MINIREVIEW

Intermolecular Interactions of Sprouty Proteins and Their Implications in Development and Disease

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Received March 4, 2009; accepted July 1, 2009

ABSTRACT

Receptor tyrosine kinase (RTK) signaling is spatially and temporally regulated by a number of positive and negative regulatory mechanisms. These regulatory mechanisms control the amplitude and duration of the signals initiated at the cell surface to have a normal or aberrant biological outcome in development and disease, respectively. In the past decade, the Sprouty (Spry) family of proteins has been identified as modulators of RTK signaling in normal development and disease. This review summarizes recent advances concerning the biological activities modulated by Spry family proteins, their interactions with signaling proteins, and their involvement in cardiovascular diseases and cancer. The diversity of mechanisms in the regulation of Spry expression and activity in cell systems emphasizes the crucial role of Spry proteins in development and growth across the animal kingdom.

The Sprouty (Spry) protein was first described by Hacohen et al. (1998) as an inhibitor of fibroblast growth factor (FGF)-stimulated tracheal branching during Drosophila melanogaster development. Subsequent work established D. melanogaster Spry (dSpry) as a widespread inhibitor of receptor-tyrosine kinase (RTK) signaling during organogenesis. For example, spry-null flies or flies harboring loss of function mutations on spry exhibit eye and wing phenotypes indicative of uncontrolled epidermal growth factor receptor (EGFR) signaling (Minowada et al., 1999).

Four mammalian spry genes have been defined based on sequence similarity with dSpry. Three homologs of dSpry were first identified in a search of the human expressed sequence tag database (http://www.ncbi.nlm.nih.gov/dbEST/) (Hacohen et al., 1998). The fourth mammalian spry homolog was originally discovered in mice (de Maximy et al., 1999). Although shorter than dSpry, all of the human homologs of Spry have a C-terminal cysteine-rich domain that is similar to the cognate domain within dSpry (Hacohen et al., 1998). However, similarity in their N termini is limited. The four human Spry proteins are products of different genes located on chromosomes 4q28.1 (spry1), 13q31.1 (spry2), Xq28/Yq12 (spry3), and 5q31.3 (spry4). Expression patterns for mammalian Spry isoforms were shown to be highly localized in embryos, some appearing near centers of FGF signaling (de Maximy et al., 1999; Minowada et al., 1999; Tefft et al., 1999; Chambers and Mason, 2000; Chambers et al., 2000; Zhang et al., 2001). These initial studies provided the first clues that growth factors may regulate the expression of Spry proteins.

Concerning the expression of Spry proteins, Su et al. (2002) performed large-scale gene analysis of both human and mouse Spry1 and Spry2. Their results indicated that Spry1 and Spry2 are expressed in a variety of cell types, including endothelial cells, smooth muscle cells, and fibroblasts, suggesting a role for Spry proteins in the regulation of RTK signaling in these cell types. In addition, Su et al. observed that Spry1 and Spry2 are expressed in the developing heart, suggesting a role for Spry proteins in cardiac development.

This work was supported by the National Institutes of Health National Institute of General Medical Sciences [Grant GM073181]. Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org. doi:10.1124/mol.109.055848.

ABBREVIATIONS: Spry, Sprouty; RTK, receptor tyrosine kinase; FGF, fibroblast growth factor; FGFR, FGF receptor; EGF, epidermal growth factor; EGFR, EGF receptor; VEGF, vascular endothelial growth factor; ERK1/2, extracellular signal-regulated kinases 1/2; Cbl, Casitas b-lineage lymphoma proto-oncogene; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PTP1B, protein tyrosine phosphatase 1B; PTEN, phosphate and tensin homolog; Grb2, growth factor receptor-bound protein 2; MAPK, mitogen-activated protein kinase; Mnk1, MAPK interacting kinase 1; DYRK1A, dual-specificity tyrosine-phosphorylated and -regulated kinase 1A; TESK1, testicular protein kinase 1; PP2A, protein phosphatase 2A; SHP2, SH2-domain containing tyrosine phosphatase 2; TKB, tyrosine kinase binding; SIAH2, seven in absentia homolog 2; CIN85, Cbl interacting protein of 85 kDa; NSCLC, non–small-cell lung cancer; aa, amino acid(s); TKB, tyrosine kinase binding; xPAPC, Xenopus laevis paraxial protocadherin.
mouse samples to create a profile of the normal physiological state of human and mouse transcriptomes (Su et al., 2002). Although Spry2 appears to be ubiquitously expressed, the expression of the other isoforms is more limited to certain organs and tissues. The expression of human and mouse Spry isoforms in fetal tissues, adult tissues, and certain cell lines can be obtained from the following web sites: http://biogps.gnf.org, and http://www.ncbi.nlm.nih.gov/sites/entrez?db=

Like dSpry, mammalian Spry proteins were shown to antagonize FGF-stimulated organogenesis (Minowada et al., 1999; Tefft et al., 1999). Since these initial findings, the number of pathways and biological processes regulated by Spry proteins has continued to expand. To understand the mechanism(s) involved in the biological actions of Spry proteins, significant emphasis has been placed on identifying the proteins that interact with them, the post-translational modifications that may alter the function of Spry proteins, and mechanisms that alter cellular levels of these regulators of RTK signaling. In this review, we discuss some of the more recent discoveries pertaining to Spry interactions with various proteins, how these interactions allow Spry to regulate different signaling pathways and biological processes, as well as how Spry expression is affected by these proteins at both the transcriptional and protein levels. Finally, the role of Spry proteins in several diseases is briefly reviewed.

