Oncogene. Author manuscript; available in PMC 2013 February 28.

Published in final edited form as:
Oncogene. 2012 August 30; 31(35): 3961–3972. doi:10.1038/onc.2011.556.

Sprouty1 is a candidate tumor suppressor gene in medullary thyroid carcinoma

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Abstract

Medullary thyroid carcinoma (MTC) is a malignancy derived from the calcitonin-producing C-cells of the thyroid gland. Oncogenic mutations of the Ret proto-oncogene are found in all heritable forms of MTC and roughly one half of the sporadic cases. However, several lines of evidence argue for the existence of additional genetic lesions necessary for the development of medullary thyroid carcinoma. Sprouty (Spry) family of genes is composed of four members in mammals (Spry1-4). Some Spry family members have been proposed as candidate tumor suppressor genes in a variety of cancerous pathologies. In this work, we show that targeted deletion of Spry1 causes C-cell hyperplasia, a precancerous lesion preceding MTC, in young adult mice. Expression of Spry1 restrains proliferation of the MTC-derived cell line, TT. Finally, we found that the Spry1 promoter is frequently methylated in MTC and that Spry1 expression is consequently decreased. These findings identify Spry1 as a candidate tumor suppressor gene in medullary thyroid carcinoma.

INTRODUCTION

Medullary thyroid carcinoma (MTC) is a rare neoplasm arising from the calcitonin-producing C-cells of the thyroid gland, which accounts for approximately 5-8% of human thyroid cancers (Matias-Guiu et al., 2004). Around 75% of MTC cases are sporadic while the remaining 25% occur as a component of the inherited multiple endocrine neoplasia type 2 (MEN 2) syndrome. MEN2 is a cancer syndrome with dominant autosomal inheritance, a variable expression pattern, and almost complete penetrance. Three types of MEN2 have been described, MEN2A, MEN2B and FMTC. MEN2A is characterized by MTC,

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CONFLICT OF INTERESTS
The authors declare no conflict of interests.

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pheochromocytoma, and hyperparathyroidism. MEN2B is characterized by MTC, pheochromocytoma, ganglioneuroma, thickened cornea nerves and marfanoid habitus. Familial MTC (FMTC) is characterized by MTC in four or more members of an affected family without involvement of adrenal and parathyroid glands. Virtually all MEN2 patients carry germline mutations of the proto-oncogene RET, while approximately 50% of sporadic cases of MTC also present oncogenic mutations of RET (Asai et al., 2006; Cerrato et al., 2009; Plaza-Menacho et al., 2006).

RET (REarranged during Transfection) was identified in 1985 as a proto-oncogene that can undergo activation by DNA rearrangement (Takahashi et al., 1985). RET encodes a receptor tyrosine kinase (RTK) which functions as a receptor for the Glial cell line-Derived Neurotrophic Factor (GDNF) family ligands (GFLs). Besides its role in MTC development, Ret signaling regulates several aspects of the peripheral nervous system and genito-urinary development. Thus, mice deficient in Ret die within hours after birth due to renal agenesis and lack of enteric neurons (Airaksinen & Saarma, 2002; Baloh et al., 2000). Despite the importance of RET mutations in the pathogenesis of MTC, both clinical and animal model data argue for the existence of additional genetic alterations necessary for tumor development (Santarpia et al., 2009). For example, FMTC patients develop monoclonal tumors even though the germline RET mutation is present in all somatic cells of the affected individual (Gagel & Marx, 2003). Variability on the age of onset and tumor spectrum in patients carrying the same RET mutation also suggest the presence of modifier genes. Moreover, about 50% of sporadic MTC do not bear RET mutations. Finally, although transgenic mice expressing oncogenic Ret mutations under the control of heterologous promoters develop MTC (Acton et al., 2000; Kawai et al., 2000; Michiels et al., 1997; Reynolds et al., 2001), knockin mice expressing MEN2A or MEN2B mutations from the Ret locus fail to develop MTC but instead exhibit C-cell hyperplasia, a precancerous lesion that precedes MTC (Smith-Hicks et al., 2000; Yin et al., 2007).

Sprouty family of genes is composed of four members in mammals (Spry1-4), orthologous to a single Drosophila Melanogaster gene (dSpry). dSpry gene product inhibits FGFR and EGFR signaling during trachea and eye development, respectively. (Hacohen et al., 1998). In mammals the situation is more complicated as Spry proteins have been shown to be activators or inhibitors of RTK signaling depending on the cellular context or the RTK analyzed (Cabrita & Christofori, 2008; Edwin et al., 2009; Guy et al., 2009; Mason et al., 2006). Spry family members have been proposed to function as tumor suppressor genes in a growing list of cancerous malignancies, including prostate and hepatocellular carcinoma, B-cell lymphoma, or neuroblastoma (Fong et al., 2006; Frank et al., 2009; Ishida et al., 2007; Kwabi-Addo et al., 2004). In this paper we sought to elucidate whether Spry1 could be functioning as a putative tumor suppressor gene in MTC. We found that Spry1 is expressed in mouse C-cells and that targeted deletion of Spry1 causes C-cell hyperplasia in young adult mice. Expression of moderate amounts of Spry1 reduced cell growth of the MTC cell line TT in vitro and its ability to generate tumors when xenografted in immunocompromised mice. Surprisingly, such reduction in cell proliferation does not appear to be related to inhibition of Ret signaling but to induction of cellular senescence. Finally, we found that the SPRY1 promoter is frequently methylated and its expression decreased in human MTC. In conclusion, our data identifies Spry1 as a candidate tumor suppressor gene in MTC.

