Time-point and dosage of gene inactivation determine the tumor spectrum in conditional *Ptch* knockouts

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Mutations in *Ptch* (Patch) have been associated with tumors characteristic both for children [medulloblastoma (MB) and rhabdomyosarcoma (RMS)] and for elderly [basal cell carcinoma (BCC)]. The determinants of the variability in tumor onset and histology are unknown. We investigated the effects of the time-point and dosage of *Ptch* inactivation on tumor spectrum using conditional *Ptch*-knockout mice. *Ptch* heterozygosity induced prenatally resulted in the formation of RMS, which was accompanied by the silencing of the remaining wild-type *Ptch* allele. In contrast, RMS was observed neither after mono- nor biallelic postnatal deletion of *Ptch*. Postnatal biallelic deletion of *Ptch* led to BCC precancerous lesions of the gastrointestinal epithelium and mesenteric tumors. Hamartomatous gastrointestinal cystic tumors were induced by monoallelic, but not biallelic *Ptch* mutations, independently of the time-point of mutation induction. These data suggest that the expressivity of *Ptch* deficiency is largely determined by the time-point, the gene dose and mode of *Ptch* inactivation. Furthermore, they point to key differences in the tumorigenic mechanisms underlying adult and childhood tumors. The latter ones are unique among all tumors since their occurrence decreases rather than increases with age. A better understanding of mechanisms underlying this ontological restriction is of potential therapeutic value.

Introduction

Tumorigenesis generally increases with age, probably reflecting the accumulation of genetic lesions due to deteriorating DNA repair capacity. A notable exception are childhood tumors, which are usually observed in young and only very infrequently in adult individuals. The mechanisms underlying this ontological restriction remain unknown. In one scenario, a childhood tumor’s cell of origin may be temporarily more prone to mutation accumulation or the number of mutations required for tumorigenesis is lower than in adult tumors. Another possibility is that the cell of origin becomes insensitive to normally tumorigenic mutations at later stages of ontogenesis. Yet another possibility is that the cell of origin is no longer present in adult organism due to differentiation or apoptosis. The discrimination between these and other possibilities would profit from model systems that allow for a maximally direct comparison between tumors of the childhood and of the elderly.

One such model could be the rare, inherited haploinsufficiency in the tumor suppressor Patched (*PTCH*) known as Gorlin syndrome showing a prevalence of 1:60 000 (1). Individuals with this syndrome develop both childhood tumors such as medulloblastoma (MB), rhabdomyosarcoma (RMS) and rhabdomyoma and tumors typical for the adulthood such as basal cell carcinoma (BCC), meningioma and ovarian fibrosarcoma. Further abnormalities comprise cysts of the skin, ovaries and mesenterium (2–4). The importance of *PTCH* is not restricted to Gorlin syndrome since somatic mutations have been detected in at least 50, 10 and 40% of sporadic forms of BCC, MB and rhabdomyoblastic tumors, respectively (5–7). Somatic alterations or mutations in *PTCH* have also been detected 2/3 in cardiac fibroma (8) and in 2/30, 2/54 and 2/7 carcinomas of the esophagus (9), bladder (10) and breast (11), respectively.

In the present work, we applied a conditional mouse model of *Ptch* deficiency (*Ptchfl/+*) (12) to investigate the mechanisms underlying the ontological restriction of tumor entities associated with its loss. Using this model, we specifically investigated the effect of the time-point (mouse embryonic development; postnatal life) and gene dosage of *Ptch* inactivation (monoallelic or biallelic) on the onset and spectrum of associated tumors. The activity status of the *Ptch* signaling was assessed as the expression of its targets Gli1 and *Pcth* itself (13). Our data point to key differences in the tumorigenic mechanisms underlying childhood and adult tumors. The *Ptch* mouse models presented here will provide an excellent tool to investigate the exact mechanisms underlying the ontological restriction of tumor formation in the future.

Material and methods

**Animals**

The used mouse strains were *Ptchfl/+* (12), *Elletcre* (14) and *Rosa26CretERT2* (14). On the genetic backgrounds employed, *Ptchfl/+* mice are entirely normal and healthy prior to the excision of the floxed *Ptch* locus by Cre-mediated recombination [see also (12)]. To achieve a germline deletion, *Ptchfl/+* mice were crossed with *Elletcre* mice, which express Cre recombinase most efficiently in mature oocytes (14). Female F1 *Ptchfl/+* females and *Elletcre*/-/- mice (14) were bred to wild-type (wt) mice and the resulting heterozygous *Ptchfl/+* mice were interbred to generate *Ptchfl/+* mice.

