Chapter 25
Reelin Gene Polymorphisms in Autistic Disorder

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Contents

1 Introduction

Migratory streams occur throughout the central nervous system (CNS) during development. Neuronal and glial cell populations migrate out of proliferative zones to reach their final location, where neurons soon establish early intercellular connections. Reelin plays a pivotal role in cell migration processes, acting as a stop signal for migrating neurons in several CNS districts, including the neocortex, the cerebellum, and the hindbrain (Rice and Curran, 2001). At the cellular level, Reelin acts by binding to a variety of receptors, including the VLDL receptors, ApoER2, and $\alpha 3\beta 1$ integrins, and also by exerting a proteolytic activity on extracellular matrix proteins, which is critical to neuronal migration (D'Arcangelo *et al*., 1999; Hiesberger *et al*., 1999; Quattrocchi *et al*., 2002). Indeed, neuronal migration is profoundly altered in *reeler* mice, lacking Reelin protein due to spontaneous deletions of the reelin

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(*RELN*) gene (D'Arcangelo *et al*., 1995). Their brains display major cytoarchitectonic alterations, yielding a behavioral phenotype characterized by action tremor, dystonic posture, and ataxic gait (Goffinet, 1984). Interestingly, despite significant interindividual differences, postmortem studies of brains of autistic patients have consistently found neuropathological evidence of altered neuronal migration, including ectopic neurons, altered cytoarchitectonics, and aberrant fiber tracts, as recently reviewed by Persico and Bourgeron (2006). Furthermore, the *RELN* gene maps to human chromosome 7q22, in a region hosting one or more autism genes, according to converging evidence from multiple genetic linkage studies (Muhle *et al*., 2004; Persico and Bourgeron, 2006). These findings provided initial suggestions that Reelin may play relevant roles in neurodevelopmental disorders, such as autism. Yet, autistic patients are not "*reeler*" humans: *RELN* gene mutations resulting in the absence of Reelin protein yield a much more severe phenotype, the Norman-Roberts syndrome (Hong *et al*., 2000). This rare autosomal recessive neurological disease is characterized by lissencephaly and cerebellar hypoplasia, with severe mental retardation, abnormal neuromuscular connectivity, and congenital lymphedema. Therefore, *RELN* gene variants potentially conferring genetic liability to neuropsychiatric disorders, such as autism and schizophrenia, were predicted to more likely modulate gene expression levels and/or protein function, rather than to produce a complete loss of function. And indeed, no mutation resulting in premature stop codons and no triplet repeat expansions halting *RELN* gene expression have been identified to date in autistic or schizophrenic patients. This chapter will thus review current knowledge on *RELN* gene polymorphisms influencing gene expression and summarize the results of studies addressing the possible genetic association between functional *RELN* gene variants and autism. Genetic and epigenetic *RELN* gene variants possibly involved in other neurodevelopmental disorders, such as schizophrenia, will be described elsewhere in this book.

2 *RELN* Gene Polymorphisms and Autism

The *RELN* gene encompasses approximately 450 kb, including 65 exons, with alternative splicing of exon 64 and two different polyadenylation sites (Royaux *et al*., 1997). Several polymorphisms present in the 5'UTR, in the coding region, and in the splice junctions have been assessed for association with autistic disorder (Table. 25.1); an additional promoter variant (G-888C) was assessed only in schizophrenia (Chen *et al*., 2002). To date, no *de novo* mutations have been identified in autistic individuals. Missense coding variants inherited by autistic children from a heterozygous parent, co-segregating with autism in the family, and not found in normal controls, include N1159K in exon 25, R1742Q and V1762I in exon 35, R2290H in exon 44, and T2718A in exon 51 (Bonora *et al*., 2003). These rare variants, though interesting for their disease-specificity, have not yet been investigated from a functional standpoint and cannot explain the linkage peak detected in the same families around chromosomal region 7q22 (Bonora *et al*., 2003). The P1703R

Table 25.1 Reelin missense, splicing, and 5'UTR variants in autistic and control samples (n.d., not determined)

Localization	Variation in the 5'UTR or amino acid change	Allelic frequencies*	
		Patients	Controls
5'UTR	GGC triplet repeat†	17.9%[a]	9.1%[a]
		9.2%[b]	6.8%[b]
		5.9%[c]	n.d.
		5.6%[d]	n.d.
Intron 5	A84446G‡ (rs607755)	49.5%[a]	54.3%[a]
Exon 10	Gly370Arg	1.8%	0
Exon 10	Val338Gly	<1%	0
Exon 15	Ser630Arg	7.2%	8.3%
Exon 22	Leu997Val (rs362691)	14.5%[e]	21.4%[e]
		11.0%[f]	n.d.
Exon 25	Asn1159Lys	1.8%	0
Exon 27	Gly1280Glu	3.6%	3.1%
Exon 34	Pro1703Arg (rs2229860)	<1%	n.d.
Exon 34	Ser1719Leu	1.8%	<1%
Exon 35	Arg1742Trp	<1%	0
Exon 35	Arg1742Gln	<1%	0
Exon 35	Val1762Ile	3.6%	0
Exon 44	Arg2290His	1.8%	0
Exon 47	Gly2480Ser	1.8%	1.6%
Exon 51	Thr2718Ala	1.8%	0

* Allelic frequencies are from: [a]Persico *et al*. (2001), [b]Zhang *et al*. (2002), [c]Li *et al*. (2004), [d]Krebs *et al*. (2002), [e]the IMGSAC sample in Bonora *et al*. (2003), [f]Serajee *et al*. (2005). Rare missense variants without footnote are from the IMGSAC sample (Bonora *et al*., 2003).

† Allelic frequencies of "long" alleles (i.e., ≥ 11 GGC repeats) are reported.

‡ bp numbering refers to GenBank acc. n. AC000121; G allele frequencies are reported.

missense variant, present in exon 34 (rs2229860), was found in one family with an autistic proband (Serajee *et al*., 2005) and was not encountered in other control samples (Bonora *et al*., 2003). An A/G transversion present in intron 5 (rs607755), 3 base pairs 5' of the exon 6 splice junction, is predicted to affect the probability of splicing and represents a common polymorphism not associated with autism (Bonora *et al*., 2003; Persico *et al*., 2001; Serajee *et al*., 2005). Finally, a polymorphic trinucleotide GGC repeat was identified in the 5'UTR, immediately adjacent to the ATG start site (Persico *et al*., 2001), and represents to date the only *RELN* gene polymorphism characterized at both the genetic and functional level.

The 5'UTR GGC triplet repeat alleles range between 4 and 23 repeats (Fig. 25.1, panel A). In our initial study, the 8 and 10 GGC repeats represented the most frequent alleles both in autistic patients (8-repeats = 44.2%; 10-repeats = 45.3%) and in normal controls (8-repeats = 44.4%; 10-repeats = 51.1%). Interestingly, longer GGC alleles (i.e., alleles encompassing 11 or more GGC repeats) were found in

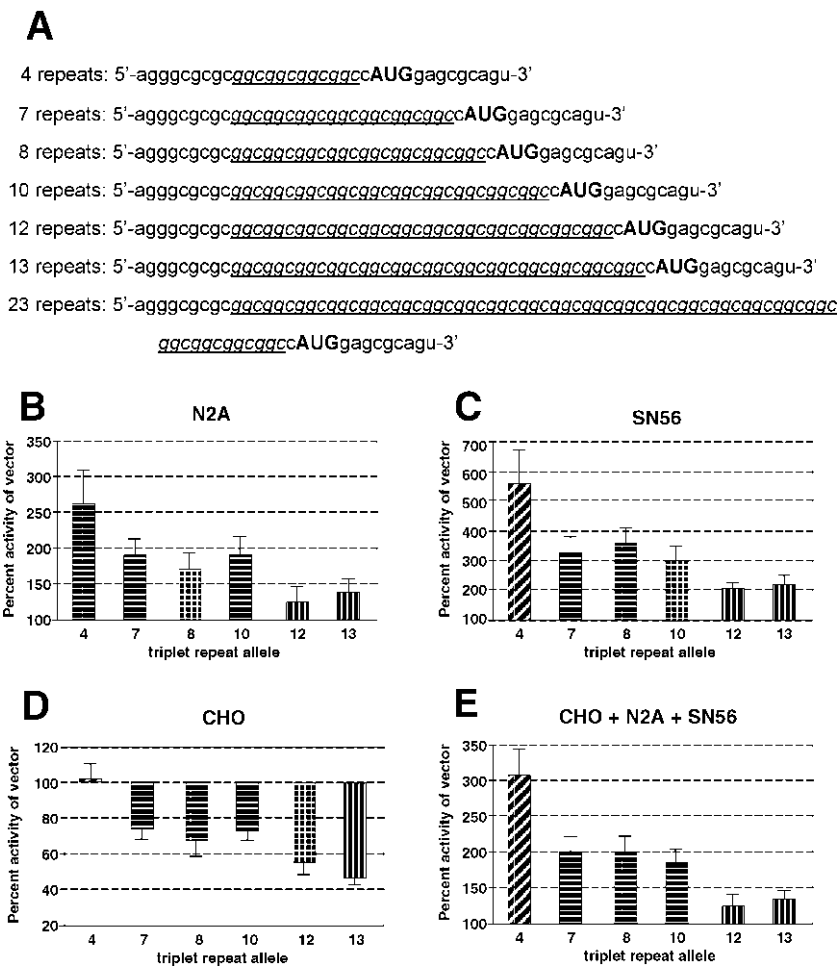

Fig. 25.1 (**A**) Schematic representation of GGC repeat alleles 4-to-23 (repeat underlined and in italics) located in the 5' UTR of the *RELN* mRNA, adjacent to the AUG translation start site, highlighted in bold. Mean luciferase activity of pGL3-Promoter Vector constructs with different 5'UTR GGC alleles in (**B**) N2A, (**C**) SN56, (**D**) CHO, and (**E**) all cell lines together (Persico *et al*., 2006). Statistically homogeneous sets of alleles differing from one another by at least $p<0.05$ are designated by oblique, horizontal, and vertical lines. Overlapping vertical and horizontal lines designate samples reaching a p value <0.1

17.9% of autistic patients versus 9.1% of normal controls ($p<0.05$). The existence of a significant association between these "long" alleles and autism was confirmed using family-based association tests, showing the preferential transmission of "long" alleles from heterozygous parents to their autistic offspring (31 transmissions versus 11 nontransmission: $p<0.05$ after Bonferroni's correction). The preferential transmission of these putative risk-conferring alleles from heterozygous

parents to their autistic offspring was paralleled in these same families by the preferential nontransmission of "long" alleles to the unaffected offspring (6 transmissions versus 13 nontransmission). Consequently, allelic transmission rates differed very significantly between autistic patients and their unaffected siblings ($p<0.001$). Also, the frequency distribution of *RELN* gene alleles marked by haplotypes estimated after genotyping the 5'UTR GGC repeat, the intron 5 SNP (rs607755), and a synonymous coding SNP found in exon 50 (rs2229864), were significantly different between 94 autistic patients and 186 normal controls ($p<0.01$). Several haplotypes encompassing "long" GGC alleles were more frequent among autistic patients than in controls, more frequently transmitted from heterozygous parents to these autistic patients, and less frequently transmitted to their unaffected siblings, than expected by chance (Persico *et al*., 2001).

