (v/v) liquid block (Amersham Biosciences) and incubated with an alkaline phosphate-coupled anti-fluorescein antibody (1:200 in block buffer, Roche). After washing three times with 100 mM Tris/HCl, 150 mM NaCl, 0.3% (v/v) Tween 20, pH 7.4, for 5 min each; twice with 100 mM Tris/HCl, 100 mM NaCl, pH 9.5, for 5 min; and three times with 50 mM Tris/HCl, 150 mM NaCl, pH 7.5, for 10 min each the detection was carried out with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche). After 5–60 min the reaction was stopped with water. The signals obtained with the specific probes revealed the FnIII domain composition of single bacterial clones. When the composition of the FnIII domains was ambiguous after dot blot hybridization the plasmids were tested via PCR using domain-specific primers. The FnIII domains are highly homologous and, thus, a stringent two-step PCR amplification protocol was applied. Using 10–20 pg of plasmid DNA and the same concentrations of ingredients as outlined above cycling was performed starting with a 160-s denaturation step followed by 33 cycles of 30 s denaturation and annealing and elongation at 72 °C together for 40 s. As positive and negative controls six plasmids were used that contain the single alternatively spliced FnIII domains. Some cloned Tnc isoforms containing plasmids were sequenced to verify the combination of FnIII domains.

Immunohistochemistry—For fixation, neurospheres were spun down and resuspended in freshly prepared 4% paraformaldehyde. After 20 min paraformaldehyde was replaced with 15% sucrose for cryoprotection for 4 °C overnight. The neurospheres were transferred to 30% sucrose overnight, embedded in TissueTek (Jung), and stored at −70 °C. Cryosections of 12 μm thickness were prepared using a conventional cryostat (Leica), mounted, and air-dried. After rehydration in PBS (1.7% NaCl) with 10% horse serum for 1 h at room temperature the neurosphere sections were incubated at 4 °C overnight with undiluted anti-Pax6 mouse hybridoma supernatant (Developmental Hybridoma Bank) or affinity purified rabbit anti-Tenascin C antibodies (KAF 14) diluted 1:300 in PBS, 0.1% BSA (6). After three 5-min wash steps with PBS the sections were incubated with a CY3-labeled anti-mouse IgG secondary antibody (1:500 in PBS, 0.1% BSA) or a CY2-labeled anti-rabbit IgG secondary antibody (1:300 in PBS, 0.1% BSA) for 2 h at room temperature (Dianova). During incubation with the secondary antibody, cell nuclei were labeled with bisbenzimide (Hoechst 33258, diluted 1 × 10⁵, Sigma). After three further washes in PBS the sections were embedded in Immumount (Thermo Science) and examined at a fluorescent microscope (Axioplan2, Zeiss).

Immunocytochemistry—Electroporated neurosphere-derived cells were plated 1 day after transfection on PORN-coated (10 μg/ml for 1 h at 37 °C) dishes for 3 h. These cultures were washed once in PBS and then fixed for 15 min in 4% paraformaldehyde. After permeabilization in PBS, 0.1% Triton X-100, 1% BSA the cells were immunostained using BLBP (1:1000, Chemicon), GLAST (1:1000, Chemicon), RC2 hybridoma supernatant (1:10, Developmental Hybridoma Bank), Pax6 (undiluted hybridoma supernatant), and GFAP (1:300, DAKO, Denmark). For detection of the 473HD epitope, the cells were live stained with 473HD antibody (1:200) for 20 min at room temperature before fixation in 4% paraformaldehyde (28). The primary antibodies were detected using the appropriate secondary antibodies from Dianova as outlined above. During incubation with the secondary antibody, cell nuclei were labeled with bisbenzimide (Hoechst 33258, diluted 1 × 10⁵, Sigma). After three further washes in PBS the sections were embedded in Immumount (Thermo Science) and examined at a fluorescent microscope (Zeiss). For quantification a total of 600–1200 Pax6-positive cells were counted for every marker under each condition in three independent experiments and expressed as mean ± S.D.

