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Sprouty2 Deficiency in Mice Leads to the Development of Achalasia

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SPROUTY2 DEFICIENCY IN MICE LEADS TO THE DEVELOPMENT OF ACHALASIA

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ABSTRACT

SPROUTY2 DEFICIENCY IN MICE LEADS TO

THE DEVELOPMENT OF ACHALASIA

Sprouty 2 (Spry2), one of the four mammalian Spry family members, is a negative feedback regulator of many receptor tyrosine kinases (RTKs) signaling including Met. It fine-tunes RTKs signaling through multiple levels of regulations starting from RTK itself to several downstream molecules that are crucial for signal transduction. To understand what role Spry2 might play during developmental processes, we created a *Spry2* conventional knockout mouse in which Spry2 expression is replaced by enhanced green fluorescent protein (EGFP) reporter. Spry2 appears to be expressed in mouse embryo and in many adult tissues, and Spry2-deficient mice have smaller body size and shorter lifespan. Pathologically, we observed that Spry2 deficiency results in impaired relaxation of the lower esophageal sphincter and mega-esophagus, a condition known as achalasia, which is likely due to malfunction of the innervated neurons where Spry2 is expressed. The Spry2-deficient mice may serve as a useful animal model for achalasia. Currently, we are investigating how loss of Spry2 leads to achalasia and whether the Met pathway plays a role in the development of this phenotype.

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CHAPTER I

INTRODUCTION

Intracellular signaling is an intensely studied topic in current cell and molecular biology research. The flow of information from the outside of the cell to the inside and the communication among the cellular components has revealed many fascinating discoveries, but much still remains unknown about the specifics of these communication pathways. One group of cellular receptors that transfers signals across the cell membrane is the receptor tyrosine kinases (RTKs). These transmembrane proteins play important roles in processes such as development and cancer progression; for example, RTKs have been shown to be integral in the functions of cell migration and proliferation, processes necessary in both a developing embryo and in cancer (Schlessinger, 2000). The pathways initiated by the receptors are regulated by a host of proteins designed to potentiate or inhibit the pathway and thereby allow close regulation of a variety of outcomes from a single receptor.

One of these regulatory proteins was discovered to be a crucial inhibitor of tracheal branching during *Drosophila* embryonic development. Excessive branching of the trachea occurs when the gene for this protein is lost, so it was given the name Sprouty (Spry) (Hacohen *et al.*, 1998). *Spry* presence among higher organisms has since been shown to be wide-spread, as homologous genes have been found in chickens (Chambers and Mason, 2000), frogs (Nutt *et al.*, 2001), mice (Tefft *et al.*, 1999), and humans (Minowada *et al.*, 1999). Tracheal branching in *Drosophila* is

normally regulated by fibroblast growth factor receptor (FGFR) signaling, so excessive branching in the absence of *Spry* gene provided the first evidence that Spry negatively regulates RTK signaling (Hacohen *et al*., 1998). Negative feedback inhibition of a signaling pathway involves the transcription of a protein that limits the same pathway that led to its own transcription. Spry has been shown to be a negative feedback regulator of multiple RTKs including epidermal growth factor receptor (EGFR), FGFR, and hepatocyte growth factor receptor (HGFR, also known as Met), whose ligands can induce Spry gene expression (Hanafusa *et al.*, 2002; Reich *et al.*, 1999; Ozaki *et al.*, 2001; Hacohen *et al.*, 1998; Lee *et al.*, 2004). Spry fine-tunes RTKs signaling input in a timely manner, thereby influencing the signaling output as manifested by changes in cellular activities such as cell proliferation, migration, etc. (Kim and Bar-Sagi, 2004).

The mitogen activated protein kinase (MAP kinase) pathway is a chain of protein-protein interactions by which the signal that was passed through the cell membrane is propagated, and terminates in regulation of gene transcription. Following the conformational change that occurs upon ligand binding, growth factor receptor-bound protein 2 (Grb2) is able to interact with the receptor as an adaptor protein, recruiting the guanine nucleotide exchange factor son-of-sevenless (Sos) to the cell membrane. The small GPTase Ras can then interact with the Grb2-Sos complex, changing from the GDP to GTP conformation and thereby becoming activated. Activated Ras binds and activates Raf, a serine-threonine protein kinase, which subsequently activates a series of kinases eventually leading to phosphorylation of a protein such as extracellular signal-regulated kinase (ERK) which translocates to the nucleus and regulates transcription factors for genes leading to cell growth and differentiation (Molina and Adjei, 2006).

Spry proteins can exert multiple levels of regulation on RTK signaling, starting from the receptor itself to several downstream signaling molecules (Kim and Bar-Sagi, 2004). One way Spry proteins inhibit Ras/MAP kinase pathway is by sequestering Grb2 so it cannot mediate the binding of Sos to the RTK, a step necessary for propagating the signal (Gross *et al.*, 2001; Hanafusa *et al.*, 2002). Sprys also bind to the protein kinase Raf through its conserved cysteine-rich domain, thereby inhibiting the activation of Raf and downstream ERK (Kim and Bar-Sagi, 2004). Spry becomes inactivated when its tyrosine residues become dephosphorylated by Shp2 phosphatase (Hanafusa *et al.*, 2004). This leads to disassociation of Spry from Grb2 and releases Spry-mediated signal inhibition (Hanafusa *et al.*, 2004).

Although Spry proteins seem to be general RTK inhibitors, the mode of Spry action on different RTKs does not appear to be exactly the same. For instance, FGF signaling is down-regulated by Spry2 while EGF signaling does not appear to be as greatly affected (Rubin *et al.*, 2005). One method of down-regulating a signaling pathway is by marking components of the pathway for degradation by the proteasome. This marking is performed by the family of E3 ubiquitin ligases, a prominent member of which is c-Cbl. Spry proteins have also been found to increase MAP kinase signaling induced by EGF and this increase is likely due to the ability of Spry to sequester c-Cbl (Egan *et al.*, 2002). Following phosphorylation, Spry can interact with the Src-homology 2-like domain of c-Cbl and block c-Cbl's binding to EGFR, thus preventing EGFR ubiquitination and degradation (Egan *et al.*, 2002; Fong *et al.*, 2003). This is not the case in FGF-induced signal transduction as Spry shows no protection against FGFR ubiquitination (Fong *et al.*, 2003). It has been suggested that the difference between FGF and EGF signaling is most likely due to the ability of FGFR but not EGFR to phosphorylate certain Spry carboxy-terminal tyrosine residues

necessary for full inhibitory function (Rubin *et al.*, 2005). This selective phosphorylation may be a key to the extent Spry plays in regulating RTK signaling originating from different receptors. The ability for receptors to target specific tyrosines on the Spry proteins allows a single Spry protein enough versatility to display a variety of responses, depending on which receptor initiates its phosphorylation.