3 Functional Studies of *RELN* GGC Alleles

The polymorphic GGC triplet repeat is immediately adjacent to the AUG translation start site and occupies an ideal position to influence gene expression rates. *In vitro* and *in vivo* studies have indeed shown that this GGC variant plays functional roles in modulating *RELN* gene expression. *In vitro* experiments were performed transfecting nonneuronal CHO and neuronal SN56 and N2A cell lines with constructs encompassing: (a) the SV40 promoter, (b) 136 bp of *RELN* 5'UTR sequence, (c) either 4, 7, 8, 10, 12, or 13 GGC repeats, and (d) the luciferase reporter gene, in this order (Persico *et al*., 2006). Both neuronal and nonneuronal cell lines showed statistically significant reductions in reporter gene expression with "long" allele, compared to the "normal" 8- and 10-repeat alleles (Fig. 25.1, panels B–E). Levels of gene expression separate GGC alleles into three statistically homogeneous subsets, namely, 4 repeats > 7–10 repeats > 12 or longer. Neural cell lines display step-like 50–60% reductions in luciferase activity with "long" 12- and 13-repeat alleles, compared to the more common 8- and 10-repeat alleles (Fig. 25.1, panels B and C); instead, CHO cells show a roughly linear, progressive decrease in luciferase activity with increasing GGC repeat number (Fig. 25.1, panel D). This progressive trend closely resembles the outcome of computer-based simulations, predicting that the number of GGC repeats would positively correlate with increasingly more stable mRNA secondary structures, characterized by progressively lower free energy (Persico *et al*., 2006). Increasing triplet repeat numbers could presumably decrease the efficiency of *RELN* gene expression in CHO cells, as ribosomes would require more time and energy to scan progressively longer and thermodynamically more stable mRNA secondary structures, before reaching the AUG translational start site. In neuronal cell lines, displaying much higher *RELN* 5'UTR-driven luciferase activity compared to CHO cells (Fig. 25.1, panels B–D), additional complexity could be contributed by the presence of neural-specific factors modulating *RELN* gene expression. Putative factors could include CAGER-1 and CAGER-2, i.e., the $(CAG)_n$ and $(CGG)_n$ repeat-binding

proteins 1 and 2, which are selectively expressed in postmitotic neurons and are known to bind single-stranded triplet repeats (Yano *et al*., 1999). Conceivably, their affinity for *RELN* mRNA could be influenced by the length of the GGC repeat, as suggested by preliminary *in vitro* experiments (Dennis Grayson and colleagues, unpublished data discussed in Zhang *et al*., 2002). Alternatively, or in synergy with cell-specific profiles of RNA-binding proteins, differential DNA methylation patterns could also contribute to differences in basal *RELN* gene expression between neuronal and fibroblast cell lines.

A significant correlation between *RELN* gene expression and the number of GGC repeats was also found *in vivo* (Lugli *et al*., 2003) by assessing archival plasma samples from a subset of autistic patients studied in our initial genetic study (Persico *et al*., 2001). Peripherally, Reelin is expressed by the hepatocytes and secreted into the bloodstream (Smalheiser *et al*., 2000). Reelin's peripheral functions have not yet been fully elucidated but may relate to the immune system, since patients with Norman-Roberts syndrome display congenital lymphedema (Hong *et al*., 2000). Reelin blood levels were initially shown to be significantly reduced in autistic individuals compared to normal controls (Fatemi *et al*., 2002). We then demonstrated a "genotype" effect superimposed on this "disease status" effect by studying 10 pairs of autistic patients matched by sex and age, while differing only at the genotypic level, with one member of each pair carrying the common 8 and/or 10 GGC allele (i.e., genotypes were 8/8, 8/10, or 10/10), while the other member had one "long" GGC allele (for example, 8/12 or 10/13). All 10 pairs consistently showed lower Reelin plasma levels in the patient carrying one "long" GGC allele: the correlation between GGC repeat genotypes and the intensity of the 310-kDa band visualized by Western blotting was highly significant ($p < 0.001$). Overall, patients carrying "long" repeat alleles displayed a mean 24.5 ± 3.8% reduction in Reelin plasma levels compared to the matched counterpart carrying "normal" GGC alleles (Lugli *et al*., 2003).

4 *RELN* GGC Alleles and Autism: Replication Studies

Following the initial genetic findings by Persico *et al*. (2001), several replication studies were performed, as summarized in Table 25.2. Three studies either replicated the initial association of "long" GGC alleles with autism or provided evidence supporting *RELN* gene contributions to autism liability through gene variants in linkage disequilibrium with these and with other known polymorphisms.

Zhang *et al*. (2002) tested the GGC repeat for association with autism using both a case–control and a family-based association test. Selecting one autistic patient per multiplex family (i.e., including two or more siblings affected with autism) and contrasting allelic and genotypic distributions in 126 patients with those of 347 unassessed controls, the authors found no significant association, but only a minor trend ("long" GGC allele frequencies: 9.2% versus 6.9% in autistic patients and controls, respectively; p=0.370). Applying the family-based

Table 25.2 Summary of genetic association studies on *RELN* gene variants and autism. All *RELN* gene polymorphisms assessed in each study are listed, except for Serajee *et al*. (2005), where only the 2 SNPs found associated with autism are reported here, out of 34 SNPs assessed; AGRE, Autism Genetic Resource Exchange

Reference	Polymorphisms	Experimental design	Race and ethnicity	Outcome
Persico *et al*. (2001)	5' UTR: GGC repeat Intron 5: rs607755 Exon 50: rs2229864	Case-control Family-based	Italians; U.S.-Caucasians	Association with GGC repeat and with haplotypes formed by GGC + rs607755 + rs2229864
Zhang *et al*. (2002)	5'UTR: GGC repeat	Case-control Family-based	Not specified (families from Canada and AGRE)	Association with GGC repeat
Krebs *et al*. (2002)	5'UTR: GGC repeat	Family-based	Mostly (94%) EU-Caucasians	No association with GGC repeat
Bonora *et al*. (2003)	5'UTR: GGC repeat Intron 5: rs607755 Exon 22: rs362691 Intron 31: RELNint31 Exon 50**:** rs2229864	Family-based	EU-Caucasians: Sample I, IMGSAC families; Sample II, German families	No association with any common variant; rare missense variants are present (see Table. 25. 1)
Li *et al*. (2004)	5'UTR: GGC repeat	Family-based	Not specified	No association with GGC repeat
Devlin *et al*. (2004)	5'UTR: GGC repeat	Family-based	Not specified (families from the NIH CPEA network)	No association with GGC repeat
Skaar *et al*. (2004)	5'UTR: GGC repeat Intron 5: rs607755 Exon 44: rs2075043 Exon 45: rs362746 Exon 50: rs2229864 Intron 59: rs736707	Family-based	U.S.-Caucasians from Duke Univ., AGRE, and Tufts Univ.	Association with GGC triplet and with specific haplotypes
Serajee *et al*. (2005)	Exon 22: rs362691 Intron 59: rs736707	Family-based	U.S.-Caucasians from AGRE	Association with rs362691 and rs736707

association test (FBAT), which uses information from both affected siblings in multiplex families, a statistically significant overtransmission of the "long" alleles to the affected offspring was found ($p < 0.05$). Autistic children without delayed onset of phrase speech tended to carry "long" alleles more frequently than children with onset of phrase speech later than 36 months (59.1% versus 78.3%, respectively; $p = 0.06$).

An association between the GGC variant and autism was also found by Skaar *et al*. (2004), who studied 371 Caucasian-American families ascertained through Duke University (217 families), AGRE (86 families), and Tufts University (68 families). In addition to the GGC variant, these authors genotyped five SNPs in the *RELN* gene, including the SNPs in intron 5 (rs607755) and exon 50 (rs2229864), previously tested by Persico *et al*. (2001), and SNPs found in the *ORC5L* and *PSMC2* genes, flanking the *RELN* gene at the 5′ and 3′ ends, respectively. The strongest single-marker family-based association was detected at the 5'UTR GGC variant ($p = 0.002$), followed by the exon 44 SNP ($p = 0.028$). Different subsamples displayed different patterns of association, with the AGRE subsample largely driving the association with the GGC variant. An overall haplotypic analysis defined an association between autism and a 6-marker *RELN* gene haplotype, encompassing ≥ 10 repeats at the GGC variant (FBAT $p < 0.002$). Unlike the work by Persico *et al*. (2001), this group found an overtransmission of the 10-repeat allele and not of "long" alleles, which were present at low frequency (approximately 5%) in these patients. This discrepancy was thus interpreted as likely stemming from interethnic differences in genetic structure and in allelic frequencies.

Serajee *et al*. (2005) investigated 34 SNPs in 196 Caucasian families from the AGRE collection. Two SNPs located in intron 59 and in exon 22 showed overtransmission to affected individuals (TDT p-values = 0.0005 and 0.03, respectively). Applying strict diagnostic criteria for autism, only the intron 59 variant remained significant, with a preferential transmission of the common C allele. These two variants had been previously reported by Skaar *et al*. (2004), who also found a significant association with the intron 59 SNP in the AGRE sample, and by Bonora *et al*. (2003), who did not further investigate the exon 22 missense variant due to its low frequency in their sample.

