Developmental and oncogenic effects of Insulin-like Growth Factor-I in \textit{Ptc1}^{+/−} mouse cerebellum

Mirella Tanori\textsuperscript{†}, Melissa Santone\textsuperscript{†}, Mariateresa Mancuso, Emanuela Pasquali, Simona Leonardi, Vincenzo Di Majo, Simonetta Rebessi, Anna Saran, Simonetta Pazzaglia\textsuperscript{*}

Abstract

\textbf{Background:} Medulloblastoma is amongst the most common malignant brain tumors in childhood, arising from neoplastic transformation of granule neuron precursors (GNPs) of the cerebellum via deregulation of pathways involved in cerebellar development. Deregulation of the Sonic hedgehog/Patched1 (Shh/Ptc1) signaling pathway predisposes humans and mice to medulloblastoma. In the brain, insulin-like growth factor (IGF-I) plays a critical role during development as a neurotrophic and neuroprotective factor, and in tumorigenesis, as IGF-I receptor is often activated in medulloblastomas.

\textbf{Results:} To investigate the mechanisms of genetic interactions between Shh and IGF signaling in the cerebellum, we crossed nestin/IGF-I transgenic (IGF-I Tg) mice, in which transgene expression occurs in neuron precursors, with \textit{Ptc1}^{+/−} knockout mice, a model of medulloblastoma in which cancer develops in a multistage process. The IGF-I transgene produced a marked brain overgrowth, and significantly accelerated tumor development, increasing the frequency of pre-neoplastic lesions as well as full medulloblastomas in \textit{Ptc1}^{+/−}/IGF-I Tg mice. Mechanistically, tumor promotion by IGF-I mainly affected preneoplastic stages through \textit{de novo} formation of lesions, while not influencing progression rate to full tumors. We also identified a marked increase in survival and proliferation, and a strong suppression of differentiation in neural precursors.

\textbf{Conclusions:} As a whole, our findings indicate that IGF-I overexpression in neural precursors leads to brain overgrowth and fosters external granular layer (EGL) proliferative lesions through a mechanism favoring proliferation over terminal differentiation, acting as a landscape for tumor growth. Understanding the molecular events responsible for cerebellum development and their alterations in tumorigenesis is critical for the identification of potential therapeutic targets.

\textbf{Background}

Normal development and tumorigenesis have several common characteristics. In particular, pediatric neoplasms of the nervous system, arising from progenitor cells which are already proliferating as part of the developmental process, are closely linked to disordered mechanisms of normal development. The delicate balance among programmed cell death, proliferation and differentiation, in fact, is crucial for normal neural development. Defects in any of the mechanisms controlling these processes could promote transformation, making developing cells prone to tumorigenesis.

Medulloblastoma is the most common pediatric brain tumor, and develops in the cerebellum of children and young adults. Expression profiling of medulloblastoma has indicated a remarkable similarity between this tumor and early postnatal cerebellum, arguing that the germinal layer of the cerebellum harbors precursor cells for medulloblastoma [1,2]. During cerebellar development, granule neuron precursors (GNPs) migrate from the rhombic lip to the external granular layer (EGL), where they postnatally undergo a proliferative burst before exiting the cell cycle and migrating inward to form the mature inner granule layer (IGL). The cerebellum undergoes an over 1000-fold increase in volume during this process [3]. Proliferation of GNPs is governed principally by the Sonic hedgehog pathway (Shh), but their expansion and survival are also promoted by insulin-like growth factors (IGFs).
Deregulation of the Shh pathway has been linked to medulloblastoma development. Approximately 15-30% of sporadic medulloblastomas contain mutations in Patched1 (Ptc1) or other elements of the Shh pathway [1,4,5]. Germline deficiency of the Shh receptor, Ptc1, is responsible of the hereditary Neviod Basal Cell Carcinoma Syndrome (NBCCS) in which patients are predisposed to medulloblastoma and other tumors. Mice with heterozygous Ptc1 mutations are also susceptible to medulloblastoma formation, and 8-40% of them develop tumors that resemble human medulloblastomas [6,7]. These mice have provided information on the early stages of tumorigenesis [8,9] and on the genes that cooperate with deregulation of the Shh pathway to promote tumor progression [10-12].

IGF-I and IGF-II act as potent survival factors expressed in a wide variety of cell types. IGF signaling is important for central nervous system (CNS) development, and increased IGF-I activity results in brain overgrowth [13,14]. Moreover, molecular oncology studies in humans and mice strongly implicate IGFs in medulloblastoma development.

In this study, to clarify the role of IGF-I in physiological (development) and pathological (tumorigenesis) settings in the cerebellum, we cross-bred transgenic mice, overexpressing IGF-I (IGF-I Tg) in neural progenitors under control of regulatory sequences from the nestin gene [15], with Ptc1+/− mice, a faithful model of human medulloblastoma.