There are four members in the mammalian Spry family. Two conserved regions among Spry protein family members account for their functions: a carboxy-terminal cysteine rich domain is responsible for translocation of Spry from its pre-stimulated location in the cytosol to the post-stimulated location at the plasma membrane (Lo *et al.*, 2004; Lim *et al.*, 2002). Phosphorylation of an amino-terminal conserved tyrosine upon growth factor stimulation is crucial for Sprys to inhibit RTK-Ras-MAP kinase signaling cascade (Hanafusa *et al.*, 2002; Gross *et al.*, 2001; Kim and Bar-Sagi, 2004).

One method of determining the role of a gene is to overexpress it *in vitro* or *in vivo*. Abnormalities that arise in the over-expression tissue often provide an indication of the gene's function. Chick Spry over-expression results in stunting of chick limb outgrowth by preventing proper bone formation (Minowada *et al.*, 1999). Over-expression of the mouse Spry2 protein *in vitro* results in decreased cell proliferation, migration, and invasion, as well as accumulation of cells at cell cycle phase G2/M (Lee *et al.*, 2004). Endogenous levels of Spry1 and Spry2 proteins negatively regulate proliferation, differentiation, and anchorage-independent cell growth (Gross *et al.*, 2001), while promoting cell adhesion and spreading (Lee *et al.*, 2004).

One common means to identify the effect a gene has on development is the creation of a knockout mouse in which the gene of interest is removed and the

development of the mouse is followed. Anomalies from standard development indicate the deleted gene may be influential in the development of the system that produced the difference observed. Spry1 knockout mice suffer from a ureteric bud defect that is attributed to loss of Spry1 in the Wolffian duct during embryogenesis, leading to multiple ureters and epithelial cysts on the kidneys (Basson *et al.*, 2005). Spry2 knockout mice have also been created and are described in the discussion section. There have been no reports of Spry3 knockout mice. Spry4 knockout mice exhibit lower body weights than wild type mice, exhibit polysyndactyly, and have mandible defects (Taniguchi *et al.*, 2007).

Spry2 has been shown to be induced in leiomyosarcoma cells by hepatocyte growth factor (HGF) and acts as a negative feedback inhibitor of the Met pathway (Lee *et al.*, 2004) which is involved in both embryogenesis and tumorgenesis (Birchmeier *et al.*, 2003). This paper also showed that overexpression of Spry2 leads to inhibition of HGF-induced AKT and ERK signaling, as AKT phosphorylation was completely abolished and the duration of ERK phosphorylation was reduced. Cell proliferation and invasion were also inhibited when Spry2 was overexpressed (Lee *et al.*, 2004). These attributes are characteristic of tumor suppressor genes, and Lee *et al.* were the first to suggest that Spry2 is a tumor suppressor gene.

Previously published data and results within our lab led us to ask questions related to the role Spr2 plays in the mouse. What happens *in vivo* when regulation of the RTKs by Spry2 is lost? Will mice without Spry2 develop tumors more quickly than mice with normal Spry2 levels? What happens to mouse development when Spry2 is lost?

We wanted to determine what role Spry2 might play during normal development; we generated a *Spry2* knockout mouse in which Spry2 alleles are

replaced by an enhanced green fluorescent protein (EGFP) reporter using a conventional gene targeting technology. In this mouse model we see evidence for Spry2 expression in several embryonic and adult tissues as indicated by EGFP fluorescence. Pathologically, Spry2-deficient mice display an enlarged esophagus and much smaller body size as well as a shorter lifespan than wild type and heterozygous littermates, likely due to developmental/functional defect in the lower esophageal sphincter which fails in relaxation or is hypercontractile, a condition known as achalasia.

CHAPTER II

RESULTS

To investigate the role of Spry2 during mouse development, we created *Spry2*-deficient mice using conventional gene knockout techniques. The entire coding region of Spry2 was replaced with an EGFP/PGK-Neo cassette, leaving EGFP expression under the control of endogenous *Spry2* promoter (Figure 1A). The expected homologous recombination of Spry2 alleles was confirmed by Southern blot analysis and PCR genotyping using genomic DNAs derived from Spry2 wild type, heterozygous and homozygous mice (Figure 1B).

We first examined the expression of Spry2 in various tissues including brain, lung, and kidney of wild type, heterozygous, and *Spry2* homozygous mice by reverse transcriptase PCR (RT-PCR). Spry2 displays highest expression in the brain, and moderate expression in the lung and kidney of Spry2 wild type mice (Figure 2A). No Spry2 expression was detected in the tissues derived from $Spry2^{-/-}$ mice, indicating a complete *Spry2* knock-out (Figure 2A). This was also confirmed by the detection of EGFP in tissues derived from $Spry2^{+/-}$ and $Spry2^{-/-}$ mice but not $Spry2^{+/+}$ mice either by RT-PCR (Figure 2A) or western blot analysis (Figure 2B). DNA, RNA, and protein results all indicate accurate homologous recombination in the Spry2 alleles.

Mouse embryo and adult tissues were also examined under a fluorescence microscope to determine the expression sites of Spry2, as seen by EGFP reporter fluorescence in the Spry2-deficient animal. $Spry2^{-/-}$ embryos show strong fluorescence in the developing ear and limbs, whereas $Spry2^{+/+}$ embryos show only background fluorescence (Figure 3A). Strong EGFP fluorescence was also observed from $Spry2^{-/-}$

adult brain, while moderate fluorescence was observed from lung, heart, and kidney of three-month old Spry2$^{-/-}$ mice (Figure 3B). These data are consistent with the results obtained from RT-PCR and western blot analysis (Figure 2).

Spry2 wild type, heterozygous and homozygous mice were born in the expected Mendelian ratio, indicating no embryonic lethality due to Spry2 deficiency. There is no obvious difference in littermate sizes at birth, but later on Spry2$^{-/-}$ mice appear to be smaller than their wild type and heterozygous littermates, indicating growth retardation is likely a result of a postnatal defect (Figure 4A). The average body weight of the Spry2$^{-/-}$ mice was only two-thirds of the Spry2$^{+/+}$ and Spry2$^{+/-}$ mice (Figure 4A). Upon gross examination, the most obvious difference that we observed was the appearance of the esophagus (Figure 4B). While other organs appear proportional to the body size, Spry2$^{-/-}$ mice have a megaesophagus with an average diameter more than twice as large as wild type or heterozygous littermates (Figure 4B). The increased diameter extends down to, but does not appear to include, the lower esophageal sphincter (LES). Longitudinal sections through the LES and adjoining esophagus reveal an enlarged lumen and dilated wall, while the LES appears to be in a contractile state (Figure 4C), mimicking a pathological condition known as achalasia in humans. This phenotype is likely due to a blockage between the esophagus and stomach resulted from either nonrelaxation or hypercontraction of the LES. Food and water cannot pass properly through the LES into the stomach due to the defect, and as a result the Spry2-deficient mice are malnourished, appear skinny, and are short-living. There is an inverse correlation between body weight and esophagus size (Figure 4A and 4B).