Western Blot—Protein lysates of E13.5 forebrain tissue or neurospheres that were grown from cortical or striatal cell suspensions of E13.5 telencephalon in the presence of 20 ng/ml EGF or bFGF for 6 days, were obtained as described previously (29). After determination of the protein content (DC Protein Microassay, Bio-Rad) 10 μg of total protein were loaded per lane onto an 4–12% NuPAGE/BisTris gel (Invitrogen) and electrohoresed at 100 V in MOPS running buffer according to the manufacturer’s instructions (Invitrogen). After semi-dry Western blotting to polyvinylidene difluoride membranes and blocking with 5% (w/v) milk powder, the blot was incubated overnight at 4 °C with the KAF14 antibody (1:5000). Detection was performed by the standard ECL method according to the manufacturer’s instructions (Amersham Biosciences) after incubation with anti-rabbit horseradish peroxidase-coupled secondary antibodies (1:5000, Dianova) for 2 h at ambient temperature.
RESULTS

Tenascin C is a multimodular extracellular matrix glycoprotein that is expressed in neural progenitor cells during early CNS development. Under defined, serum-free conditions neural stem/progenitor cells can be cultivated in the presence of the growth factors EGF or bFGF, which leads to the formation of free-floating cellular aggregates called neurospheres (22). We suspected Tnc expression in neurospheres because its mRNA has been detected previously in gene array approaches (30, 31). Indeed, cortical and striatal neurospheres derived from E13.5 single cell suspensions and grown for 6 days in the presence of EGF and bFGF strongly express Tnc on the protein level (Fig. 1). The Western blot analysis revealed that Tnc expression levels are stronger when the neurospheres were grown in bFGF compared with EGF (Fig. 1C). As more than one major Tnc band could be revealed the data indicated the presence of several Tnc isoforms or differentially glycosylated forms of Tnc. This pattern of Tnc expression appeared comparable with the one observed in Western blots of E13.5 forebrain tissues (Fig. 1C).

To reveal Tnc isoform expression in neural stem cells, we performed RT-PCR analysis using primers 5s and 6as located at the beginning of the 5th and the end of the 6th constitutive FnIII domain, respectively (Fig. 2A). This strategy leads to the detection of up to seven amplicons in ethidium bromide-stained agarose gels corresponding to Tnc isoforms without alternatively spliced FnIII cassettes and Tnc isoforms containing one to six additionally inserted FnIII domains (Fig. 2B). This amplification pattern for Tnc isoforms was confirmed using primers 5f and 6r, which are located at the end of the 5th and the beginning of the 6th constitutive FnIII domain, respectively, and are directly flanking the alternatively spliced region of Tnc (Fig. 2A). This led to the detection of six amplicons representing one to six additional FnIII domains (Fig. 2C). However, with the exception of the Tnc isoform containing all six alternatively spliced FnIII domains this pattern does not resolve which combinatorial code of alternatively spliced FnIII domains are represented in individual Tnc isoforms.

