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John NICHOLLS
S.I.S.S.A., Neurobiology, Trieste, Italy

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Differential Expression of Genes at Stages When Regeneration Can and Cannot Occur after Injury to Immature Mammalian Spinal Cord

Miranda Mladinic,[1,4] Marie Wintzer,[1,4,5] Elaine Del Bel,[2] Cristina Casseler,[1] Dejan Lazarevic,[1] Sergio Crovella,[3] Stefano Gustincich,[1] Antonino Cattaneo,[1] and John Nicholls[1,6]

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SUMMARY

Comprehensive screens were made for genes that change their expression during a brief critical period in development when neonatal mammalian central nervous system (CNS) loses its capacity to regenerate.

1. In newly born opossums older than 12 days regeneration ceases to occur in the cervical spinal cord. It continues for 5 more days in lumbar regions. The mRNA's expressed in cords that do and do not regenerate were analyzed by polymerase chain reaction-based subtractive hybridization. The mRNAs extracted from cervical cords of animals aged 9 and 12 days were subtracted reciprocally, old from young and young from old.

2. Additional subtractions were made between lumbar regions of 12 day-old cords (which can regenerate) and cervical regions (which cannot). Mini libraries of approximately 2000 opossum cDNA clones resulted from each subtraction. Many sequences were novel. Others that were expressed differentially were related to cell growth, proliferation, differentiation, motility, adhesion, cytoskeleton and extracellular matrix.

3. A major task was to narrow the search and to eliminate genes that were not associated with regeneration. Clones from different subtractions were cross-hybridized. After those common to regenerating and nonregenerating cords were rejected, approximately 284 sequences of interest remained. Our results revealed novel sequences, as well as genes involved in transcription, cell signaling, myelin formation, growth cone motility, liver regeneration, and nucleic acid and protein management as the candidates important for neuroregeneration.

4. For selected genes of potential interest for regeneration (for example cadherin, catenin, myelin basic protein), their temporal and spatial distributions and levels of expression in the CNS were measured by Northern blots, semiquantitative and real-time RT-PCR, and *in situ* hybridization.

[1] Neurobiology Sector, SISSA, Trieste 34014, Italy.
[2] Departamento de Fisiologia, University of Sao Paolo, Ribeirao Preto, Brazil.
[3] Genetic Service, Children's Hospital Burlo Garofolo, Trieste 34137, Italy.
[4] Contributed as first authors to this work.
[5] Department of Neuroscience, Karolinska Institute, Stockholm, Sweden.
[6] To whom correspondence should be addressed at Neurobiology Sector, SISSA, Trieste 34014, Italy; e-mail: nicholls@sissa.it.

5. Our experiments set the stage for testing the efficacy of candidate genes in turning on or off the capacity for spinal cord regeneration. Opossum spinal cords *in vitro* provide a reliable and rapid assay for axon outgrowth and synapse formation.

KEY WORDS: spinal cord injury; trophic molecules; inhibitory molecules; gene expression; *Monodelphis domestica*; development; opossum.

INTRODUCTION

In birds and mammals, the adult CNS has little or no capacity for functionally useful regeneration. By contrast, peripheral nerves regenerate successfully with recovery of function after injury, as do connections in the CNS of fish, amphibians, reptiles and invertebrates (von Bernhardi and Muller, 1995; Nicholls *et al.*, 2001; Blackshaw *et al.*, 2004). That immature mammalian CNS can repair itself, has been shown in embryonic or neonatal chicks, hamsters, opossums and rats (Nicholls and Saunders, 1996; McBride *et al.*, 2003; Kawaguchi *et al.*, 2004). Molecular mechanisms underlying regeneration are poorly understood, even though numerous molecules that influence regeneration have been identified. Such molecules include proteins that promote neurite outgrowth and myelin associated proteins that inhibit regeneration (Bregman *et al.*, 2002; Schwab, 2004; Properzi and Fawcett, 2004; Wintzer *et al.*, 2004). There is evidence that inhibitory molecules do not on their own account for the failure of regeneration in mature mammalian spinal cord (Kim *et al.*, 2003).