Four studies have failed to replicate the initial association findings. Krebs *et al*. (2002) performed a family-based study with 117 simplex families (i.e., only one affected individual per family) and 50 multiplex families, mainly recruited throughout Europe. These authors genotyped only the GGC repeat and found no significant overtransmission of the "long" alleles to affected children. In a thorough mutational search of the entire *RELN* gene performed on two separate samples encompassing IMGSAC and German families, Bonora *et al*. (2003) identified the missense variants described above, concluding that their low frequencies could not explain the strong linkage results on 7q22 obtained in these same families. Furthermore, these authors found no evidence of association between autism and more common *RELN* gene polymorphisms, including the polymorphic GGC repeat. Nonetheless, all affected individuals carrying the rare missense variants displayed severe language impairment, possibly providing evidence converging on the genotype–phenotype correlation between *RELN* gene variants and language development, initially proposed by Zhang *et al*. (2002). Finally, two U.S.-based studies also described a lack of association between the polymorphic GGC repeat and autism: Li *et al*. (2004) assessed the GGC repeat and two SNPs located in the 3′ UTR of the RELN gene in 107 multiplex families, whereas Devlin *et al*. (2004) assessed the GGC repeat in a larger sample, comprising 202 simplex and 183 multiplex families recruited by the NIH Collaborative Programs

of Excellence in Autism (CPEA) Network. The latter study also found the GGC repeat associated neither with age at first word nor with the age at first phrase.

Several methodological issues must be briefly considered in order to evaluate these studies and their outcome. First, a case–control design is more powerful than family-based designs, but it is also less reliable in the presence of population substructure (i.e., interethnic differences in linkage disequilibrium and allelic frequencies). In the latter scenario, false-positive and false-negative differences in allelic or genotypic distributions between cases and controls could reflect differences in the ethnic composition of the case and control samples, rather than the existence of a true genetic association. Therefore, studies using both approaches, and where both approaches display a significant association or at least similar trends, are more reliable than studies using only either approach. Second, sample size and statistical power are a major issue, especially with family-based designs: most replication studies performed to date, with the exception of studies by Bonora *et al*. (2003), Skaar *et al*. (2004), and Devlin *et al*. (2004), lack the power necessary to find modest-to-moderate-size single-gene contributions within the framework of a polygenic disorder, like autism. Third, the genetic underpinnings of multiplex families may partly differ from those of simplex families. Multiplex families likely encompass patients with more genetically driven forms of autism, whereas simplex families may represent a mix of families with more environmentally driven forms of the disease, and families that, despite prominent genetic liability, have not evolved to become multiplex only due to stoppage (i.e., parents choosing to have no more children once their first child is diagnosed with autism). Merging multiplex and simplex families into a single sample may thus not be entirely appropriate. Finally, several groups have assessed subsets of families recruited by the AGRE consortium (Geschwind *et al*., 2001), without providing a complete list of their AGRE family identification numbers. It is thus impossible to determine to what extent different studies have really assessed "independent" samples and to perform a reliable meta-analysis of all available data.

The most plausible interpretation of genetic, biochemical, and neurodevelopmental studies of Reelin in autism is that *RELN* gene variants may provide contributions to autism pathogenesis, neither necessary nor sufficient to cause the disease (Bartlett *et al*., 2005). *RELN* gene variants could enhance susceptibility in interaction with gene variants at other loci and/or with environmental factors. In particular, "long" GGC alleles are functionally correlated with decreased *RELN* gene expression, but several studies have pointed toward risk haplotypes encompassing "normal" alleles (Persico *et al*., 2001; Skaar *et al*., 2004). Genetic contributions to autism pathogenesis could thus come not only from "long" GGC alleles but also from additional polymorphisms in linkage disequilibrium with SNPs located in intron 1 or more toward the 3′ end of the *RELN* gene, or perhaps even in the nearby *ORC5L* gene, which is in linkage disequilibrium with the 5´ GGC repeat (Skaar *et al*., 2004). In particular, the large intron 1 present in the *RELN* gene could host functionally relevant sequences, similar to intronic sequences exerting profound influences on gene expression at the acetylcholinesterase (De Jaco *et al*., 2005) and β-casein loci (Lenasi *et al*., 2006).

5 Modeling *RELN* Gene Contributions to Autism: The Challenge of Complexity

Genetic and clinical heterogeneity is often seen as the main source of nonreproducibility in psychiatric genetics. Syndromic autism can, indeed, be produced by a variety of genetic and environmental causes (Persico and Bourgeron, 2006). Consistent with the hypothesis of genetic heterogeneity, in our initial study, "long" *RELN* GGC alleles were present only in approximately 20% of autistic patients (Persico *et al*., 2001). We thus concluded that this variant could play a role in a relatively limited subset of patients. In order to move now from the generic notion of "genetic heterogeneity" to more heuristic and hypothesis-generating pathogenetic models, it may be critical to consider gene × gene and gene × environment interactions, which display some geographical and ethnic specificity, possibly contributing to the discrepancies recorded among association studies. In our original study, the genetic association was almost entirely carried by our Caucasian-American families, with only a modest nonsignificant trend present in our Italian families (odd ratios=19.2 and 1.6 for Caucasian-Americans and Italians, respectively) (Persico *et al*., 2001). One possible interpretation of this interethnic difference is offered by *in vitro* studies, showing that Reelin exerts a proteolytic activity which is crucial for neuronal migration; this proteolytic activity is inhibited by diisopropylphosphofluoridate (Quattrocchi *et al*., 2002), one of many toxic organophosphate (OP) compounds routinely used as pesticides in agriculture and as household insecticides. Based on this observation and on the significantly more widespread use of OPs inside American homes compared to Europe, we proposed a gene × gene × environment interaction model (Fig. 25.2), which predicts that individuals carrying genetic or epigenetic variants resulting in reduced *RELN* gene expression, if exposed prenatally to OPs during critical periods in neurodevelopment, will more likely suffer from altered neuronal migration resulting in autistic disorder (Persico and Bourgeron, 2006). Additional evidence in favor of this model comes from the demonstration that autism is associated, again in our Caucasian-American but not in our Italian families, with genetic variants of the *PON1* gene encoding for paraxonase, the organophosphate-detoxifying enzyme present in human serum bound to HDL (D'Amelio *et al*., 2005). Furthermore, we have recently shown that the *PON1 R192* allele associated with autism yields in autistic patients, but not in normal controls, prominent reductions in serum PON1 arylesterase activity (Gaita and Persico, 2006), as predicted by our model (Fig. 25.2).

The biological roles of the Reelin protein are likely broader than currently appreciated and may eventually justify or even require that other models be generated, in addition to or in substitution of this proposed model (Fig. 25.2). As an example, recent neuroanatomical and brain imaging studies have provided intriguing evidence supporting an abnormal activation of the immune system in autism (Laurence and Fatemi, 2005; Vargas *et al*., 2005; Petropoulos *et al*., 2006). These findings are sur-

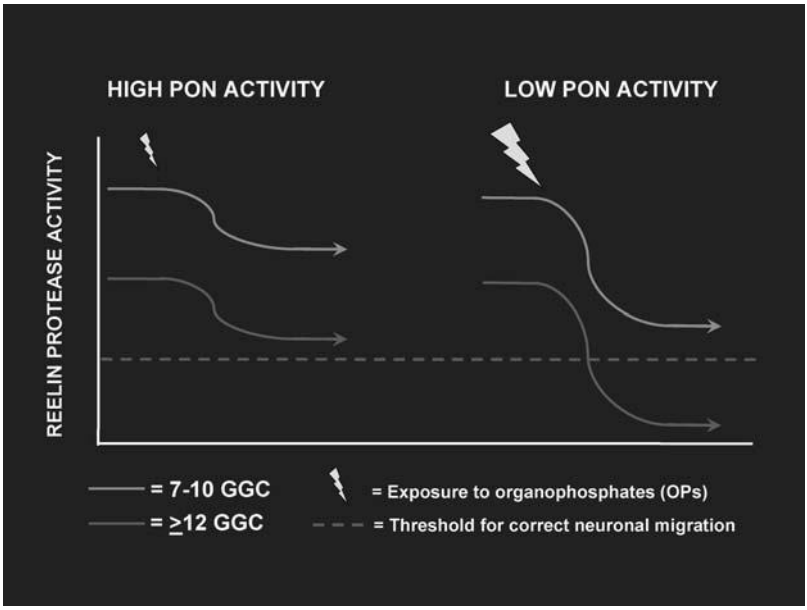

Fig. 25.2 Putative gene–environment interaction model involving the Reelin and PON1 genes, and prenatal exposure to organophosphates (OPs). Reelin gene variants genetically determine normal or reduced levels of Reelin, associated with normal or "long" GGC alleles, respectively. Both conditions are compatible with normal neurodevelopment, but prenatal exposure to OPs can transiently inhibit Reelin's proteolytic activity, which may or may not fall below the threshold critical to neuronal migration, also depending on baseline levels of Reelin. Furthermore, exposure to identical doses of OPs can affect Reelin to a different extent, depending on the amount and affinity spectrum of the OP-inactivating enzyme paraoxonase produced by the *PON1* gene alleles carried by each subject (Gaita and Persico, 2006; Persico and Bourgeron, 2006). (Modified from *Trends Neurosci*., Vol. 29, Persico, A.M., and Bourgeron, T., Searching for ways out of the autism maze: genetic, epigenetic and environmental clues, pages 349–358, copyright 2006, with permission from Elsevier) (*See Color Plates*)

prisingly convergent with our results indicating that macrocephaly (i.e., a head circumference > 97th percentile) is significantly correlated with a positive history of "allergies" both in the autistic patient and in his/her first-degree relatives (Sacco *et al*., 2006). Also, the significant decrease in serum PON1 enzymatic activity we find in autistic patients and first-degree relatives (Gaita and Persico, 2006) parallels similar decreases found in the presence of viral hepatitis C (Ferré *et al*., 2005; Kilic *et al*., 2005), influenza (Van Lenten *et al*., 2002), and HIV infections (Parra *et al*., 2007). These results are thus compatible with the presence of a persistent, virally triggered immune reaction in a subgroup of genetically predisposed autistic children displaying macrosomic features. Within this scenario, the proteolytic activity exerted by Reelin on extracellular matrix proteins, such as fibronectin, could play multiple roles in modulating inflammatory mechanisms at the extracellular level and the

recruitment of lymphocytes to the site of inflammation. The latter phenomenon is interestingly mediated by the binding of α4β1 integrin receptors found on lymphocyte membranes, with the CS-1 fragment of fibronectin, present on the membranes of endothelial cells (Munoz *et al*., 1997).