When examining the location of Spry2 expression in the Spry2$^{-/-}$ esophagi under a fluorescent microscope, we observed a bright EGFP fluorescence, indicating

of Spry2 expression, along the longitudinal nervous tissues of the esophagus running to the LES region (Figure 4D). This suggests that a potential defect in the neuronal tissues innervating the LES may result in achalasia in the Spry2$^{-/-}$ mice.

To determine if the esophageal phenotype is due to an unregulated Met signaling in neuronal tissues, we then asked if eliminating Met activity will rescue or alleviate the Spry2-deficient phenotype. Selective knockout of genes in specific tissues can be obtained using the cre-lox conditional knockout system (Sternberg and Hamilton, 1981; Nagy, 2000) in which regions of DNA flanked by LoxP sites can be deleted only in tissues expressing the cre enzyme. Only one copy of cre recombinase (*cre*$^{tg/-}$) is necessary and sufficient to excise any regions flanked by LoxP sites. Since complete knockout of *Met* is embryonically lethal (Bladt *et al.*, 1995), we conditionally knocked out the *Met* allele in neuronal tissues by crossing *Met*$^{LoxP/LoxP}$ mice (Borowiak *et al.*, 2004) to *nestin-cre*$^{tg/-}$ mice in which cre recombinase is specifically expressed in the neuronal tissues under the control of nestin gene promoter (Burns *et al.*, 2007). Using the mating scheme outlined in Figure 5, we aimed to obtain animals with *Met*$^{LoxP/LoxP}$*; nestin-cre*$^{tg/-}$*; Spry2*$^{-/-}$ genotype to achieve *Spry2*-deficiency in conjunction with Met alleles specifically knocked out in neuronal tissues. However, no animals of this genotype have been observed thus far.

To determine how loss of Spry2 affects RTK signal transduction *in vitro*, we established mouse embryonic fibroblast (MEF) cell lines from *Spry2* wild type, heterozygous, and homozygous embryos. A more robust ERK phosphorylation was observed in Spry2$^{-/-}$ MEF cells upon EGF stimulation, compared to that in the Spry2$^{+/+}$ and Spry2$^{+/-}$ cells (Figure 6), indicating that releasing negative regulation from Spry2 may enhance the RTK signaling.

CHAPTER III

DISCUSSION

While most studies have focused on *in vitro* analysis of the pathways Spry2 affects (Lee *et al.*, 2004; Chambers and Mason, 2000; Hanafusa *et al.*, 2002), we aimed to examine the role of Spry2 *in vivo* and to determine how loss of Spry2 may affect mouse development. In this study we created and characterized a *Spry2* knockout mouse in which the Spry2 gene is replaced by an EGFP reporter. We observed that loss of Spry2 results in a postnatal defect in the esophagus as manifested by a severely dilated and enlarged esophagus and nonrelaxation or hypercontraction of the lower esophageal sphincter (LES) (Figure 4), a condition known as achalasia in humans (Richter, 2010). Spry2 appears to be expressed in the enteric nerve tissues of the esophagus, especially around the LES, as evidenced by the EGFP fluorescence (Figure 4D). Identification of endogenous Spry2 presence in the esophageal neuronal tissue suggests a possible role for Spry2 in controlling sphincter function. The LES appears to be an important location of Spry2 activity, which is consistent with published data showing LES contraction in Spry2 null mice to be five times as strong as wild type mice (Taketomi *et al.*, 2005).

Spry2 is the most closely studied isoform among all Spry family members for its role in cancer (Lo *et al.*, 2004; McKie *et al.*, 2005; Fong *et al.*, 2006). Even though Spry2 is highly expressed in the brain and moderate in tissues such as lung and kidney (Figure 3), we observed no spontaneous tumor formation in Spry2-deficient mice, suggesting that loss of Spry2 alone is not sufficient for tumorigenesis. Other labs publishing Spry2 knockout mice phenotypes have not described tumor formation as a

result of Spry2 loss either. It is also possible that other Spry family members may be compensating somewhat for Spry2 loss, preventing or suppressing tumor formation while other phenotypes of Spry2 deficiency are still visible. It remains to be seen if the diffenential phosphorylation of Spry2 residues in response to various growth factors plays a role in tumor formation.

Two other research groups have reported phenotypes lacking *Spry2* in mice: one described a severe hearing impairment (Shim *et al.*, 2005), and the other showed an esophageal phenotype similar to the one we observed (Taketomi *et al.*, 2005). The hearing impairment defect in *Spry2*-deficient mice has been attributed to excessive FGF signaling which leads to the development of an extra pillar cell in the inner ear (Shim *et al.*, 2005). Glial cell derived neurotrophic factor (GDNF) signaling was reported to be responsible for developing Spry2-deficient esophageal phenotype (Taketomi *et al.*, 2005). In the latter case, the authors conclude that loss of Spry2 results in enteric nerve hyperplasia, based on counting immunostained neurons from mouse esophagus and colon (Taketomi *et al.*, 2005). Injection of neutralizing antibody against GDNF or an antagonist of Ret (the receptor tysosine kinase for the GDNF ligand) into neonatal Spry2$^{-/-}$ mice alleviated some of the esophageal phenotype (Taketomi *et al.*, 2005), although the animals were followed for only a short time window that may not have taken into account the incomplete penetrance we see in our Spry2-deficient animals. Ret germline inactivating mutations are the cause of about half the cases of a human condition known as Hirschsprung's disease. This condition is manifested by megacolon due to a defect in the enteric innervation known as intestinal aganglionosis (Arighi *et al.*, 2005). It seems that the esophagus is spared from defect due to Ret deficiency, as knocking out Ret in mice causes intestinal aganglionosis but only partially affecting enteric innervation in the esophagus

(Durbec *et al.*, 1996). Yet, another report showed that neural cells in the esophagus are Ret-dependent (Yan *et al.*, 2004). There seems to be no *direct* evidence showing that the Spry2-deficient esophageal phenotype is caused by GDNF-Ret pathway. A direct approach to test this hypothesis would be to cross Spry2 knockout mice with Ret-deficient mice and determine if loss of Ret activity rescues achalasia resulted from Spry2 deficiency.