RESULTS

Spry 1 is expressed in mouse thyroid C-cells

To ascertain whether Spry1 is expressed in the murine thyroid gland, we performed real time RT-PCR on different tissues of newborn mice using a probe against Spry1 and normalizing the data to GAPDH expression. As shown in Figure 1A, relative mRNA levels of Spry1 in

murine thyroid were high when compared to other organs known to express Spry1 such as heart or lungs and only below those found in kidney, where it is known to play a critical role. To ascertain whether expression in the thyroid gland persisted in adulthood, we checked its levels in tissues from 1 month-old mice. As depicted in Figure 1A, very high levels of Spry1 mRNA were found at that age, similar to those found in lungs, suggesting that Spry1 plays an important role in the thyroid during adult life.

The thyroid gland is composed of two main cell types, follicular cells and C-cells. The former are by far the most abundant of the two, are organized in follicles and secrete thyroxine and triiodothyronine. The latter comprise less than 5% of cells, are scattered among follicular cells and in spaces between follicles, and secrete calcitonin. C-cells arise from the ultimobranchial bodies (UB), whereas follicular cells are derived from a diverticulum of the prospective pharynx (reviewed in (Fagman & Nilsson, 2010)). To begin to elucidate whether Spry1 is expressed in C-cells or their precursors, we performed in situ hybridization on paraffin sections of E10.5 mouse embryos. As shown in Figure 1B, expression of Spry1 was detected on the fourth pharyngeal pouch, which gives rise to the UB, indicating that Spry1 is expressed by C-cell precursors. To specifically check whether Spry1 was present in the C-cells of the mature thyroid, we tested several different commercially available antibodies using mouse embryonic fibroblasts (MEFs) from wild type and Spry1 knockout mice. Unfortunately, none of the antibodies proved specific in our hands (Supplemental Figure 1). To circumvent this problem, we analyzed the expression of bacterial β-galactosidase in frozen sections from Spry1$^{LacZ/+}$ knockin mice, which express LacZ from the endogenous Spry1 locus (Thum et al., 2008). Double staining using antibodies to bacterial β-galactosidase and calcitonin revealed that indeed Spry1 is found at notably high levels in thyroid C-cells and more moderately in follicular cells (Figure 1C).

Spry1 knockout mice develop C-cell hyperplasia

To elucidate whether Spry1 could be acting as a tumor suppressor in medullary thyroid carcinoma, we examined the thyroids from 1 to 3 month-old wild type and Spry1 knockout littermates. Although thyroids from 3 month-old knockout mice were noticeably bigger than those from wild type littermates, they had normal shape and showed no external signs of malignant growth (data not shown). However, thyroid glands form Spry1 knockout mice had almost twice as many C-cells as their wild type littermates, as judged by calcitonin staining (Figures 2A and 2B). The premature death of Spry1 knockout mice owing to renal problems (Basson et al., 2005) precluded further analysis at older ages. To determine whether C-cell hyperplasia developed in the adult or reflected an increase in the number of C-cells at earlier ages, we counted calcitonin-immunoreactive cells in thyroids from newborn wild type and Spry1 knockout mice. As shown in Figure 2C, we found no significant differences between the numbers of calcitonin-immunoreactive cells in wild type and Spry1$^{-/-}$ newborn mice. Moreover, no obvious signs of hyperplasia in the UB at E12.5 were observed (Figure 2D), further supporting the notion that the increased numbers of C-cells found in Spry1 knockout mice are due to increased proliferation of C-cells during adulthood rather than a developmental defect. In conclusion, targeted deletion of Spry1 causes C-cell hyperplasia, a precancerous lesion that precedes development of medullary thyroid carcinoma

Expression of Spry1 reduces proliferation of MTC cells *in vitro* and *in vivo*

The observed effects on postnatal increase in the number of C-cells in Spry1 null mice could be explained by cell-autonomous and non-cell autonomous mechanisms. To address this issue, we experimentally manipulated levels of Spry1 in the MTC-derived cell line, TT. We first measured the expression levels of Spry1 in this cell line by means of real time RT-PCR, and compared it to other tissues or cell lines known to express it. As shown in Figure 3A, and as expected for a putative tumor suppressor, Spry1 was found to be expressed at very

low levels in TT cells. For comparison, we used two kidney-derived cell lines, namely HEK293 and HK2. We therefore decided to generate stable cell lines expressing different amounts of Spry1 in TT cells by means of lentiviral infection, as such system allows for stable expression of quasi-endogenous levels of transgenes. We generated two different stable cell lines expressing Spry1, termed Spry1A and Spry1B. Real time RT-PCR was used to monitor levels of Spry1 mRNA whereas gene copy number was assessed by real time PCR of genomic DNA. As shown in Figure 3A, Spry1A cells expressed roughly ten times more Spry1 than vector-infected cells, matching levels of Spry1 found in whole thyroid, whereas Spry1 levels in Spry1B cells were around twenty times higher than control. Such mRNA levels corresponded to an average of one extra copy of Spry1 inserted on the genome of Spry1A TT cells, whereas Spry1B cells harbored approximately two extra copies of Spry1 inserted on their genomes (Figure 3B). We noticed that re-expression of Spry1 caused a morphological change on TT cells, whose cell bodies rounded, turned phase-bright, and extended a few cellular processes (Figure 3C).