Results
Expression of IGF-I transgene and nestin in the cerebellum
To examine the impact of IGF-I overexpression on normal development and tumorigenesis in the cerebellum, we crossed Ptc1+/− mice with IGF-I Tg mice [15]. Expression of the human IGF-I transgene, quantified by reverse-transcription PCR in cerebella at P5, was evident in the cerebellum of Ptc1+/−/IGF-I Tg and Ptc1+/−/IGF-I Tg mice, whereas it was absent in Ptc1+/+ and Ptc1+/− cerebella (Figure 1A). Since the spatial expression pattern of nestin/IGF-I transgene was reported to be consistent with that of the nestin native gene [16], to localize the expression of the transgene, sections of

![Figure 1](image-url)
cerebellum from mice at P5 were immunostained for nestin. As shown in Figure 1B, nestin was strongly expressed in GNPs of the EGL, as well as in other layers of the developing cerebellum. In addition, by immunostaining, with an antibody that specifically recognizes human IGF-I, we detected IGF-I expression in cerebellum of IGF-I Tg mice but not in mice lacking the IGF-I transgene (Figure 1C and 1D). Thus, by using the nestin/IGF-I mouse model, we accomplished our goal to target IGF-I overexpression to the neural precursors of the cerebellum, the potential medulloblastoma progenitor cells [17].

Effects of IGF-I transgene expression on P5 cerebellum

Next, we analyzed proliferation of GNPs in the setting of altered Shh and IGF signaling in vivo. These analyses were performed at postnatal day 5 (P5), when the IGF-I transgene is expressed at high level in the cerebellum [15]. We assessed the number of proliferating cells by immunohistochemistry using antibodies to the antigen encoded by the Mki67 gene (Ki-67) and the Proliferating Cell Nuclear Antigen (PCNA). The presence of the IGF-I transgene caused a significant increase in the number of Ki-67 positive GNPs in Ptc1+/− (1.14 vs. 0.57%; \( P = 0.0091 \)) but not in Ptc1+/+ mice (1.12 vs. 0.8%; \( P = 0.11 \); Figure 2A and 2C). Moreover, the presence of IGF-I transgene significantly increased the frequency of PCNA positive GNPs in both Ptc1+/− (41.6 vs. 29.7%; \( P = 0.0078 \)) and Ptc1+/+ mice (44.6 vs. 34.0%; \( P = 0.0055 \); Figure 2B and 2D). No significant differences in numbers of Ki-67 and PCNA positive cells were observed between Ptc1+/− and Ptc1+/+ mice. By immunoblotting of isolated cerebellar extracts the expression of cyclin D1, a cell cycle regulatory protein was also increased, although not significantly, in the cerebellum of Ptc1+/− and Ptc1+/+ mice carrying the IGF-I transgene (Figure 2E and 2F). Taken together, these data indicate that the IGF-I transgene strongly stimulates cell proliferation in the cerebellum.

During neural embryogenesis, about 50-70% of neural cells undergo programmed cell death leading to a massive loss of granule cells during active neurogenesis in the first three postnatal weeks of cerebellar development [18]. Several growth factors, including IGF-I, have been shown to modulate cell death in this population [19]. An inhibition of naturally occurring GNPs death may therefore represent a possible mechanism to sustain cell proliferation and tumor growth. We assessed the number of cells undergoing programmed cell death in the EGL by immunostaining using an antibody against cleaved caspase-3. The presence of the IGF-I transgene caused a significant reduction in the number of caspase-3 positive GNPs in Ptc1+/−/IGF-I Tg mice compared with the non transgenic mice (0.5% in Ptc1+/−/IGF-I Tg vs. 0.11% in Ptc1+/−; \( P < 0.005 \); 0.09% in Ptc1+/−/IGF-I Tg vs. 0.17% in Ptc1+/−; \( P < 0.005 \); Figure 2G and 2H). No significant difference was observed between Ptc1+/− and Ptc1+/− mice. Overall, our data indicate that the presence of IGF-I transgene increased proliferation rate and decreased programmed cell death in GNPs.

Combined IGF-I transgene expression and Ptc1 mutation counteract differentiation of neural precursors

Among the pleiotropic IGF-I activities during neurogenesis, IGF-I also affects neuronal differentiation, as well as possibly influencing neural stem cells. To study the effect of IGF-I transgene on GNPs differentiation we examined morphologic abnormalities in H&E-stained sagittal sections of the cerebellum at P15. At this age, in the cerebellum of wild-type mice GNPs have almost completed their migration into the IGL, and the EGL has ceased to exist (Figure 3A). Instead, a thin 1-cell layer of EGL was detected in the cerebellum of IGF-I Tg (Figure 3B) and Ptc1+/− mice (Figure 3C). A thicker EGL layer of 2-3-cells was observed in the cerebellum of double mutants (Figure 3D). These results indicate that Ptc1 mutation and transgenic IGF-I expression delayed differentiation of EGL progenitors in the cerebellum.

To evaluate the effect of IGF-I overexpression on neurogenesis, we examined the expression of NeuN, which marks postmitotic mature granule neurons in the IGL, by immunoblotting of isolated cerebellar extracts at P5. As shown in Figure 3E and 3F, the presence of IGF-I transgene in Ptc1+/− mice significantly decreased NeuN expression in the cerebellum of compound mutants (\( P < 0.05 \)). In addition, immunostaining showed a strong decrease in expression of NeuN in Ptc1+/−/IGF-I Tg compared with Ptc1+/− mice (Figure 3G and 3H). Accordingly, quantization of NeuN positive neurons in the IGL showed a significant reduction in Ptc1+/−/IGF-I Tg (27%) compared with Ptc1+/− mice (42%; \( P = 0.0003 \); Figure 3I). This strongly suggests that IGF-I signaling cooperates with Shh deregulation in suppressing differentiation of granule progenitor cells from the active pool in the EGL. In this regard, a link between IGF signaling pathway and stem or progenitor cell potency has been recently highlighted by the finding that the number of cells expressing Sox9, a stem/progenitor cell biomarker, is decreased in intestinal crypts of IRS-1−/−/Min compared with IRS-1−/−/Min mice [20].