Interestingly, it has also been reported that GDNF can induce Met phosphorylation in Ret-deficient cells (Popsueva *et al.*, 2003), suggesting that Met may mediate Ret-independent GDNF signaling in certain cell contexts. This option of GDNF signaling through the Met receptor opens the possibility of crosstalk between the HGF-Met and GDNF-Ret pathways. If this is the case in Spry2-deficient mice, the results of the anti-GDNF experiment in the paper by Taketomi *et al.* may have been dependent on Met signaling also.

The *Met* gene codes for an RTK that has been shown to play an important role both in embryonic development and in cancer metastasis. Met has a key function in placenta formation, liver development, myogenic precursor cell spread, and integration of the nervous system (for reviews, see Birchmeier *et al.*, 2003; Trusolino *et al.*, 2010). HGF binds the Met receptor and initiates several signaling pathways that lead to proliferation, migration, invasion, or other cellular outcomes (Birchmeier *et al.*, 2003).

Since Spry2 is a negative feedback regulator of HGF-Met signaling pathway, we asked if Met plays a role in the development of achalasia phenotype in Spry2-deficient mice. *Met* knock-in (Met^{ki}) mouse lines in which a cassette derived from *Met* cDNA was introduced into the *Met* locus have been produced (Graveel *et al.*, 2005). The mice with this insertion but lacking Met activation mutations developed severe

megaesophagus, however various constitutive activating mutations in the cassette rescued the phenotype (unpublished results). Although it could be predicted that loss of Spry2 in the presence of hyperphosphorylated active Met would enhance the esophageal phenotype, crossing our Spry2 line to a line with one of these activating mutations did not significantly alter the esophageal diameter or the lifespan of the Spry2-deficient mice (unpublished data). Crossing Spry2-deficient mice to the Met^{ki} line with no mutations should answer the question if $Met^{ki/ki}$; $Spry2^{-/-}$ mice have an even larger esophagus or if they are even viable at all.

HGF is a chemoattractant for motor neurons during development and guides neuron outgrowth (Maina and Klein, 1999). HGF also patterns the axonal outgrowth, and HGF mutant mice have been found to have defective motor axon growth (Miana *et al.*, 2001). In addition, Met has been reported to play an important role in axonal development, with Met mutant mice developing shorter nerves that have fewer branching points than wild type mice (Miana *et al.*, 1997). Anti-HGF antibodies can stunt the differentiation of sympathetic neurons, and survival and growth of these neurons are significantly enhanced with HGF (Miana *et al.*, 1998).

Two major pieces of evidence pointed us toward examining the role of Spry2 in mouse development: 1) HGF has been shown to upregulate Spry2 expression (Lee *et al.*, 2004), and 2) the HGF-Met pathway is crucial in neuronal development (Miana *et al*., 1997; Miana *et al*., 1998; Miana *et al.*, 2001). Given the Met signaling importance in neuronal development and its negative regulation by Spry2, we speculated that Met might play a role in the development of achalasia in $Spry2^{-/-}$ mice. Therefore, we hypothesized that Met signaling is responsible for the migration of neural crest cells into the esophagus. Absence of Spry2 leads to enhanced Met

signaling in the neural crest cells, likely leading to hyperganglionosis in the esophagus.

To test our hypothesis, we asked if eliminating Met activity rescues the esophageal phenotype developed by the Spry2-deficient mice. If Met is the driver of this phenotype, eliminating Met should restore the normal esophageal phenotype. Since complete knockout of either Met or HGF results in embryonic lethality (Bladt *et al.*, 1995; Schmidt *et al.*, 1995; Uehara *et al.*, 1995), we took an approach to conditionally knockout Met in the neuronal tissues by crossing Met-flox mice (Borowiak *et al.*, 2004) with nestin-cre transgenic mice in the absence of Spry2 (see Figure 5 for breeding scheme). However, we have not yet obtained any mice exhibiting either a $Met^{LoxP/+}$;*Nestin-* $cre^{tg/-}$; $Spry2^{-/-}$ or $Met^{LoxP/LoxP}$;*Nestin-* $cre^{tg/-}$; $Spry2^{-/-}$ genotype. Albumin-cre mice express cre recombinase in the liver (Postic *et al.*, 1999), and crossing these mice with $Met^{LoxP/LoxP}$, $Spry2^{-/-}$ mice can produce a $Met^{LoxP/LoxP}$; *albumin-*$cre^{tg/-}$; $Spry2^{-/-}$ mouse, ruling out a possible background effect due to the cre mouse strain. $Met^{LoxP/LoxP}$;*Nestin-* $cre^{tg/-}$; $Spry2^{+/+}$ mice are also viable, indicating that the loss of Met in the neuronal tissue alone is not lethal. Two possibilities may account for the lack of the two mouse genotypes: 1) there have not been enough pups born to see the genotypes we are seeking, or 2) those two genotypes are embryonically lethal. We are currently investigating these possibilities.

Taken together, we show that Spry2 is expressed in the enteric nerve innervations of the esophagus and its deficiency results in the development of esophageal achalasia. We are aiming to gain further insight into how this condition is developed in the absence of Spry2 and whether Met plays a role in its onset. The Spry2 knockout mouse can be a useful model for understanding the underlying mechanism of achalasia and for developing therapies for this condition.

CHAPTER IV

MATERIALS AND METHODS

Generation of Spry2 Knockout Mouse

To construct a Spry2 targeting vector as shown in (Figure 1A), a 2-kb genomic fragment (including partial intron 1 and partial exon 2) and a 4.6-kb genomic fragment downstream of exon 2 were inserted into the pPNT vector (Tybulewicz *et al.*, 1991) ahead of and behind an EGFP-PGK-NEO cassette, respectively. In the resulted vector, the EGFP reporter uses the ATG codon from Spry2 gene as its starting codon. Once homologous recombination takes place, the entire coding region of Spry2 is replaced by the EGFP-PGK-NEO cassette, and the EGFP reporter is under the control of Spry2 promoter for its expression. To generate ES cells carrying the $Spry2^{+/-}$ genotype, ES cells were electroporated with a linearized targeting vector and selected with neomycin. Two established $Spry2^{+/-}$ ES clones were used to generate Spry2 knockout mice. Successive homologous recombination was confirmed by Western Blot analysis and PCR genotyping as detailed below.

Southern Blot Analysis

Mouse tails were clipped at weaning and were submitted to automatic DNA extraction by an AutoGenPrep 960 machine (AutoGen). DNA was then digested with restriction enzymes NdeI and HindIII, separated via electrophoresis, and transferred to a Hybond XL membrane. The Sy2-5' probe (Figure 1A) was radiolabeled with ^{32}P, hybridized with the membrane, and detected on a Kodak x-ray film.