Regulation of Biological Processes by Spry Proteins

Morphogenesis, Organogenesis, and Development. Tubular networks, which comprise the vascular system, lungs, and kidneys, require tight control to develop and maintain a hierarchical structure (Horowitz and Simons, 2008). Similar to the findings in D. melanogaster, mammalian Spry proteins have also been shown to play a critical role in tubular morphogenesis associated with tracheal/lung development, uteretic budding, and angiogenesis. The function of individual Spry proteins in these processes are elegantly reviewed elsewhere (Cabrita and Christofori, 2008; Horowitz and Simons, 2008; Warburton et al., 2008). FGF signaling, in particular, is crucial to initiating/regulating tubular morphogenesis and is known to up-regulate Spry expression in D. melanogaster (Hacothen et al., 1998), mice, chicks (Minowada et al., 1999), and zebrafish (Fürthauer et al., 2001). In addition, a recent report of FGF signaling in antherozoan cnidarians (Nematostella vectensis) revealed the expression of three FGF ligands, two FGF receptors, and two orthologs of vertebrate spry genes, highlighting the importance of the conservation of FGF/antagonist signaling loops among species (Matus et al., 2007). When an intraspecies comparative genomic analysis of the human spry genes was performed, investigators were able to show the linkage of spry4 and spry1 genes to the fgf1 and fgf2 genes, respectively (Katoh and Katoh, 2006). Except for the nematodes (which, interestingly, contain no spry genes), a conservation of function for FGF signaling implies a crucial role for Spry in development and growth across the animal kingdom.

Besides the role of Spry proteins in tubular morphogenesis (Hacothen et al., 1998), limb development (Minowada et al., 1999), patterning of the midbrain, and anterior hindbrain (Lin et al., 2005), recent reports have provided additional evidence for Spry protein involvement in craniofacial and trunk development. Because the functions of Spry proteins in embryonic development have been reviewed by others (Cabrita and Christofori, 2008; Horowitz and Simons, 2008; Warburton et al., 2008), we have focused mainly on the role of Spry proteins in craniofacial features. As early as 2001, a hint of Spry’s role in maintaining epithelial-mesenchymal interactions for craniofacial and trunk development in vertebrates became apparent after examining the expression profiles of Spry1, -2, and -4 during mouse embryogenesis (Zhang et al., 2001). Although spry4 knockout mice exhibited growth retardation and sustained FGF-mediated extracellular signal regulated kinase (ERK) activation (Taniguchi et al., 2007), mice deficient in spry2 exhibited clefting of the palate, excessive cell proliferation, and aberrant expression of downstream target genes of FGF receptor signaling (Welsh et al., 2007). Moreover, Spry2-BAC transgenic mice were able to rescue palate defects of mice with a deletion of spry2 in a dosage-dependent manner (Welsh et al., 2007). On the other hand, overexpression of Spry2 did not disrupt FGF signaling during facial development of avian embryos, and craniofacial defects such as cleft palate were still observed, suggesting that overexpression of Spry2 may mimic the actions of Spry deficiency (Goodnough et al., 2007). A role for Spry2 in facial development is also suggested by a report identifying cleft palate candidate genes in which D20A and K68N point mutations in Spry2 were revealed (Vieira et al., 2005). So far, however, no studies suggest that the D20A or K68N substitutions in Spry2 alter its ability to regulate growth factor signaling. It is noteworthy that spry2/4 double-knockout mice were embryonic lethal with severe craniofacial, limb, and lung abnormalities (Taniguchi et al., 2007), suggesting that Spry2 and Spry4 may each compensate to some extent for the other’s functions.

The pleiotropic effects of Spry proteins in mouse development also include a role for Spry2 during inner ear development (Shim et al., 2005), lens morphogenesis (Spry1 and -2) (Boros et al., 2006), tooth elongation (Spry4 together with Spry1 or -2) (Klein et al., 2008), and tooth development (for review, see Tummers and Thesleff, 2009). In the case of inner ear development, both Spry2 and the FGF receptor 3 (FGFR3) are required for normal hearing in the mouse (Shim et al., 2005). spry2-null mice exhibit impaired hearing with an increase in the numbers of outer hair cells and supporting cells in the organ of Corti. Whereas decreasing fgf8 gene dosage was able to rescue hearing in these mice, decreasing fgf8 gene dosage in the spry2(--/-) mice did not alter the extra numbers of sensory and nonsensory cells (Shim et al., 2005). Although no definitive studies of Spry protein participation in lens development have been performed, expression profiles of Spry2 in the developing lens suggests a role for Spry proteins in this process (Boros et al., 2006). Other developmental processes involving Spry proteins, not reviewed in this section, are delineated in Table 1.

Cellular Proliferation and Migration. Because of their ability to regulate RTK signaling, the role of Spry proteins in regulating cellular proliferation and migration has been the focus of several studies. A number of papers were published in 2001 concerning Spry’s effect on cell proliferation and migration. Yigzaw et al. (2001) showed that Spry2 inhibited the proliferation and migration of HeLa cells in response to a number of growth factors including EGF, FGF, VEGF, and platelet-derived growth factor. This study also showed that the microtubule association and membrane translocation domains described by Lim et al. (2000) are necessary for the antiproliferative and antimigratory actions of Spry2. This
Recent reports of Spry role in organogenesis and development are listed, including relevant growth factor, ligand, or signaling pathway. Additional information describing the Table 1: Role of Sprouty proteins in organogenesis and development

<table>
<thead>
<tr>
<th>Biological Event</th>
<th>Experimental System</th>
<th>Sprouty Isoform(s)</th>
<th>Growth Factor/Ligand</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placental vili sprouting</td>
<td>Placenta</td>
<td>hSpry1, -2, -3</td>
<td>FGF4, FGF10</td>
<td>Antebay et al., 2005</td>
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<tr>
<td>Placental vili sprouting</td>
<td>Placenta</td>
<td>hSpry1, -2, -3</td>
<td>FGF10</td>
<td>Natanzon-Yaron et al., 2007</td>
</tr>
<tr>
<td>Kidney development</td>
<td>Embryonic kidney explants</td>
<td>mSpry1</td>
<td>Gdnf/Ret/Wnt11</td>
<td>Gross et al., 2003</td>
</tr>
<tr>
<td>Ureteric branching</td>
<td>spry-null mice</td>
<td>mSpry1</td>
<td>Wnt11/FGF/7 Gdnf</td>
<td>Bausson et al., 2005, 2006</td>
</tr>
<tr>
<td>Kidney development</td>
<td>Human renal mesenchymal cells</td>
<td>mSpry2</td>
<td>LIF/FGF2/TGFα</td>
<td>Chi et al., 2004</td>
</tr>
<tr>
<td>Male sex organogenesis</td>
<td>Testes</td>
<td>mSpry2</td>
<td>FGF9</td>
<td>Price et al., 2007</td>
</tr>
<tr>
<td>Neuronal differentiation</td>
<td>Immature neurons</td>
<td>mSpry2</td>
<td>BDNF</td>
<td>Chi et al., 2006</td>
</tr>
<tr>
<td>Muscle regeneration</td>
<td>Soleus muscle</td>
<td>hSpry1, -2, -4</td>
<td>FGFl</td>
<td>Laxiz et al., 2007</td>
</tr>
<tr>
<td>Pancreas development</td>
<td>Pancreatic beta cells</td>
<td>mSpry4</td>
<td></td>
<td>Jaggi et al., 2008</td>
</tr>
</tbody>
</table>