Phenotype of nestin/IGF-I transgenic brains

To evaluate the effect of the IGF-I transgene on brain growth, brains from Ptc1+/−, Ptc1+/−/IGF-I Tg, Ptc1+/−, and Ptc1+/−/IGF-I Tg mice of both sexes, at 3, 5 and 8 weeks of age were excised and weights determined. IGF-I acted to increase both size and weight of the
developing brain. The largest difference in brain size was observed between Ptc1+/+ and Ptc1+/IGF-I Tg mice at 8 weeks, as shown by representative H&E-stained sagittal sections (Figure 4A and 4B). Importantly, the cerebellum was among the brain regions showing a more marked overgrowth. Compared with Ptc1+/+ littermates, the presence of IGF-I transgene produced significant brain weight increases at all time points examined (9.3-13.2%; P ≤ 0.005; Figure 4C and 4D). In comparison with Ptc1+/+ littermates, significant increments were also produced by heterozygosity of the Ptc1+/- gene (11.6-13.2%; P ≤ 0.05). In addition, the presence of IGF-I transgene in Ptc1+/+ mice caused a further significant increase in brain weight compared with Ptc1+/+.
Figure 3 Delayed differentiation in neural precursors caused by IGF-I altered expression. (A) Morphologic analysis of H&E-stained sagittal sections of mouse cerebellum at P15, showing physiological absence of EGL in the cerebellum of Ptc1+/+ mice. A thin 1-cell layer of EGL was present in the cerebellum of Ptc1+/-/IGF-I Tg (B), and Ptc1+/+ mice (C). (D) A thicker 2-3-cells layer was observed in the EGL of Ptc1+/-/IGF-I Tg mice. (E) Western blot analysis showing the level of NeuN (48 and 46 kDa, solid and open square, respectively) expression in cerebellum from Ptc1+/+, Ptc1+/-/IGF-I Tg, Ptc1+/-, and Ptc1+/-/IGF-I Tg mice at P5, with relative β-actin to control protein loading. (F) Graphic representation of densitometric analysis. (G and H) Immunohistochemical analysis showing a decrease in the expression of NeuN in the IGL of the cerebellum of Ptc1+/-/IGF-I Tg mice (H) compared to Ptc1+/- mice (G). (I) Frequency of NeuN positive neurons in the IGL of Ptc1+/+ and Ptc1+/-/IGF-I Tg mice. More than 5 x 10^3 granule neurons from 12 randomly selected digital images of the IGL (2 mice per genotype) have been examined.
Despite the striking effect of the IGF-I transgene on brain size, the overall neural development was relatively normal and transgenic mice showed normal appearance and behavior. On the whole, these results indicate that the presence of either the IGF-I transgene or of the Ptc1 heterozygous mutation leads to macrocephaly, as brains were larger and weighted significantly more compared with littermate controls. Furthermore, the increment in brain weight observed in Ptc1+/− mice carrying the IGF-I transgene suggests an independent and cooperative effect of Shh and IGF-I pathways in brain development.

IGF-I has tumor promotion activity in medulloblastoma tumorigenesis

As a next step, we determined the frequency of early and fully developed medulloblastoma stages in the cerebellum of the F1 progeny of crosses between Ptc1+/+ and IGF-I Tg mice. During postnatal cerebellar development,
differentiating GNPs complete their migration from the EGL to IGL by the third postnatal week. The presence of EGL remnants in the cerebellum of Ptc1<sup>+/−</sup> mice, which persist after the 3rd week of age, is considered indicative of a differentiation defect of GNPs, suggestive of a preneoplastic condition [8,9,12]. Notably, ectopic EGL areas from 3-week old Ptc1<sup>+/−</sup> mice markedly expressed nestin, thus assuring the expression of the nestin/IGF-I transgene from the initial steps of the tumorigenic process (Figure 5A). To determine whether IGF-I affects early tumor development, brains from asymptomatic Ptc1<sup>+/−</sup>, Ptc1<sup>+/+</sup>/IGF-I Tg, Ptc1<sup>+/−</sup> and Ptc1<sup>+/−</sup>/IGF-I Tg mice at 3, 5 or 8 weeks were histologically examined and cerebellar pathology was assessed (Figure 5B). At 3 weeks of age, medulloblastoma precursor lesions were evident in 50% (9/19) of Ptc1<sup>+/−</sup>/IGF-I Tg and (9/18) Ptc1<sup>+/−</sup>/IGF-I Tg mice. At 5 weeks, 52.2% (12/23) of Ptc1<sup>+/−</sup>/IGF-I Tg mice presented cerebellar abnormalities compared with 35.3% (6/17) of Ptc1<sup>+/−</sup> mice. The largest effect of IGF-I transgene was evident at 8 weeks, when a significant increase of preneoplastic lesions was observed in Ptc1<sup>+/−</sup>/IGF-I Tg compared with Ptc1<sup>+/−</sup> mice (85.7%, 18/21, vs. 40%, 6/15; P = 0.01). No ectopic EGL areas were observed in the cerebellum of Ptc1<sup>+/−</sup> and Ptc1<sup>+/−</sup>/IGF-I Tg mice. These findings suggest that the IGF-I transgene, by protraying the susceptible phase of the cerebellum to development of preneoplastic areas in the cerebellum of Ptc1<sup>+/−</sup> mice, fosters de novo formation of EGL lesions.