A starting point for our experiments is the idea that regeneration of mammalian CNS axons is promoted or blocked by interactions of multiple molecules derived from oligodendrocytes, astrocytes, microglia, neurons and extracellular matrix (Pearse *et al.*, 2004). During development, the balance of such molecules is in turn influenced by transcription; key proteins could change their expression and function in time and space.

What one can expect is that most of the genes expressed by a neuron or glial cell are responsible for maintaining essential properties, while those that control regeneration are likely to be of low abundance. How is one to identify those few genes out of the immense gene pool expressed by each neuron? Our approach has not been directed toward a search for a single candidate gene that could be responsible for failure or success of regeneration. Rather we have begun a comprehensive analysis of all the genes that are up or down regulated in regenerating or nonregenerating CNS tissues.

A favorable preparation for the analysis of gene expression in relation to regeneration is the spinal cord of the neonatal opossum (*Monodelphis domestica*). Newborn opossums are born at a highly immature stage, corresponding roughly to E14 mouse or rat embryos, and a critical period of development has been defined at which regeneration stops being possible (Nicholls and Saunders, 1996; Saunders *et al.*, 1992, 1998; Terman *et al.*, 2000; Ito *et al.*, 2001). Thus, the cervical spinal cords of newborn *Monodelphis domestica* pups that have been completely transected or crushed, repair with complete functional recovery, whereas in opossums older than 12 days, as in other adult mammals, axons in the cervical spinal cord fail to grow

across lesions. Particularly useful is that lumbar segments of the spinal cord are less mature than cervical, and regeneration continues there until 17 days of age (Varga *et al.*, 1995). Hence the developing opossum presents a variety of spinal cord preparations (old-young, cervical-lumbar), that do and do not regenerate after injury, depending on a 3-day difference in age.

Polymerase chain reaction (PCR)-based subtractive hybridization was used in neonatal opossum spinal cord to study changes in gene expression during the critical period. This technique was chosen because it allows enrichment of rare differentially expressed sequences. Two different mRNA populations are compared so as to obtain clones of genes that are differentially expressed (Diatchenko *et al.*, 1996). The method combines normalization and subtraction of cDNAs in a single procedure. Unlike oligonucleotide chip technology, subtractive PCR also allows one to identify unknown genes.

To select genes involved in regeneration and exclude the others that are not relevant for this process, we isolated mRNAs from regenerating and nonregenerating opossum spinal cords of animals that differed in age by only three or 4 days. We supposed that the detection of genes connected with regeneration would be enhanced by keeping the window of time as short as possible. This would lower the background of other genes expressed differentially during development. In addition, subtractions were made from preparations consisting of cervical and lumbar regions of spinal cords of animals aged 12 days, tissues with very similar characteristics except for their ability to regenerate.

Our results have revealed novel sequences, as well as those encoding proteins already supposed to play a role in regeneration. These include molecules involved in nucleic acid management (transcription factors and regulators, RNA splicing factors), protein synthesis and processing, control of the cell growth, structure and motility, cell signaling, and extracellular matrix molecules and their receptors. In a further step we have analyzed the expression of certain candidate genes in the developing spinal cord and brain. The results obtained from different subtractions were compared by cross-hybridization, and this allowed us to identify those sequences exclusively expressed or up-regulated in spinal cord regions that could and could not regenerate.

MATERIALS AND METHODS

Dissection of the CNS

Opossums (*Monodelphis domestica*) were bred and maintained as described previously (Nicholls *et al.*, 1990). Pups were removed from their mothers 9 or 12 days after birth. The animals were anesthetized with metofane and then killed by rapid excision of the heart and lungs; the entire CNS was removed while immersed in oxygenated Basal Medium Eagles (BME) containing Earle's salts (Lepre *et al.*, 1998). Brains were separated from spinal cords, which were divided into cervical and thoracic/lumbar regions, fresh frozen and stored at -80°C until use.

RNA Extraction

Poly(A)$^+$ RNA was extracted from the fresh frozen opossum spinal cord tissue by use of Oligotex Direct mRNA kits (Quiagen GmbH, Hilden, Germany). The Poly(A)$^+$ RNA was quantified by spectrophotometry and its quality was checked on denaturating RNA agarose gels. Approximately 5 μg of the Poly(A)$^+$ RNA was obtained from 100 mg of tissue.