To obtain *Ptchfl/fl* (*EIIaCretERT2*-/-, *Ptchfl/fl* (*EIIaCretERT2*-/-/-) mice and *Ptchfl/fl* *EIIaCretERT2*-/-/- mice and to time specifically inactivate *Ptch*, *Ptchfl/fl* mice were bred to *EIIaCretERT2*-/- mice that ubiquitously express a tamoxifen-inducible Cre recombinase from the Rosa26 locus (15). The efficiency of the *EIIaCretERT2* deleter has been demonstrated in embryos between E7.5 and E10, where it is expressed in all embryo-derived tissues. In the adult, *EIIaCretERT2* expression has been demonstrated in several organs including skin, heart, kidney, liver, bladder and organs of the gastrointestinal tract, although not in brain and cerebellum (15). All experiments using animals were performed with consideration of all necessary legal requirements.

Genotyping was performed using the primers, Neo-R/p910F.A, Neo-F/p1011R.2 or mPTCNx_f/mPTCNx_r and p910F.A/p1011R.2 or mPTCNx_f/mPTCNx_r, to discriminate between the floxed and wt *Ptch* loci, respectively (12). To detect the Cre-mediated *Ptch* deletion, which removes the floxed exons 8 and 9, mice were genotyped by polymerase chain reaction (PCR) using the primer combination Exon 7F/NeoR, Neo-F/p1011R.2 (12) or mPTCNx_f/mPTCNx_r or mPTCNx_A/mPTCNx_r, to discriminate between the floxed and wt *Ptch* loci, respectively (12). To detect the Cre-mediated *Ptch* deletion, which removes the floxed exons 8 and 9, mice were genotyped by PCR using the primer combination EIIa-Cre F/EIIa-Cre R and RosCre ER-F/RosCre ER-R, respectively. All primers used for genotyping are shown in the supplement I (available at Carcinogenesis Online).

The predominant genetic background was Balb/c, as indicated in Table I. The projected observation period for the mice was at least 200 days.

**Abbreviations:** AR, artificial reference; BCC, basal cell carcinoma; CTX, carboxyterminus; EIIa, hedgehog; i.m., intramuscularly; i.p., intraperitoneally; MB, medulloblastoma; PCR, polymerase chain reaction; *Ptch*, patched; RMS, rhabdomyosarcoma; RT–PCR, reverse transcription–polymerase chain reaction; SP, side population; wt, wild-type.

¹These authors contributed equally to this work.

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Table I. Mice generated for the study