Pax6 Regulates Tnc Isoform Expression

The domain composition of the different Tnc isoforms in neurospheres was examined further after the individual PCR products were subcloned after amplification using the primers 5f and 6r. To eliminate false positives, all clones were checked by PCR. Plasmids representing Tnc clones were dot blotted onto nylon membranes and seven replica membranes were hybridized with FnIII domain probes specific for A1, A2, A4, B, C, D, and 6. The probe against FnIII domain 6 served as an internal negative control because this part should not be included in any amplified sequence. As positive control a plasmid was applied on every membrane that contained all alternatively spliced domains and was produced by subcloning the largest PCR product generated with the primer pair 5f, 6r. More than 250 clones were analyzed and revealed a complex diversity of Tnc isoforms being expressed in neurospheres that were generated from the medial ganglionic eminence of E13 mice. In total, we detected 20 different Tnc isoforms of different sizes in E13 neurospheres (Table 1). When only one single FnIII domain was spliced between the fifth and sixth constitutive FnIII domain of Tnc it represented domain D in more than 90% of the clones analyzed. We also found some clones containing the FnIII domain A1 but signals for the domains A2, A4, B, or C were undetectable. In the clones containing two alternatively spliced Tnc cassettes we preferentially found the combinations A1/D and C/D with similar ratios, with a few clones containing the alternatively spliced FnIII domains A1/A2. The widest variety of different FnIII domain compositions was observed for the medium-sized Tnc isoforms containing three or four alternatively spliced FnIII domains. The three FnIII domain containing Tnc isoform A1/C/D was the most prominent and detected in three out of four clones. The Tnc isoform containing A1/A2/D was found in 16% of the clones, whereas Tnc isoforms A1/B/D and B/C/D were rarely detected and seem to be under-represented (Table 1). No further Tnc isoforms with three additional FnIII domains were revealed. In more than half of the clones containing four additional FnIII cassettes the combination A1/A2/A4/B was recorded. In about 10% of those clones we found the domain combination A1/A4/B/D that has not been described before (3) and, thus, represents a novel Tnc isoform. Some clones also contained the forms A1/A2/C/D, A1/B/C/D, A1/A2/B/D, or A1/A2/A4/D (Table 1). When only one FnIII domain is missing in the alternatively spliced region of the Tnc sequence, we revealed in all cases the absence of the FnIII domain C. We also analyzed some clones containing all six alternatively spliced domains and all turned out to have the expected FnIII domain combination A1/A2/A4/B/C/D. Taken together, the systematic investigation of Tnc isoform expression in neurospheres revealed an unexpected complexity with one novel Tnc mRNA and 19 additional alternatively spliced Tnc transcripts.

Regulation of Tnc Isoform Expression by Pax6—The above findings prompted us to address the regulation of Tnc isoform
complexity. We chose Pax6 overexpression, because previous data showed that Tnc expression during forebrain development depends on functional Pax6, and because signals for Tnc are hardly detected in the telencephalon of *sey* mice (20), which are naturally occurring *Pax6* mutants. The overexpression of mouse Pax6 was achieved by nucleofection of E13 neurospheres that developed in cell suspensions derived from the MGE (Fig. 3). These neurospheres were chosen because they were observed to have the lowest endogenous Pax6 mRNA expression levels in comparison to neurospheres derived from cortical or lateral ganglionic eminence tissues (data not shown). This finding was confirmed by the immunohistochemical stainings, where few cells in control neurospheres were expressing moderate levels of Pax6 (Fig. 3E). As expected, after electroporation of a *Pax6* expression vector many cells within the neurosphere were strongly immunopositive, suggesting the presence of a functional nuclear Pax6 protein (Fig. 3F). Because not all cells within neurospheres were immunopositive for Pax6 and neurospheres are heterogeneous in cell type composition (28, 31), we examined which cell types were overexpressing Pax6. As expected, 1 day after electroporation the majority of the Pax6-positive neurosphere-derived cells were neural stem/progenitor cells that expressed the radial glia cell marker BLBP (48 ± 11%, n = 3), GLAST (46 ± 4%, n = 3), and RC2 (31 ± 6%, n = 3; Fig. 4A). The co-expression with markers of differentiated cell types was very low (e.g. GFAP, Fig. 4B), suggesting that neural stem/progenitor cells are the primary targets of Pax6 overexpression.