Suppression Subtractive Hybridization (SSH) and cDNA Library Construction

Both forward and backward subtractions were performed between Poly(A)$^+$ RNAs extracted from 9 day and 12 day cervical spinal cord tissue (Subtractions 1 and 2). Other subtractions were made between cervical and thoracic/lumbar spinal cord at 12 days (Subtractions 3 and 4, see Table I). cDNA synthesis and SSH were carried out using a PCR-Select^{TM} cDNA Subraction kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. 2 μg of poly (A)$^+$ RNA from each group were used for cDNA synthesis of tester and driver. After digestion with Rsa I, the tester cDNA preparations were divided into two subpopulations, which were ligated with different adaptors. The tester subpopulations were then hybridized with an excess amount of driver cDNA, after which they were combined and hybridized again in the presence of tester cDNA, without denaturing the DNA before the second hybridization. Following the second hybridization, two PCR rounds were performed to enrich and amplify the differentially expressed sequences. The resultant cDNA obtained (61.2%) from subtractions 1 and 2 was cloned into a pT-Adv vector (Clontech) and transformed into TOP10F' *E. coli* cells. 1309 and 1958 clones were obtained from SUB 1 and SUB 2, respectively.

cDNA Library Arrays

All the clones from the two subtracted cDNA libraries were grown in 96-well liquid culture. Inserts from the clones were PCR-amplified using adaptor-specific primers (Nested primer 1 and Nested primer 2R, Clontech). The PCR-amplified inserts were denatured in 0.2 M NaOH and incubated at 37°C for 15 min. Two microliters of each denatured insert was spotted onto nylon membranes (Hybond^{TM}-N, Amersham) and fixed by crosslinking with the UV light (120 mJ/cm^2).

Table I. Classification OF PCR-Based Suppressive Subtractive Hybridization Experiments in Which mRNAs from Different Preparations Were Subtracted One from Each Other (Tester-Driver)

Subtraction	Tester	Driver
1 P 9 – P 12 cervical	9 day old cervical spinal cord (can regenerate)	12 day old cervical spinal cord (cannot regenerate)
2 P 12 – P 9 cervical	12 day old cervical spinal cord (cannot regenerate)	9 day old cervical spinal cord (can regenerate)
3 P 12 lumbar–P 12 cervical	12 day old thoracic/lumbar spinal cord (can regenerate)	12 day old cervical spinal cord (cannot regenerate)
4 P 12 cervical–P 12 lumbar	12 days old cervical spinal cord (cannot regenerate)	12 day old thoracic/lumbar spinal cord (can regenerate)

Hybridization of the Arrayed Membranes

Nylon membranes were incubated in a prehybridization solution consisting of 5× SSC, 5× Denhardt's, 0.5% SDS, 100 μg/ml nested PCR primers (to prevent background hybridization between nested sequences present at the end of each amplified cDNA insert and the probes) at 60°C for 20 h. Radioactive probes were generated from the subtracted cDNA obtained after the second PCR round. The DNA was ^{32}P-labeled using the Prime-It®II Random Primer Labeling kit (Stratagene) according to the user's manual. The probe was added to the prehybridization solution to a final concentration of $\sim 10^7$ c.p.m./ml and the membranes were washed with 2× SSC, 0.1% SDS, 1× SSC, 0.1% SDS and then 0.1× SSC, 0.1% SDS, for 2 times, 20 min each at 60°C. Membranes were then exposed to a X-OMAT, BioMax, Kodak film for 24 to 72 h.

Northern Blotting

Two micrograms of poly(A)$^+$ RNA from 9 day and 12 day cervical and thoracic/lumbar spinal cord, and from 9 days and 12 days brain were electrophoresed on an agarose-formalhehyde gel and blotted onto a nylon membrane (Hybond™-N, Amersham). The membrane was prehybridized for 4 h in ExpressHyb hybridization solution (Clontech) at 60°C. [α^{32}P] dATP-labelled cDNA probes were synthesized using the Strip-EZ PCR™ Synthesis Kit (Ambion), according to the manufacturer's instruction and using the M13-reversed/M13-forward PCR amplified cloned fragments as the templates. The probe was added to the prehybridization solution to a concentration of 1–5 $\times$ 10^6 c.p.m./ml. The membrane was hybridized for 12 to 18 h at 60°C. Following incubation, the membrane was washed in 2× SSC, 0.05% SDS and in 0.1× SSC, 0.1% SDS twice for 20 min each at 50°C, and exposed to Phosphor Screens (Molecular Dynamics/Amersham Pharmacia Biotech) for 4 to 24 h. Signals were analyzed using Storm 820 Phosphoimager and the ImageQuant software (Amersham Pharmacia Biotech).