LIF, leukemia inhibitory factor; BDNF, brain-derived neurotrophic factor.
tions between Spry proteins and the adapter proteins that bind activated RTKs. Further rationale to pursue such investigations was provided by studies in D. melanogaster S2 cells that demonstrated that Spry acts downstream of FGF receptor and either at or above Ras and Raf1 (Casci et al., 1999). D. melanogaster Spry was found to interact with Drk, an SH2-SH3 domain containing adaptor protein homologous to mammalian Grb2 and Gap1, a Ras GTPase-activating protein.

Fig. 1. Schematic representation of the proteins that interact with human Spry2. The functional consequences of each interaction are represented as colored rectangular boxes and indicated by solid arrows. Some of the known phosphorylation sites (Tyr55, Ser1112/Ser121, Thr75) are shown, and the kinases or phosphatases that modify these sites are provided in the key box. The adaptor protein CIN85 binds to more than one PXXXPR motif on Spry2. The interaction sites of proteins such as Hrs and SHP2 are not clear; therefore, these proteins are not placed next to Spry2. Proteins that are known to interact with the N-terminal or C-terminal regions of Spry2 without knowledge of the precise sites are located on the brackets that demark the N and C termini. Proteins such as PTP1B and PTEN have been shown to be involved in Spry2 function. However, their physical interaction with Spry2 has not been observed. MTD, membrane translocation domain.

TABLE 2
Effect of overexpressed Sprouty Isoforms on ERK1/2 activation by different growth factors

<table>
<thead>
<tr>
<th>Sprouty Isoform</th>
<th>EGF</th>
<th>FGF</th>
<th>PDGF</th>
<th>VEGF</th>
</tr>
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<tbody>
<tr>
<td>Sprouty1</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>?</td>
</tr>
<tr>
<td>Sprouty2</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>▼</td>
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<tr>
<td>Sprouty3</td>
<td>?</td>
<td>↑</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Sprouty4</td>
<td>?</td>
<td>↑</td>
<td>?</td>
<td>▼</td>
</tr>
</tbody>
</table>

EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

PDGF, platelet-derived growth factor; ↑, overexpression of sprouty-activated ERK activation; ↓, overexpression of sprouty-inhibited ERK activation; *, overexpression of sprouty did not affect ERK activation; ?, no current reports.
(Casci et al., 1999). Because Drk (Grb2) and Gap1 are important components of RTK signaling pathways, Spry, by binding these proteins, may negatively affect Ras activation. Further support for this hypothesis was provided in mammalian system by studies showing that Spry1, Spry2, and Spry4 inhibit the Ras/Raf/MAPK pathway by preventing Ras activation (Gross et al., 2001; Leekama et al., 2002). Although two earlier studies suggested that Spry2 and Spry4 bind Raf1 (Tefft et al., 2002; Sasaki et al., 2003), these findings have not been consistently reproduced by others (Gross et al., 2001); therefore, the role of Raf1 association with Spry proteins in regulation of downstream activation of Erk1/2 is debatable. On the other hand, among proteins upstream of Ras, the interactions of Spry2 with Grb2 have been more consistently observed, and these interactions are enhanced by growth factors that augment the tyrosine phosphorylation of Spry proteins (Gross et al., 2001; Tefft et al., 2002; Lao et al., 2006; Martinez et al., 2007). Because one of the critical tyrosine residues phosphorylated on Spry proteins is the residue corresponding to Tyr55 on Spry2, studies have investigated the role of this tyrosine phosphorylation in the interactions between Spry2 and Grb2. Using the Y55F mutant of Spry2 and a tyrosine phosphorylated octapeptide corresponding to the residues around Tyr55 on Spry2, one study concluded that phospho-Tyr55 and surrounding residues form a site for the SH2 domain of Grb2 (Hanafusa et al., 2002). This study also suggested that Spry1, which is phosphorylated on a cognate site (Tyr53), and also has surrounding residues that are similar to those around Tyr55 on Spry2, binds Grb2. However, direct binding of SH2 domain of Grb2 to the residues surrounding Tyr55 on Spry2 or Tyr53 on Spry1 was not shown in this study. Moreover, others have not observed an interaction between Spry1 and Grb2 (Gross et al., 2001; Lao et al., 2006). Thus, the residues surrounding Tyr55 seem not to be the Grb2 binding site. More likely, as suggested by Lao et al. (2006), growth factor-mediated phosphorylation of Tyr55 on Spry2 alters its conformation to reveal a cryptic PXPPXR motif (aa 304–309) in the extreme C terminus of Spry2 that serves to bind the SH3 domain of Grb2. Spry2 binding to Grb2 sequesters Grb2 away from Sos, thereby inhibiting the Erk1/2 pathway (Lao et al., 2006). It is noteworthy that the PXPPXR motif in the C terminus of Spry2 is not present in the other Spry isoforms, and therefore may explain why Spry2 is more effective at inhibiting Erk1/2 activation by FGF (Aranda et al., 2008). This interaction and Thr75 phosphorylation of Spry2 by DYRK1A seem to negatively affect the function of Spry2 as an antagonist of RTK signaling, because substitution of Thr75 with Ala increased the ability of Spry2 to inhibit Erk1/2 activation by FGF (Aranda et al., 2008). However, the role of Thr75 phosphorylation has to be further substantiated by other approaches including silencing or knockout studies of DYRK1A. Likewise, although the phosphorylation of Tyr227 by FGF, but not by EGF, has been shown to interact with and phosphorylate Spry2 on Thr75 (Aranda et al., 2008). This interaction and Thr75 phosphorylation of Spry2 by DYRK1A seem to negatively affect the function of Spry2 as an antagonist of RTK signaling, because substitution of Thr75 with Ala increased the ability of Spry2 to inhibit Erk1/2 activation by FGF (Aranda et al., 2008). However, the role of Thr75 phosphorylation has to be further substantiated by other approaches including silencing or knockout studies of DYRK1A. Likewise, although the phosphorylation of Tyr227 by FGF, but not by EGF, has been shown to augment the ability of Spry2 to inhibit the FGF-activated Erk1/2 cascade (Rubin et al., 2005b), this sole report needs to be explored further to determine the mechanisms, including structural changes, by which phosphorylation of Tyr227 may augment the inhibitory actions of Spry2.