Finally, geographically diversified environmental factors could also differentially influence epigenetic mechanisms including DNA methylation, histone acetylation, and higher-order chromatin organization. It is well known that DNA methylation is profoundly influenced by factors like nutrition, smoking habits, and aging (Jaenisch and Bird, 2003; Feil, 2006). Epigenetic mechanisms have been demonstrated to play a role in the pathogenesis of several neurological disorders often associated with autism. In fragile-X syndrome, a CGG triplet repeat expansion present in the 5'UTR of the *FMR1* gene is accompanied by an aberrant *de novo* methylation of the CpG islands located in the *FMR1* promoter, yielding transcription silencing (Chelly and Mandel, 2001). Mutations in the methyl CpG binding protein 2 (MeCP2) are associated with Rett syndrome, an X-linked pervasive developmental disorder characterized by autism, loss of speech, seizures, microcephaly, and hand wringing (Amir, 1999). A recent study by Horike *et al*. (2005) identified Dlx5 as a direct target of MeCP2 and showed substantial chromatin differences at the Dlx5-Dlx6 locus between MeCP2 null and wild-type mice. In particular, MeCP2 seems to mediate the formation of a silent-chromatin loop through histone modifications, which was absent in MeCP2 null mice. There is strong *in vitro* evidence that the methylation status of the promoter modulates *RELN* gene transcription rates (Chen *et al*., 2002). Downregulation of Reelin mRNA and protein levels in schizophrenic patients have been directly correlated to *RELN* promoter hypermethylation (Abdolmaleky *et al*., 2005). These observations point toward the possibility that genetic polymorphisms associated with autism may mark *RELN* gene alleles carrying abnormal epigenetic variants. Experiments are ongoing in our laboratory to test this hypothesis.

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Chapter 26
Alzheimer's Disease and Reelin

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Contents

1 Introduction

Alzheimer's disease (AD) is the most common cause of dementia among elderly people and is characterized by loss of memory and cognitive functions. The pathological hallmarks include extensive synaptic and neuronal loss, astrogliosis, and accumulation of fibrillar deposits. The amyloid plaques are extracellular deposits mainly composed of a small insoluble protein called β-amyloid protein or Aβ that is derived from the β-amyloid precursor protein (APP) (Masters *et al*., 1985). The neurofibrillary tangles are composed of intracellular paired helical filaments containing an abnormally phosphorylated form of the tau protein (Grundke-Iqbal *et al*., 1986). Specific genetic factors are also linked closely to AD. Thus, despite the occurrence of missense mutations in APP, the most common mutations in AD to date are in presenilin (PS1 and PS2) genes, membrane proteins which play a critical role in the γ-secretase processing of APP (Selkoe, 2001). Whereas these mutations are quite infrequent causes of AD, the major known genetic risk factor for the disorder in the typical late-onset period is the ε4 allele of apolipoprotein E (ApoE) (Strittmatter *et al*., 1993).

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S. H. Fatemi (ed.), *Reelin Glycoprotein: Structure, Biology and Roles in Health and Disease.*

As extensively reviewed in this book, Reelin is a signaling protein that regulates the migration of neurons during encephalic development and is essential for the correct organization, development, and plasticity of the cerebral cortex. The Reelin pathway involves a cascade of intracytoplasmic events that ends with limitations of the extent to which the tau protein is phosphorylated. Reelin binds to the transmembrane liporeceptors apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR) (D'Arcangelo *et al*., 1999; Hiesberger *et al*., 1999), which relay the signal into the cell via the adapter Dab1 (disabled-1; Bar and Goffinet 1999; Cooper and Howell, 1999; Trommsdorff *et al*., 1999). Co-receptors, such as cadherin-related neuronal receptors (Senzaki *et al*., 1999), $\alpha 3\beta 1$ integrin protein (Dulabon *et al*., 2000), are also likely involved in this process, as well as the c-Jun N-terminal kinase (JNK)-interacting proteins (JIP)-1 and -2 (Stockinger *et al*., 2000; Verhey *et al*., 2001).

Although the role of Reelin pathway in the adult brain is not precisely known, the complex pattern of cellular and regional Reelin expression is consistent with Reelin having multiple roles in adult mammalian brain function (Ikeda and Terashima, 1997; Alcántara *et al*., 1998; Pesold *et al*., 1998; Rodriguez *et al*., 2000; ; Smalheiser *et al*., 2000; Martínez-Cerdeño *et al*., 2002; Roberts *et al*., 2005). In addition, recent studies have suggested a connection between the Reelin/ApoE receptor system and human neuropsychiatric disorders, which will have exhaustive review in other chapters of this book. The possibility of an involvement of the Reelin signaling pathway in neurodegeneration has merited extensive review (D'Arcangelo *et al*., 1999; Bothwell and Giniger, 2000; Herz and Beffert, 2000; Rice and Curran, 2001; Grilli *et al*., 2003; Fatemi, 2005). Recent studies have also shown that Reelin itself can modulate synaptic function and that disruption of Reelin receptors results in learning and memory deficits (Weeber *et al*., 2002; Beffert *et al*., 2005), suggesting that impairment of Reelin/ApoE receptor-dependent neuromodulation may contribute to cognitive impairment and synaptic loss in AD.

The purposes of this chapter are to review the links between Reelin and elements of its signaling pathway with the main hallmarks of AD pathology and summarize our recent findings, including the first evidence of altered Reelin expression in the AD brain.

2 Altered Reelin Expression in Brains of Subjects with Alzheimer's Disease and Transgenic Mouse Models

The first evidence for the association of Reelin with AD features comes from a transgenic mouse model. In APP/PS1 double transgenic mice, Reelin immunostaining was found together with human APP in the neuritic component of many AD-like plaques (Wirths *et al*., 2001). However, in a second APP/PS1 transgenic mouse model, despite the occurrence of occasional Reelin-immunoreactive AD-like plaques, the distribution and intensity of Reelin immunoreactivity in the hippocampal formation was similar to that in the wild-type (Miettinen *et al*., 2005). This puzzling scenario is completed by a couple of studies in the human AD brain, both focused on

Reelin-immunoreactive Cajal-Retzius cells. First, Riedel *et al*. (2003) did not find Reelin immunoreactivity in neuritic plaques and described that Cajal-Retzius cells appear marginally affected by the formation of paired helical filaments in the AD brain, suggesting that these subtle changes are a result rather than a cause of the pathogenetic cascade of AD. On the other hand, Baloyannis (2005) reported a dramatic decline of the number of Cajal-Retzius cells in early cases of AD and suggested that Reelin loss may be implicated in the synaptic pathology and the multifactorial pathogenetic pathways of AD. Despite these controversial reports, the expression of Reelin in cortical interneurons of the AD brain warrants further study.

In this context, using SDS-PAGE and Western blotting, the presence of detectable levels of the three Reelin forms (full-length 420-, and 310- and 180-kDa N-terminal fragments) was reported in cerebrospinal fluid (CSF) (Sáez-Valero *et al*., 2003). A significant increase of 180-kDa Reelin levels was found in AD patients compared to healthy individuals (Sáez-Valero *et al*., 2003). Increased levels of this major 180-kDa CSF-Reelin fragment have recently been confirmed in a different cohort of AD samples (Botella-López *et al*., 2006; see also Fig. 26.1). The concentrations of the full-length 420-kDa Reelin and 310-kDa bands in CSF from AD patients did not differ significantly from those of nondemented subjects, demonstrating that altered Reelin processing is unlikely to account for the increased abundance of this protein in the CSF of patients affected by AD. Another study failed to confirm our previous report of altered levels of the 180-kDa CSF-Reelin fragment in AD samples (Ignatova *et al*., 2004). However, the smaller sample sizes analyzed and the handling of the samples may contribute to this divergence of results. In fact,

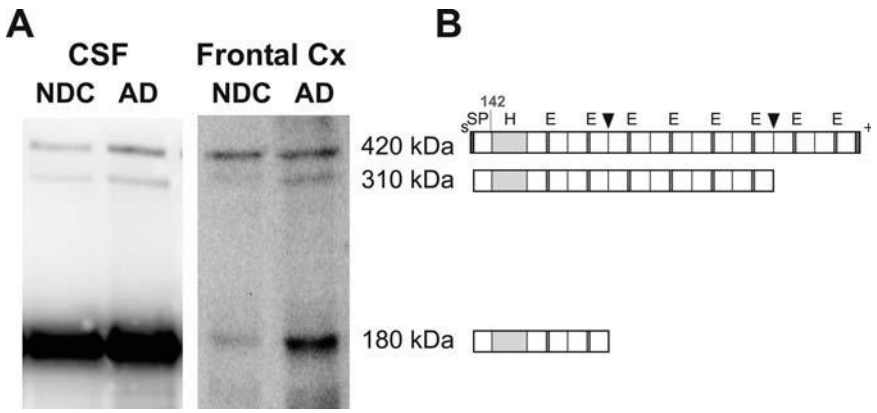

Fig. 26.1 (**A**) Comparison of the banding pattern of CSF and brain (frontal cortex extracts) Reelin identified with antibody 142 (for details see Botella-López *et al*., 2006), from AD and nondemented control individuals (NDC). The N-terminal 180-kDa Reelin was always the predominant fragment, although faint 420- and 310-kDa bands were also stained in most cases. (**B**) Schematic representation of the Reelin protein, its 310- and 180-kDa fragments generated by the two main processing sites (arrowheads), and recognized by the 142 antibody (the epitope is approximately located as indicated). s, signal peptide; SP, spondin similarity region; H, unique region; E, EGF-like motifs which separated two related subdomains in the eight internal repeats; +, terminal basic region

and in good agreement with the results obtained for human plasma by Lugli *et al*. (2003), methodological factors, such as storage temperature, thawing–freezing cycles, and heating before electrophoresis, influenced Reelin level assessment (Botella-López *et al*., 2006).