Semiquantitative RT-PCR

500 ng mRNA from each preparation was digested with DNase I and then reverse transcribed with AMV-reverse transcriptase. PCR amplification was performed with specific set of primers for each gene. Specific primers were designed by using the Mac Vector software. Primer sequences (1) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were, up-ACGCTTGCTTCACCACCTTCT, down-ATTATCTCTGCCCCCTCTGCT, (2) for Myelin basic protein up-GCACCC TTGTATCCCTTGTGAG, down- TCAAGAACATTGTGTCACCTCGG, (3) for CNP up- TGGGCAATGGGCACTGGAAGC, down-TAGTGAGAAAGGAAA GGGGAAAA. To normalize variations between levels of total cDNA template across different samples, GAPDH was used as an internal control. To be sure that amplification was within exponential range, 5 μl of each semiquantitative RT-PCR reaction was collected at 17, 22 and 28 cycles. PCR product was analyzed on 2% agarose gel.

Real-Time PCR

Total RNA was extracted from fresh tissue using TRIZOL Reagent (GibcoBRL) and Rneasy Mini kit (Quiagen). cDNA was reversely transcribed from 2 μg of RNA using SuperScript Reverse Transcriptase (Invitrogen). Expression of NF-kB gene was analyzed by two-step quantitative PCR using the 1× Taqman Universal Master Mix (Applied Biosystems). Primers and probes set for NF-kB were designed using Primer Express 2.0 software (Applied Biosystems) and were as follows: Forward 5′-TGTGATCTCAAGAAATGAAATTCGA-3′, Reverse 5′-TGTCCTCTTTGAT CTGCTGTTGATA-3′, MGB Probe 5′-6-FAM-TCTTTCAGCAGAAGAAG-3′. Real-Time quantitative PCR was performed using the following temperatures: initial hold at 95°C for 10 min then 40 cycles of 95 °C for 15 s and 60°C for 60 s. Two replicates of each Real-TimePCR were run on ABI 7700 Sequence Detection System (Applied Biosystems). GAPDH (primers and probe were respectively: forward 5′–CTCTGCTGATGCCCCAATGT–3′, reversed 5′ – GAGTCCTTCCACAATGC-CGA –3′, 6–FAM–ATAACTCCCTCAAGATCGTCA–3′) was used as reference in order to normalize the quantitative data. Differences in gene expression were quantified by the comparative Ct method with PE Relative Quantification Software 1.0 (Applied Biosystems).

Nonradioactive *in situ* Hybridization, Using Digoxigenin-Labeled RNA Probes

Brains and spinal cords of 9-day and 12-day opossums were embedded in Tissue-Tek O.C.T. embedding compound (Sakura Finetek USA, Inc. Torrance, Ca, USA) and frozen on dry ice. 10 μm cryostat sections were cut, thaw-mounted onto poly-L-lysine coated slides and allowed to air dry. The slices were fixed and the *in situ* hybridization performed as previously described by Mladinic *et al.*, 1999.

Radioactive *in situ* Hybridization

In situ radioactive hybridization histochemistry was performed as described before (Del-Bel *et al.*, 2002). In brief, 12 μm frozen sections were cut, fixed, dehydrated, delipidated and allowed to dry. The base sequence of the cloned opossum Myelin basic protein insert used for the design of sense and antisense probe was 5′–GCAGGT-GGTGTCCGAGGTGACACAATGTTCTTGAAGAAGAGAG–3′ (antisense). The 40-base oligonucleotide was 3′-tail labeled with [^{35}S] dATP (Amersham) using terminal deoxynucleotidyl transferase from a kit (NEN Dupont). Hybridization buffer consisted of 4 × SSC (600 mM NaCl and 60 mM tri-sodium-citrate pH 7.0), 50% deionized Formamide, 1× Denhardt's solution (Sigma) and 200 mg/ml of denatured salmon sperm (Sigma). Labeled probe was spread over each section. Slides were incubated for 18 h at 37°C, dipped in 1 × SCC to remove hybridization buffer, washed in 1 × SCC 55 °C for 20 min, 1 × SCC for 1 h at room-temperature and then dehydrated before exposure to Hyperfilm (Amersham), stored at −70°C and developed after 3 weeks. In control experiments sections were hybridized with labeled sense strand.