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Backgrounda</th>
<th>Drug; age at application</th>
<th>N</th>
<th>Age (days) rangeb; median</th>
<th>Tumors: % animals (absolute numbers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ptchflox/flox</td>
<td>1</td>
<td>—</td>
<td>78</td>
<td>27–471; 254</td>
<td>Kidney cysts: 2% (1/57), ovarian cyst: 2% (1/57)</td>
</tr>
<tr>
<td>Ptchflox/flox</td>
<td>Balb</td>
<td>—</td>
<td>21</td>
<td>87–204; 145</td>
<td></td>
</tr>
<tr>
<td>Ptchflox/flox</td>
<td>Balb</td>
<td>—</td>
<td>68</td>
<td>23–471; 194</td>
<td></td>
</tr>
<tr>
<td>Ptchflox/flox</td>
<td>B6</td>
<td>—</td>
<td>68</td>
<td>47–383; 203</td>
<td></td>
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<tr>
<td>Ptchflox/flox</td>
<td>2</td>
<td>6/50 embryos</td>
<td>53</td>
<td>25–416; 102</td>
<td></td>
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<tr>
<td>Ptchflox/flox</td>
<td>2</td>
<td>—</td>
<td>110</td>
<td>65–200; 148</td>
<td></td>
</tr>
<tr>
<td>Ptchflox/flox; ERT2+/−</td>
<td>3</td>
<td>5 mg TA i.p., 8 weeks</td>
<td>74</td>
<td>34–78; 73</td>
<td>tPtch−/− phenotype: 100% (74/74)</td>
</tr>
<tr>
<td>Ptchflox/flox; ERT2+/−</td>
<td>4</td>
<td>100 μg TA i.m., 6–8 weeks</td>
<td>10</td>
<td>93–365; 205</td>
<td>BCC: 100% (10/10)</td>
</tr>
<tr>
<td>Ptchflox/flox; ERT2+/−</td>
<td>4</td>
<td>100 μg TA i.m., 6–8 weeks +1 μmol CTXa</td>
<td>10</td>
<td>73–205; 171</td>
<td>BCC: 100% (10/10)</td>
</tr>
<tr>
<td>Ptchflox/flox; ERT2+/−</td>
<td>4</td>
<td>50 μg TA i.m., 2 weeks</td>
<td>10</td>
<td>83–291; 160</td>
<td>BCC: 100% (10/10)</td>
</tr>
<tr>
<td>Ptchflox/flox; ERT2+/−</td>
<td>3</td>
<td>5 mg TA i.p., 8 weeks</td>
<td>32</td>
<td>82–440; 376</td>
<td>Cystc: 13% (4/32), fibroma: 3% (1/32), epidermal cyst: 3% (1/32)</td>
</tr>
<tr>
<td>Ptchflox/flox; ERT2+/−</td>
<td>4</td>
<td>100 μg TA i.m., 6–8 weeks</td>
<td>10</td>
<td>242–423; 326</td>
<td></td>
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<tr>
<td>Control lines</td>
<td>Ptchflox/flox</td>
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<td>5 mg TA i.p., 8 weeks</td>
<td>9</td>
<td>372–441; 417</td>
</tr>
<tr>
<td>Ptchflox/flox</td>
<td>4</td>
<td>100 μg TA i.m., 6–8 weeks +1 μmol CTXa</td>
<td>10</td>
<td>92–365; 338</td>
<td></td>
</tr>
<tr>
<td>Ptchflox/flox</td>
<td>4</td>
<td>50 μg TA i.m., 2 weeks</td>
<td>10</td>
<td>182–192; 228</td>
<td></td>
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<tr>
<td>Ptchflox/flox</td>
<td>2</td>
<td>5 mg TA i.p., 8 weeks</td>
<td>5</td>
<td>441; 41</td>
<td></td>
</tr>
<tr>
<td>Ptchflox/flox; ERT2+/−</td>
<td>3</td>
<td>Vehicle i.m., 6–8 weeks</td>
<td>5</td>
<td>95–466; 286</td>
<td>Cystd: 11% (1/9)</td>
</tr>
<tr>
<td>Ptchflox/flox; ERT2+/−</td>
<td>4</td>
<td>Vehicle i.m., 2 weeks</td>
<td>5</td>
<td>293–365; 317</td>
<td></td>
</tr>
<tr>
<td>Ptchflox/flox; ERT2+/−</td>
<td>3</td>
<td>Vehicle i.m., 6–8 weeks</td>
<td>7</td>
<td>275–399; 277</td>
<td></td>
</tr>
</tbody>
</table>


*From birth on.

†Intrapertioneally localized blood-filled cystic tumors of unknown origin.

*For tPtch−/− phenotype see text.

‡CTX was applied i.m. 2 and 28 days after tamoxifen injection into the same muscle.

§Tamoxifen.

Induction of ERT2 activity by tamoxifen injection

Cre activity was induced in 6- to 8-week-old or 14-day-old (hereafter P14) Ptchflox/flox; ERT2+/− or Ptchflox/flox mice by tamoxifen (10 mg/ml), a solution of sterile ethanol: sunflower oil, 1:10). Adult 6- to 8-week-old mice were injected intraperitoneally (i.p.) with 1 mg tamoxifen on five consecutive days (cumulative dose 5 mg) or once in the musculus tibialis anterior intramuscularly (i.m.) with 100 μg of the drug. P14 mice were injected once i.m. with 50 μg tamoxifen. For estimation of Cre-efficiency in skeletal muscle, several mice were killed 12–14 days after injection. All other mice were observed over a period of time indicated in Table I.

Muscle injury model

In order to activate muscle regenerative processes and thus proliferation of Ptchflox-ablated muscle cells, we injected 100 μl of a 10 μM cardiotxin (CTX) solution from Naja mossambica mossambica (Sigma–Aldrich, Steinheim, Germany) in the musculus tibialis anterior of adult 6- to 8-week-old mice 2 days after i.m. application of tamoxifen. The muscles were harvested at various times 70–200 days after injection. Uninjected left muscles were used as non-CTX-injected controls.