By SDS-PAGE analysis of brain extracts, a marked increase (~40%) in Reelin protein levels was also found in AD frontal cortex (see also Fig. 26.1) compared to nondemented controls (Botella-López *et al*., 2006). The increase in protein levels was accompanied by a similar increase (~60%) in Reelin transcript levels, supporting the notion that Reelin levels are intrinsically altered in this pathology. In contrast, the protein and mRNA for Reelin appeared unaffected in the cerebellum of AD, an area which normally does not have compact neuritic plaques, demonstrating that Reelin expression is only affected in brain areas targeted by AD. Finally, an abnormality in the glycosylation pattern of Reelin in AD CSF was identified. The pattern of Reelin–lectin binding was altered for two mannose-specific lectins, *Lens culinaris* agglutinin (LCA) and *Canavalia ensiformis* lectin (Con A). Whether the altered glycosylation pattern in AD is a direct consequence of altered metabolism or reflects changes in differentiation state warrants further study.

Taken together, these results suggest an altered Reelin expression in AD. However, these results do not distinguish whether altered Reelin signaling is a mechanism participating in the pathogenesis or whether it is secondary to the degenerative process itself. Interestingly, other types of neurological disorders, such as frontotemporal dementia and progressive supranuclear palsy, which, together with AD, belong to the group of diseases referred to as tauopathies, also show increased Reelin levels in CSF compared to nondemented controls (Sáez-Valero *et al*., 2003; Botella-López *et al*., 2006). Whether this reflects the participation of Reelin in the pathogenesis of these diseases, and the potential molecular mechanisms by which Reelin contributes to AD, remains to be elucidated. The next step will be to demonstrate whether Reelin overexpression in adult brains is involved in the sequence of events that occurs during AD. The generation and analysis of transgenic mice overexpressing Reelin may shed light on new signaling pathways associated with Reelin, and may also improve our understanding of the mechanisms related to AD and other neurodegenerative diseases.

3 Reelin, Amyloid, and Neurodegeneration

The relation between Reelin and AD has been highlighted by recent findings suggesting that Reelin signaling affects APP processing or Aβ deposition. Interestingly, it has been reported that Reelin can function as a serine protease of the extracellular matrix (Quattrocchi *et al*., 2002). Based on this proteolytic activity of Reelin, it has been suggested that Reelin may be involved in the proteolytic processing of APP or in the clearance of amyloid aggregates generated by APP processing (Grilli *et al*., 2003). However, this serine protease activity of Reelin is currently doubted and questioned (Quattrocchi *et al.,* 2004). Alternatively, sprouting of Reelin-expressing interneurons might be induced by Aβ.

Dab1, which is considered an essential component of the Reelin signaling pathway, also binds to APP (Trommsdorff *et al*., 1998; Howell *et al*., 1999). Recent studies examined the effect of Dab1 on APP processing. Whereas Parisiadou and Efthimiopoulos (2006) found that Dab1 increases surface APP and its processing by secretases, Hoe *et al*. (2006) reported that APP and secreted Aβ decrease in Dab1 transfected cells. More interestingly, Reelin treatment increases cleavage of APP and ApoER2 and decreases production of Aβ, suggesting that the effect of Dab1 on APP and ApoER2 trafficking and processing is influenced by Reelin (Hoe *et al*., 2006). Finally, in the context of AD, it has been recently proposed that increased formation of APP cytoplasmic domain in the cytosol released after cleavage of Aβ could inhibit the Reelin signaling pathway and influence synaptic plasticity (Hoareau *et al*., 2008).

Perhaps the most intriguing indication thus far that Reelin is involved in AD is the potential relationship between disturbed synaptic plasticity and loss of differentiation control in neurodegenerative processes. Considerable effort is concentrated on gaining insights into the basic mechanisms of the Reelin/ApoE pathway in adult synaptic transmission and plasticity. Conversely, AD appears as a disorder of brain self-organization associated with morphodysregulation at the synaptic level and is characterized by a complex reactivation of developmental molecular mechanisms and synaptic dysregulation (Bothwell and Giniger, 2000; Arendt, 2003; Grilli *et al*., 2003). Reelin and ApoE receptors fulfill critical functions during brain development and may influence the pathogenesis of AD as part of this common mechanism of aberrant neuronal plasticity which changes neuronal morphology and synaptic contacts.

Moreover, presenilins are involved in the Notch and Wnt/beta-catenin signaling pathways linking many of the players involved in neuronal maturation and neurodegeneration. Interestingly, in agreement with models in which neuronal migration disorders have been linked to a defect in Reelin-expressing Cajal-Retzius cells, the altered properties and loss of most of these cells in PS1-deficient mice lead to cortical dysplasia (Hartmann *et al*., 1999; Kilb *et al*., 2004). Thus, the link between presenilins and Reelin appears to be of interest.

4 Reelin Signaling Pathway, ApoE Receptors, and the Regulation of Tau Phosphorylation

The most robust circumstantial evidence linking Reelin with neurodegeneration is the binding of Reelin to ApoE receptors and the identity of the downstream target of the Reelin pathway as mediators of tau hyperphosphorylation. Indeed, Reelin binding to the ApoE receptors leading to a cascade of phosphorylations ultimately inhibits glycogen synthase kinase-3β (GSK3β) (Beffert *et al*., 2002), an enzyme that regulates tau phosphorylation (Mandelkow *et al*., 1992; Ishiguro *et al*., 1993). The cyclin-dependent kinase 5 (CDK5), another kinase that can phosphorylate tau (Baumann *et al*., 1993), is speculatively considered to be a downstream partner for the Reelin signaling pathway (Rice and Curran, 2001). Thus, lack of Reelin is associated with increased tau phosphorylation (D'Arcangelo *et al*., 1999; Hiesberger *et al*.,

1999; Tueting *et al.,* 1999), and this hyperphosphorylation apparently leads to cytoskeletal disruption and neuronal degeneration. Mutations that prevent the Reelin-dependent induction of Dab1 tyrosine phosphorylation also cause tau hyperphosphorylation (Brich *et al*., 2003).

On the other hand, the *in vitro* interaction of Reelin with lipoprotein receptors is inhibited in the presence of ApoE3 and ApoE4 alleles (D'Arcangelo *et al*., 1999), and the presence of ApoE also limits phosphorylation of tau protein and protein kinase activity in Reelin-deficient mice (Ohkubo *et al*., 2003). It has further been shown that a secreted soluble isoform of ApoER2 can also inhibit Reelin signaling (Koch *et al*., 2002). A direct role for ApoER2 in amyloid deposition and neurodegeneration in AD has also been suggested (Motoi *et al*., 2004).

All of the findings together raise the intriguing possibility that Reelin, ApoE, and its receptors contribute to some of the complex processes involved in neurodegeneration.

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Chapter 27
Reelin and Stroke

Kunlin Jin

Contents

1 Introduction

In 1951, Falconer reported the spontaneous occurrence of a disorder that produced ataxia, incoordination, and tremor in mice, and which came to be designated the *reeler* phenotype (Falconer, 1951). These mice showed neuropathological changes consisting of malpositioned neurons in a variety of brain regions, including cerebral neocortex, hippocampus, and cerebellum (D'Arcangelo *et al*., 1995). The gene defect was discovered subsequently to affect reelin (Reln), a serine protease produced by developing neurons and found in the extracellular matrix (ECM). Reln expression regulates the migration and settling of central neurons in the developing brain (Tissir and Goffinet, 2003) and spinal cord (Yip *et al*., 2000). Mutations in the *RELN* gene on chromosome 7q22 in patients account for rare cases of autosomal recessive, Norman-Roberts type lissencephaly with developmental delay, epilepsy, and nystagmus (Hong *et al*., 2000). At least two allelic variants have been reported: a splice acceptor site mutation (IVS37AS, G-A, -1) and an exon deletion (EX43 DEL).

In addition to its developmental role, Reln appears to function in the adult brain, and decreased *RELN* expression has been implicated in some cases of temporal

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lobe epilepsy (Haas *et al*., 2002). In this chapter, we review effects of Reln on proliferation and migration of neural stem/progenitor cells (NPCs) in normal and ischemic rodent brain and on outcome from experimental stroke.

2 Stroke

Clinical stroke usually results from cerebral ischemia due to occlusion of a cerebral blood vessel, most often an artery. Less common causes include venous occlusion and intracerebral hemorrhage. The three main classes of *in vivo* rodent models of cerebral ischemia are global ischemia, focal ischemia, and combined hypoxia/ischemia. In the latter, which is typically used to study neonatal brain ischemia, vascular occlusion is combined with hypoxia (Levine, 1960). Focal ischemia, a model of stroke, is usually modeled by middle cerebral artery occlusion (MCAO), and gives rise to localized brain infarction (Ginsberg and Busto, 1989). This model incorporates pathophysiological and histopathological features of clinical stroke, although drugs that protect mice or rats in this model have often failed in clinical trials of stroke therapy. Global ischemia, which produces pancerebral hypoperfusion leading to death of selectively vulnerable neurons, such as those in the CA1 region of the hippocampus, recapitulates the neuropathology that may follow cardiac arrest.

In focal ischemia, all cells (neurons, glia, and endothelium) within the affected vascular territory are deprived of oxygen and glucose, leading to energy failure, glutamate release, and loss of transmembrane ion gradients. These cells may survive or die, depending on the severity and duration of the insult and the efficacy of endogenous neuroprotective programs (Lipton, 1999). Brain injury from experimental focal ischemia is characteristically quantified in terms of infarct volume (Osborne *et al*., 1987), although this does not necessarily correlate with the degree of functional neurological impairment, as measured by neurobehavioral tests of cognitive or sensorimotor performance.

3 Reln and Neurogenesis in Normal Adult Brain

Altman first observed the proliferative potential of adult rodent brain in the 1960s (Altman, 1962; Altman and Das, 1965). It is now generally accepted that the subventricular zone (SVZ) surrounding the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) are active proliferative regions that generate neurons (Chiasson *et al*., 1999), astrocytes (Doetsch *et al*., 1999), and oligodendrocytes (Chiasson *et al*., 1999; Johansson *et al*., 1999) throughout life in mice (Yoshimura *et al*., 2001), rats (Jin *et al*., 2001), nonhuman primates (McDermott and Lantos, 1991), and humans (Eriksson *et al*., 1998). Neural precursor cells (NPCs) in these regions of adult brain can be identified by administering

[^{3}H]thymidine or the thymidine analog 5-bromo-2´-deoxyuridine-5´-monophosphate (BrdU), which are incorporated into DNA during S-phase of the cell cycle (Altman and Das, 1965; del Rio and Soriano, 1989). Because these labels are nonspecific with regard to cell type, identification of newborn neurons requires the use of cell type-specific markers as well.