PCR Genotyping

For PCR genotyping of Spry2, three primers (P1, P2, and P3) were used in a mixed reaction to distinguish wild type and mutant alleles (Figure 1A). The forward primer P1 (5'-GACTTGACCGATACGTTGTAGG-3') is common to both wild type and mutant alleles. The reverse primer P2 (5'-ATCAGGTCTTGGCAGTGTGTTC-3') is specific for wild type allele, while P3 (5'-ATGAACTTCAGGTCAGCTTGC-3') is specific for mutant allele. PCR amplification used an annealing temperature of 58°C and an elongation period of two minutes for 30 cycles. Genotypes were determined by separating the PCR products on a 1.2 % agarose gel containing ethidium bromide, followed by visualization under UV light.

Other Mouse Strains

Nestin-cre animals were purchased from Jackson Laboratories (www.jax.org). Met-flox mice were obtained from Dr. Carmen Birchmeier (Max Delbrück Center for Molecular Medicine, Germany).

RT-PCR

Brain, lung, and kidney tissues were harvested and homogenized in 4 mL of TRIzol reagent (Sigma-Aldrich). RNA extraction proceeded according to the TRIzol protocol. A One-Step RT-PCR Kit (Invitrogen) was employed according to the manufacturer's recommendations, using primers as follows: 5'-CAGATGTGTTCTAAGCCTGCTG-3' and 5'-GAGCAACAATCCCTATTACTGC-3' for *Spry2*; 5'-AAGGACGACGGCAACTACAAG-3' and 5'-CGGATCCGCTCGAGCGCCTTAAGATACATTGATGAG-3' for EGFP; 5'-TCCAGTATGACTCCACTCACG-3' and 5'-ACAACCTGGTCCTCAGTGTAG-3'

for GAPDH. PCR products amplified using an annealing temperature of 55 °C and an elongation time of one minute (30 cycles) were run on a 1 % agarose gel containing ethidium bromide and visualized under UV light.

Tissue Culture

Mouse Embryonic Fibroblasts (MEFs) were established from day-14 individual embryos and cultured in DMEM (Invitrogen) supplemented with 10% Fetal Bovine Serum (HyClone) and 1% Penicillin/Streptomycin. Following establishment, the MEF cells genotypes were determined by PCR.

Western Blot Analysis

Mice were sacrificed and the brain, lung, and kidney of each mouse was homogenized separately in RIPA buffer [50 mM Tris-HCl, pH 7.4, 1 % NP-40, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/mL Aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM Na3VO4, 1 mM NaF, Complete Protease Inhibitor Tablet (Roche Applied Science)]. Subsequently, samples were frozen in liquid nitrogen to rupture the cells, thawed at 4 °C, and centrifuged at 13,500 rpm for fifteen minutes.

To collect tissue culture lysates, cells were rinsed with phosphate buffered saline (PBS) and RIPA was added. Following 15 minute incubation at 4°C, cell debris was removed by centrifugation at 13,500 rpm for 15 min. Protein lysates were quantified using the DC Protein Assay (Bio-Rad) according to manufacturer's specifications, and diluted to equal concentrations in 2X Laemmli Sample Buffer (Sigma). Proteins were denatured by boiling for five minutes and then loaded into a 10 % Tris-Glycine PAGE gel (Invitrogen) in equal concentrations. The gel was run at

125 volts for approximately two hours, and transferred to a PVDF membrane (Invitrogen) via electrophoresis at 25 volts for two hours. The membrane was blocked with 5 % dry milk for one hour, and probed overnight at 4 °C with the primary antibody. Membranes were washed with Tris Buffered Saline with Tween (TBST), probed with the appropriate anti-rabbit or anti-mouse secondary antibody (Amersham Biosciences RPN2108) for one hour at room temperature, and then washed again. Blots were developed on X-ray film using an Amersham Biosciences ECL kit (RPN2108). The primary antibodies used were: GFP (AbCam ab290, 1:1000), phospho-ERK (Cell Signaling #9101S, 1:1000), and β-actin AC-17 (Sigma-Aldrich A1978, 1:10,000).

Paraffin Embedded Tissue Slide Preparation

Tissues were fixed overnight in formalin, embedded in paraffin wax, cut in 5 µm sections, and stained with hematoxylin and eosin.

Fluorescence Microscopy

Ectopic Spry2 expression was investigated by examining EGFP fluorescence in embryo and adult tissues. Organs extracted from sacrificed mice or whole embryos (day-15 old) were directly viewed under UV illumination using a Leica MZ FLIII fluorescence stereomicroscope for EGFP fluorescence. The esophagus was opened longitudinally and splayed for examination under a Zeiss LSM510 META Confocal Microscope.

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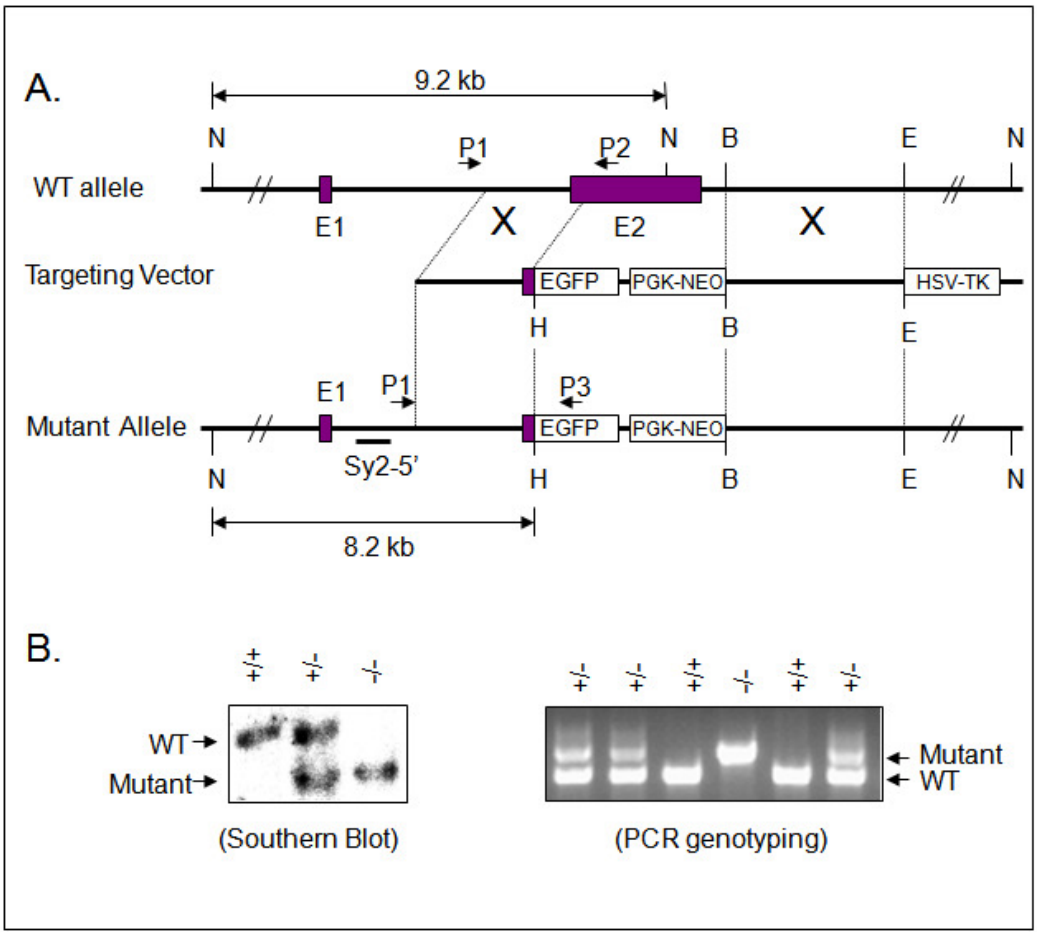