Isolation of mononucleated muscle cells

Mononucleated muscle cells were isolated using a standard protocol (http://www.bcm.edu/labs/goodell/protocols.html). In brief, the tibialis anterior of 6-week-old mice was excised, minced and digested at 35°C in 0.2% collagenase type II ( Worthington Biochemical Company, Lakewood, NJ) for 30–60 min. The digestion was inactivated by adding 20 ml Hanks’ balanced salt solution (+) buffer pre-warmed to 37°C. The reaction mixture was centrifuged at 1900 g for 5 min at 25°C. The pellet was carefully resuspended in prewarmed 5 ml Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and filtered through 40 μm cell strainers (BD Biosciences Europe, Erembodegen, Belgium). Cells were spun down again (see above), resuspended in 3 ml pre-warmed Hanks’ balanced salt solution (+) buffer, loaded onto a Percoll gradient (40–70%) and centrifuged at 1300 g for 20 min at 25°C with breaks turned off. Mononucleated cells were carefully removed (middle band) and washed once with 10 ml pre-warmed Hanks’ balanced salt solution (+) buffer at 1900 g for 5 min at 25°C.

Histological examination

Tissues samples were derived from formalin-perfused animals or were fixed in formalin immediately after excision from killed animals. The 0.5 μm paraffin sections were examined upon staining with hematoxylin and eosin.

Dissection of tumor tissue and isolation of DNA

Formalin-fixed, paraffin-embedded tissue sections were cut in 5 μm-thick sections. For macrodissection, neoplastic tissue was dissected from non-neoplastic tissue under a microscope using a scalpel. For microdissection, the Arcturus XTmin PtcxCell II Microdissection System (Arcturus Engineering Inc., Sunnyvale, CA) was used. This system consists of a 655 nm infrared laser coupled to an inverted microscope. Approximately 5000 cells were microdissected from BCC lesions. DNA was isolated using the DNA extraction Kit from AlphaMetrix Biotech (Rödermark, Germany) according to the manufacturer’s description.

In situ hybridization

Sections of 3 μm thickness were prepared from mouse BCC and normal skin and hybridized with Digoxigenin-uridine triphosphate-labeled RNA probes. The probe sequences for mouse Glil and Gl2 have been described previously (16). A 477 bp (spanning Ptcx exons 2–6) and 250 bp (spanning Ptcx exons 8 and 9) partial complementary DNA fragment of Ptcx was generated by reverse transcription–polymerase chain reaction (RT–PCR), cloned into pBluescriptII and sequenced to verify the identity for generation of antisense riboprobes. Sense riboprobes were applied as negative controls. In situ hybridization was performed as described elsewhere (16).

Quantification of ERT2-mediated recombination at the Ptchflox locus

The efficiency of ERT2-mediated recombination at the floxed Ptcx locus in microdissected BCC, in mononucleated cells and whole muscle was analyzed.
on genomic DNA isolated from Ptchflox/flox ERT2+/−/− mice. DNA from muscle was isolated 12–14 days after the first tamoxifen injection from hind limbs. Recombination efficiency was quantified by real-time PCR. Primers mPTCdelNx_f/mPTCNx_r amplify a 160 bp fragment after Cre-mediated excision of the floxed locus. A 6-carboxylfluoresceine-labeled probe (mPTCdelNx_S1) was used for detection of the deleted Ptchdel allele. The primer combination mPTCNx_f/mPTCNx_r amplifies a 150 bp fragment of

Fig. 2. Analysis of transcripts derived from Ptchflox and Ptchdel alleles. RT–PCR analyses of Ptch expression in different organs from wt and Ptchflox/ERT2+/−/− mice in comparison with Ptchdel/+ mice harboring a Ptch germline mutation and with tamoxifen-treated Ptchflox/+ ERT2+/−/− and Ptchflox/ERT2+/−/− mice. Amplification of wt Ptch and Ptchdel transcripts result in a 731 bp and in a 451 bp fragment, respectively. In skeletal muscle of Ptchflox/+ mice, we observed an elevated level of a normally occurring Ptch messenger RNA splice variant (wt PtchD10) in which exon 10 is excised by alternative splicing of exon 9 into exon 11 (19) (confirmed by sequencing). Amplification of mGapd served as control. The respective tamoxifen treatment is depicted on the left side of each panel (i.m., intramuscular injection; i.p.). li, liver; ki, kidney; sp, spleen; lu, lung; he, heart; sm, skeletal muscle; ce, cerebellum; br, brain; th, thymus; sk, skin; sm (i.m.), skeletal muscle locally injected with tamoxifen.

