***Sprouty1* haploinsufficiency accelerates pheochromocytoma development in *Pten*^{+/-} mice**

Dear Editor,

Pheochromocytomas (PCCs) are rare catecholamine-secreting tumors arising from the chromaffin cells in the adrenal medulla. Closely related paragangliomas share developmental origin but arise extra-adrenally in the paraganglia that belongs to the sympathetic and parasympathetic ganglia. Most PCCs are sporadic; however, around 25–30% of them are associated with familial syndromes caused by germ line mutations of at least ten genes (Gimenez-Roqueplo et al. 2012). The low incidence of pheochromocytomas poses difficulties to the development of diagnostic or prognostic markers as well as effective therapies, and thus, the development of novel animal and cellular models for the study of the disease is needed. *Sprouty* (*Spry*) family of genes is composed of four members of feedback inhibitors of receptor tyrosine kinase signaling that specifically targets the MAPK pathway. As such, they have been proposed as tumor suppressors in several cancerous pathologies such as tumors of the prostate, thyroid, or liver (Masoumi-Moghaddam et al. 2014). Here, we present a novel mouse model of pheochromocytoma consisting of double-heterozygous mice for *Pten* and *Sprouty1* (*Spry1*). These animals develop pheochromocytomas that appear at earlier onset and grow at a higher rate than those from *Pten*^{+/-} mice.

In a previous report, we have found that mice bearing a *Spry1*-null mutation in the context of *Pten* haploinsufficiency developed thyroid hyperplasia at higher frequencies and earlier onset than *Pten*^{+/-} littermates (Macià et al. 2014). While examining thyroid glands from these animals, we noticed that *Pten*^{+/-}; *Spry1*^{+/-} mice also developed thyroid hyperplasia with a frequency comparable to that of *Pten*^{+/-}; *Spry1*^{-/-} mice at 3 months of age (M Vaquero, A Macià, C Anerillas, A Velasco, X Matias-Guiu, J Ribera and M Encinas, unpublished observations), indicating that deletion of a

single *Spry1* allele was enough to accelerate thyroid tumorigenesis in the context of *Pten* haploinsufficiency. We therefore looked for tumor formation in tissues from *Pten*^{+/-}; *Spry1*^{+/-} double-heterozygous mice in other tissues, including the adrenal gland, which expresses high levels of *Spry1* by real-time PCR (M Vaquero, A Macià, C Anerillas, A Velasco, X Matias-Guiu, J Ribera and M Encinas, unpublished observations). Histological analysis of mice at 5 months of age revealed that roughly one third (9/28; 32%) of adrenal glands from double-heterozygous mice presented pheochromocytoma compared with 17% (3/18) of adrenals from *Pten*^{+/-} mice (Fig. 1A and B). In these lesions, the alveolar or ‘nested’ pattern found on normal medullae was replaced by more densely packed sheets or cords of cells that compressed the adjacent cortical parenchyma (Fig. 1A). Most of the remaining adrenal glands of double-heterozygous mice (15/28; 50%) developed hyperplastic nodules (Tischler et al. 2004) in their medullae (Fig. 1A and B), which were similar in morphology to pheochromocytomas but did not compress the adrenal cortex. In contrast, only four out of 18 glands (22%) from *Pten*^{+/-} mice developed nodular hyperplasia at this age, being the remaining 60% normal. Overall, at five months of age, more than 80% of double-mutant mice compared with around 40% of *Pten*^{+/-} mice showed neoplastic lesions of the adrenal medulla. Medullae from *Spry1*^{+/-} mice were normal in all cases and at all ages analyzed (Fig. 1A). Tumors from both *Pten*^{+/-} and double-heterozygous mice stained positive for tyrosine hydroxylase, demonstrating their chromaffin origin (Fig. 1C). We next conducted loss of heterozygosity analysis by PCR, using primers flanking a FRT site present in the knockout but not the wild-type allele (Basson et al. 2005). Capillary electrophoresis fragment analysis indicated that the wild-type allele was retained

Figure 1

(A) Representative images of hematoxylin–eosin-stained adrenal glands from 5-month-old mice of the indicated genotypes. Arrowhead points to a hyperplastic nodule, noted as N in the lower panel. (B) Frequencies of pheochromocytoma, nodular hyperplasia, or normal medullae in 5-month-old mice of the indicated genotypes. (C) Tyrosine hydroxylase staining of adrenal glands from 5-month-old mice of the indicated genotypes. (D) Representative results of loss of heterozygosity analysis of DNA from tumoral vs normal tissues from two 9-month-old double-heterozygous mice. (E) The amount of SPROUTY1 in pheochromocytomas from mice of the indicated phenotypes was assessed by immunoblot. The two upper panels are different exposures of the same blot to show lack of immunoreactivity in the first lane, corresponding to adrenal medulla from *Spry1* knockout mice. Equal loading was assessed by actin immunoblot. NS, non-specific band.