The observation that IGF-I promotes the initial steps of medulloblastoma growth in Ptc1<sup>+/−</sup> mice prompted us to examine its influence on development of advanced tumors. To this aim, the F1 progeny of crosses between Ptc1<sup>+/−</sup> mice and IGF-I Tg mice was placed on a lifetime study and brain tumor development was monitored. Notably, the IGF-I transgene produced a significant acceleration of medulloblastoma development (Figure 5C). By 15 weeks, 7 of 28 (25.0%) Ptc1<sup>+/−</sup>/IGF-I Tg mice had developed medulloblastoma compared with 1 of 30 (3.3%) Ptc1<sup>+/−</sup> mice. At the end of the experiment, 20 of 28 (71.4%) Ptc1<sup>+/−</sup>/IGF-I Tg mice developed medulloblastomas compared with 13/30 (43.3%) Ptc1<sup>+/−</sup> mice (P < 0.05). These data highlight the influence of the IGF-I transgene on the malignant potential of preneoplastic EGL. Up to 50% of young (3-8 wks) Ptc1<sup>+/−</sup> mice show presence of precursor lesions in cerebellum, and given a final medulloblastoma incidence of 43%, about 86% of these preneoplastic areas have potential to give rise to full tumors. The IGF-I transgene caused a significant 2-fold increase in the frequency of preneoplastic lesions (86%) in Ptc1<sup>+/−</sup>/IGF-I Tg mice. Based on a final medulloblastoma incidence of 71%, the conversion rate of preneoplastic lesions was unaltered compared to Ptc1<sup>+/−</sup> mice (83% vs. 86%). This observation suggests that the IGF-I transgene results in de novo formation of preneoplastic lesions but does not modify their rate of progression to full tumors. However, IGF-I per se does not exert a tumor initiating activity in vivo, as no medulloblastomas developed in Ptc1<sup>+/−</sup>/IGF-I Tg mice.

Histology of medulloblastomas revealed no major morphological differences with respect to the presence of IGF-I transgene (data not shown). Immunohistochemistry of tumors from Ptc1<sup>+/−</sup>/IGF-I Tg mice showed a strong expression of p-IGF-IR - the active IGF-IR form - localized on the outer part of the tumors, suggesting that IGF signaling is required for medulloblastoma growth in the Ptc1<sup>+/−</sup> mouse model (Figure 5D). Interestingly, tumors from Ptc1<sup>+/−</sup>/IGF-I Tg mice revealed a strong and uniform p-IGF-IR staining throughout the tumor mass (Figure 5E). This probably reflects a generalized expression of the nestin/IGF-I transgene that follows the spatial expression pattern of the nestin native gene throughout the tumor (Figure 5F). We also examined by immunohistochemistry the expression of IRS1, Akt/Pkb and Erk1/2 kinases, downstream mediators of the IGF-I signaling pathway, in medulloblastoma samples from Ptc1<sup>+/−</sup> and Ptc1<sup>+/−</sup>/IGF-I Tg mice (n = 3). All the tumors from single and compound mutants showed IRS1, Akt and Erk 1/2 expression (Figure 5G, H, L and 5N), indicating that IGF signaling is required to maintain tumor growth in vivo. By immunoblotting, we determined IRS1 expression, as well as total and phosphorylated Akt/Pkb and Erk 1/2 protein levels. All the tumors strongly expressed IRS1 irrespective of the presence of IGF transgene, and showed a large intertumor variability in the activation of Akt/Pkb and Erk 1/2 protein that did not correlate with transgenic IGF-I expression (Figure 5I, N and 5O). To further investigate whether the IGF-I transgene influences the mechanisms of tumorigenesis, we assayed loss of the wild-type Ptc1 allele, a prerequisite for the biological switch to malignancy of early cerebellar lesions in Ptc1<sup>+/−</sup> mice [9], in medulloblastoma from compound mutants. Sequence analysis of tumor DNA showed that, similar to medulloblastomas from Ptc1<sup>+/−</sup> mice, tumors from Ptc1<sup>+/−</sup>/IGF-I Tg mice (n = 3) also showed lack of wild type Ptc1 (data not shown). Altogether, these data indicate that IGF-I strongly modulates the penetrance of medulloblastomas but not the molecular pathogenesis of tumors in the Ptc1<sup>+/−</sup> mouse model.