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the Ptch\(^{flox}\) locus. A Yamika Yellow-labeled probe (mPTCNs_S2) detected the Ptch\(^{flox}\) allele. A pelota gene-specific quantitative PCR-assy (primer combination Pelo-F1/Pelo-R, probe Pelo-Sonde2) was used for data normalization. Standard curves for amplification of the Ptch\(^{flox}\) and the Ptch\(^{del}\) allele were prepared from serial dilutions of artificial references (ARs). For generation of ARs, 10 pmol of the primers AR-NxF.1 and AR-NxR.1 or AR-delNxF.1 and AR-delNxr.1 were annealed and 5\(^{\text{th}}\) overhangs were filled in using Klenow enzyme. The reactions were brought to a final volume of 1 ml by adding 900 µl H\(_2\)O. Standard curves were prepared from serial dilutions starting from a 10\(^{-4}\) dilution of the stock solution.

The data were analyzed using the standard curve method for relative quantification. The deletion efficiency was calculated as the ratio of the values for the deleted allele to the total value from a Ptch\(^{flox}\) mouse plus the deleted allele and is expressed in percent (for primer and probe sequences and their modifications see supplement 1, available at Carcinogenesis Online).

**RNA-isolation and RT–PCR**

Total RNA was extracted using PqgGOLD TriFast\(^{\text{TM}}\) reagent (PegLab, Erlangen, Germany). Reverse transcription of total RNA was performed using random hexamers and SuperScriptII reverse transcriptase (Invitrogen, Karlsruhe, Germany). To analyze the expression of transcripts derived from the Ptch\(^{flox}\) as well as from the deleted Ptch\(^{del}\) locus, PCR was carried out using the primers mPtC11 and mPtC7R that are localized in exons 6 and 11 of the murine Ptch transcript, respectively (12). To analyze transcription levels of Gli1, the primer combination mGliF8 and mGliR9 were used (for primers see supplement 1, available at Carcinogenesis Online).

**Blood values**

Ethylenediaminetetraacetic acid blood samples were collected by heart puncture and were centrifuged at 3000 r.p.m. for 10 min. Glucose, cholesterol, triglycerides, glutamate-pyruvate-transaminase, total bilirubin, blood urea nitrogen and total protein were determined in the plasma by enzymatic *in vitro* assays for the quantitative determination of the parameters. The assays were performed on Roche automated clinical chemistry analyzers (Roche/Hitachi MODULAR P).

**Results**

**Homozgyous germline deletion of the Ptch\(^{flox}\) allele results in embryonic lethality**

To achieve germline deletion, Ptch\(^{flox}\)/Ptch\(^{flox}\) mice were crossed with EllaCre mice, which express Cre-recombinase in mature oocytes (14). All homzygous Ptch\(^{del}\)/Ptch\(^{del}\) mutants died in *utero* before or around embryonic day 9.5. At day 9.5 only 50% of Ptch\(^{del}\)/Ptch\(^{del}\) embryos are available for analysis and showed severe closure defects of the neural tube (data not shown), a phenotype identical to that of a homozygous non-conditional Ptch deletion (17,18).

**Heterozygous germline deletion of the Ptch\(^{flox}\) allele results in RMS, MB and hamartomatous gastrointestinal cytis tumors**

Heterozygous mutant (Ptch\(^{del+/-}\)) mice were obtained from crosses of Ptch\(^{flox}\)/Ptch\(^{flox}\) mice with EllaCre mice. Similar to what was observed in conventional Ptch\(^{flox}\)/Ptch\(^{flox}\)-knockout mice (18), the number of heterozygous mice was 25% lower than expected, indicating pre- or perinatal lethality. By 200 days of age, 21 and 8% of heterozygotes have developed RMS and MB, respectively (Figure 1A; Table I). RMS overexpressed Gli1 and mutant Ptch\(^{del}\) transcripts (Figure 1B, lower panel). Mutant Ptch\(^{del}\) transcripts in which exon 7 is spliced into exon 7 are never observed in tissues derived from wt or Ptch\(^{del}\)/Ptch\(^{del}\) mice (Figure 2). Since no wt Ptch transcripts were detected in tumor tissue (Figure 1B, lower panel), we investigated whether the wt Ptch allele was lost in RMS. However, as shown in Figure 1B (upper panel), the wt Ptch allele was retained. The data indicate that, similar to RMS of conventional Ptch\(^{flox}\)/Ptch\(^{flox}\) mice (20,21), the wt Ptch allele is silenced in these tumors.