Then, we performed a semi-quantitative RT-PCR analysis to compare mRNA expression levels between *Pax6*-transfected and control MGE neurospheres. In the non-transfected control cells both splice variants of *Pax6* were faintly expressed and contributed to a double band in which the larger one was only slightly weaker than the smaller one (Fig. 5). When the same RT-PCR was carried out with *Pax6*-transfected neurospheres, a strong band for the smaller *Pax6* isoform but no signal for the larger isoform including exon 5 was seen (Fig. 5). This provided evidence that the shorter isoform of *Pax6* encoded by the expression vector was much more strongly expressed than the alternate exon 5 insert form of *Pax6* (32) that could no longer become faithfully amplified. The exogenous *Pax6* expression therefore appeared predominant over the endogenous *Pax6* expression. Next, the expression of *Tnc* isoforms after Pax6 overexpression was studied as described before. We also observed a ladder of six or seven amplicons, respectively, with a clear increase in the *Tnc* isoforms containing four, five, and six additional FnIII domains (Fig. 2, B and C). The relative increase was quantified by comparing band intensities of corresponding amplicons. This semi-quantitative RT-PCR approach indicated that the *Tnc* isoforms containing four, five, and six additional FnIII domains were 3–4-fold up-regulated after Pax6 overexpression (Fig. 2D). We

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**FIGURE 2. Regulation of Tnc isoform expression by Pax6.** A, the schematic representation of Tnc and the localization of the primers used in this study for the amplification of the *Tnc* isoforms are shown. Tnc is a multimodular protein with a cysteine-rich Tnc assembly domain, 14.5 EGF-like domains, several fibronectin type III (FnIII) domains, and a fibronogen-like domain. In the shortest variant there are eight constitutive FnIII domains. Up to six additional domains can be inserted between the fifth and sixth domain by alternative splicing. The primer pair 5s, 6as binds to the 5′ end of FnIII domain 5 and the 3′ end of FnIII domain 6 so that the smallest amplicon contains only the constitutive FnIII domains 5 and 6. Every additional domain increased the amplicon size by 273 bp (data shown in B). The primers 5f and 6r bind to the 3′ end of FnIII domain 6, respectively. The smallest amplicon therefore contained one of the alternatively spliced domains. Every additional domain increased the amplicon size by 273 bp (data shown in C). B and C, ethidium bromide-stained agarose gels revealing the *Tnc* isoform pattern in Pax6 overexpressing neurospheres and in non-transfected controls analyzed by RT-PCR are shown using the above primers as indicated. Note that upon Pax6 overexpression in neurospheres the large *Tnc* isoforms containing four, five, and six alternatively spliced FnIII domains are up-regulated, whereas the small *Tnc* isoforms without or with one additional domain are down-regulated compared with the non-transfected controls. D, graphical representation of the relative expression levels of the corresponding *Tnc* amplicons. The data were quantified and compared by measuring the intensities of the individual amplicons using the ImageJ program and were expressed as mean ± S.E. from three independent experiments. Note the 4-fold increase of the largest *Tnc* isoforms containing five and six additional FnIII domains.
TABLE 1
Tenascin C isoform expression pattern in neural stem cells

Control neurospheres were analyzed for their expression pattern of different Tnc isoforms by dot blot hybridizations and compared to neurospheres overexpressing Pax6. The table depicts the presence of FnIII domains that are alternatively spliced between the fifth and sixth constitutive FnIII domain with their relative abundance in percent. The total number of clones analyzed is also indicated and varied in a way that closely correlated with the intensities of the respective amplicons in RT-PCRs (see Fig. 2). In total, 20 different Tnc isoforms were found in neural stem cells including one new isoform containing the alternatively spliced FnIII domains A1, A4, B, and D. The pattern of Tnc isoform expression in Pax6-transfected neurospheres appeared not significantly different. The results from two independent experiments are summarized.

| Number of alternatively spliced fnIII domains | Fraction of analysed clones |
|---------------------------------------------|-----------------------------|
|                                             | Control (93 %) | 92 % | 84 % |
| 1                                           | A1             | D    | 7 %  | 64 % |
| 2                                           | A1 A2          | D    | D    | 3 %  |
| 3                                           | A1 A2 A3 A4 A5 | D    | D    | 3 %  |
| 4                                           | A1 A2 A3 A4 A5 | D    | D    | 3 %  |
| 5                                           | A1 A2 A3 A4 A5 | D    | D    | 3 %  |
| 6                                           | A1 A2 A3 A4 A5 | D    | D    | 3 %  |