Figure 1. Targeted disruption of mouse *Spry2* wild type alleles. (A) Diagram of the gene targeting vector for the *Spry2* knockout mouse, and the genomic structures of the wild type (WT) and mutant alleles, respectively. After homologous recombination, the entire coding region within exon 2 (E2) was deleted and replaced with a cassette containing EGFP and PKG-NEO. The EGFP sequence was linked to the leftover short fragment of E2 and its expression is under the control of *Spry2* gene promoter. The indicated restriction enzyme sites are: B, BamH I; E, EcoR I; H, Hind III; N, Nde I. (B). Determining *Spry2* WT and mutant alleles by Southern Blot analysis and PCR genotyping. The mouse genomic DNA was digested by Nde I and Hind III, and the blot was hybridized with ^{32}P-labeled Sy2-5' probe. A 9.2 kb WT band and an 8.2 kb

mutant band were detected accordingly. PCR genotyping was performed using primer pair P1-P2 for WT allele and P1-P3 for mutant allele.

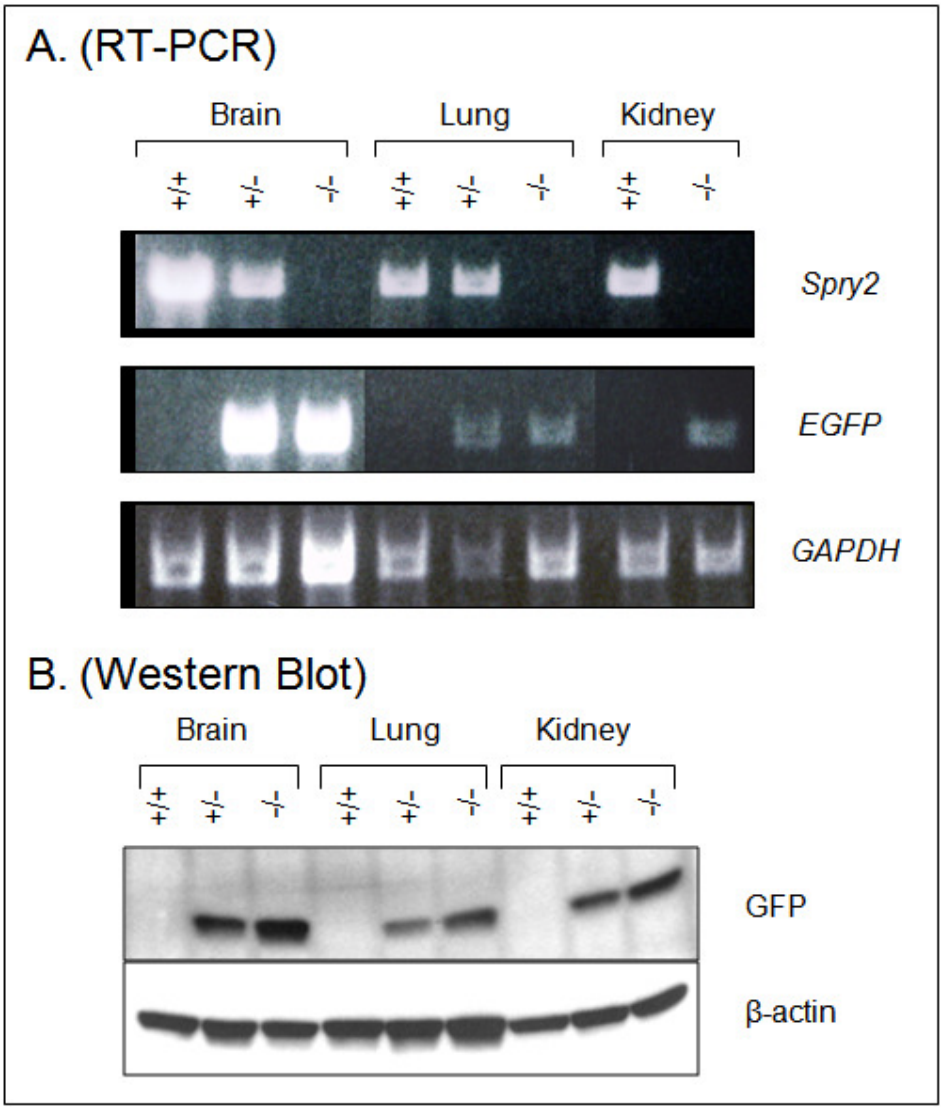

Figure 2. EGFP replaces Spry2 expression in Spry2 heterozygous and homozygous mice. (A) Reverse Transcriptase-PCR detection of *Spry2* and *EGFP* in mouse tissues. Total RNA was prepared from brain, lung, and kidney derived from *Spry2*$^{+/+}$, *Spry2*$^{+/-}$, and *Spry2*$^{-/-}$ mice. RT-PCR was performed using primer pairs specifically for mouse *Spry2* and EGFP. GAPDH was also amplified as an internal control. (B) Detection of EGFP protein in Spry2 heterozygous and homozygous mouse tissues. Whole protein extract was prepared from the indicated mouse tissues, subjected to Western Blot analysis, and probed with anti-GFP and anti-β-actin antibodies.