**Discussion**

Normal proliferation of GNPs in the cerebellum is dependent upon Shh and IGF-I signaling, and deregulation of both pathways is implicated in medulloblastoma [4,5,21-23]. Constitutive activation of the Shh pathway - frequently due to inactivating mutations of Ptc1 - has
Figure 5 Acceleration of tumor development in the cerebellum of Ptc1+/− mice expressing IGF-I transgene under control of the nestin promoter. (A) Representative immunohistochemical analysis of nestin in preneoplastic lesions detected in Ptc1+/− cerebellum at 3 weeks of age. (B) Frequency of preneoplastic medulloblastoma lesions in cerebellum of Ptc1+/− and Ptc1+/−/IGF-I Tg mice of 3, 5 and 8 weeks of age. (C) The IGF-I transgene caused a significant enhancement in medulloblastoma development (71% in Ptc1+/−/IGF-I Tg mice vs. 43% in Ptc1+/− mice; P < 0.05). (D and E) Representative immunostaining for phosphotyrosine 1316 (pY1316) IGF-IR in medulloblastomas from Ptc1+/− mice and Ptc1+/−/IGF-I Tg mice. Insets in (D) and (E), higher magnification (100×). (F) Analysis of nestin expression in medulloblastomas from Ptc1+/− mice. (G) Representative immunostaining for IRS1 in medulloblastomas from Ptc1+/− mice. (H) Higher magnification (100×). (I) Immunoblotting of IRS1 in medulloblastomas from Ptc1+/− and Ptc1+/−/IGF-I Tg mice, with relative HSP-70 to control protein loading, and graphic representation of densitometric analysis (lower panel). (L and M) Representative immunostaining for Akt and p-Erk1/2 in medulloblastomas from Ptc1+/− mice. Evaluation of Akt/Pkb (N) and Erk1/2 (O) phosphorylation status by immunoblotting in medulloblastomas from Ptc1+/− and Ptc1+/−/IGF-I Tg mice, and relative graphic representation of densitometric analysis.
been shown in approximately 30% of human medulloblastomas [1]. Molecular oncology studies in humans and mice strongly implicated IGFs as additional causative factors for medulloblastoma. In fact, increased expression levels of IGF-II have been shown in human medulloblastomas, and overexpression of IGF-IR and IGF-I mRNA was observed in medulloblastoma cell lines [24,25]. In addition, a strong synergy between IGF and Shh signaling pathways has been demonstrated by using the RCAS/tv-a system, in which combined expression of IGF-II and Shh was shown to induce medulloblastoma at a significantly higher incidence compared with Shh alone [23]. In this system, however, gene transfer is performed in the cerebella of newborn mice, thus hampering investigations on the early effects of such a synergy on neural precursors, the proposed cells of origin of medulloblastoma. In the present study, we crossbred nestin/IGF-I Tg mice, in which transgene expression starts prenatally and is detectable in the cerebellar primordium as early as embryonic day 13 [15], with Ptc1+/− mice, a faithful model of medulloblastoma recapitulating the histopathology of the human tumor. Importantly, the use of this novel genetic cross offers the opportunity to study how interactions between Shh and IGF-I signaling, starting during embryonic life, affect development and neoplastic growth of neural precursors in neonatal cerebellum.

As already reported in a different line of IGF-I Tg mice [19], we show here that transgenic expression of IGF-I in the cerebellum during development produced a hyperplastic EGL, characterized by neural precursors exhibiting increased proliferation and decreased programmed cell death. We also report a novel effect of the IGF-I transgene in our system, i.e., a marked differentiation defect, as shown by a reduced expression of NeuN and a delayed disappearance of neural progenitors from the EGL pool. Furthermore, in line with a previous report that IGF-I promotes brain overgrowth by stimulating neural cell proliferation and inhibiting apoptosis in the cerebral cortex [15], we found that nestin/IGF-I Tg mice exhibited a marked generalized brain overgrowth that also includes the cerebellum. Moreover, we provide evidence that IGF-I overexpression in cerebellum cooperates with deregulation of the Shh pathway to further enhance brain overgrowth in double mutants, and to accelerate medulloblastoma development by significantly increasing the incidence of early, as well as full medulloblastoma stages in Ptc1+/−/IGF-I Tg compared with Ptc1+/− mice. These findings identify a novel synergy of IGF-I and Shh signaling pathways during cerebellum development and confirm, in this new genetic cross, the robust cooperation between IGF-I and Shh signaling in medulloblastoma tumorigenesis.

Our findings also suggest that brain overgrowth and increased tumor formation may stem from a common mechanism favoring survival and proliferation of neural precursors over terminal differentiation, thus stressing the link between aberrant activation of developmental pathways and tumorigenesis. On the other hand, we have previously shown that the overexpression of PC3, a gene that acts as a switch from proliferative to neuron-generating cell fate, causing a marked increase of differentiation in neuronal precursors and impairment of cerebellar development [26], significantly inhibited medulloblastoma tumorigenesis in Ptc1+/− mice [27]. Taken together these findings indicate that sets of genes controlling cell growth and differentiation may coordinately modulate developmental patterns and susceptibility to cancer in CNS. In this respect, it is also worth noting that height and weight at birth, relating to IGF-I concentration in umbilical cord [28], have been found to positively correlate with increased cancer risk in humans [29-32].

Medulloblastomas from Ptc1+/− and Ptc1+/−/IGF-I Tg mice both express active IGF-1R, although with a different staining pattern probably reflecting the generalized expression of the nestin/IGF-I transgene in tumors from double mutants. Medulloblastomas from Ptc1+/− and Ptc1+/−/IGF-I Tg mice also express IRS1, and show Akt and Erk 1/2 activation, demonstrating a functional role for the IGF-I signaling system in medulloblastoma formation. Furthermore, IGF-I transgenic expression does not influence the morphological characteristics of the tumors, nor the genetic events in tumorigenesis, as Ptc1 inactivation represents the critical event in medulloblastoma development in both Ptc1+/− and Ptc1+/−/IGF-I Tg mice. Altogether, these results indicate that IGF-I modulates tumor development in CNS of Ptc1+/− mice but does not alter the pathogenesis of tumor development.