These data implied that larger Tnc isoforms should be diminished or lost in the absence of Pax6. Therefore, we analyzed the expression of Tnc isoforms in different CNS regions of E13.5 sey mice, which lack a functional Pax6 protein (33). In accordance with our expectations (20) the large Tnc isoforms could not be recorded by RT-PCR using telencephalic cDNAs obtained from sey forebrains, whereas a residual expression of the smallest Tnc isoform was still detectable (Fig. 6). In hindbrain, where Pax6 is not expressed in the ventricular zone, a wild type pattern of Tnc isoforms was amplified when cDNA from sey mice was used (Fig. 6). Thus, Pax6 appears to be essential for the expression of Tnc isoform complexity. It remained unclear, however, if the observed up-regulation of Tnc isoforms after forced Pax6 expression in neural stem/progenitor cells is cell type-specific or a general consequence of Pax6 overexpression.

To further control for the specificity of the differential regulation of Tnc isoforms by Pax6 overexpression, we performed semi-quantitative RT-PCR analysis for Tnc mRNA expression.

FIGURE 3. Overexpression of Pax6 in neurospheres. Phase-contrast photomicrographs of neurospheres derived from E13 MGE cell suspensions are shown in A (control) and B (transfected). The neurospheres were co-transfected with expression plasmids encoding for Pax6 and EGFP. Control neurospheres showed no EGFP fluorescence (C) and only weak to moderate endogenous Pax6 immunoreactivity after immunolabeling of cryosections (E). Transfected neurospheres showed EGFP fluorescence (D) and cryosections of such neurospheres were highly Pax6 immunoreactive (F). The scale bars are 50 μm.

These data implied that larger Tnc isoforms should be diminished or lost in the absence of Pax6. Therefore, we analyzed the expression of Tnc isoforms in different CNS regions of E13.5 sey mice, which lack a functional Pax6 protein (33). In accordance with our expectations (20) the large Tnc isoforms could not be recorded by RT-PCR using telencephalic cDNAs obtained from sey forebrains, whereas a residual expression of the smallest Tnc isoform was still detectable (Fig. 6). In hindbrain, where Pax6 is not expressed in the ventricular zone, a wild type pattern of Tnc isoforms was amplified when cDNA from sey mice was used (Fig. 6). Thus, Pax6 appears to be essential for the expression of Tnc isoform complexity. It remained unclear, however, if the observed up-regulation of Tnc isoforms after forced Pax6 expression in neural stem/progenitor cells is cell type-specific or a general consequence of Pax6 overexpression. To address this point, we transfected MEF and the astrocytic cell line A7 (34) with the Pax6 expression plasmid and determined the pattern of Tnc isoform expression by RT-PCR. We recorded strikingly different patterns of Tnc isoform expression in the two cell types that were, however, both unaffected by Pax6 overexpression (Fig. 7). In addition, we noted a strong additional band of unexpected size between the Tnc amplicons without additional FnIII domains and those with one FnIII domain, which was also seen in Fig. 2B (control lane). This band often appears when amplification of the Tnc isoforms is performed with the primer pair 5s, 6as. We have subcloned and sequenced this amplicon, which contains the first 102 bp of the 5th FnIII domain linked to the last 84 bp of the FnIIIC domain of Tnc. We interpret the occurrence as a PCR artifact. However, we presently cannot rule out that this amplicon represents an unusual Tnc isoform because the reading frame is maintained resulting in a hybrid FnIII domain that would be smaller than the average FnIII domains (62 amino acids instead of 90). Nevertheless, the regulation of Tnc isoforms by Pax6 appears to be cell type-specific as it was selectively observed in neural stem/progenitor cells.