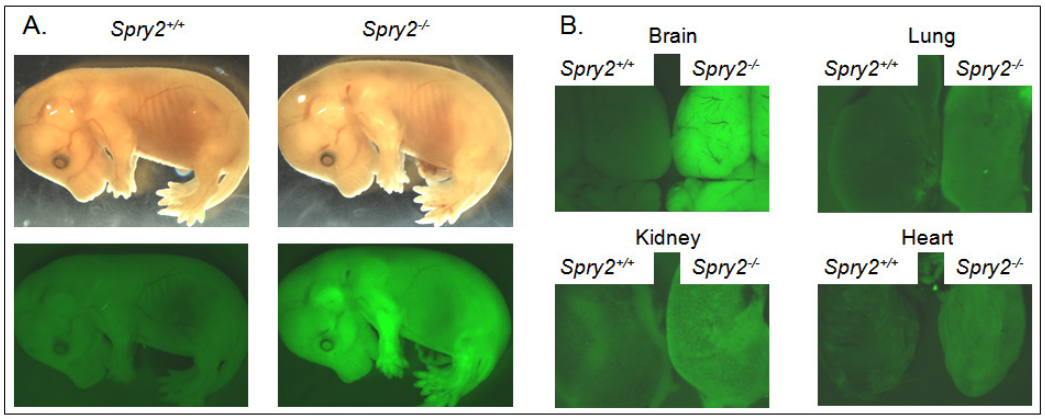

Figure 3. Visualization of Spry2 expression in embryo and adult tissues of *Spry2*-deficient mice revealed by EGFP fluorescence. (A) *Spry2*$^{+/+}$ and *Spry2*$^{-/-}$ embryos at embryonic day 15. Green fluorescence represents EGFP expression. (B) Adult brains, lungs, kidneys and hearts derived from three months old *Spry2*$^{+/+}$ and *Spry2*$^{-/-}$ mice were visualized under a Leica FLUO III microscope.

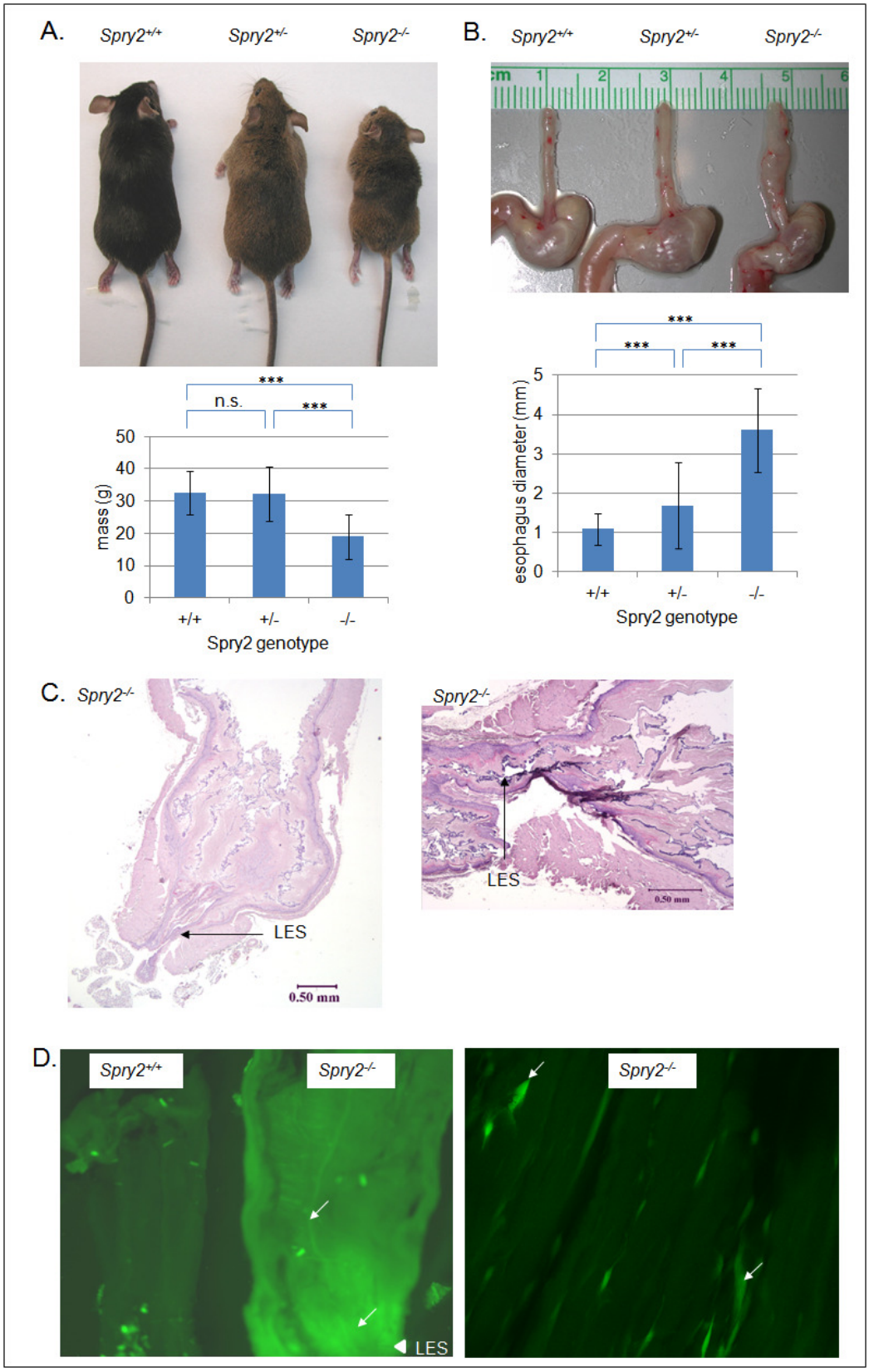
A.
Spry2+/+
Spry2+/-
Spry2-/-
n.s.
***
***
mass (g)
50
40
30
20
10
0
+/+
+/-
-/-
Spry2 genotype
B.
Spry2+/+
Spry2+/-
Spry2-/-
cm 1 2 3 4 5 6
***
***
***
esophagus diameter (mm)
5
4
3
2
1
0
+/+
+/-
-/-
Spry2 genotype
C.
Spry2-/-
LES
0.50 mm
Spry2-/-
LES
0.50 mm
D.
Spry2+/+
Spry2-/-
LES
Spry2-/-