In addition to being regulated by kinases, Spry interactions with kinases may also regulate their functional activity. In this respect, testicular protein kinase 1 (TESK1) was identified from a human fetal liver cDNA library as a binding partner of hSpry4 (Leekama et al., 2002). TESK1 is a serine/threonine kinase that phosphorylates coflin and plays a critical role in integrin-mediated actin cytoskeletal reorganization and cell spreading. The Spry4-TESK1 interaction increases upon growth factor stimulation, and the two proteins...
colocalize in apparent cytoplasmic vesicles (Leeksmas et al., 2002). Spry4, by binding through the C-terminal cysteine-rich region, inhibits the kinase activity of TESK1 and suppresses integrin- and TESK1-mediated collagen phosphorylation during the spreading of cells on laminin (Tsumura et al., 2005). More recently, TESK1 has been shown to interact with Spry2 and localize the protein to vesicular compartments including endosomes (Chandramouli et al., 2008). TESK1, independent of its kinase activity, attenuates the ability of Spry2 to inhibit growth factor actions, primarily by interfering with Spry2/Grb2 interactions and dephosphorylation of Ser residues by PP2A (Chandramouli et al., 2008).

Using electron-capture dissociation mass spectrometry, a recent study has identified 15 Ser/Thr/Tyr phosphorylation sites on Spry2 (Sweet et al., 2008). Besides Tyr55, three of these Ser/Thr phosphorylation sites were previously identified as phosphorylation sites (DaSilva et al., 2006; Lao et al., 2007). However, the other 11 sites have not previously been documented as phosphorylation sites on Spry2. It is noteworthy that some of the sites on Spry2, including Thr75, Tyr227, Ser112, and Ser121 discussed above in this section were not documented as phosphorylation sites on Spry2. It is noteworthy that the N-terminal region (aa 50–60) around Tyr55 is also the region on Spry2 that binds c-Cbl binding site discussed under Ubiquitin Ligases (cCbl and SIAH2). It is noteworthy that the N-terminal region (aa 50–60) around Tyr55 is also the region on Spry2 that binding site discussed under Ubiquitin Ligases (cCbl and SIAH2).

As suggested for PP2A, in forced-expression studies, the Src homology-2 containing phosphotyrosine phosphatase (SHP2) has also been implicated in regulating the biological activity of Spry proteins. Hanafusa et al. (2004) showed that constitutively active SHP2 resulted in dephosphorylation of Spry1 and Spry2 on Tyr53 and Tyr55, respectively. Decreased phosphorylation of Tyr55 was suggested to decrease binding of Grb2 and, therefore, to decrease the inhibitory actions of Spry2 on receptor tyrosine kinase signaling. Although one study has shown that the association between SHP2 and Spry1 is enhanced by activated growth factor receptors (Jarvis et al., 2006), a previous report showed that Spry2 association with SHP2 was decreased by FGF activation (Tefft et al., 2002). The reports suggesting that dephosphorylation of Spry1 on Tyr53 and Spry2 on Tyr55 decrease their functional activity and relieve the inhibitory effect on RTK signaling (Hanafusa et al., 2004; Jarvis et al., 2006) may provide a tenable mechanism for SHP2-mediated augmentation of growth factor signaling (Noguchi et al., 1994; Xiao et al., 1994; Tang et al., 1995; Yamauchi et al., 1995; Bennett et al., 1996; Deb et al., 1998; Wu et al., 2006). However, the discrepancies between whether growth factor activation increases or decreases interactions between SHP2 and Spry proteins (Tefft et al., 2002, Jarvis et al., 2006), as well as the lack of any data concerning the role of endogenous SHP2 in regulating the ability of Spry proteins to modulate growth factor signaling, call into question the physiological significance of SHP2 in modulating the function of Spry proteins. Moreover, as discussed by Chan et al. (2008), spry1- and spry2-null mice do not show the phenotype of the active SHP2 transgenic mice.

It is noteworthy that by sequestering c-Cbl, Spry proteins can also augment RTK signaling (Egan et al., 2002; Wong et al., 2002; Fong et al., 2003; Guy et al., 2003; Hall et al., 2003; Rubin et al., 2003; Mason et al., 2004; Haglund et al., 2005). Because Tyr55 on Spry2 has to be phosphorylated to bind c-Cbl, SHP2, by dephosphorylating this tyrosine, would be expected to decrease the ability of Spry2 to sequester c-Cbl. This would then diminish the protection of RTKs from downregulation by c-Cbl. This scenario predicts that by decreasing Spry2 function, SHP2 would also diminish cell surface growth factor receptors and signaling capacity if these receptors were c-Cbl substrates. However, to our knowledge, no
experimental evidence supports such a scenario, perhaps because SHP2/Spry2 interactions may be transitory and may not affect long-term trafficking of the EGFR.

The interactions of tumor suppressor phosphatase and tensin homolog (PTEN), another phosphatase, with Spry proteins have been difficult to show, but, like PTP1B, PTEN activity is necessary for the biological function of Spry proteins. In HeLa cells, overexpression of Spry2 increases the total amount of cellular PTEN (Edwin et al., 2006). Overexpression of Spry2 also decreased the amount of PTEN that is phosphorylated (Edwin et al., 2006). Because dephospho-PTEN is more enzymatically active than phospho-PTEN, Spry2 expression increases PTEN activity by both increasing its amount as well as decreasing its phosphorylation. The resultant increase in PTEN activity decreases activation of AKT and downstream signaling (Edwin et al., 2006). Most importantly, by silencing PTEN or by performing experiments in PTEN-null mouse embryonic fibroblasts, PTEN was found to be necessary for Spry2-mediated inhibition of cell proliferation (Edwin et al., 2006).