We next analyzed how expression of Spry1 affected proliferation of TT cells *in vitro*. Cell numbers of vector-infected control, Spry1A and Spry1B cells were counted over the indicated periods of time in culture, demonstrating that increasing amounts of Spry1 caused a gradual reduction in cell growth rate (Figure 4A). Clonogenic assays corroborated the above observations (Figure 4B). We conducted 5′-Bromo-deoxyuridine uptake experiments and Hoechst staining of nuclei to ensure that such reduction in growth was truly due to a decrease in proliferation and not an increase in the rate of apoptosis. Figure 4C shows that cells bearing one or two extra copies of Spry1 underwent a reduction of the uptake of 5′-Bromo-deoxyuridine, whereas Hoechst staining detected no significant amounts of apoptotic nuclei on either cell line (Figure 4D). Quantification of apoptotic nuclei revealed no significant differences between cell lines (Vector 1.1±1.4%, Spry1A 0.5±0.4%, Spry1B 0.1±0.1%; P=0.5 Vector vs. Spry1A and P=0.3 Vector vs. Spry1B by Student's t-test). Taken together, these results indicate that Spry1 induces a reduction in cell proliferation of TT cells in vitro. Moreover, the effect on proliferation was almost maximal when a single extra copy of Spry1 was inserted on the genome of TT cells. Therefore, we conducted the subsequent experiments using only the Spry1A cell line.

TT cells are capable of generating subcutaneous tumors when xenografted in immunocompromised mice. To ascertain whether expression of Spry1 reduces the ability of these cells to form tumors in vivo, we injected control and Spry1A cells subcutaneously in SCID mice. As shown in Figures 5A and 5B, expression of Spry1significantly reduced tumor growth in xenografts. Expression of Spry1 was maintained in these tumors as revealed by real-time RT-PCR (Figure 5C).

Spry1 does not antagonize Ret signaling in TT cells

As stated in the introduction, oncogenic mutations of Ret underlie development of MTC. One of the observations that prompted us to investigate the role of Spry1 in the development of MTC was that some Spry family members have been shown to antagonize Ret signaling in vivo. Thus, targeted deletion of Spry1 in mice causes abnormal renal development that can be rescued by ablation of one copy of Gdnf or hypomorphic mutation of Ret (Basson et al., 2005; Michos et al., 2010; Rozen et al., 2009). On the other hand, targeted deletion of Spry2 leads to enteric nervous system hyperplasia that can be prevented by GDNF function-blocking antibodies (Taketomi et al., 2005). To begin to elucidate whether expression of Spry1 in TT cells reduced proliferation by inhibiting Ret signaling, we first aimed to identify putative downstream targets of Ret signaling, and then assess the effect of Spry1 on their activation or expression. Extracellular-regulated kinases 1 and 2 (ERK1/2) are phosphorylated by endogenously expressed oncogenic Ret in TT cells. Moreover, Wnt11 and Calcitonin have been shown to be expressed in response to Ret signaling during kidney

development (Basson et al., 2006; Majumdar et al., 2003) and in MTC cell lines (Akeno-Stuart et al., 2007), respectively. We confirmed these findings by silencing Ret expression in TT cells by means of lentiviral expression of a shRNA and monitoring both phosphorylation of ERK1/2 (Figure 6A) and expression of Wnt11 and Calcitonin genes (Figure 6B). We next assessed the status of these downstream targets of Ret in Vector and Spry1A cells. To our surprise, expression of Spry1 did not reduce but if anything caused a modest increase in phosphorylation of ERK1/2 (Figure 6C). Expression levels of both Wnt11 and Calcitonin were unchanged by Spry1 expression (Figure 6D) as it was Ret phosphorylation (Figure 6C). Taken together, these findings suggest that Spry1 does not reduce proliferation of TT cells by inhibiting Ret signaling.

Spry1 induces expression of the CDKN2A locus and cellular senescence

Having ruled out inhibition of Ret signaling as the mechanism by which Spry1 reduces proliferation of TT cells, we examined the differential mRNA levels of key cell cycle regulatory proteins in Vector and Spry1A cells by means of a qPCR array (Supplemental Table 1). We detected a three-fold increase in the expression of the CDKN2A locus and a more modest increase in the expression of CDKN2B. The CDKN2A locus generates at least two different mRNA that give rise to two unrelated proteins by alternative splicing, namely $p16^{INK4a}$ and $p14^{Arf}$. As the probe used in the qPCR array for CDKN2A could not distinguish between $p16^{INK4a}$ and $p14^{Arf}$ mRNAs, we next specifically measured their expression using specific probes for either transcript. We found that mRNA for $p16^{INK4a}$ was up-regulated by approximately 8-fold in Spry1A cells, whereas mRNA for $p14^{Arf}$ and $p15^{INKb}$ (encoded by CDKN2B) were more modestly induced (Figure 7A, left). In contrast, mRNA coding for members of the CIP family of cdk inhibitors $p21^{CIP/WAF1}$ and $p27^{KIP1}$ remained largely unchanged (Figure 7A, right). These findings were confirmed at the protein level by immunoblot (Figure 7B). Although $p16^{INK4a}$ and $p14^{Arf}$ have no sequence similarity, they both cause cells to enter a state of growth arrest known as cellular senescence. Cellular senescence can be consequence of telomere attrition (replicative senescence) or can be induced by oncogenic insults (oncogene induced senescence, OIS). Interestingly, OIS has emerged as a potent tumor suppresive mechanism in many cancerous pathologies. Besides expression of $p16^{INK4a}$ and $p14^{Arf}$, the most widely used marker of cellular senescence is staining for senescence-associated β-galactosidase activity (SA-β-gal). As shown in figure 7C, expression of Spry1 caused a robust increase in the number of cells that stained positive for SA-β-gal activity. We confirmed these findings by staining sections of xenografts with either antibodies to $p16^{INK4a}$ (Figure 7D, top panels) or by assaying senescence-associated β-galactosidase activity (Figure 7D, bottom panels). Taken together, these findings strongly suggest that Spry1 reduces proliferation of TT cells by causing cellular senescence.