In addition to MB and RMS, 8% of Ptch\(^{del+/-}\)/Ptch\(^{del+/-}\) mice developed i.p. localized hamartomatous gastrointestinal cytis tumors, which were attached to the wall of the stomach, of the intestine or were located within pancreatic ducts. Due to the presence of smooth muscle, epithelium and fibrous tissue (Figure 1C, upper panel), these tumors are reminiscent of hamartomatous lesions in humans. As in RMS, the wt Ptch allele was retained in the cystic wall (Figure 1C, lower panel).

**Monoallelic loss of Ptch in adult Ptch\(^{flox}\)/Ptch\(^{flox}\) ERT2/+- mice results in hamartomatous gastrointestinal cytis tumors**

The 6- to 8-week-old Ptch\(^{flox}\)/ERT2/+- mice were treated i.p. with 1 mg tamoxifen for five consecutive days. Except for brain and cerebellum, this dose resulted in an inefficient recombination of the targeted Ptch locus in the examined tissues (Figure 2). Within an observation period of 12 months, 6/32 (19%) animals developed tumors including four hamartomatous gastrointestinal cytis tumors, one epidermal cyst and one fibroma of the skin (Table I). One of the five vehicle-injected Ptch\(^{flox}\)/ERT2/+- controls developed a cyst of the kidney. Except for these tumors, no additional conspicuous macroscopic or histological changes were observed. These data show that monoallelic inactivation of Ptch in the adult organism does not result in RMS or BCC, whereas cystic tumors developed at low incidence.

**Biallelic Ptch loss in adult Ptch\(^{flox}\)/Ptch\(^{flox}\) ERT2/+- mice induced by high dose of tamoxifen results in hyperproliferative lesions of the skin, stomach epithelium and mesenterium**

Next, we determined the effects of biallelic Ptch inactivation in the adult organism. For this purpose, 6- to 8-week-old Ptch\(^{flox}\)/ERT2/+- mice were treated with a cumulative dose of 5 mg tamoxifen i.p. (hereafter also called tPtch/-/- mice). As shown in Figure 2, with the exception of cerebellum and brain, Ptch ablation was efficiently induced in all tissues examined. The diminished amplification of the mutant Ptch transcript in lung and skeletal muscle in comparison with Ptch\(^{flox}\)/ERT2/+- mice might have been caused by a less efficient RT–PCR reaction. Besides the already reported defect in T and B cell development (12), all animals showed progressive hair loss that was more marked ventrally than dorsally (Figure 3A). Histological examination revealed severe skin abnormalities, including hyperproliferation areas associated with either the interfollicular or follicular epidermis (Figure 3B). Hyperproliferation was also detected in the non-glandular cutaneous stomach and the glandular mucous stomach (Figure 3C). In addition, small tumors composed of spindle cells were visible in the mesenterium of all animals examined (Figure 3D). Mesothelioma or leiomyoma was excluded due to lack of smooth muscle actin (SMA), CD34 or cytokeratin expression (data not shown). Based on their macroscopic and histological appearance, these tumors are also not related to the hamartomatous gastrointestinal cytis tumors that occur after monoallelic Ptch inactivation (see Figure 1C). Although heart, lung, kidney, liver, pancreas and skeletal muscle appeared normal (data not shown), increased blood urea nitrogen and decreased blood glucose levels (supplement 2 is available at Carcinogenesis Online) suggested an impaired kidney function and defects of either the digestive tract or an endocrine organ, respectively. Due to their poor general condition, tPtch/-/- mice were killed not later than 23 days after Cre-activation.

**Biallelic loss of Ptch induced in adult Ptch\(^{flox}\)/Ptch\(^{flox}\) ERT2/+- mice by low dose of tamoxifen results in BCC**

Longer follow-up observation time was made possible by injecting tamoxifen as a low, single dose i.m. in 6- to 8 week (100 µg) or P14 (50 µg) old Ptch\(^{flox}\)/Ptch\(^{flox}\) ERT2/+- mice. All animals developed tumors...
Fig. 4. In situ hybridization and Ptch status in BCC of Ptchflox/ERT2−/− mice after i.m. injection of tamoxifen. (A) Upregulation of Hh target genes in BCC from Ptchflox/ERT2−/− mice. For detection of Ptch transcripts, two probes were used. The 477 bp probe identifies both wt and mutant Ptch transcripts. The 250 bp probe exclusively binds to wt Ptch transcripts. In normal skin, wt Ptch and Gli1 transcripts are expressed in the hair follicle. BCC highly overexpresses Gli1 and mutant Ptch transcripts, as signals were exclusively obtained using the Ptch 477 bp probe. (B) PCR analysis on DNA derived from microdissected BCC shows that the Ptchflox allele is efficiently deleted in BCC nodules (61–76%).
of the skin on tails and ears (Table I). The tumors were grossly visible 90 days after tamoxifen injection and they expressed elevated levels of Gli1 (Figure 4A), which indicated hedgehog (Hh)-signaling activity. All tumors had features of human nodular BCCs since they were composed of nests of basoloid cells with large nuclei and a few mitotic figures (supplement 3 is available at Carcinogenesis Online). In addition, the tumors showed peripheral palisading, were well circumscribed, non-invasive and did not metastasize (Figure 4A). Furthermore, we observed structures resembling retention cysts of the sebaceous glands.