**Ubiquitin Ligases (c-Cbl and SIAH2).** A prominent binding partner of Spry is c-Cbl. Cbl proteins are ubiquitin ligases, which, through mono- and polyubiquitination of the RTKs, initiate their endocytosis and/or lysosomal/proteosomal degradation (Levkowitz et al., 1998; Miyake et al., 1998). c-Cbl and b-Cbl interact through their SH2-like tyrosine kinase binding (TKB) domain with the N-terminal region of Spry proteins that encompass the critical tyrosine residue equivalent to Tyr55 on Spry2 (Wong et al., 2001; Fong et al., 2003; Rubin et al., 2003). This interaction requires phosphorylation of the tyrosine equivalent to Tyr55 on Spry2 (Fong et al., 2003; Hall et al., 2003; Mason et al., 2004).

In a detailed structural study using Cbl TKB domain and peptides of Cbl substrates, Ng et al. (2008) showed that the region encompassing Tyr55 on Spry2 has the highest affinity for Cbl TKB. The cognate region on Spry4 binds weakly to Cbl TKB, perhaps because of the lack of a conserved Thr residue adjacent to the conserved tyrosine residue in Spry4 that is phosphorylated (Ng et al., 2008).

Because of its high affinity for Cbl TKB, Spry2 effectively sequesters Cbl and protects receptors such as the EGFR from ubiquitination, endocytosis, and degradation. Stabilized cell surface EGFR are then able to sustain EGF-induced Erk signaling that culminates in the differentiation of PC12 cells (Wong et al., 2001; Egan et al., 2002; Rubin et al., 2003). The competitive interplay between Cbl, Spry, and EGFR seems to occur in the endosomal compartments, and it regulates the amplitude and longevity of intracellular signals. A more direct study on this aspect showed that hSpry2 interferes with the trafficking of activated EGFR specifically at the step of progression from early to late endosomes (Kim et al., 2007). This effect seems to be mediated by the binding of Spry2 to the endocytic regulatory protein hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) (Kim et al., 2007). Thus, Spry proteins can augment RTK signaling and are therefore referred to as modulators of RTKs rather than inhibitors (for reviews, see Guy et al., 2003; Li et al., 2003; Kim and Bar-Sagi, 2004; Rubin et al., 2005a; Cabrita and Christofori, 2008). By binding Cbl and protecting growth factor receptors from down-regulation, Spry2 also enhances the antiapoptotic actions of serum (Edwin and Patel, 2008) as well as Erk signaling by the EGF receptor (Wong et al., 2001; Egan et al., 2002; Rubin et al., 2003). One of the caveats with these studies is that they either used forced expression of Spry2 or were performed with high concentrations (>100 ng/ml) of EGF. As described by Sigismund et al. (2005), at low physiological concentrations of EGF, the EGFR is not ubiquitinated and is internalized via a clathrin-mediated pathway, whereas at high EGF concentrations, the EGFR undergoes an ubiquitination and lipid raft-mediated internalization process. At present, whether Spry2 alters the EGFR internalization and degradation at low physiological concentrations of EGF remains unknown.

Despite the presence of Tyr residue equivalent to Tyr55 in Spry2, Spry4 does not interfere with down-regulation of the EGFR (Wong et al., 2002). This is perhaps related to the fact that the Spry4 does not contain the Thr residue immediately next to Tyr55 that is important for Cbl binding. Although Spry4 binds the c-Cbl TKB domain with moderate affinity in vitro, its binding to c-Cbl in intact cells is limited because Spry4 is poorly phosphorylated (Ng et al., 2008). Moreover, in the case of Spry2, additional adaptor molecules may be involved to modulate receptor down-regulation. For instance, Cbl recruits endocytic complexes, including the Cbl-interacting protein of 85 kDa (CIN85/endophilins), to activated RTKs to facilitate endocytosis (Petrelli et al., 2002; Souberyan et al., 2002; Szymkiewicz et al., 2002). Haglund et al. (2005) identified CIN85 as a new interacting partner of Spry2 that plays an important role in Cbl-mediated down-regulation of growth factor receptors and EGF-induced differentiation of PC12 cells. The CIN85 SH3 domains A and C bind specifically to Pro/Arg-rich motifs present in the N- (aa 59–64, PTVPVR) and C-terminal (aa 304–309, PTVPVR) regions of Spry2, respectively (Haglund et al., 2005); these regions are not present in other Spry isoforms. In addition, the SH3C domain of CIN85 may bind the Pro-rich region encompassed by amino acids 67 to 72 on Spry2 (Haglund et al., 2005). Substitution of Arg309 and Ala markedly decreased the association of Spry2 with CIN85 SH3A domain, whereas R64A substitution modestly decreased interaction with SH3A domain. On the other hand, when Arg309 and Arg64 were simultaneously substituted, the association between CIN85 SH3A domain and Spry2 was abolished, whereas this double mutant did not affect the binding to CIN85-SH3C domain, suggesting a multivalent mode of binding between these proteins (Haglund et al., 2005). Moreover, substitution of R309A on CIN85 abrogated the effects of Spry2 on EGFR down-regulation (Haglund et al., 2005). Thus, in addition to the interaction with Cbl, Spry2/CIN85 association seems necessary to down-regulate the EGFR. The importance of the C-terminal CIN85 binding region on Spry2 in facilitating the antiapoptotic actions of serum was also shown by the findings that wild-type Spry2, but not its mutant with disrupted CIN85 binding site (aa 304–309), could rescue the functions of Spry2 when the endogenous Spry2 was silenced (Edwin and Patel, 2008).