SPRY1 promoter is frequently methylated in MTC

Recently, the human SPRY1 locus was shown to be silenced in prostate cancer by means of promoter methylation. More specifically, a CpG island between exons 1b and 1a, whose methylation dramatically affects SPRY1 expression on reporter assays, has been described. We checked whether such CpG island was methylated in samples of genomic DNA obtained from human MTC by methylation-specific PCR (see Supplemental Table 2 for details on the MTC series). As shown in Figure 8A, most of the MTC samples were found to be methylated, suggesting that silencing of SPRY1 gene could be achieved by methylation of its promoter in MTC. In contrast, promoter methylation of normal thyroid was undetectable under the same conditions (Figure 8A). We next assessed whether SPRY1 expression levels were reduced in human MTC, as it would be expected from a tumor suppressor gene. Real time RT-PCR conducted on cDNA from the same samples (except for sample 04T241 from which we had no cDNA) of human MTC showed that SPRY1 levels were frequently low

when compared to whole human thyroid (Figure 8B). Only 2 out of the methylated samples showed high SPRY1 expression levels, whereas the remaining had medium to low levels. Consistently, the two samples which showed no methylation of the promoter displayed high expression levels. Other samples such as samples 08T31, 08T49 and especially 09T133 showed low expression of SPRY1 but weak methylation of the promoter, suggesting that additional mechanism might be responsible for such reduced expression. We finally used the hypomethylating agent 5′-Aza-deoxycytidine (5′-Aza-dC) to confirm a causal relationship between promoter methylation and Spry1 expression. As expected, TT cells treated with 5′-Aza-dC showed an increase of approximately 6-fold in the levels of Spry1 mRNA when compared to vehicle-treated cells (Supplemental Figure 2).

DISCUSSION

Our data indicate that Spry1 is abundantly expressed in the whole thyroid gland, and that such expression increases with age, suggesting an important role of Spry1 in the function of adult thyroid. Consistently, C-cell hyperplasia found in Spry1 knockout mice does not appear to be caused by a developmental defect during morphogenesis of the embryonic thyroid, but rather seems to progress during postnatal life. We found that young adult Spry1 knockout mice failed to develop MTC but instead exhibited C-cell hyperplasia. One possible explanation for such observation is that our analysis was conducted too early, but as stated above renal complications cause premature death of Spry1 null mice within the first fifth months of life (Basson et al., 2005). In agreement with this notion, transgenic mice carrying MEN2A or MEN2B transgenes under different promoters develop MTC at ages ranging from eight to fourteen months or more (Acton et al., 2000; Kawai et al., 2000; Michiels et al., 1997; Reynolds et al., 2001).. Alternatively, genetic ablation of Spry1 *per se* could not be sufficient for MTC development but might require additional genetic insults (see below).

We have found that expression of Spry1 reduces proliferation of TT cells, which bear a oncogenic Ret mutation, not by specifically inhibiting Ret signaling but by inducing cellular senescence. Given that Spry family members have been proposed to function as tumor suppressors in a wide spectrum of tumors, we suggest that Spry-mediated senescence could be a general mechanism of tumor suppression. In fact, our preliminary data suggests that induction of cellular senescence by Spry1 is a general event as MEFs from the Spry1 knockout appear to escape senescence (data not shown). During the last years it is becoming more evident that oncogene induced senescence (OIS) poses a barrier to tumoral transformation in vivo. Thus, expression of activated Ras, E2f3, or B-Raf oncogenes in mice induces cellular senescence in premalignant tumors of the lung, pancreas, mammary gland, pituitary gland, and melanoma. Interestingly, loss of senescence responses induced by genetic ablation of *Cdkn2a* or *Trp53* leads to malignant progression of these tumors (reviewed in (Collado & Serrano, 2010; Prieur & Peeper, 2008). Likewise, Rb haploinsufficiency in mice leads to development of bening C-cell adenomas that progress to adenocarcinomas when N-Ras is deleted. Again, it has been shown that N-Ras protects C-cell adenomas from malignant transformation by induction of cellular senescence (Shamma et al., 2009). In light of this scenario one likely possibility is that C-cell hyperplasia observed in oncogenic Ret knockin mice could progress to MTC by deletion of Spry1 and thus attenuation or abrogation of senescence responses. We are currently generating double mutants expressing a oncogenic Ret mutation and lacking Spry1 to test this hypothesis.

We do not currently know how expression of Spry1 induces cellular senescence. One simple explanation could be that the very modest activation of the ERK pathway seen upon Spry1 expression would be enough to elicit senescence responses. The role of ERK1/2 activation in triggering senescence is however controversial. Initial studies showed that the ERK pathway was both necessary and sufficient to promote senescence of human fibroblasts (Lin

et al., 1998; Zhu et al., 1998). It is also well known that transcription of the CDKN2A locus is positively regulated by the ERK targets Ets1 and Ets2 (Lanigan et al., 2011). However, more recent reports have found that negative feedback mechanisms (including expression of Spry1) in response to activation of the Ras/Raf/ERK pathway are indeed responsible for the senescence response (Courtois-Cox et al., 2006). It is also possible that Spry1 causes senescence independently of the ERK pathway. A recent report describes that the kinase Dyrk1A promotes cellular senescence via phosphorylation of LIN52 and assembly of the DREAM transcriptional repressor (Litovchick et al., 2011). Interestingly, Dyrk1A has been shown to interact with Spry2 in a two hybrid screening (Aranda et al., 2008). Moreover, in another two hybrid assay Spry1 was identified as a binding partner of E1A, which is known to inhibit Ras-induced senescence of normal human fibroblasts (Zaremba et al., 2011). In conclusion, there is a plethora of putative mechanisms by which Spry can induce cellular senescence that deserves extensive future research.