A key question relative to the mechanism of cancer promotion by IGF-I is whether it involves (i) tumor initiation, through a pro-survival effect, leading to survival of a mutated cell, or (ii) malignant conversion, through a mitogenic effect that facilitates progression of precancerous stages [33]. In this respect, our novel mouse cross has proven useful. Through analysis of preneoplastic cerebellar lesions we show that, although IGF-I overexpression is not by itself carcinogenic in CNS, it can nevertheless increase tumor penetrance in a genetically susceptible model of human medulloblastoma by increasing the number of mice bearing medulloblastoma precursor lesions. On the contrary, we show that the rate of conversion of early to fully malignant tumor stages is not modified by IGF-I. Collectively, these findings suggest that IGF-I may have a role as a risk factor in susceptible individuals. Therefore, IGF-I levels should be regarded as a tumor modifying factor.
concurring to determine individual susceptibility to cancer. From a more general standpoint, if such a basic science findings translate to the human population, they might have important general implications for tumorigenesis. In fact, IGF-I signaling is also relevant to neoplasia in a number of other tissues such as peripheral nervous system, skin, and prostate [34-38], and epidemiological studies have linked high circulating levels of IGF-I with increased cancer risk in breast, prostate and colon cancer [39-44]. Interestingly, the hypothesis of IGF-I as modifier of disease risk is supported by a recent report showing a strong association of IGF1 CA repeat polymorphism and early onset of colorectal cancer in hereditary non-polyposis colorectal cancer patients [45]. These observations provide a solid foothold to pursue this topic further.

Conclusions
In summary, we made use of a novel genetic mouse cross of deregulated Shh and IGF-I signaling to show that brain growth patterns and tumor growth are modulated by IGF-I host physiology. We have also identified increased survival and proliferation and suppression of differentiation in neural precursors as the underlying biological mechanisms linking IGF-I signaling with brain overgrowth and tumor development in a powerful mouse model of medulloblastoma. Finally, we have shown an important role of IGF-I altered expression in the initiation and maintenance of early lesions en route to medulloblastoma.

Understanding the molecular events responsible for the normal developmental process of neural progenitor cells, and how these are altered to sustain the tumorigenic process is a necessary first step towards identification of potential targets for therapeutic intervention.

Methods
Animals and genotyping
Mice lacking one Ptc1 allele (Ptc1neo6-7/+), named Ptc1+/-, throughout the text) generated through disruption of exons 6 and 7 in 129/Sv embryonic stem cells [46] and maintained on C57BL/6 background were crossed to IGF-I transgenic mice maintained on the same background and overexpressing Homo Sapiens IGF-I (A.J. D’Ercole, University of North Carolina at Chapel Hill). The mouse lines and F1 progeny resulting from cross-breeding were genotyped using primers specific for the human IGF-I transgene: 5' - TGG ATG CTC TTC AGT TCG TG - 3' and antisense 5' - CCT GCA CTC CCT CTA CTG GC -3' corresponding to the Homo Sapiens IGF-I transgene cDNA, yielding a 265-bp product.

Histological analysis and tumor quantification
Mice were observed daily for their whole lifespan. Upon decline of health (i.e., severe weight loss, paralysis, ruffling of fur, or inactivity), they were euthanized and autopsied. Brains were fixed in 4% buffered formalin. Samples were then embedded in paraffin wax according to standard techniques, sectioned and stained with H&E. Medulloblastoma incidence was expressed as the percentage of mice with the tumor.

Tissue collection
Asymptomatic Ptc1+/+, Ptc1+/+/IGF-I Tg, Ptc1+/-/ and Ptc1+/-/IGF-I Tg mice were euthanized at P5 or P15 and brains were fixed in 4% buffered formalin and/or preserved at -80°C. For determination of preneoplastic stages, asymptomatic mice were also euthanized at 3, 5 or 8 weeks. The brains were removed, weighted and fixed in 4% buffered formalin to evaluate the incidence of hyperplastic areas in the cerebellum. In all, 18 sections were examined for each cerebellum with an interval of 70 μm.

Immunohistochemistry and immunoblotting analysis
Immunohistochemistry analysis was carried out on 4-μm thick paraffin sections of cerebellum at P5 or on sections of medulloblastoma samples. Antibody-antigen complexes were visualized using a horseradish peroxidase-conjugated secondary antibody and the DAB chromogen system (Dako North America, Inc, Carpinteria, Ca). Immunohistochemistry analysis of monoclonal antibody against NeuN (Millipore Billerica, MA) and PCNA (Ab-1/PC-10, Calbiochem, Germany) was performed using the Histomouse MAX Kit (Zymed Laboratories, San Francisco, CA) according to the manufacturer’s instructions.

For immunoblotting, proteins (30 μg) were extracted from a pool of 2 cerebella (P5) per genotype, and from medulloblastomas developed in Ptc1+/- and Ptc1+/-/IGF-I Tg mice [47]. Proteins were visualized with horseradish
peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) followed by chemiluminescence detection (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL). Protein levels were quantified by densitometric analysis using Scion Image Beta 4.02 software package (Scion Corporation, Frederick, MD). We used mouse anti-β-actin or anti-Heat Shock Protein 70 (HSP-70) antibody (Sigma-Aldrich Inc., St Louis, MO) to control protein loading. Two to three blots were run for each set of samples.

Other antibodies used include rabbit polyclonal antibody against nestin (Abcam Ltd, Cambridge, UK), Ki-67 (Novocastra, Novocastra Laboratory, Newcastle, UK), cleaved caspase-3 (Asp175), IGF-I receptor β, p-IGF-I receptor β, Erk1/2, p-Erk1/2, p-Akt (Ser473), total Akt, all from Cell Signaling (Beverly, MA), IRS-1 (Santa Cruz Biotechnology), goat polyclonal antibody against human IGF-I antibody (R&D System, MN), monoclonal antibody against cyclin D1 (Santa Cruz Biotechnology).