Besides binding Spry, C-Cbl ubiquitinates Spry in a growth factor-dependent manner (Hall et al., 2003; Rubin et al., 2003). The c-Cbl-mediated ubiquitination of Spry2 targets the protein for degradation by the 26S proteasome (Hall et al., 2003; Rubin et al., 2003). This process may also be regulated by the phosphorylation of other residues on Spry2. Hence, phosphorylation of Ser112 and Ser121 on Spry2 may decrease the phosphorylation of Tyr55 and, therefore, bind-
Phospholipase C and Protein Kinase C. A formal association of Spry proteins with either phospholipase C or protein kinase C has not been demonstrated. However, Spry4 overexpression decreases VEGF-A-mediated hydrolysis of PtdIns(4,5)P_2, thereby decreasing the formation of inositol,1,4,5-trisphosphate and diacylglycerol with a resultant decrease in elevation of intracellular Ca^{2+} concentrations and activation of protein kinase C. This action of Spry4 probably results from binding of PtdIns(4,5)P_2 to the C terminus of Spry4, thereby limiting or altering substrate availability to PLC (Ayada et al., 2009). As with all overexpression studies, the physiological significance of these findings remain to be determined.

Paraxial Protocadherin. The intracellular cytosolic domain of Xenopus laevis paraxial protocadherin (xPAPC) has been shown to interact with Spry1 and -2 (Wang et al., 2008). This interaction negatively affects the inhibitory function of Spry, consequently enhancing the planar cell polarity signaling and thus gastrulation movements in X. laevis. Interestingly, RhoA activation, as well as the membrane localization of Dishevelled and protein kinase C_6, were inhibited upon overexpression of xSpry1 in X. laevis; however, expression of xPAPC rescued both RhoA activation and the recruitment of Dishevelled and protein kinase C_6 to the membrane. The antagonistic properties of xPAPC and xSpry1 on the planar cell polarity signaling pathway suggests that sprouty/xPAPC interactions may play a role in β-catenin independent Wnt-signaling (Wang et al., 2008).

Hetero-Oligomers of Spry Proteins. In recent years, it has become evident that the different Spry isoforms can interact with each other to form homo- and heterodimers or -oligomers (Ozaki et al., 2005). This interaction among the different isoforms seems to occur via their Cys-rich C-terminal regions. Because the Spry isoforms interact with different signaling proteins and in some cases with the same protein but with various affinities, the finding that Spry proteins form hetero-oligomers has the following significance. First, when studying the interactions of a given isoform of Spry with another protein, it is possible that the interacting protein is associated with a Spry isoform other than the one being investigated. Second, by binding different proteins in a pathway, two Spry isoforms may have a greater effect than a single isoform by itself. Indeed, Ozaki et al. (2005) showed that the coexpression of Spry1 and Spry4, which interact with Grb2 and SOS, respectively, inhibited FGF2-elicited activation of Erk1/2 to a greater extent compared with each individual isoform; similar results were also obtained when endogenous Spry isoforms in Swiss 3T3 cells were silenced (Ozaki et al., 2005). A third implication of the hetero-oligomerization of Spry proteins is that the differential expression of the four isoforms in various cell types or tissues may modulate a given pathway to different extents. This could be biologically important in fine-tuning the actions of Spry proteins during development of different organs. Perhaps the different phenotypes observed with spry2 or spry4 single knockouts versus double knockout of these genes (reviewed above under Morphogenesis, Organogenesis, and Development and in Mason et al., 2006; Cabrita and Christofori, 2008) may be due to oligomerization of these Spry isoforms.

Regulation of Spry Proteins. As modulators of RTK signaling, the cellular content of Spry proteins would be expected to regulate the extent of their actions. As with all proteins, the cellular content of the Spry proteins is determined by the balance between its degradation and synthesis. Concerning degradation, the regulation of cellular amounts of Spry proteins by ubiquitination-mediated proteosomal degradation has been most extensively studied for Spry2 and, so far, involves the ubiquitin ligases c-Cbl and Siah2. The regulation of Spry proteins by transcriptional control is reviewed in the following section.

Transcriptional Regulation. Transcriptional regulation of Spry in response to growth factor stimulation was one of the first mechanisms revealed to alter sprouty expression (Hacohen et al., 1998; Minowada et al., 1999). In D. melanogaster, Spry’s ability to act as a negative feedback inhibitor is due in part to its up-regulation in response to growth factor stimulation (Hacohen et al., 1998). This phenomenon was also observed in vertebrates (discussed in the Introduction) with studies by Minowada et al. (1999) showing the regulation of Spry expression in both mice and chicks through FGF signaling in various tissues. Besides growth factors, Ozaki et al. (2001) found that activation of protein kinase C by phorbol 12-myristate-13-acetate and active Raf kinase were also able to induce spry gene expression. Although MAPK signaling has been shown to effectively stimulate Spry induction by several mechanisms, Abe and Naski (2004) showed that Spry expression could be regulated by other pathways, such as through calcium-dependent and PLCγ signals.

Several transcription factor-binding sites have been identified in the spry2 (Ding et al., 2003) and spry4 promoters (Ding et al., 2004); however, little work has centered on identifying regulatory sequences in the promoters of spry genes. Although DNA methylation was shown to down-regulate Spry4 (Wang et al., 2006) and Spry2 in prostate cancer (McKie et al., 2005), no such preferred methylation status was identified in the Spry2 promoter of breast cancer samples to account for the reduced Spry2 levels observed in both a cancer profiling array study and reverse transcription-polymerase chain reaction study of breast cancer samples (Lo et al., 2004). Likewise, although Spry2 levels are decreased in prostate cancer, no hypermethylation of the Spry2 promoter was observed (Fritzsche et al., 2006). Is it possible that
other sites in the promoter of Spry genes participate in their regulation in normal cell development and disease progression? Recent papers suggest that this is possible. For example, a report by Lagha et al. (2008) revealed that Pax3, a transcription factor crucial for myogenesis and progenitor cell survival (Buckingham and Relaix, 2007), may target Spry1 in progenitor cells. In their study, transcripts corresponding to Spry1 were detected in Pax3-positive progenitor cells within the dermomyotome and myotome. Pax3(−−−) somite cells, however, displayed reduced Spry1 and increased phosphorylation of Erk, indicating the loss of Spry1 function (Lagha et al., 2008). In addition, another study (Katoh and Katoh, 2006) identified two Wnt-mediated-TCF/LEF-β-catenin sites in the 5′ promoter of Spry4 in the human, chimpanzee, rat, and mouse sequences. This is interesting because non–small-cell lung cancer (NSCLC) samples exhibit decreased Wnt-7a expression (Winn et al., 2005). In fact, overexpression of Wnt-7a reverses cell transformation, decreases anchorage-independent growth, and, in conjunction with Fzd-9 (Wnt receptor), induces Spry4 expression (Winn et al., 2005). This suggests that the tumor suppressor function of Wnt7a/Fzd-9 signaling in NSCLC may be reduced as a result of low levels of Wnt-7a mRNA (Winn et al., 2005), which in turn may affect Spry expression and function.