As shown in Figure 2, the injected low doses of tamoxifen resulted in an efficient deletion of Ptcch in the skin. Efficient inactivation of Ptcch in tumor tissue was also demonstrated in DNA from microdissected tumor tissue, which revealed a ~60 to 75% deletion of the Ptcch alleles in BCCs (Figure 4B) and by in situ hybridization using two probes. The 477 bp probe hybridizes to Ptcch exons 2–6 and identifies both wt and mutant Ptcch transcripts. The 250 bp probe spans exons 8–9 and detects exclusively wt Ptcch transcripts. As shown in Figure 4A, hair follicles of normal skin stained with either probes. In contrast, follicular tumors hybridized exclusively to the probe comprising exons 2–6. These data show that wt Ptcch is lost in BCC cells and, as a result of the recombination and loss of negative feedback, leads to overexpression of mutant Ptcch transcripts.

In contrast to the skin of ears and tails, follicular tumors were detected rarely on the trunk and paws. At the latter locations, follicular tumors were more differentiated and showed a trichoblastoma-like histology (data not shown). No other macroscopic or histological overt tumors or proliferative changes were observed in any other tissues examined until the age of 400 days.

Since i.m. injection of tamoxifen in Ptcchfloxflox ERT2+/- mice did not result in the formation of muscle tumors, recombination at the floxed Ptcch locus was examined more thoroughly in the skeletal muscles of five P14 Ptcchfloxflox ERT2+/- mice injected i.m. with 50 μg tamoxifen. As shown in Figure 5A, between 53 and 87% of the Ptcchfloxflox alleles were converted into Ptcchdel alleles. Analysis of mononuclear muscle cells (including side population (SP) and satellite cells (22)) revealed recombination efficiencies between 5% and 9% (Figure 5B). Efficient Ptcch deletion was also confirmed by RT–PCR using the primers mPtc11 and mPtc7R, which are located in exons 6 and 11 of Ptcch, respectively (Figure 5C). However, Hh signaling remained similar between mutant and control muscle as revealed by measurements of Gli1 expression (data not shown).

Finally, we activated muscle regeneration and thus proliferation of resident muscle stem cells by means of CTX injection 2 days after local Ptcch ablation. Although CTX injection caused the typical myopathic picture including fiber degeneration, intensive mononuclear cell infiltration and centralization of nuclei in regenerating muscle fibers (23) (data not shown), neither RMS nor morphological change resembling this tumor developed over 200 days in the injected muscles of the 10 Ptcchfloxflox ERT2+/- mice (Table I).

In summary, these data indicate that deletion of both Ptcch alleles in the adult skeletal muscle is neither sufficient to activate Hh signaling nor to induce tumor formation, also upon combination with a CTX challenge.

Discussion

The mechanisms underlying ontological restriction of tumor formation are unknown. Conditional Ptcchfloxflox, knockout mice are a perfect tool to study these mechanisms. Thus, the impact of the mutation location is kept to an absolute minimum since it is predetermined by the targeting strategy and is in consequence constant. In addition, Ptcch can be inactivated in a time-specific and dose-dependent manner and Ptcch mutations are necessary for formation of tumors of childhood (RMS) and adulthood (BCC) both in men and mice (13). Ptcch is assumed to act as a tumor suppressor gene (13). However, like with many other tumor suppressor genes, it is not clear whether mono- or biallelic mutations and/or the time-point of the mutational hit determine the occurrence of a specific tumor.

Our data show that the histological identity and the ontological stage of the tumors are affected by number of inactivated Ptcch alleles, by the time-point of inactivation and by its mechanism. The results, which are schematically summarized in the supplement 4 (available at Carcinogenesis Online), point to key differences in the tumorigenic mechanisms underlying childhood and adulthood tumors.