To further control for the specificity of the differential regulation of Tnc isoforms by Pax6 overexpression, we performed semi-quantitative RT-PCR analysis for Tnc mRNA expression.
using two pairs of primers that are located in the constitutive regions of the molecule. It appeared as if the total Tnc mRNA expression level was unaltered (Fig. 5), which is in accordance with the observation that the long and short Tnc isoforms were up- and down-regulated, respectively. In addition, the mRNA expression level of the unrelated extracellular matrix molecule DSD-1-PG/phosphacan in MGE neurospheres was unaffected by Pax6 overexpression (Fig. 5). Thus, Tnc isoform expression appears to be under precise control of Pax6 that dramatically influences Tnc isoform size but not composition. These findings prompted us to analyze the mRNA expression of Tnc receptor molecules in neurospheres. We focused on F3/Contactin and the integrins α7 (Itga7) and β1 (Itgb1) because their binding sites on Tnc have not only been mapped to the alternatively spliced region (1) but, more importantly, in a functional context (5, 6). All three mRNAs were detectable in neurosphere cultures (Fig. 5). Surprisingly, Pax6 overexpression selectively increased the mRNA levels of Itga7 6 h after electroporation, whereas Itgb1 and F3/contactin were unchanged. This implies the integrin receptor α7 subunit not only as a Pax6 target gene but also as a potentially important candidate for mediating isoform-specific Tnc function in neural stem/progenitor cells.

DISCUSSION

In the present study, we have investigated the complexity of Tnc isoform expression in neural stem/progenitor cells grown as neurospheres and revealed the presence of 20 different Tnc isoforms. The expression of short and long Tnc isoforms was differentially regulated by Pax6 overexpression in a cell type-specific fashion, with smaller Tnc isoforms being reduced, whereas longer Tnc isoforms were more abundant. However, the total Tnc mRNA level and the complexity of Tnc isoform composition appeared largely unchanged. In sey mutant forebrain tissues the large Tnc isoforms were selectively lost. These findings imply Pax6 as a modulator of the spliceosome, which is the first time that a homeobox protein has been found to affect alternative splicing.

Tnc is an alternatively spliced glycoprotein of the extracellular matrix. During mouse brain development 27 Tnc isoforms were reported to be present (3). Interestingly, the pattern of Tnc isoform complexity observed in neurospheres derived from ventral telencephalic neural stem/progenitor cells at E13 was similar to that described for the early postnatal cerebellum at P6 (3). At the latter stage Bergmann glia or Golgi epithelial cells, which are the radial glia cells of the cerebellum, are the only identified source of Tnc (12, 35). The observation of a largely overlapping pattern of Tnc isoform complexity between P6 cerebellum and E13 forebrain neurospheres was unexpected, because we initially assumed a neural stem cell-specific signature of Tnc isoform expression. However, our findings could
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FIGURE 5. Regulation of extracellular matrix molecules and Tnc receptors after transfection of Pax6. Representative, ethidium bromide-stained agarose gels of RT-PCR experiments using cDNAs from control and Pax6 overexpressing neurospheres (Nsphs) are shown. Left panels, in control neurospheres both Pax6 isoforms are moderately expressed. Note that after transfection of Pax6 expression plasmids encoding the shorter Pax6 isoform, the alternate exon 5 insert form of Pax6 could no longer be faithfully amplified (top panel). The total Tnc mRNA levels appeared unaltered after Pax6 transfection when primer pairs located in the constitutive domains of Tnc were used (middle panel). Also, the same expression level of DSD-1-PG/phosphacan as in non-transfected controls was observed (bottom panel). The expression levels of Tnc were analyzed semi-quantitatively after normalizing the intensity of the PCR bands to those of β-actin and plotted in the bar chart (n = 3, data are expressed as mean ± S.D.). Note that Pax6 overexpression did not alter the total mRNA level of Tnc. Right panels, Pax6 overexpressing neurospheres and non-transfected controls were analyzed by RT-PCR using primers that allow the amplification of Tnc receptors with known affinities for the alternatively spliced region of Tnc. Please note that integrin α7 (Itga7) mRNA levels are selectively increased after Pax6 overexpression (top panel), whereas integrin β1 (Itgb1) and F3/contactin (Cntn1) mRNA levels are unchanged in comparison to non-transfected control neurosphere cultures after 6 h (upper and lower middle panels, respectively). Amplicons for β-actin (Actb) served as controls (bottom panel).