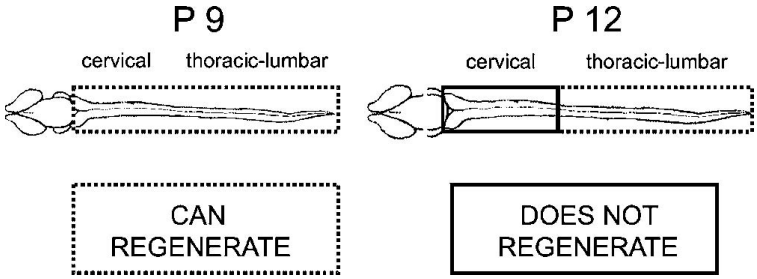

Fig. 1. Regional differences in capacity for regeneration. At postnatal day 9 the entire spinal cord can regenerate while at postnatal day 12 only more caudal, less mature thoracic-lumbar segments continue to regenerate.

RESULTS

To detect genes that are differentially expressed in developing opossum spinal cord during the critical period when regeneration stops being possible, we performed the suppression subtractive hybridizations shown in Fig. 1 and Table I. 1309 clones were obtained from subtraction 1 (9 day minus 12 day; cords that regenerate) and 1958 genes from subtraction 2 (12 day minus 9 day; cords without regeneration). These numbers were far too large for a detailed analysis of each gene, one at a time.

Diversity of Genes Obtained by Subtractions and Homology of Opossum Sequences to Mouse and Human

To determine the diversity of the libraries, about 40 clones were randomly picked from each library and sequenced.

In both libraries about 50% of the clones represented novel *M.domestic* specific sequences, showing no significant homologies with the sequences in the database.

A large number of the sequences that were identified displayed various interesting functions. Moreover those 44 out of more than 1000 clones picked at random from subtractions 1 and 2 displayed a remarkable series with little overlap. Among them are candidates that could play a part in regeneration. For example, in subtraction 1 (sequences amplified from 9 day old cervical opossum spinal cords that could perhaps promote regeneration) we found: RIG protein (involved in liver regeneration), G-protein involved in insulin regulation and liver regeneration, death-associated protein kinase 1 (tumor suppression candidate), alpha spectrin (calmodulin and calpain binding), Ca^{2+}-calmodulin-dependent protein (CaM kinase II, alpha-subunit), tenascin C (extracellular matrix glycoprotein expressed by astrocytes involved in axon growth guidance), beta thymosin (modulator of actin cytoskeleton, upregulated in regenerating retinal ganglion cells), alpha tubulin (cytoskeleton).

By contrast in subtraction 2 (amplified from 12 day old cervical spinal cords) we found: BTG 1 (anti-proliferative factor), KIAA1001 (sulfatase) and 0768 (latrophilin 3, G-protein coupled receptor signaling pathway), neural PTB protein (splicing regulator), Reticulon 3 (member of the reticulon family that includes NOGO),