Analyses of proliferation and programmed cell death
Paraffin sections of cerebellar tissue of pups at P5 were cut at 4 μm thickness. Immunohistochemical analysis of Ki-67, PCNA and caspase-3 were performed on brain samples. Digital images of the entire midsagittal cerebellar section from 3 mice were collected by IAS image-processing software (Delta Sistemi, Rome, Italy). Ki-67-, PCNA- and caspase-3 positive cells in the EGL were counted. Rates of proliferation and apoptosis were calculated as the percentage of positively stained cells relative to the total number of cells of the EGL.

LOH analysis at the Ptc1 locus
DNA was extracted from tumors and normal tissue of Ptc1−/− (n = 3) and Ptc1+/−/IGF-I Tg mice (n = 3) using Wizard SV Genomic DNA Purification System (Promega). LOH analysis was performed as described [47].

Statistics
Statistical comparisons were made using Student’s t-test and Fisher exact test. P values < 0.05 were considered statistically significant.

Acknowledgements
We thank Dr AJ D’Ecole (University of North Carolina at Chapel Hill, North Carolina) for his generous gift of IGF-I transgenic mice. This work was supported by “Fondo per gli Investimenti della Ricerca di Base (FIRB), project R8N04P4ET.

Authors’ contributions
All authors participated in the design of the study. MS, MT, EP and SL performed the experimental work. MM, VDM, SR and AS contributed to data analysis and interpretation. SP conceived the study and wrote the manuscript. All the authors read, revised and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 23 November 2009 Accepted: 9 March 2010
Published: 9 March 2010

References
1. Lee Y, Miller HL, Jensen P, Hernan R, Connelly M, Wetmore C, Zindy F, RousSEL MF, Curran T, Gilbertson RJ, McKinnon PJ: A molecular fingerprint for medulloblastoma. Cancer Res 2003, 63:5428-37.
2. Ko AT, Zhao Q, Cai Z, Butte AJ, Kim JY, PomeroY SL, Rowitch DH, Kohane I: Conserved mechanisms across development and tumorigenesis revealed by a mouse development perspective of human cancers. Genes Dev 2004, 18:629-40.
3. Goldowitz D, Harne K: The cells and molecules that make a cerebellum. Trends Neurosci 1998, 21:375-82.
4. Pietsch T, Waha A, Koch A, Kraus J, Albrecht S, Tonn J, SörensE N, Berthold F, Henk B, Schmandt N, Wolf HK, von Deimling A, WarnRt W, ChenEvix-Trench G, Wiestler OD: Sticking C and Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of Drosophila patched. Cancer Res 1997, 57:2085-8.
5. Thompson MC, Fuller C, Hogg TL, Dalton J, Finklestein D, Lau CC, Chintagumpala M, Adesina A, Ashley DM, Kellei SJ, Taylor MD, Curran T, Gajjar A, Gilbertson RJ: Genomics identifies medulloblastoma subgroups that are enriched for specific genetic alterations. J Clin Oncol 2006, 24:3294-31.
6. Pazzaglia S, Mancuso M, Atkinson MJ, Tanori M, Rebessi S, Di Majo V, Covielli V, Hahn H, Saren A: High incidence of medulloblastoma following X-ray-iradiation of newborn Ptc1 heterozygous mice. Oncogene 2002, 21:7580-4.
7. Pazzaglia S, Pasquali E, Tanori M, Mancuso M, Leonardi S, Di Majo V, Rebessi S, Saren A: Physical, heritable and age-related factors as modifiers of radiation cancer risk in patched heterozygous mice. Int J Radiat Oncol Biol Phys 2009, 73:1203-10.
8. Oliver TG, Read TA, Kessler JD, Mehrmeh A, Wells JF, Huynh TT, Lin SM, Wechsler-Reya R: Loss of patched and disruption of granule cell development in a pre-neoplastic stage of medulloblastoma. Development 2005, 132:2435-39.
9. Pazzaglia S, Tanori M, Mancuso M, Gessi M, Pasquali E, Leonardi S, Oliva MA, Rebessi S, Di Majo V, Covielli V, Giangaspero F, Saren A: Two-hit model for progression of medulloblastoma preneoplasia in Patched heterozygous mice. Oncogene 2006, 25:5575-80.
10. Wetmore C, Eberhart DE, Curran T: Loss of p53 but not ARF accelerates medulloblastoma in mice heterozygous for patched. Cancer Res 2001, 61:513-6.
11. Uziel T, Zindy F, Sherr CJ, RousSEL MF: The CDK inhibitor p18Ink4c is a tumor suppressor in medulloblastoma. Cell Cycle 2006, 5:363-S.
12. Tanori M, Mancuso M, Pasquali E, Leonardi S, Oliva MA, Rebessi S, Di Majo V, Covielli V, Giangaspero F, Saren A: PARP-1 cooperates with Ptc1 to suppress medulloblastoma and basal cell carcinoma. Carcinogenesis 2008, 29:1911-9.
13. Carson MJ, Behringer RR, Brinster RL, McMorriss FA: Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. Neuron 1993, 10:729-40.
14. Ye F, Carson J, D’Ecole AJ: In vivo actions of insulin-like growth factor-I (IGF-I) on brain myelination: studies of IGF-I and IGF binding protein-1 (IGFBP-1) transgenic mice. J Neurosci 1995, 15:7344-56.
15. Popken GJ, Hodge RD, Ye P, Zhang J, Ng W, O’Kusky JR, D’Ecole AJ: In vivo effects of insulin-like growth factor-I (IGF-I) on prenatal and early postnatal development of the central nervous system. Eur J Neurosci 2004, 19:2056-68.
16. Zimmerman L, Parr B, Lendahl U, Cunningham M, McKay R, Gavin B, Mann J, Vassileva G, McMahon A: Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron 1994, 11:21-4.
17. Read TA, Fogarty MF, Markant SL, McLendon RE, Wei Z, Ellison DW, Fiebbo PC, Wechsler-Reya R: Identification of CD15 as a marker for tumor-propagating cells in a mouse model of medulloblastoma. Cancer Cell 2005, 10:135-47.
In situ labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. Neuron 1993, 11:621-32.