The expression of different Spry family members has been shown to be downregulated in a variety of human cancers when compared to normal, adjacent tissue. Thus, Spry1 and Spry2 levels are decreased in prostate and breast cancer (Fritzsche et al., 2006; Kwabi-Addo et al., 2004; McKie et al., 2005), whereas downregulation of Spry2 has been described in hepatocellular carcinoma, B-cell lymphoma or endometrial carcinoma, among others (Fong et al., 2006; Frank et al., 2009; Velasco et al., 2010) A complication of such expression studies when working with MTC is that C-cells constitute only 5% of the thyroid tissue in which they are embedded. Consequently, comparing tumoral to normal cells is impossible by techniques such as real time RT-PCR or western blot. Moreover, the lack of specific antibodies precluded microscopic analyses involving tissue micro-arrays. Therefore, we could only compare expression of Spry1 in MTC to that of whole thyroid or of cells that are known to express it. Although not ideal, we believe that such comparison is legitimated by the observation that murine C-cells from Spry1$^{lacZ/+}$ mice express higher levels of beta-galactosidase than their neighboring follicular cells. We also show that SPRY1 promoter is methylated in a high percentage of human MTC and that such methylation correlates well with decreased expression of the gene. Epigenetic silencing of the SPRY1 and SPRY2 appears to be the preferred way by which tumor cells repress their expression. Thus, methylation of the SPRY1 promoter has been demonstrated in prostate carcinoma (Kwabi-Addo et al., 2009), and methylation of the SPRY2 promoter has been described in prostate carcinoma and B-cell lymphoma (Frank et al., 2009; McKie et al., 2005). Alternatively, loss of heterozygosity on the SPRY2 locus occurs in prostate cancer (McKie et al., 2005). A recent study using high resolution array-comparative genomic hybridization found allelic losses at 4q28.1, where Spry1 gene maps, in 8 out of 30 (~27%) human MTC samples (Ye et al., 2008). Interestingly, the same work found genomic losses at 5q31.1, where Spry4 gene maps, in ~33% MTC. Finally, a very recent work indicates that expression of the microRNA mi-R-21 in prostate tumors might be responsible for reduced expression of SPRY1 in prostate tumors that show no promoter methylation (Darimipourain et al., 2011). In conclusion, our data indicate that Spry1 is a candidate tumor suppressor gene in MTC.

MATERIAL AND METHODS

Real time RT-PCR

Taqman® technology from Applied Biosystems was used for real-time RT-PCR analyses. Probes Mm01285700_m1 (Spry1) and Mm99999915_g1 (GAPDH) were used for mouse tissues. Probe Hs01083036_s1 (SPRY1) was used for detection of SPRY1 in MTC samples, whereas a custom probe located within the open reading frame of SPRY1 was used for detection in TT cells. Other probes used included: Hs00182986_m1 (Wnt 11), Hs01100741_m1 (Calcitonin), Hs02902543_m1 (p16^{INK4a}), Hs00924091_m1 (p14^{Arf}), Hs00355782_m1 (p21$^{CIP/WAF1}$), and Hs001532277_m1 (p27^{KIP1}). To analyze cell cycle

regulators, an Array PCR kit was used (Human cyclins and cell cycle regulation TaqMan array 96 Applied Biosystems #4414123). In all cases probe Hs99999905_m1 (GAPDH) was used for data normalization. Results were calculated by the $2^{-\Delta\Delta CT}$ method.

In situ hybridization

RNA in situ hybridization on paraffin sections was performed as described (Yaguchi et al., 2009) using a digoxygenin-labeled probe for Spry1.

Immunofluorescence and immunocytochemistry

Immunofluorescence was carried out using standard procedures with antibodies to β-galactosidase (Abcam, #ab9361) and calcitonin (DAKO, #A0576). Immunocytochemistry on paraffin sections was conducted as described (Gallel et al., 2008). Calcitonin-positive cells from a single thyroid lobe were counted every other section by a blind observer. The final number of C-cells was estimated by multiplying the resulting number by a factor of four. Anti-calcitonin antibody was from DAKO (#IR515), whereas $p16^{IKN4a}$ was stained with the CINtec®p16 kit (MTM labs, #9511).

Cell culture and infection

TT cells were maintained in DMEM supplemented with 10% FBS (Invitrogen). Infection with lentiviruses was conducted as described (Llobet et al., 2008). The sequence of the shRNA to human Ret has been described (Gallel et al., 2008). For growth curves, 10^4 cells were seeded into 96 well plates in quadruplicate and cell numbers were determined at the indicated time points using a haemocytometer. 5′-Bromo-deoxyuridine (BrdU) labeling was performed as described (Eritja et al., 2010). For clonogenic assays, TT cells were seeded in triplicate at clonal density (420 cells/cm^2). After 10 days of culture, colonies were visualized by staining with MTT.

Xenografts

10^7 cells of the indicated TT cell lines were subcutaneously inoculated in the right flank of 8 weeks-old female SCID mice. Tumor growth was measured weekly using a digital caliper, until tumor volume reached a maximum of 2.5 cm^3. Tumor volume was calculated according to the formula $d^2D/2$, where d and D are the shortest and the longest diameter, respectively.

Immunoprecipitation and Western blot

Immunoprecipitation and western blot were conducted as described (Encinas et al., 2008). For Ret immunoprecipitations, a mixture of antibodies to Ret9 (C19G, Santa Cruz Biotechnology) and Ret51 (C20G, Santa Cruz Biotechnology) was used. For western blot, anti-Ret9 (C-19G), anti-Ret51 (C-20G), and anti-$p15^{INK4b}$ (C-20) were purchased from Santa Cruz Biotechnology, antibodies to phospho-ERK1/2 (#9101S) and $p16^{INK4a}$ (#4824) were from Cell Signaling Technologies; antibodies to $p14^{Arf}$ (#P2610) and tubulin (#T5168) were from Sigma, anti-phosphotyrosine (Clone 4G10, Millipore) and anti-p21CIP/WAF1 (#05-453) were from Millipore, and anti-p27KIP1 (#61041) was from BD Biosciences.