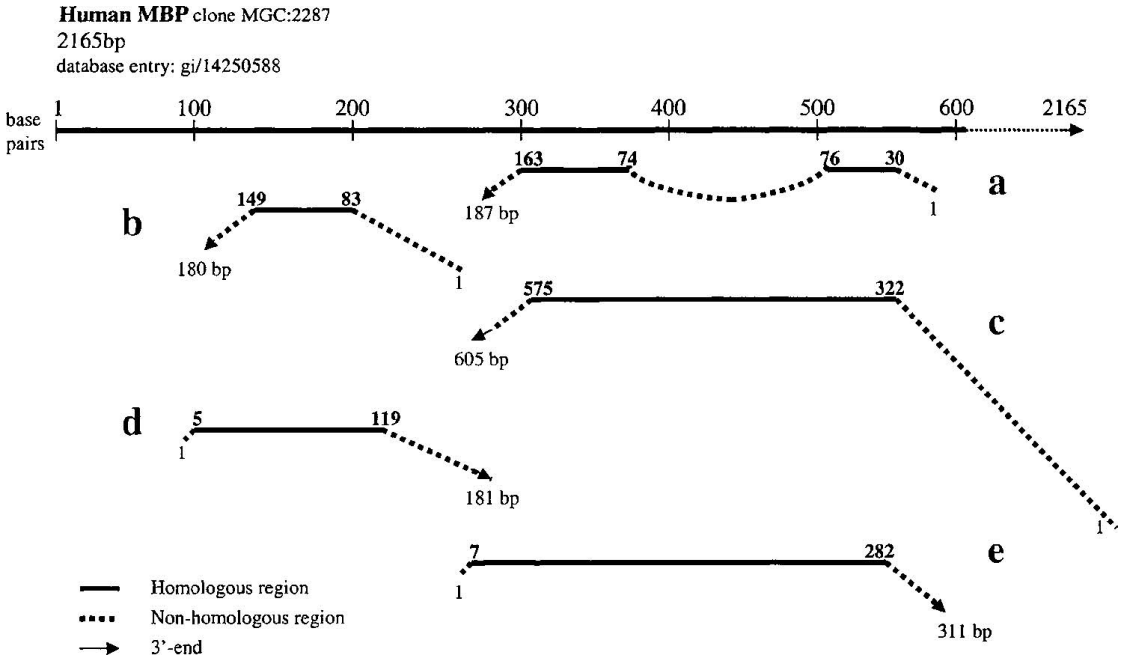

Fig. 2. Homology of opossum myelin basic protein clones to a human sequence from the NCBI database. Scheme of sequence homology between the 5 independent MBP clones obtained from SUB 2 with the human MBP sequence (clone MGC: 2287, database entry gi/14250588).

calmodulin (signal transducing), profilin 2 (actin-binding), carboxypeptidase E (neuropeptide processing), ARA70 (known to inhibit the growth of cancer cells).

The differential pattern of expression of these clones needs further validation with complementary techniques (see below) because some of them represent well-known housekeeping genes including: Na/K - ATPase subunits, cytochrome oxidase and NADH dehydrogenase, suggesting an incomplete subtraction.

An encouraging feature was the sequence homology between opossum and human genes. This is shown in Fig. 2 for Myelin basic protein, which, as shown later, was clearly up-regulated in mature spinal cord regions that failed to regenerate.

Narrowing Down the Search for Genes Relevant for Regeneration

A next important step was to distinguish housekeeping genes and genes that are developmentally regulated between days 9 and 12 from those that actually play a role in regeneration. To find out which of the genes found in subtractions 1 and 2 could play a part in regeneration, we compared the results obtained from various regions of cord at various times. Would a group of genes be seen in common in all those preparations that could regenerate, and be different from the population found in those that could not regenerate?

The procedure was to make comprehensive dot blot arrays of all the hundreds of clones from subtraction libraries 1 and 2. These arrays were then hybridized with radioactively labeled probes derived from each of the other subtractions. Thus the blots of genes apparently over-expressed in subtraction 1 (9 minus 12 day cords)

were hybridized first with those from subtraction 2 (12 minus 9 day) and subtraction 4 (12 day cervical minus 12 day lumbar). In principle, subtractions 2 and 4 should not hybridize since those were from preparations that could not regenerate.

Subsequently subtraction 3 was tested (12 day lumbar minus cervical): in principle that group of clones should hybridize and allow genes favorable for regeneration to stand out. All genes that were identical in the reciprocal subtractions (i.e. present in both 1 and 2, and in both 3 and 4) were eliminated for the time being as false positives. 20–30% of those genes were abundant genes that had escaped the process of subtraction (see discussion).

A small group of clones (about 14%) from subtractions 1 and 2 gave clear signals when cross hybridized with their appropriate counterparts, (subtractions 3 and 4 respectively), but not with their reciprocal (subtractions 2 or 1).

Cross-hybridizations between different subtractions reduced the number of interesting genes by a factor of about 10. Thus, there remained 164 potentially interesting clones from subtraction 1 (9 minus 12 days) and 120 from subtraction 2 (12 minus 9 days). These clones were sequenced. They are shown in Table II and represent a manageable series for further investigation. Many were identified as genes that influence regeneration or neurite outgrowth as shown by experiments made in other systems.