Cells derived from the adult SVZ or SGZ appear to be capable of developing into mature, functional neurons, although this normally occurs in small numbers. Evidence for neuronal differentiation of these cells includes the observations that they: (1) express neuronal markers such as neuron-specific enolase (NSE) (Cameron *et al*., 1993; Jin *et al*., 2001), (2) receive synaptic inputs (Bayer, 1985), develop neuronal electrical properties and synaptic transmission (Song *et al*., 2002), (3) have axons that can be backfilled (Cameron *et al*., 1993), (4) exhibit rapid, reversible increases in intracellular Ca^{2+} in response to depolarization with K^+, typical of neuronal voltage-gated Ca^{2+} channels (Kirschenbaum *et al*., 1994), and (5) develop neurotransmitter-specific phenotypes *in vitro* and *in vivo* (Sawamoto *et al*., 2001).

The production of new neurons in adult brain is regulated by physiological factors, such as exercise (van Praag *et al*., 1999) and stress (Gould *et al*., 1998), as well as by pathological conditions, such as stroke (Jin *et al*., 2001) and epilepsy (Yoshimura *et al*., 2001). We found that Reln may also be involved in neurogenesis in the hippocampal DG of normal adult brain (Won *et al*., 2006). Proliferation of NPCs in DG of *reeler* mice (B6C3Fe-a/a-Reln^{rl}) was identified by BrdU incorporation and immunostaining for doublecortin (Dcx), a microtubule-stabilizing factor found in newborn and migrating neurons, and a generally reliable marker of new neurons in the adult rodent brain (Nacher *et al*., 2001). Knockdown of Dcx expression inhibits the transit of newborn neurons from the SVZ along the rostral migratory stream (RMS) en route to the olfactory bulb (Jin *et al*., 2004). We found that the number of BrdU-labeled cells that also expressed Dcx was reduced in the SGZ, but not in the SVZ, of *reeler* mice. In contrast to wild-type mice, *reeler* mice showed an aberrant, disorganized distribution of BrdU-labeled cells in the hippocampus, consistent with the absence of an anatomically identifiable SGZ. The mechanism for impaired hippocampal neurogenesis in adult *reeler* mice is unclear, but could relate to the absence of normal SGZ-derived signaling.

4 Reln and Migration of Neural Stem/Progenitor Cells in Normal Adult Brain

NPCs arising in SGZ and SVZ of adult brain must migrate to the regions in which they will become functional neurons. The SGZ, located between the hilus and the granule cell layer (GCL) of the hippocampal DG, retains the potential to form new neurons into adulthood (Gage *et al*., 1998; Cameron and McKay, 1999). These

cells migrate into the GCL, where they become granule neurons. In the SVZ, immature neurons aggregate in a network of neuroblast chains that line the lateral wall of the lateral ventricles (Doetsch and Alvarez-Buylla, 1996), and form a restricted migratory route, the RMS, from the anterior SVZ into the olfactory bulb (OB). Unlike the radial glia-guided migration of young neurons during early brain development (Rakic, 1990), chain migration in the adult SVZ/RMS involves interactions between migrating cells and tubelike structures formed by specialized astrocytes (Lois *et al*., 1996). When neuroblasts enter the OB, they differentiate into interneurons (Luskin, 1993; Kornack and Rakic, 2001). However, the OB is not essential for proliferation or directed migration of these cells, since the number of cells in the RMS is not significantly affected after olfactory bulbectomy (Kirschenbaum *et al*., 1999). In adult *reeler* mice, the number of BrdU- and Dcx-positive cells is reduced, and the normal chainlike structure of the RMS is lost, suggesting that migration of neural stem/progenitor cells in the SVZ-to-OB pathway via the RMS is impaired (Won *et al*., 2006).

Reeler mice (Rakic and Caviness, 1995), mouse mutants deficient in VLDLR and ApoER2, and mice deficient in Disabled-1 (Dab1) (Howell *et al*., 1997; Borrell *et al*., 1999; Trommsdorff *et al*., 1999) all show dentate granule cell migration defects, consistent with an important role for the Reln signaling pathway in the neuronal migration during cerebral cortical development (Forster *et al*., 1998). Reln binding to VLDLR and ApoER2 on migrating neurons induces tyrosine phosphorylation of Dab1, activating PI3K/Akt and inhibiting glycogen synthase kinase-3β (GSK3β) (Tissir and Goffinet, 2003). PI3K/Akt is implicated in neuronal migration during both development (Bock *et al*., 2003) and adulthood (Katakowski *et al*., 2003), and inhibiting PI3K impairs migration of new neurons from adult rat SVZ explants *in vitro*. GSK3β has not been clearly implicated in SVZ neurogenesis, but GSK3β activation reduces granule neuron migration *in vitro* (Tong *et al*., 2001). Consequently, the effects of Reln deficiency on PI3K and GSK3β signaling could account for impaired migration of newborn neurons in the RMS of adult brain.

5 Reln and Neurogenesis After Stroke

There is substantial evidence for increased proliferation of NPCs in the adult brain after brain injuries. In global cerebral ischemia in the gerbil, neurogenesis was increased in the SGZ (Liu *et al*., 1998), with enhanced BrdU labeling of cells coexpressing the neuronal markers NeuN, MAP-2, and calbindin. These cells migrated into the GCL, where they took on phenotypic attributes of mature neurons. Global ischemia also enhanced proliferation of BrdU-labeled NPCs in mouse DG (Takagi *et al*., 1999). MCAO selectively affects the ipsilateral hemisphere, leaving the contralateral hemisphere for a yoked control. MCAO increased the proliferation of NPCs, identified by BrdU labeling and the

expression of Dcx and cell proliferation (PCNA) markers, in both DG and SVZ (Jin *et al*., 2001). Notably, neurogenesis occurred in areas that were not themselves affected by the injury, requiring the existence of a mechanism linking injury to neurogenesis and operating at a distance. In addition, unilateral injury increased neurogenesis bilaterally. Other studies have demonstrated increased numbers of BrdU/Musahi1 (an RNA-binding protein that is highly expressed in NPCs), immunopositive cells in DG (Takasawa *et al*., 2002), or of BrdU- or PSA-NCAM-immunoreactive cells in SVZ and cerebral cortex ipsilateral to MCAO (Zhang *et al*., 2001). At postischemic intervals of 1–2 months, BrdU-labeled cells that coexpressed neuronal marker proteins were also increased in cerebral cortex (Jiang *et al*., 2001; Takasawa *et al*., 2002). Although SGZ neurogenesis was reduced in *reeler* mice, ischemia-induced SVZ neurogenesis was preserved (Won *et al*., 2006).

6 Effects of Reln on Migration of Neural Stem/Progenitor Cells After Stroke

Following ischemia, newborn neurons migrate from SVZ into affected brain areas. In transient global forebrain ischemia in rats, BrdU/NeuN-immunopositive cells appeared to migrate to the hippocampus and replace CA1 pyramidal neurons damaged by ischemia (Nakatomi *et al*., 2002). Transient MCAO in the rat was associated with migration of BrdU-labeled cells that coexpressed Dcx, and later NeuN, from SVZ into the ischemic striatum (Arvidsson *et al*., 2002). In another rat MCAO study, SVZ neurogenesis, identified by BrdU labeling and immunostaining for neuronal markers, was markedly increased 10–21 days postischemia, and newborn neurons appeared to migrate in chains from the SVZ to the ischemic striatum. Within the striatum, some of these cells expressed markers of medium spiny neurons, which are preferentially affected in ischemia, suggesting differentiation toward the phenotype of dead or damaged cells (Sato *et al*., 2001; Parent *et al*., 2002). We examined the ipsilateral hemisphere of rat brain after MCAO using dual-label immunohistochemistry and found new neurons migrated from SVZ, either directly or via RMS, to the striatum. A time-course mapping study showed a progressive increase in the number of newborn neurons and the extent of their penetration into the striatum over 72 hours (Jin *et al*., 2003). In the contralateral hemisphere, this was not the case (Arvidsson *et al*., 2002; Jin *et al*., 2003). In other studies, SVZ-derived cells have been shown to migrate in a "ventral migratory mass" via the nucleus accumbens into the basal forebrain (De Marchis *et al*., 2004) and, following MCAO, into the cortex (Jin *et al*., 2003). These observations suggest the existence of endogenous guidance mechanisms that are mobilized in the ischemic brain. However, in *reeler* mice, Dcx-positive migrating cells were not observed in the cortical penumbra after MCAO, confirming that postischemic neuromigration is impaired.

7 Effects of Reln on Functional Outcome After Stroke

In *reeler* mice subjected to MCAO, neurobehavioral deficits were more severe, and cerebral infarcts were larger than in wild-type mice (Fig. 27.1) (Won *et al*., 2006). The explanation for this finding is unclear, but several possibilities merit consideration. Impaired neuroproliferation and migration might contribute to worsened outcome, because ischemia appears to stimulate the generation of functional neurons (Nakatomi *et al*., 2002), and because ablation of SGZ neurogenesis in guinea pigs by whole-brain ionizing radiation (two 5-Gy doses separated by 7 days) produced a less favorable outcome after ischemia (Raber *et al*., 2004). This schedule of radiation seems to inhibit neurogenesis without affecting microvascular morphology or dendritic profiles (Monje *et al*., 2002; Mizumatsu *et al*., 2003). Another possibility is that postischemic neurogenesis does not yield new functional neurons but does lead to the release of neuroprotective growth factors.