Figure 4. Spry2 deficiency results in growth retardation and mega-esophagus in $Spry2^{-/-}$mice. (A) Upper: Appearances of three months old $Spry2^{+/+}$, $Spry2^{+/-}$ and $Spry2^{-/-}$ mice. $Spry2^{-/-}$ mice display a much smaller body size than the $Spry2^{+/+}$ and $Spry2^{+/-}$ littermates of the same age. Lower: Average mouse mass as measured at the time of necropsy. (B) Upper: Gross anatomy of esophagus and stomach derived from $Spry2^{+/+}$, $Spry2^{+/-}$ and $Spry2^{-/-}$ mice, respectively. The $Spry2^{-/-}$ mouse esophagus is twice as large in diameter as that of wild type or heterozygous littermates. The bottom part of the mutant esophagus at the lower esophageal sphincter (LES) region does not appear enlarged. Lower: Average esophagus diameter at the time of necropsy. n.s. = no significant difference, *** = $p < 0.001$ by Student's T-test, $Spry2^{+/+}$ n=26, $Spry2^{+/-}$ n=58, $Spry2^{-/-}$ n=96, error bars = standard deviation (C) Hematoxylin and eosin staining of the $Spry2^{-/-}$ mutant esophagus revealed enlarged lumen space with marked hyperkeratosis. The esophagus-stomach junction does not appear to be enlarged. (D) Left panel shows gross visualization of EGFP fluorescence in the esophagus derived from 3 months old $Spry2^{+/+}$ and $Spry2^{-/-}$ mice. Right panel shows high-power confocal image of EGFP fluorescence revealing the neuronal network in the mutant esophagus. Arrowhead indicates the location of LES; arrows indicate nerve tissues with strong fluorescence across the mutant esophagus and especially around the LES region.

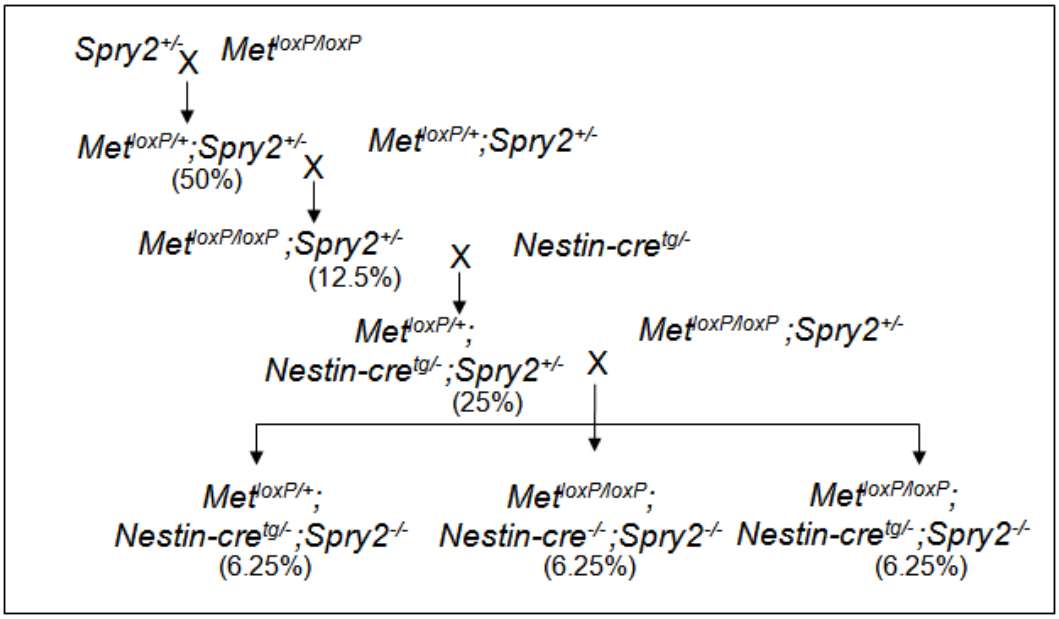

Figure 5. Mating scheme for conditional knockout of Met in the neuronal tissues of Spry2-deficient mice. *Spry2*$^{+/-}$ mice are crossed to *Met*$^{loxP/loxP}$ and *Nestin-cre*$^{tg/-}$ mice to generate *Met*$^{loxP/loxP}$*;Nestin-cre*$^{tg/-}$*;Spry2*$^{-/-}$ mice for determining the role of Met in the development of the *Spry2*$^{-/-}$ esophageal phenotype. Values in parentheses are the expected percentages of offspring with the indicated genotype.

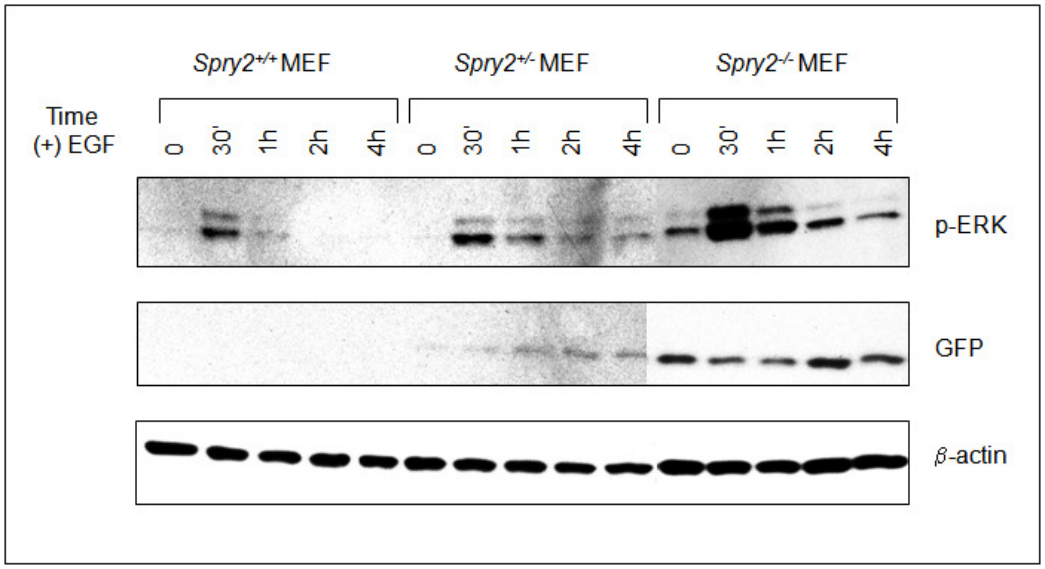

Figure 6. Loss of Spry2 causes ERK/MAPK hyperactivation in response to growth factor stimulation in *Spry2*$^{-/-}$ MEF cells. MEF cell lines were established from prenatal *Spry2*$^{+/+}$, *Spry2*$^{+/-}$ and *Spry2*$^{-/-}$ mouse embryos (day 15). Cells were plated in 6 well dishes, serum starved overnight, and then stimulated with EGF (50 ng/mL) for the indicated times. Whole cell lysates were prepared and subjected to Western Blot analyses. The blots were probed with phospho-p44/42 MAPK (p-ERK), GFP, and β-actin antibodies sequentially.