In the following paragraphs we describe key examples of clones for which we measured expression by Northern blots, semiquantitative RT-PCR, and Real-time PCR, as well as their distribution in the CNS by *in situ* hybridization.

Candidate Genes Obtained after Screens of Spinal Cords Capable of Regenerating: Northern Blots, RT-PCR, Real-Time PCR and *in situ* Hybridization

We have focused on four genes that are up regulated at day 9 compared to 12 days and that could be candidates for promoting regeneration. Northern blots are shown in Fig. 3 for Catenin which provides a link between the cytoskeleton and cadherin. It is over-expressed in growth cones of regenerating axons, in injured spinal cord and in regenerating liver (Shibuya *et al.*, 1996). Other genes that were up-regulated included: (a) Cadherin, a calcium dependent intercellular adhesion molecule that has been shown to play a part in axonal navigation, target recognition and synapse formation during development (Hatoko *et al.*, 2001); (b) KIAA0938, also known as neuronal navigator 3 (NAV3), which is a novel gene with homology to cell guidance genes from *C. elegans* (Maes *et al.*, 2002). KIAA clones isolated from human adult and fetal brain have been implicated in cell signaling, nucleic acid and protein management, as well as cell motility. (c) Nuclear factor-kappa B (NF-kB) DNA binding factor is an inducible transcription factor that responds to various cellular signals. It has been shown in a different model systems that NF-kB levels increase as a consequence of brain injury (Salminen *et al.*, 1995; Yang *et al.*, 1995).

Blots for cadherin, catenin, NAV3 and NF-kB all showed clear up-regulation in tissues of opossum spinal cord that can regenerate after injury (results of tests repeated 2 or 3 times on RNA samples isolated from different batches of animals).

Table II. List of Genes Cloned from Subtractions 1 (P9C-P12C) and 2 (P12C-P9C) Selected after Cross-Hybridizations

Category	Sub 1 (P9–P12 Cervical)	Sub 2 (P12–P9 Cervical)
Novel sequences	79	62
Apoptosis	Pro-thymosin alpha	Ubiquitin-protein ligase E3 MDM2 (p53 binding) (antiapoptotic)
	Apoptosis regulatory protein Siva	
Transcription and translation	Matrin 3	*Paired box protein (PA × 6)
	Similar to TAF9-like RNA polymerase II: TATA box binding protein	RuvB-like 2 Reptin 52 (recombination and transcription)
	Similar to SMART/HDAC1 associated repressor protein (SHARP)	Eukaryotic translation initiation factor 3 (TGF-beta receptor interacting protein)
	LINE-1 retrotransposon	KIAA 0669 (regulation of transcription)
	Nuclear receptor subfamily 2, group F, member 1	PABP-interacting protein-2 (translation initiation)
	Smad4 (two different clones)	
	*Nuclear factor-kappa B(NF-kB)	
	KIAA 0938: interacts with Sp1 transcription factor	
Cell signaling and signal transduction	*Beta-catenin (cadherin binding protein)	14-3-3 protein zeta/delta (regulator of kinase mediated cell signaling) (2 different clones)
	RAS family GTP-binding protein RAP-1B (small GTPase)	Rab GDP-dissociation inhibitor alpha (2 overlapping clones)
	GTP-binding RAB2 protein (small GTPase)	Serine/threonine kinase PINK1
	Protein kinase C inhibitor (14-3-3)	Calmodulin 1 (2 identical clones)
	Intracellular hyaluronan-binding protein p75	Amyotrophic lateral sclerosis 2 (ALS2)
	Tyrosine 3 monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3)	Amyloid-like protein 2 precursor
	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (PIK3R1)	
	Syntenin: syndecan binding	
	Serine/threonine protein phosphatase 2A, beta isoform	
	Serine/threonine protein phosphatase 2A, alpha isoform	
Cell adhesion	*Protocadherin alpha	Reelin
	Adhesion regulating molecule 1 (Arm 1, 2 different clones)	Neural cell adhesion molecule 1 (NCAM)
	Tenascin (hexabrachion, cytotactin)	Tetraspan 3
Myelin		*Myelin Basic protein (5 different clones)
		*2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP)
RNA processing and transport	Splicing coactivator subunit SRm300	RNA-binding protein TIA-1 (2 different clones)
	RAS-GTPase activating protein binding protein 2: mRNA transport	Ras-GTPase activating protein SH3 domain-binding protein (mRNA transport)