Finally, Reln deficiency could worsen outcome after stroke by reducing inhibitory control of excitatory transmission, thereby exacerbating excitotoxic damage

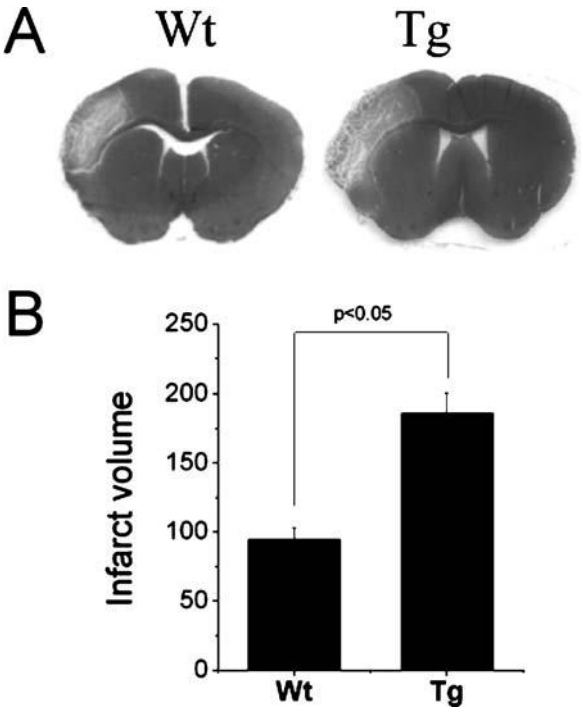

Fig. 27.1 Ischemic brain injury in wild-type (Wt) and transgenic mice with Reln deficiency (Tg) following focal cerebral ischemia. (**A)** HE staining shows an increased area of ischemic injury in *reeler* mice compared to WT mice. (**B)** Quantification of infarct volume in WT and *reeler* mice. $p<0.05$ compared to WT (Student's *t*-test) (*See Color Plates*)

(Costa *et al*., 2004). A mechanism for such an effect is suggested by the observation that GABA turnover is decreased in cerebral cortex, hippocampus, and striatum of *reeler* mice (Carboni *et al*., 2004).

8 Summary

Reln is an extracellular matrix-associated serine protease, which helps to regulate the migration of newborn neurons in development and adulthood. Neurogenesis is decreased in the dentate gyrus but not the subventricular zone of *reeler* mice, and neuromigration in the rostral migratory stream is also reduced. Similar findings pertain to the ischemic *reeler* brain, which fails to mount a normal neuroproliferative and neuromigratory response to injury. Unexpectedly, infarct volume after MCAO was also increased in *reeler* compared to wild-type mice, and neurobehavioral deficits were increased. The latter finding suggests that Reln exerts a neuroprotective effect in the adult brain, the basis for which remains to be determined.

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Chapter 28
Reelin and Pancreatic Cancer

Kimberly Walter[1] and Michael Goggins

Contents

1 Overview: Reelin and Pancreatic Cancer

Classically, the *RELN* gene has been known for its role in neuronal migration and positioning during central nervous system development. Absence of *RELN* expression results in the characteristic reeler phenotype in rodents, marked by severe defects in cortical layer formation and an uncoordinated, unsteady gait. In humans, loss of reelin expression causes a type of lissencephaly with severe cortical and cerebellar malformation. *RELN* is also expressed in peripheral tissues, including the liver, kidney, adrenal glands, and pancreas, suggesting an additional role for reelin in development and possibly in structural maintenance of these organs

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(Smalheiser *et al*., 2000). Recent findings indicate that *RELN* is expressed in the normal duct cells of the adult pancreas, and that *RELN* expression is frequently lost in pancreatic ductal adenocarcinomas and in precursor neoplasms in association with epigenetic silencing (Sato *et al*., 2006). *In vitro* studies suggest that loss of *RELN* contributes to the ability of pancreatic cancer cells to migrate and invade surrounding tissues. These findings support the notion that the effect of reelin pathway status on cell migration may depend on the cell type affected, perhaps depending on the downstream effects of reelin-mediated signaling on the cell's cytoskeleton. For example, reelin loss stimulates migration in some cell types (Gong *et al*., 2007), even though the phenotype of RELN gene inactivation in the brain is a failure of migration (Kim *et al*., 2002; Trommsdorff *et al*., 1999). Although epigenetic mechanisms appear to be responsible for *RELN* silencing in pancreatic neoplasms, the mechanism directing this epigenetic silencing of *RELN* expression is uncertain (Sato *et al.,* 2006).

2 Epigenetic Alterations in Cancer

Cancer is initiated and driven by genetic changes that include mutations in tumor-suppressor genes and oncogenes, and chromosomal abnormalities, such as deletions, amplifications, and rearrangements. A growing body of evidence indicates that in addition to these genetic modifications, cancer is also driven by epigenetic alterations—heritable modifications in DNA-associated information that do not involve the primary DNA sequence itself (Baylin and Ohm, 2006). One of the best-characterized epigenetic alterations is DNA methylation, which occurs particularly at gene promoter sequences and may result in changes in gene expression that can drive tumorigenesis. Methylation occurs at CpG islands, regions of DNA that contain a high frequency of CG dinucleotides, and leads to a closed chromatin state which results in repression of gene transcription. The mechanisms responsible for the aberrant DNA methylation in pancreatic and other cancers are still not well understood, but multiple mechanisms are likely to be responsible for the full spectrum of methylation alterations associated with cancer.

Overall, it is likely that alterations in methylation in cancers likely reflect the combined effects of aging, nutritional and environmental influences, imprinting effects, genetic alterations associated with tumor development, and cell environmental changes due to tumor stromal interactions or chronic inflammation (Anway *et al*., 2005; Bachman *et al*., 2003; Blewitt *et al*., 2006; Dolinoy *et al*., 2006; Feinberg *et al*., 2006; Fraga *et al*., 2005; Huusko *et al*., 2004; Ishihara *et al*., 2006; Morgan *et al*., 1999; Pruitt *et al*., 2006; Waterland and Jirtle, 2003). Some of these influences act by regulating normal DNA methylation and chromatin modifications; others influence changes at the local gene level in the context of normal epigenetic machinery. DNA methylation likely initially arises in discrete CpG sites independent of gene expression but then spreads into promoter CpG islands, presumably through a loss of balance between factors that promote and those that

protect against methylation spreading (Song *et al.,* 2002). There is also a close interplay between DNA methylation and histone modifications. In some instances, cancer-associated DNA methylation changes may be a secondary event that occurs as a consequence of genetic or other events, such as loss of transcription factor(s) that alter the transcriptional activity of an affected promoter (Di Croce *et al*., 2002; Huusko *et al*., 2004). In addition, certain sequences in the human genome are probably more prone to DNA methylation (Bock *et al*., 2006). For example, transposons are prone to methylation, as exemplified by the transposon that undergoes variable methylation in the agouti mouse (Blewitt *et al*., 2006; Dolinoy *et al*., 2006; Morgan *et al*., 1999; Waterland and Jirtle, 2003). The variable methylation at this locus influences expression of the agouti gene and results in the variable coat color phenotype in these mice (Morgan *et al*., 1999). Importantly, nutritional or environmental influences on DNA methylation can influence agouti phenotype (Morgan *et al*., 1999). In addition, certain chromosomal regions are targeted for DNA methylation in cancer cells (Frigola *et al*., 2006). In contrast, in certain model systems histone modifications (methylation of histone H3 lysine-9) are the primary events that occur prior to DNA methylation (Bachman *et al*., 2003). In addition, a certain overall histone code (the presence of bivalent and trivalent lysine 9 methylation in H3 histones) appears to predispose to the development of DNA methylation in cancers (Ohm *et al*., 2007). These findings have suggested that, at least at some genetic loci, initial silencing events lead to chromatin modifications that may predispose promoter CpG islands to hypermethylation. In certain experimental settings, alterations in chromatin insulator function can also give rise to methylation of imprinted or other genes (Ishihara *et al*., 2006). That chromatin alterations can lead to changes in DNA methylation is interesting not least because it is well known that signaling pathways can alter chromatin modifications (Cha *et al*., 2005). Therefore, in theory, environmental influences that mediate changes in signaling pathways and thus chromatin modifications can leave permanent epigenetic marks (West and van Attikum, 2006).

3 Reelin Silencing and Inflammation

One important change in cell environment that could lead to epigenetic alterations is chronic inflammation. Previous studies have found that maternal inflammation can suppress Reelin expression in the postnatal brain (Meyer *et al*., 2006). Reduced Reelin expression in the cortex and hippocampus has been reported in neonatal offspring from dams having been infected with influenza virus at midpregnancy (Fatemi *et al*., 1999, 2002). The cellular mechanism by which *RELN* expression is reduced by inflammation is not known. Since several inflammatory conditions are associated with altered HDAC expression (Ito *et al*., 2005), it is possible that inflammatory stimuli alter histone acetylation marks on the *RELN* promoter causing gene silencing that may be associated with promoter methylation. Indeed, *RELN* promoter methylation has previously been shown to be reduced in cells treated with

HDACs (Mitchell *et al*., 2005). The role of inflammation in *RELN* silencing may be relevant to cancer development because the risk of pancreatic and other cancers increases in the setting of chronic inflammation, such as chronic pancreatitis (Lowenfels *et al*., 1997).

4 Epigenetic Silencing of *RELN* in Human Cancer

Several genomewide strategies are available to identifying genes which are targets of epigenetic silencing in cancer. One approach involves treating cancer cell lines with epigenetic modifying drugs, followed by microarray expression analysis. Genes which are silenced by epigenetic mechanisms in cancers are reactivated on treatment with modifying drugs. By this strategy, *RELN* was identified as a gene that is silenced by aberrant methylation in the majority of pancreatic cancers (Sato *et al*., 2006). Sato *et al*. compared the gene expression patterns between 17 pancreatic neoplasms (including 5 pancreatic cancer cell lines and 12 primary pancreatic neoplasms) and 5 normal pancreatic ductal epithelial samples (Sato *et al*., 2006). *RELN* was underexpressed in pancreatic neoplasms and its expression, methylation status, and functional significance were further examined in additional pancreatic neoplasms and pancreatic cancer cell lines. These studies revealed that *RELN* silencing in pancreatic cancers is associated with promoter methylation, as demonstrated by methylation-specific PCR, combined bisulfite restriction analysis (COBRA), and bisulfite genomic sequencing. Hypermethylation of *RELN* was detected in 14 (61%) of 23 pancreatic cancer cell lines, 17 (85%) of 20 high-grade (carcinoma *in situ*) IPMNs (intraductal papillary mucinous neoplasm), and 9 (47%) of 19 pancreatic cancer xenografts (Sato *et al.,* 2006). Thus, aberrant methylation of the *RELN* promoter is a frequent epigenetic alteration in pancreatic cancer and its precursor lesions, and probably mediates gene silencing.

The role of *RELN* alterations in the pathogenesis of other cancers is largely unknown. Increased *RELN* transcripts have been demonstrated in esophageal cancer cells and tissues (Wang *et al*., 2002). Recently, reelin expression was shown to be present in about one-half of prostate cancers of advanced grade but not in normal prostate or in low-grade prostate cancers (Perrone *et al*., 2007). Although these findings raise the possibility that reelin pathway alterations contribute to prostate and esophageal cancer progression, it is not yet known if the reelin pathway is active in these cancers or if the aberrant expression of reelin is simply a manifestation of nonspecific alterations in gene expression associated with cancer.