To arrive at these conclusions, we first had to characterize the spectrum of abnormalities following the different modes of Ptcch deletion. The conditional Ptcch deletion early in embryonal development resulted in a phenotype identical to that observed in conventional heterozygous Ptcch knockouts (17,18). Thus, all homozygous Ptcchdel/del mutants died before E10 in utero, whereas Ptcchdel/+ mice survived and developed MB, RMS and hamartomatous gastrointestinal cystic tumors at incidences similar to those observed in conventional Ptcch knock-out mice on the same genetic background [(24,25) and unpublished data by A. Uhmann, A. Zibat and H. Hahn]. These similarities are consistent with the efficient targeting in our new model of exons common to all known Ptcch transcripts (19,26).

The conditional Ptcch inactivation in adult mice broadened the spectrum of tumors and other abnormalities in comparison with a conventional heterozygous Ptcch deletion. Most strikingly, we observed BCC upon biallelic Ptcch inactivation, particularly on the hairless skin. The mechanisms underlying childhood and adulthood tumors.
epithelium resembling gastric cancer precursor lesions. Aberrant Ptch transcripts were detected in several other tissues in the absence of accompanying histological abnormalities. Some of the defects might have been very minute and gone undetected, which might have been the cause in the kidney, which appears normal, although blood urea nitrogen is increased. Taken together, some organs appear much less sensitive to Ptch deletion than others. Since wt Ptch mice express Ptch in all organs examined (Figure 2), this is most probably not connected with a general low Ptch expression in the less sensitive organs. It rather points to a tissue-specific modulation of Ptch protein function.

Both onset and histology of the tumors were determined by a complex interplay between the time-point, the gene dosage and the parental mode of Ptch inactivation. The importance of the time-point is best illustrated by the comparison of RMS and BCC. RMS arises following a prenatal Ptch deletion, whereas BCC do not. Conversely, postnatal Ptch inactivation with low tamoxifen doses results in BCC, but never in RMS. Thus, RMS appear to be initiated exclusively before and BCC after birth, at least in terms of the contribution of Ptch deletion. The exclusive prenatal RMS induction appears, at least at a first glance, to contrast a recent report that RMS develop post-natally and probably originate from terminally differentiating myoblasts (27). However, in our model, RMS resemble the embryonic subtype, whereas RMS induced by conditional overexpression of a Pax3-Fkhb fusion protein by Keller et al. (27) are of the alveolar subtype. Besides differentiating myoblasts, embryonic RMS could originate from so-called satellite cells, which present a specific class of muscle stem cells (28), from SP cells which can be isolated from the mononuclear fraction of muscle cells (29,30) and which are either bone marrow-derived (cells of the bone marrow-derived side population, BMSP) or of somatic origin (cells of the muscle-derived side population, mSP) (31) or from circulating myeloid cells or mesenchymal stem cells of the bone marrow (32,33). Since all these cell types exist before and after birth (31,34,35), the ontogenetic restrictions of RMS formation might have been caused by the cell of origin becoming non-permissive. Alternatively, RMS may originate exclusively from prenatal cells that are at present unknown. Yet another possibility is that one Ptch allele needs to be silenced by a mechanism that is not present in adult animals. In any case, the absence of RMS unlikely resulted from inefficient recombination of floxed Ptch alleles since the latter one approached 90% in the muscle tissue. The identity of the tumor was furthermore affected by the gene dosage of Ptch inactivation. Thus, BCC were observed in tamoxifen-treated mice carrying two, but never one, floxed Ptch alleles. The latter one approached 90% in the muscle tissue.

Regardless of the exact underlying mechanism, this fundamental difference between RMS and BCC is strongly supported by BCC, but not RMS formation requiring the genetic deletion of both Ptch alleles. The two separate mechanisms of Ptch inactivation (deletion and silencing) appear to affect the tumor spectrum in addition to the time-point and gene dosage.

These data demonstrate that the time-point, the gene dose and mode of inactivation of a tumor suppressor may strongly affect the spectrum of the associated tumors. They also point to key differences in the tumorigenic mechanisms underlying adult and childhood tumors. The latter ones are unique among all tumors since their occurrence decreases rather than increases with age. The mechanisms underlying this ontological restriction are poorly understood, but they are of potential therapeutic utility.

Supplementary material
Supplements 1–4 can be found at http://carcin.oxfordjournals.org/.

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References

Time-point of initiation of Ptch associated tumors

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