in ten out of ten pheochromocytomas from double-heterozygous mice analyzed (Fig. 1D). Moreover, immunoblot analysis of SPROUTY1 levels from

pheochromocytomas using a specific antibody (D9V6P; Cell Signaling Technologies) clearly showed that expression of SPROUTY1 was not lost in five out of six

tumors from double-heterozygous mice (Fig. 1E). Interestingly, SPROUTY1 protein levels were reduced in pheochromocytomas from double-heterozygous mice when compared with *Pten*^{+/-} mice, likely reflecting that *Spry1* behaves as a dosage-sensitive gene. This is in agreement with our previous findings indicating that *Spry1* also behaves as a dosage-sensitive gene during genitourinary development, as deletion of a single allele of *Spry1* partially rescues renal agenesis caused by *Ret* mutation (Rozen et al. 2009). In conclusion, loss of a single allele of *Spry1* in the context of *Pten* haploinsufficiency significantly ($P=0.01$, χ^2 test) accelerated the appearance of neoplastic lesions of the adrenal medulla. Immunocytochemistry and western blot analysis also indicated that PTEN expression was frequently lost in pheochromocytomas from both

genotypes (M Vaquero, A Macià, C Anerillas, A Velasco, X Matias-Guiu, J Ribera and M Encinas, unpublished observations). By 9 months of age, virtually all double-heterozygous mice examined presented either pheochromocytoma (21/32; 66%) or nodular hyperplasia (9/32; 28%), whereas 14/30 (47%) and 12/30 (40%) of *Pten*^{+/-} adrenal glands did (Fig. 2C). These differences were not statistically significant ($P=0.29$, χ^2 test); however, adrenal glands from double-heterozygous mice were on average almost four times bigger than those from *Pten*^{+/-} mice (Fig. 2A and B). Thus, adrenal glands from *Spry1*^{+/-} mice had a mean volume of $0.79 \pm 0.19 \text{ mm}^3$ (mean $\pm$ standard error) compared with $2.64 \pm 0.51 \text{ mm}^3$ (*Pten*^{+/-}) and $8.28 \pm 1.77 \text{ mm}^3$ (*Pten*^{+/-}; *Spry1*^{+/-}). Differences in adrenal size between *Pten*^{+/-} and *Pten*^{+/-}; *Spry1*^{+/-} mice were highly significant ($P=0.0001$,

Figure 2

(A) Macroscopic images, (B) gland volume, (C) tumor frequency, and (D) number of mitoses per 10 high power fields of adrenal glands from 9-month-old mice of the indicated genotypes. (E) Tyrosine hydroxylase and synaptophysin staining showing capsular invasion of pheochromocytoma cells of 9-month-old *Pten*^{+/-}; *Spry1*^{+/-} mice.

Mann–Whitney test). The number of mitotic figures per 10 high power fields was also significantly higher in pheochromocytoma from double-heterozygous mice than those from *Pten*^{+/-} mice ($P=0.033$, Mann–Whitney test), Fig. 2D). Of 14 *Pten*^{+/-} pheochromocytomas, five (36%) presented more than three mitoses per 10 high power field, whereas 15 out of 21 (71%) *Pten*^{+/-}; *Spry1*^{+/-} did. These differences presented a trend toward but did not reach statistical significance ($P=0.07$, χ^2 test). Finally, tyrosine hydroxylase or synaptophysin staining revealed that larger glands from double-heterozygous mice but not from *Pten*^{+/-} mice often presented chromaffin cells invading the adrenal cortex or breaking through the adrenal capsule (Fig. 2E). Although tumor-associated criteria reliably predicting malignancy in pheochromocytoma are not available, tumor size, local invasion, or mitotic index have been proposed as signs of malignancy by several authors (Mete et al. 2014). In conclusion, our data demonstrate that loss of a single allele of *Spry1* accelerates both the onset of pheochromocytoma formation and the growth rate of these tumors. We currently do not know by which mechanism *Spry1* restrains growth of pheochromocytoma cells; however, several possibilities deserve future attention. Transcriptomic analysis of human pheochromocytomas and paragangliomas reveals two different clusters characterized by distinct expression signatures (Gimenez-Roqueplo et al. 2012). Cluster 1 (comprising mutations of *SDHx*, *FH*, *VHL*, or *HIF2A* (*EPAS1*)) presents a pseudo-hypoxia signature characterized by a switch from oxidative phosphorylation to aerobic glycolysis (Warburg effect), whereas cluster 2 (mutations of *RET*, *NF1*, *TMEM127*, or *MAX*) reflects aberrant activation of the MAPK and mTOR pathways. One mechanism worth exploring would be ectopic activation of the MAPK kinase pathway upon *Spry1* loss. Interestingly, *Spry1* and *Spry2* antagonize *Ret* signaling during the development of renal and enteric nervous system, via inhibition of the MAPK (and the PI3-K) pathways (Masoumi-Moghaddam et al. 2014). However, our previous work points to a role of *Spry1* in inducing cellular senescence as a mechanism of tumor suppression in the thyroid gland (Macià et al. 2012, 2014). Similarly, induction of cellular senescence could account for the protective effects of *Spry1* in pheochromocytoma development. In line with this hypothesis, You et al. (2002) have demonstrated that pheochromocytoma incidence in *Pten*^{+/-} mice dramatically increases upon concomitant deletion of the *Ink4a/Arf* locus, a master regulator of cellular senescence. Recent studies

demonstrate that escape from senescence is accompanied by a metabolic switch from oxidative phosphorylation to aerobic glycolysis (Kaplon et al. 2013), much like the pseudo-hypoxic response found in cluster 1. In any case, further studies are required to determine whether *Spry1* restricts pheochromocytoma growth by inhibiting the MAPK pathway, by inducing cellular senescence, or a combination of both.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grants from Ministerio de Economía y Competitividad (BFU2010-17628 and BFU2013-47175) to ME and funding from Suport als Grups de Recerca (2014 SGR 138) from Generalitat de Catalunya to ME and XM-G. MV is supported by a predoctoral fellowship from AGAUR (Generalitat de Catalunya). AM was supported by predoctoral fellowships from Universitat de Lleida and Fundació Alícia Cuello de Merigó. CA is supported by a predoctoral fellowship from Universitat de Lleida.

Acknowledgments

We thank Lidia Parra, Cristina Girón, and Maria Santacana for technical support.

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Received in final form 5 January 2016

Accepted 28 January 2016

Accepted Preprint published online 1 February 2016