Spry Proteins in Pathological States

Cancer. Evidence that the expression of Spry proteins is dysregulated in a number of cancers has been accumulating over the past few years (Table 3). These reports have suggested the potential of using cellular Spry content as tumor markers. A recent review (Lo et al., 2006) has comprehensively described past progress in this area; therefore, the discussion here is limited to the most recent findings pertinent to Spry proteins in cancer biology.

Consistent with a role for Spry2 in inhibiting both Erk1/2 signaling as well as proliferation, Spry2 amounts are decreased in hepatocellular carcinomas (Fong et al., 2006) and lung carcinomas (Sutterlüty et al., 2007). In mice, inhibition of the biological activity of Spry2 by hydrodynamic injection of Spry2 Y55F accelerated activated β-catenin-induced Erk1/2 activation and neoplastic phenotype in the liver (Lee et al., 2008), suggesting that loss of Spry2 function contributes to hepatic tumorigenesis. Likewise, in patients with NSCLC, lowered Spry2 expression was reported, and ectopically expressed Spry2 not only significantly reduced the proliferation of cell lines derived from these tumors but also blocked tumor formation from these cell lines in mice (Sutterlüty et al., 2007). In this study, Spry2 inhibited proliferation of mutant K-Ras-transformed NSCLC cells without inhibiting Erk1/2 activation, suggesting that Spry2 inhibits tumor formation by modulating other pathways (Sutterlüty et al., 2007). Further support for a role of Spry2 in modulating lung tumors is derived from the observations that specific overexpression of Spry2 in mouse lung epithelium significantly inhibited urethane-induced lung tumorigenesis (Minowada and Miller, 2009). Fewer tumors with a smaller diameter were observed in Spry2-overexpressing mice exposed to urethane (Minowada and Miller, 2009). The tumor-suppressing effect of Spry2 is apparently exerted at a level below K-Ras mutation, because Spry2 overexpression did not alter the frequency of occurrence of K-Ras mutations in urethane-induced tumors (Minowada and Miller, 2009). Through an as-yet-unknown mechanism, the loss of Spry2 function increases the number and size of lung tumors of mice harboring the K-RasG12D germline mutation (Shaw et al., 2007).

As mentioned under Ubiquitin Ligases (cCbl and SIAH2), by sequestering c-Cbl, Spry2 can protect certain RTKs such as the EGFR from down-regulation and degradation and sustain their signaling. In theory, this mode of regulation should have a positive effect on tumor formation. Indeed, in H-Ras(V12)-transformed fibroblasts, the expression of EGFR receptors and Spry2 was found to be elevated (Lito et al., 2008). The EGFR receptor was necessary for oncogenic H-Ras-transformed cells to grow in the absence of growth factors and form large anchorage independent colonies (Lito et al., 2008). Silencing of Spry2 in these H-Ras(V12)-transformed fibroblasts markedly attenuated their ability to form large colonies in agarose or form tumors in athymic mice (Lito et al., 2008). The effects of Spry2 were found to be specific for oncogenic Ras transformed cells because in control, nononcogenic H-Ras-transformed cells, Spry2 inhibited H-Ras and Erk1/2 activation (Lito et al., 2008). These data suggest that in oncogenic H-Ras-transformed fibroblasts, Spry2 may act as a tumor promoter by protecting EGFR receptor down-regulation, whereas in control fibroblasts, it acts upstream of Ras to inhibit the Erk1/2 cascade. Another situation in which up-regulation of Spry2 has been observed is in melanoma cell

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<td>Down-regulated spry levels</td>
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<td>Hepatocellular carcinoma</td>
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<td>Melanoma (B-Raf and N-Ras mutated cell lines)</td>
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<td>TDII thanatophoric dysplasia II</td>
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lines with mutant B-Raf and N-Ras (Bloethner et al., 2005). However, there were several other genes also up-regulated in these cells; whether Spry2 acts as a tumor promoter in these cells is not entirely clear. Nevertheless, given our understanding of how Spry proteins both positively and negatively regulate signaling and biological actions of growth factor receptors, the cellular context and repertoire of changes in signaling elements in a given type of cancer may determine whether Spry proteins may act as tumor suppressors or tumor promoters.

**Cardiovascular Diseases.** Evidence linking Spry proteins to the regulation of cardiovascular diseases was derived from the studies of Zhang et al. (2005), which showed that Spry2 by inhibiting vascular smooth muscle cell proliferation and migration also diminished neointimal hyperplasia after balloon angioplasty of rat carotid artery. This study also showed that the expression of Spry2 in the carotid artery was markedly decreased immediately after balloon angioplasty and was then elevated 1 to 2 weeks after injury. Moreover, Huebert et al. (2004) showed that human heart samples obtained from patients with left ventricular assist device implants had increased levels of Spry1 mRNA and protein levels. Increased Spry1 levels were shown in the microvasculature and appeared to be responsible for a decrease in VEGF-induced proliferation of the endothelial cells (Huebert et al., 2004), confirming the findings that Spry proteins inhibit the proliferation of cultured endothelial cells (Impagnatiello et al., 2001). However, the mechanism(s) involved in the up-regulation of Spry proteins by left ventricular assist device remain unidentified.