Senescence associated β-Galactosidase activity (SA-β-GAL)

SA-β-Gal staining was carried out with X-gal solution (20mg/ml X-Gal [Sigma], 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, and 2mM MgCl2) in PBS at pH 6.0 during at least 2 hours at 37°C.

Methylation analysis

Genomic DNA from MTC samples was converted with the MethylCode™ Bisulfite conversion kit (Invitrogen) as per manufacturer's instructions. Primers for methylation-specific PCR were picked with the MethPrimer software (Li & Dahiya, 2002) at http://www.urogene.org/methprimer/index1.html. Primers for methylated DNA were "M2 Fwd": 5′-GTTGTTGTTCGAGTTAGGATTTC-3′ and "M2 Rev": 5′-AAAAAAAACCCTATCCTATTACGTT-3′. Primers for unmethylated DNA were: "U2 Fwd": 5′-GTTGTTGTTTGAGTTAGGATTTTG-3′ and "U2 Rev": 5′-AAAAAAAACCCTATCCTATTACATT-3′.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Esteban J. Rozen for his initial contribution to the development of this work. We also thank Ana Velasco and Carme Guerris for technical assistance. This work was supported by grants from Ministerio de Educación y Ciencia (BFU2007-67619 and BFU2010-17628) and Fundació La Marató de TV3, and funding from Suport als Grups de Recerca (2009 SGR 740) from Generalitat de Catalunya to M.E; grants 2009SGR794, RD06/0020/1034 and programa de intensificación de la investigación, Instituto Carlos III to X.M.-G.; grants from Fondo de Investigaciones Sanitarias (PI080883) and Fundación Mutua Madrileña (AP2775/2008) to M.R.; and from the Medical Research Council (G0601104) to M.A.B. Tumor samples were obtained with the support of Xarxa catalana de Bancs de Tumors, the Tumor Banc Platform of RTICC and RD09/0076/00059. A.M. is supported by a predoctoral fellowship from Universitat de Lleida and was supported by a fellowship from a Fundació Alícia Cuello de Merigó. M.V. was recipient of a "beca d'introducció a la recerca" fellowship from Universitat de Lleida.

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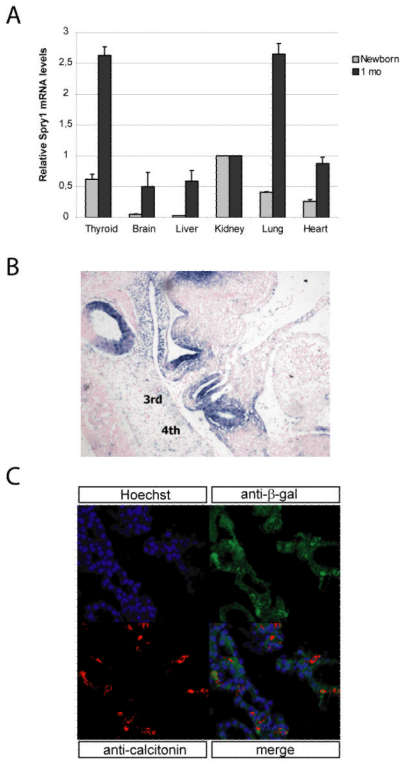

Figure 1.

Spry1 is expressed in murine C-cells of the thyroid. (A) Real time RT-PCR of Spry1 in the indicated organs of newborn and 1 month-old mice. (B) In situ hybridization showing expression of Spry1 mRNA in the fourth pharyngeal pouch, where thyroid C-cells originate. (C) Beta-galactosidase staining of thyroids from 1 month-old Spry1$^{LacZ/+}$ mice. Note expression in both follicular and especially C-cells of the gland.

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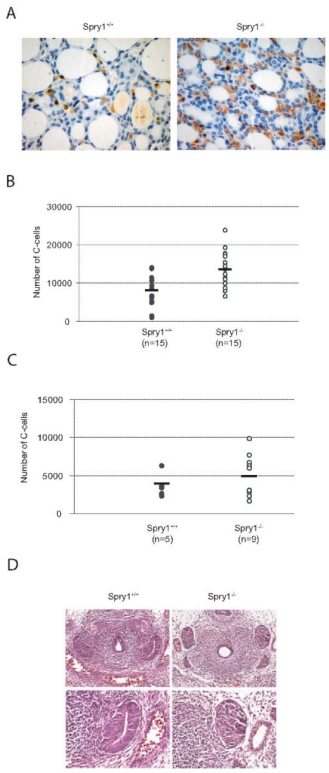

Figure 2.

C-cell hyperplasia in Spry1 knockout mice. (A) Representative picture showing calcitonin staining of thyroids from wild type (left) and Spry1 knockout mice (right) 3 month-old mice. (B) Quantification of the number of C-cells in thyroids of the indicated genotypes ($p<0.01$ by Student's t-test). Results are from the indicated numbers of animals of ages ranging from 1 to 3 month-old. (C) No significant differences in the number of C-cells in thyroid from newborn wild type and Spry1 knockout mice. P=0.14 by Student's t-test. (D) No signs of hyperplasia in ultimobranchial bodies from Spry1 knockout mice. Hematoxylin-eosin stained sections of E12.5 embryos from the indicated genotypes at low (top) and high (bottom) magnification.