5 Silencing of Downstream Reelin Pathway Genes

Mutations in *DAB1*, an intracellular adapter protein which mediates the *RELN* signaling pathway, result in a phenotype similar to the reeler mouse phenotype. Therefore, we investigated the possibility that *DAB1* is also a target of silencing in pancreatic cancer. Interestingly, the *DAB1* promoter CpG island is unmethylated in

normal pancreas and hypermethylated in 14 (64%) of 22 pancreatic cancer cell lines, 10 (59%) of 17 pancreatic xenografts, and 15 (71%) of 21 primary pancreatic adenocarcinomas (Sato *et al*., 2006). Promoter hypermethylation correlated with loss of expression of *DAB1*. This observation represents a unique phenomenon in cancer epigenetics, where two members of the same signaling pathway are both targets of epigenetic inactivation. Typically, cancers only harbor gene mutations or inactivation of one member of a particular signaling pathway. Since the loss of expression of one gene is normally sufficient to inactivate the pathway of that gene, inactivation of other genes in the same pathway is not likely to confer any additional growth advantage to the tumor. The reason for the *RELN/DAB1* silencing pattern is not clear; however, it suggests that either reelin signals through alternative downstream targets other than DAB1, or that the *RELN* pathway has additional functions which can be inactivated only through silencing of multiple pathway members.

6 Reelin Parallel Pathways in Human Cancer

In addition to control by the reelin pathway, neuronal migration is also regulated by cyclin-dependent kinase 5 (cdk5) and its coactivators p35 and p39. While cdk5 is ubiquitously expressed, p35 and p39 are neuronal proteins. cdk5 is a serine-threonine kinase that phosphorylates Dab1 independently of reelin signaling (Keshvara *et al*., 2002). It also phosphorylates other cytoskeletal proteins to contribute to the regulation of cell motility. p35 and p39 are neuronal proteins, whereas cdk5 is ubiquitously expressed. Therefore, this pathway was previously thought to be active only in neuronal tissue. However, we recently observed overexpression of p35 by immunohistochemistry and RT-PCR analysis in pancreatic cancer cell lines and primary pancreatic cancers. We observed an absence of p35 expression in normal pancreas and in a normal pancreatic duct (HPDE) immortalized cell line (unpublished data). Furthermore, specific inhibition of cdk5 in MiaPaCa2, a pancreatic cancer cell line lacking reelin expression, resulted in a marked decrease in cell migration. Although the mechanism of p35 overexpression in pancreatic cancers has not yet been determined, these data suggest that genes in both the RELN and cdk5 pathways are targets for aberrant expression and may contribute to the migratory ability of pancreatic cancer cells.

7 Effects of *RELN* Silencing in the Pancreas

RELN is expressed in normal duct cells and in normal islet cells of the pancreas (Sato *et al*., 2006). However, the functional role of RELN in normal pancreas has not been elucidated. The receptor VLDLR and downstream effectors of RELN, including DAB1 and LIS1, are expressed in normal pancreatic ductal cells, suggesting that the RELN signaling pathway is active in pancreas and may play a regulatory role in cell positioning, as it does in neuronal tissues (Sato *et al*., 2006; unpublished

data). In fact, targeted knockdown of RELN expression in pancreatic cancer cells which retain RELN expression results in increased migration and invasion of these cells *in vitro* (Sato *et al*., 2006). By measuring migration of a pancreatic cancer cell line (Su8686) in a Transwell system, we observed a 35-fold increase in the migratory capacity of RELN-siRNA-transfected cells compared to cells transfected with control nontargeting siRNA (Sato *et al.,* 2006). The ability of these cells to invade through a reconstituted basement membrane (Matrigel) in a Boyden chamber assay was increased by ~15-fold when transfected with RELN-siRNA. These data suggest a functional role for reelin in controlling cell migration and inhibiting pancreatic tumorigenesis. Further studies will help to better define the role of reelin expression in normal pancreas and the functional effects of *RELN* silencing on carcinogenesis.

8 Reelin and Pancreatic Cancer Precursor Lesions

The finding of epigenetic silencing of RELN in the majority of primary pancreatic cancers raises the question of whether RELN silencing occurs in early or late stages of pancreatic ductal carcinogenesis. IPMN and PanIN (pancreatic intraepithelial neoplasm) are two distinct precursor lesions of pancreatic ductal adenocarcinoma. Immunohistochemical analysis of RELN in IPMN lesions revealed that 17 (85%) of 20 high-grade (carcinoma in situ) IPMNs had lost RELN expression (Sato *et al*., 2006).

Preliminary analysis of PanIN lesions including PanIN1a, PanIN1b, PanIN2, and PanIN3 suggest that RELN expression is also lost in a percentage of these precancerous lesions. Examples of reelin expression in pancreatic precursor lesions are shown in Fig. 28.1. Furthermore, *RELN* knockdown increases the ability of pancreatic cancer cells to form colonies *in vitro* (Sato *et al*., 2006). Thus, epigenetic inactivation of RELN occurs in relatively early stages of pancreatic ductal carcinogenesis.

The effect of RELN silencing on the ability of pancreatic cancer cells to migrate and invade *in vitro* suggests that RELN loss could also facilitate metastasis of primary pancreatic cancers. Future studies including immunohistochemical analysis of tissue microarrays, including matching primary and metastatic pancreatic cancer cases, as well as functional analyses of the effects of *RELN* silencing *in vivo*, are needed to address this question.

9 Epigenetic Modifying Drugs as Cancer Therapeutics

Epigenetic silencing of gene transcription occurs through a sequence of events which convert the DNA from an open, accessible conformation to a compact, heterochromatic state. These events are initiated by methylation of CpG dinucleotides by

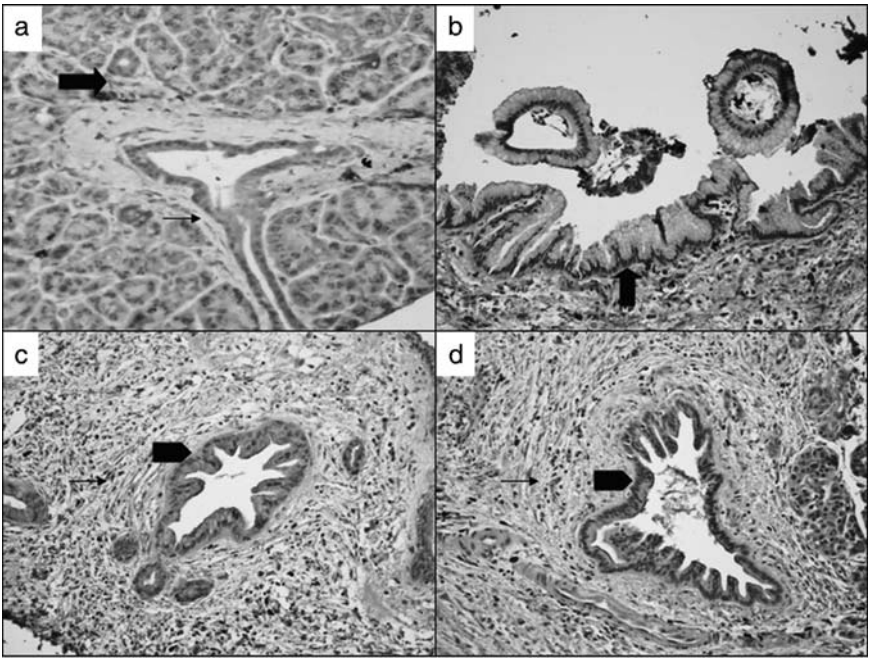

Fig. 28.1 Immunohistochemical analysis of RELN in normal pancreas (**a**) and IPMN (**b**) and PanIN lesions (**c**,**d**). The thin arrow in **a** is pointing to pancreatic ductal epithelium, while the thick arrow is pointing to pancreatic acinar cells. In **b**, the arrow is pointing to the abnormal ductal epithelium of an IPMN; in **c** and **d**, the thick arrowhead is pointing to the abnormal ductal epithelium of a PanIN. The thin arrow in **c** and **d** is pointing to the surrounding fibrosis (*See Color Plates*)

DNA methyltransferases (DNMT). Specific proteins, such as MeCP2, can then bind the CpG dinucleotides and recruit transcriptional corepressors including histone deactylases (HDAC). Cancer-associated epigenetic alterations are attractive therapeutic targets because such epigenetic alterations, unlike genetic changes, are potentially reversible. Inhibitors of DNA methylation and HDAC have been shown to suppress tumor growth *in vitro* and *in vivo*, and some of the inhibitors are being tested in clinical trials for patients with different types of solid and hematological cancers (Egger *et al*., 2004). For example, the DNMT inhibitor decitabine is an FDA-approved drug useful for the treatment of myeloid neoplasms (Oki *et al*., 2007). HDAC inhibitors are also undergoing extensive clinical trials for a variety of cancers (Marks and Jiang, 2005). For example, SAHA (suberoylanilide hydroxamic acid) has been FDA approved for the treatment of cutaneous lymphoid neoplasms (http://www.fda.gov/ohrms/dockets/98fr/84n-0102-lst0101-01.pdf). Most aberrantly hypermethylated genes require DNMT inhibition in order to reverse their epigenetic silencing; inhibition of HDACs alone is usually insufficient. Interestingly, epigenetic silencing of reelin can be reversed by HDAC inhibitors, such as SAHA and valproic

acid, as well as by DNMT inhibitors, raising the possibility that the DNA methylation status of the gene depends on its chromatin modifications (Sato *et al*., 2006). Similarly, in the brain HDAC inhibitors can limit the effects of methyl donors on *RELN* promoter methylation by demethylating the promoter (Dong *et al*., 2007).

In summary, the recent findings that pancreatic cancers frequently lose reelin expression in association with epigenetic silencing and that pancreatic cancer cell motility is influenced by the reelin pathway highlight an important overlap between the biology of neurodevelopmental disorders and cancer development. Understanding the mechanisms of *RELN* silencing may provide clues as to the environmental influences that predispose to cancer development. The ability of epigenetic modifying drugs to modulate the expression of reelin pathway components highlights the potential of these drugs to treat patients with pancreatic and other cancers.

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