Ye P, Xing Y, Dai Z, D’Ecole AI: In vivo actions of insulin-like growth factor-I (IGF-I) on cerebellum development in transgenic mice: evidence that IGF-I increases proliferation of granule cell progenitors. Brain Res Dev Brain Res 1996, 95:44-54.

Ramocki NM, Wilkins HR, Magnes SS, Simmons JG, Scull BP, Lee GH, McNagnathy RK, Lund PK: Insulin receptor substrate-1 deficiency promotes apoptosis in the putative intestinal crypt stem cell region, limits Apcmin tumors, and regulates Sorbtl. Endocrinology 2008, 149:261-267.

Dearth RK, Cui X, Kim HJ, Hadsell DL, Lee AV: Oncogenetic transformation by the signaling adapter proteins insulin receptor substrate (IRS)-1 and IRS-2. Cell Cycle 2007, 6:705-13.

Del Valle L, Enam S, Lakasak A, Wang JY, Croul S, Khalili K, Reiss K: Insulin-like growth factor I receptor activity in human medulloblastomas. Clin Cancer Res 2002, 8:1822-30.

Rao G, Pedone CA, Del Valle L, Reiss K, Holland EC, Fults DW: Sonic hedgehog and insulin-like growth factor signaling synergize to induce medulloblastoma formation from nestin-expressing neural progenitors in mice. Oncogene 2004, 23:6156-62.

Wang JY, Del Valle L, Gordon J, Rubini M, Boadi E, Lee AV: Insulin-like growth factor II is involved in the proliferation control of medulloblastoma and its cerebellar precursor cells. Am J Pathol 2005, 166:1153-62.

Canzioaere D, Farioli-Vecchioli S, Comi F,riotti MT, Tata AM, Augusti-Tocco G, Matti E, LalikanBH, Kizhatnovskv J, Reeves SA, Giovannini R, Bosco D, Servadia A, Ben-Arie N, Tirone S, Irono F: Dual control of neurogenesis by PC3 through cell cycle inhibition and induction of Math1. J Neurosci 2004, 24:1355-69.

Farioli-Vecchioli S, Tanori M, Micheli L, Mancuso M, Leonardi L, Saran A, Comi F, Ferretti E, Gulino A, Pazzaglia S, Tirone F: Inhibition of medulloblastoma tumorigenesis by the antiproliferative and pro-differentiative gene PC3. FASEB J 2007, 21:2215-25.

Vatten LJ, Nilsen ST, Odegård RA, Romundstad PR, Austgulen R: Insulin-like growth factor I and leptin in umbilical cord plasma and infant birth size at term. Pediatrics 2002, 109:1131-5.

McCormack VA, dos Santos Silva I, De Stavola BL, Mohsen R, Leon DA, Lithell HO: Fetal growth and subsequent risk of breast cancer: results from long term follow up of Swedish cohort. BMJ 2003, 326:248.

Sandhu J, Li BD, Dai Q, Cheng JR, Berkel H, Zheng W: Insulin-like growth factors and breast cancer risk in Chinese women. Cancer Epidemiol Biomarkers Prev 2002, 11:705-12.

Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Mchaud DS, Deroo B, Rosner B, Speizer FE, Pollak M: Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. Lancet 1998, 351:1393-6.

Ma J, Pollak MN, Giovannucci E, Chan JM, Tao Y, Hennkens CH, Stamper MJ: Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. J Natl Cancer Inst 1999, 91:620-4.

Reeves SG, Rich D, Meldrum CJ, Colyas K, Kurzawski G, Suchy J, Lubinski J, Scott RJ: IGF1 is a modifier of disease risk in hereditary non-polyposis colorectal cancer. Int J Cancer 2008, 123:1393-43.

Hahn H, Wojnowski L, Zimmmer AM, Hail J, Miller G, Zimmer A: Rhabdomyosarcomas and radiation hypersensitivity in a mouse model of Gorlin syndrome. Nat Med 1998, 4:619-22.

Pazzaglia S, Tanori M, Mancuso M, Rebessi S, Leonardi S, Di Majo V, Covielli V, Atkinson MJ, Hahn H, Saran A: Linking DNA damage to medulloblastoma tumorigenesis in patched heterozygous knockout mice. Oncogene 2006, 25:1165-73.

Kaplan-Leitko PJ, Sutherland BW, Evangelou AI, Hadsell DL, Barrios RJ, Foster BA, Demayo F, Greenberg NM: Enforced epithelial expression of IGF-1 causes hyperplastic prostate growth while negative selection is requisite for spontaneous metastagenesis. Oncogene 2008, 27:2868-76.