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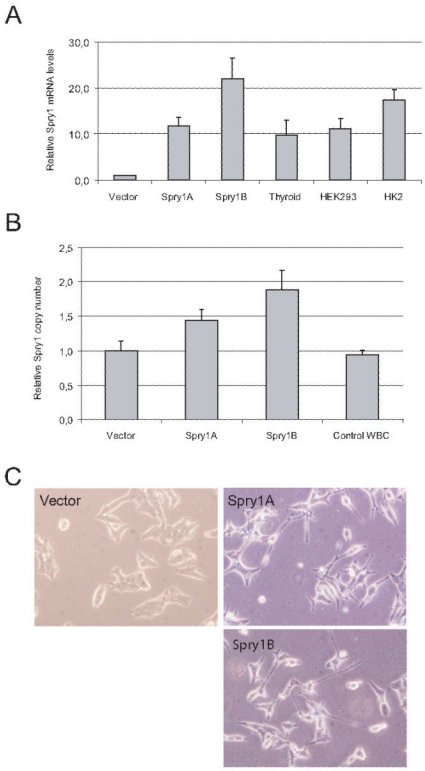

Figure 3.

Characterization of TT cell lines expressing different amounts of Spry1. (A) Relative Spry1 mRNA levels in the indicated TT-derived cell lines (Vector, Spry1A and Spry1B). For comparison, levels of Spry1 in human whole thyroid and kidney-derived human HEK293 and HK2 cell lines are shown. (B) Genomic copy number of the indicated TT-derived cell lines. Genomic DNA from normal white blood cells (WBC) was used for comparison (C) Representative pictures of the indicated cell lines.

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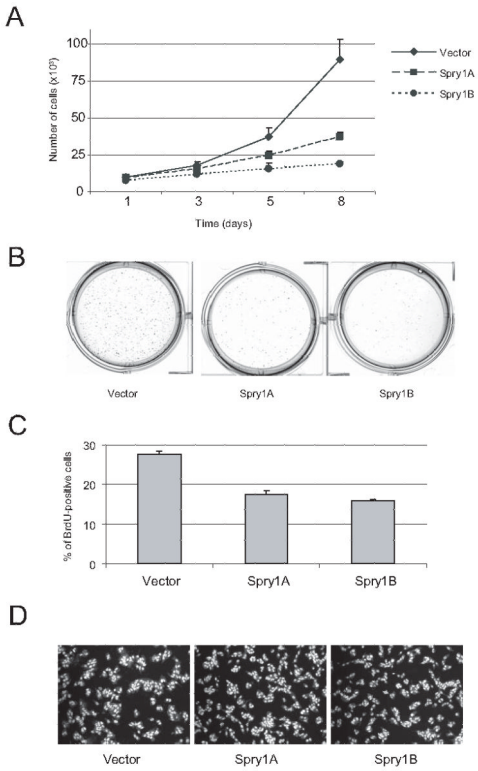

Figure 4.

Spry1 reduces cell proliferation in vitro. (A) Growth curve of the indicated TT-derived cell lines. (B) Clonogenic assays of the indicated TT-derived cell lines. (C) 5′-Bromo-deoxyuridine uptake of the indicated TT-derived cell lines. Note that in all three cases moderate expression of Spry1 (as in Spry1A cell line) is enough to greatly reduce proliferation. (D) Nuclear Hoechst staining of the indicated cell lines reveals virtually no apoptotic cell death in either cell line.

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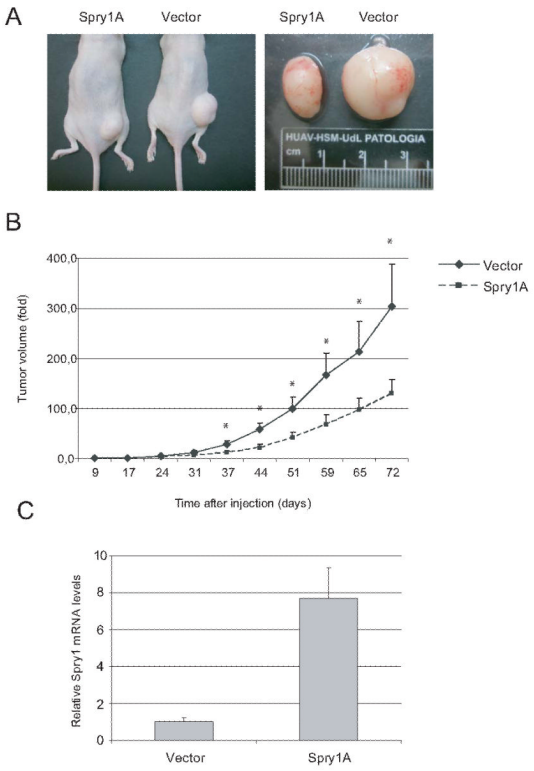

Figure 5.

Spry1 reduces tumor growth. (A) Representative pictures of SCID mice injected with the indicated TT-derived cell lines. (B) Tumor growth over time of SCID mice xenografted with Vector (n=8) or Spry1A (n=10) TT-derived cell lines. Asterisks denote significant differences between cell lines ($p<0.05$ by Student's t-test). (C) Real time RT-PCR showing levels of Spry1 in xenografts derived from either cell line.