More recently, studies have linked the expression of Spry proteins to changes in microRNAs that are up-regulated during cardiac hypertrophy and heart failure. MicroRNAs, which are non–protein-coding RNAs, participate in gene regulation in mammalian cells by repressing protein translation (for review, see Cannell et al., 2008). MicroRNA21 (miR21) is of particular interest because it is one of the microRNAs commonly up-regulated in different forms of cancer (Volinia et al., 2006; Meng et al., 2007) and in cardiac hypertrophy (Tatsuguchi et al., 2007; Sayed et al., 2008). In effect, Spry2 was identified as a potential target of miR21 in cardiac myocytes and colon cancer (SW 480) cells (Sayed et al., 2008). Overexpression of miR21 repressed Spry2 expression and induced the formation of branch-like outgrowths in cardiac myocytes that formed gap junctions between cells (Sayed et al., 2008). These morphological changes in cultured cardiac myocytes may represent the increased connectivity and conductance velocity observed in early stages of hypertrophy (Cooklin et al., 1998). A similar morphological change was also observed in cardiac myocytes after silencing of Spry2. Furthermore, β-adrenergic receptor activation, by increasing miR21 expression and decreasing Spry levels, also led to the branching of cardiomyocytes to form intracellular gap junctions (Sayed et al., 2008). This effect of β-adrenergic receptor activation was abrogated by the expression of "eraser miR21" that suppressed miR21 levels and resulted in increased expression of Spry2 (Sayed et al., 2008). Likewise, in colon cancer (SW480) cells, suppression of miR21 amounts increased cellular Spry2 content and inhibited cell migration. Given these findings, it is tempting to speculate that the down-regulation of Spry proteins in certain cancers (reviewed above under Cancer and in Table 3) may be secondary to the overexpression of miR21.

Another recent report has also shown that miR21 levels are increased in fibroblasts derived from failing hearts (Thum et al., 2008). In this study, miR21 levels were not altered in the cardiomyocytes of failing hearts. Although at first sight this seems to be at odds with the findings of Sayed et al. (2008), the findings from the two laboratories may not be inconsistent. Although Sayed et al. (2008) observed an increase in miR21 expression in cardiac myocytes of hypertrophied hearts, the expression of miR21 was decreased during the compensation stage (i.e., in the failing heart). Like its effect in cardiac myocytes, the increase in miR21 in cardiac fibroblasts decreases the expression of Spry1 and thereby up-regulates the activation of the Erk1/2 cascade (Thum et al., 2008). This increase in the Erk1/2 signaling in the failing heart contributes to fibrosis and remodeling of the heart. Overall, the two studies show the involvement of miR21 in the regulation of Spry2 and Spry1 in cardiomyocytes and cardiac fibroblasts of the hypertrophied and failing heart, respectively, suggesting that there are coordinated temporal changes in miR21 expression in different cell types in the hypertrophic and failing heart. Thus, during early stages of cardiac hypertrophy, miR21 is increased in cardiac myocytes, and as the heart progresses toward decompensation and failure, miR21 levels in the cardiomyocytes are no longer elevated but instead become elevated in cardiac fibroblasts. The increase in miR21 in cardiac myocytes, by decreasing Spry2, may augment the activation of the Erk1/2 pathway that is known to play a critical role in induction of cardiac hypertrophy. The increase in miR21 in the later stages (i.e., decompensation) may contribute to the fibrosis and remodeling of the failing heart.

**Other Pathological States or Correlates.** By regulating the strength and duration of growth factor signaling, Spry proteins may also play a critical role in the etiology or progression of other diseases. In this context, Spry2 levels are elevated in chondrocytes of mice and humans with thanatophoric dysplasia type II (Guo et al., 2008). Thanatophoric dysplasia type II is a lethal form of chondrodysplasia. Chondrodysplasias result from inherited activating mutations in FGFR3, which negatively regulates proliferation and terminal differentiation of chondrocytes in growth plates, leading to abnormal linear bone growth. It has been suggested that the increase in Spry2 content in chondrocytes of thanatophoric dysplasia type II, by sequestering c-Cbl and protecting FGFR3 from down-regulation, may exacerbate the progression of the disease (Guo et al., 2008).

A decrease in the amount of Spry2 in prefrontal cortex of schizophrenic and bipolar patients has been correlated with a decrease in brain-derived neurotrophic factor (Pillai, 2008). However, whether these changes contribute to the etiology or progression of the disease is not known. Because Spry proteins modulate the proliferation of cells, one study has examined Spry2 expression after electroconvulsive therapy (Onğur et al., 2007). The findings from these studies show that electroconvulsive therapy decreases Spry2 expression, augments glial cell proliferation and thereby, increases neuronal plasticity (Onğur et al., 2007).
Concluding Remarks

As evident from the literature reviewed here, remarkable progress has been made in understanding the functional role of a group of proteins that was discovered just a decade ago. The Spry proteins are clearly involved in the pathogenesis of a number of cancers and in cardiovascular and other diseases. Although significant progress has been made toward understanding some of the molecular mechanism(s) by which Spry proteins fine-tune the biological actions of RTKs, future studies will have to consider the following: 1) that the different Spry family members associate with different signaling molecules; 2) that the Spry isoforms themselves may form homo- or heterodimers/oligomers, depending on which isoforms are expressed in a given cell type or tissue; and 3) that some of the Spry interacting partners also associate with key regulators of other pathways, permitting Spry proteins to affect more than one signaling pathway. Hence, the modulation of a given biological function or pathway by a specific Spry isoform will depend on the repertoire of its interacting partners. Elucidating the precise composition of the Spry complexes (Spry signalosomes) to regulate a given biological outcome will be fruitful ground for future investigations.

Moreover, it should be noted that the Spry proteins also are similar to the Spre family of proteins in the conserved cysteine rich domain (for review, see Bundschu et al., 2006, 2007). Whether the coexpression of the Spred family of proteins fine-tune the biological actions of RTKs, future studies will have to be more sophisticated to better understand how the Spry proteins, sometimes referred to as “master modulators of receptor tyrosine kinases,” mediate their functions in different tissues and cell types. Although Spry proteins have been implicated in different diseases, presently no “small molecule” alter their functions. Hence, future efforts may have to be directed toward development of drugs that alter Spry function to treat pathological conditions associated with aberrant Spry expression.

References