FIGURE 6. Large Tnc isoforms are absent from sey forebrain tissues. An ethidium bromide-stained agarose gel is shown that revealed the pattern of Tnc isoforms in neural tissues obtained from E13.5 sey mutants and wild type (wt) littersmates after RT-PCR using the primers 5s and 6as. Note that the large Tnc isoforms are lost in the cortical and striatal (GE) telencephalon of sey mice in comparison to control animals. This is particularly evident in the GE samples probably because endogenous Tnc expression is most prominent at the cortico-striatal boundary at this stage, which is difficult to dissect precisely. The pattern of Tnc isoforms in the hindbrain of sey mutants is indistinguishable from normal wild type mice, which served as an internal control because Pax6 is not expressed by ventricular zone precursors in the hindbrain. Cor, cortex; GE, ganglionic eminence; HB, hindbrain; sey, small eye; WT, wild type.

relate to the fact that in neurospheres many radial glia-like neural precursor cells are found (28). Consequently, we rather may have revealed a radial glia-specific Tnc isoform expression pattern, which is also interesting because the number of radial glia cells is reduced in Tnc-deficient mice (14). It should be men-
tioned that oligodendrocyte progenitors were also found to express large Tnc isoforms, although the complexity has not been investigated (4). However, this may reflect a rather close relationship between neural stem cells and oligodendrocyte progenitors (36). Nevertheless, it would be interesting to compare the Tnc isoform complexity between radial glia cells and astrocytes that represent the most prominent source for Tnc in the early to young postnatal forebrain (7). This appears particularly relevant in light of the finding that the expression of Tnc in the small eye mutant (sey) appears to be lost in radial glia cells but is maintained in postnatal astrocytes of the forebrain (20). Indeed, we could reveal the loss of alternatively spliced Tnc isoforms in the forebrain of sey mice, whereas the constitutive form of Tnc was still detectable at low levels. Interestingly, a dynamic regulation from larger toward smaller Tnc isoforms has been reported during chicken cornea (37) and mouse kidney development (38).

Because the sey mutation has been identified as a loss of function allele of Pax6 (33), we chose to study the consequence of Pax6 overexpression in neurospheres, which has been reported to cause neuronal differentiation of neural stem/progenitor cells (39). An increased level of neurogenesis compared with control cultures was also observed in our study (data not shown). However, the number of neurons obtained after transient transfection was clearly smaller than the ones obtained after retroviral transduction of Pax6 (39). The selective regulation of Tnc by Pax6 indicates that larger Tnc isoforms correlate more closely with a neurogenic cell fate in comparison to the down-regulated small Tnc isoforms that revealed an astrocytic signature (4) as also observed in the A7 cell line. Pax6 appears to generate a neurogenic radial glia Tnc profile, which is likely to be due to direct transcriptional control, because six Pax6 consensus sites are found in the Tnc locus within 5 kb upstream of the start codon. Thus, the larger Tnc isoforms could belong to the relevant target genes regulated by Pax6 that cause the dramatic change in cell fate from astrocyte to neuron after Pax6 overexpression (40). There is no direct evidence that large Tnc isoforms are involved in cell fate decisions. However, the increased number of βIII-tubulin-positive cells observed in differentiation assays using Tnc-deficient neurospheres could be reversed by the addition of purified Tnc