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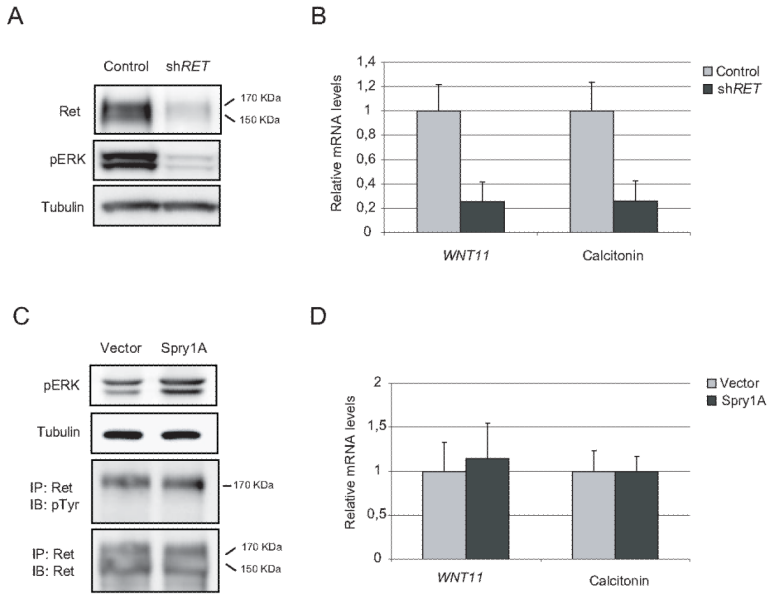

Figure 6.

Spry1 does not inhibit Ret signaling. Knockdown of Ret expression via a shRNA reduces ERK phosphorylation (A) and Wnt11 and Calcitonin expression (B) of TT cells. (C) Expression of Spry1does not block ERK phosphorylation downstream of Ret. (D) Expression of Spry1 does not reduce expression of Wnt11 or Calcitonin.

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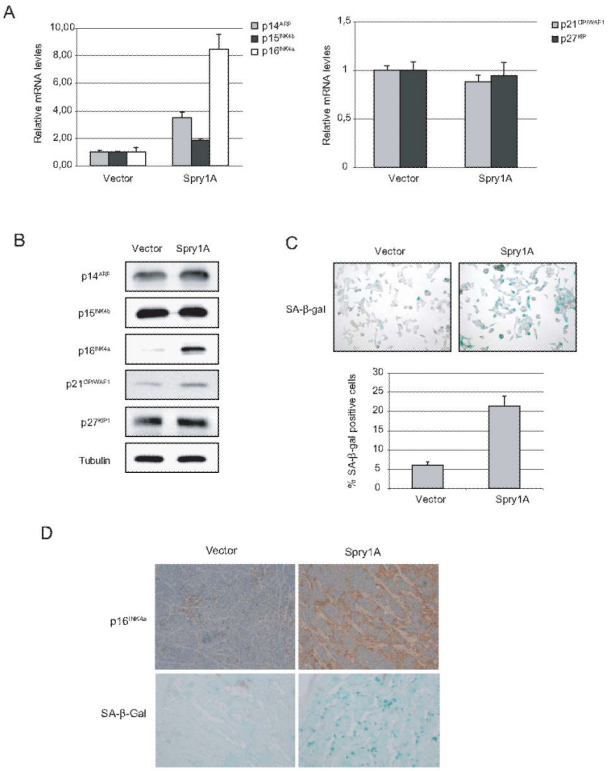

Figure 7.

Spry1 induces expression of the CDKN2A locus and cellular senescence. (A) Real time RT-PCR shows increases in the expression of mRNA coding for $p16^{INK4a}$ and, to a lesser extent, $p14^{ARF}$ and $p15^{INK4b}$ (left panel). Members of the CIP family of cdk inhibitors do not change upon Spry1 expression (right panel). (B) Protein expression levels of the indicated cdk inhibitors in cultured Vector or Spry1A TT cells. (C) Senescence-associated β-galactosidase activity of the indicated cell lines. (D) Immunostaining against $p16^{INK4a}$ and senescence-associated β-galactosidase activity in xenografts derived from the indicated cell lines.

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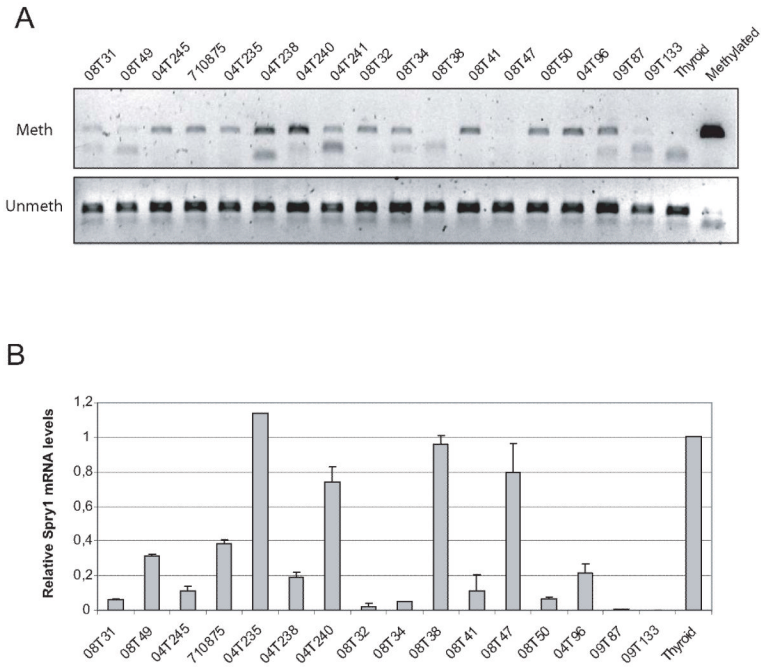

Figure 8.

Promoter methylation and reduced expression of SPRY1 in human MTC. (A) Methylation-specific PCR of the Spry1 promoter in 17 MTC samples. For comparison genomic DNA from normal thyroid is shown. Enzymatically methylated DNA is included as positive control. (B) SPRY1 levels from 16 samples of human MTC were measured by real time RT-PCR and normalized to GAPDH expression. For comparison, SPRY1 levels from whole human thyroid are shown.

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