5 U. Egbers, L. Bulow, A. von Holst, and A. Faissner, unpublished observations.
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FIGURE 7. Pax6 overexpression does not alter the pattern of Tnc isoforms in other cell types. A, ethidium bromide-stained agarose gels are shown that revealed the pattern of Tnc isoforms in MEF after RT-PCR using the primers 5f and 6r. Note that Tnc isoforms containing five alternatively spliced FnIII domains predominate and that the pattern of Tnc isoforms is unaltered in MEF after Pax6 overexpression. B, ethidium bromide-stained agarose gels are shown that revealed the pattern of Tnc isoforms in the astrocytic cell line A7 using the primers 5s and 6as. Note that Tnc isoforms containing one or no alternatively spliced FnIII domain predominate, which is also reported for primary astrocytes, and that the pattern of Tnc isoforms is unaltered in A7 cells after Pax6 overexpression. Successful transfection is seen in the middle panels using primers for Pax6. Amplicons for β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as controls (lower panels).

(14), which is enriched for larger Tnc isoforms because the purification protocol depends on an antibody that has been mapped to the FnIIID domain (6). In more general terms, the function of the alternatively spliced FnIII domains of Tnc has been puzzling since their original description. The only clear cut data that have been obtained implicate the D domain as an essential neurite outgrowth promoting entity. Depending on the flanking FnIII domains, the neurite outgrowth promoting activity is mediated by binding to F3/contactin in the absence of the neighboring FnIII domain C (6) or by interaction with the α7/β1 integrin receptor in the presence of the C domain (41). Thus, the activity of individual Tnc FnIII domains appears to be context dependent. In light of the recent progress in the field of cancer stem cells in brain tumors (42), it is worth mentioning that large Tnc isoforms have been associated with neoplasias in general (43). In particular, the FnIII domain C has been described as a marker selectively associated with proliferating cells in glioblastomas (44). We may speculate that the alternatively spliced FnIII domains of Tnc are involved in normal proliferation of neural stem/progenitor cells, which is in line with the reduced number of bromodeoxyuridine-incorporating cells in the CNS of Tnc-deficient mice (10) and in neurosphere cultures derived from these animals (14).

The integrin α7 subunit, which binds to the Tnc domain FnIIID (5), was selectively up-regulated by Pax6. Here we found the presence of five Pax6 consensus sites in the Itga7 locus within 5 kb upstream of the start codon. (6) It is of interest that β1 integrins have been shown to regulate proliferation and survival in neural stem/progenitor cells (45). β1 integrins are also involved in the maintenance of neural stem cells and the authors have suggested that laminin α2 is an important ligand in the neural stem cell niche signaling via α6/β1 integrin receptors (46). We propose that Tnc isoforms containing the alternatively spliced FnIIID domain might regulate neural stem cell behavior in a comparative mode by signaling through α7/β1 integrin receptors. This might be of particular relevance because the α7 integrin is the only α subunit present in the adult SVZ (47), where Pax6 regulates the generation and specification of neuronal progenitors (21).

It will be interesting to learn which transcription factors control Tnc expression outside the Pax6 expression domain because Tnc appears to be present in the germinal layers throughout the anterior-posterior axis of the developing CNS including regions that lack Pax6. Also, it remains to be investigated whether the complexity of Tnc isoforms is subjected to regional changes in addition to the reported developmental dynamics (3). The only reported domain-specific in situ hybridization data have used the Tnc FnIIID-domain C in E15 mouse embryos (35) or domains A, AD1, AD2, B, C, and D in the optic tectum of E10 chicken embryos (48). On a histological basis the Tnc isoform expression pattern has never been systematically investigated and the regional profile of Tnc complexity has so far remained elusive. Our findings on the intricate regulation of Tnc isoform expression by Pax6 in neural stem/progenitor cells suggest that the fine-tuning of a possible Tnc code could play an important role in the neural stem cell niche during development and in the adult brain.

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