		Nuclear cap binding protein subunit 2
DNA binding	Structure-specific recognition protein 1	RNA helicase-related protein homolog
Protein synthesis	Ribosomal protein L4	Ribosome receptor p180
	Ribosomal protein L5	
	Ribosomal protein L6 (2 different clones)	
	Ribosomal protein L21	
	Ribosomal protein L24	
	Ribosomal protein L35	
	Ribosomal protein L27 (3 different clones)	
	Ribosomal protein L7a	
	Ribosomal protein L27a (2 different clones)	
	Ribosomal protein L30 (2 different clones)	
	Ribosomal protein L35a	
	Ribosomal protein L37a	
	Ribosomal protein S3a (2 different clones)	
	Ribosomal protein S4	
	Ribosomal protein S6	
	Ribosomal protein S14	
	Similar to acidic ribosomal phosphoprotein PO	
Protein processing and degradation	DNAJ homolog (chaperone)	Ubiquitin-activating enzyme E1C
	Translation initiation factor 3, subunit 7 (2 overlaping clones)	Proteasome subunit, beta type 1
	Vitiligo associated protein (VIT-1)	
Protein transport	Transthyretin	Copine 1 (calcium dependent phospholipid binding protein)
	Clathrin, heavy chain	Von Hippel-Lindau binding protein (cytosolic chaperone)
	Adaptin Ap2b1 (clathrin complex)	
	Coatomer protein complex, subunit beta 2, beta prime	
	ATP-binding cassette transporter ABCG2	
Ion transport	Plasma membrane calcium ATPase isoform 2 (calcium transport)	Voltage-dependent calcium channel gamma-2 subunit
	Solute carrier family 12 (potassium/chloride transporter)	
	Voltage dependent anion channel (VDAC-1)	
Neurotransmission		Sodium- and chloride-dependent GABA transporter 3 (GAT-3)
		GABA (A) receptor-associated protein-like 2
Mitosis, cell cycling	Nuclear ubiquitous casein kinase and cyclin dependent kinase substrate (NUCKS)	Septin 10 (2 overlapping clones)

Table II. Continued

Category	Sub 1 (P9–P12 Cervical)	Sub 2 (P12–P9 Cervical)
Axon guidance	Fasciculation and elongation protein zeta (zygin 1)	*Autotaxin
	*Neuron navigator 3 (NAV3, KIAA 0938)	Plexin B1 (semaphorin 3A receptor)
Cytoskeleton	Dynein: axonal transport	Beta Tubulin
	Beta-thymosin	Actin
	Alpha-spectrin	Alpha tubulin 1
	Alpha-tubulin	
	Microtubule-associated protein 1b	
Cell growth	Secreted acidic cystein-rich glycoprotein: Sparc or osteonectin	
Mithocondria	Serine/threonine kinase PINK1, mitochondrial	Cytochrome C oxidase subunit VIb
	Malate dehydrogenase 1 (tricarboxylic acid cycle)	Mitochondrial DNA containing the 12S rRNA: NADH dehydrogenase activity
	Ubiquinol-cytochrome C reductase core protein (UQCRC1)	Mitochondrial DNA for tRNA-Ala nuclease activity
	Voltage-dependent anion channel 1 (mitochondrial porin)	Cytochrome C oxidase subunit VIIa3
	Cytochrome C oxidase subunit 3 (2 identical and 1 different clone)	Calcium-binding mitochondrial carrier protein Aralar1
Cancer	Ewing sarcoma gene (transcription regulation)	Colon-cancer associated protein MIC1
Cell metabolism	Fructose-bisphosphate aldolase A (glycolysis)	KIAA 1001 (Arylsulfatase G)
	Fructose-bisphosphate aldolase C (glycolysis)	Phosphoglycerate kinase 1 (glycolysis)
	Ferritin heavy chain (iron storage)	5′-biphosphate nucleotidase 1
	ATPase inhibitor	Enolase-phosphatase E-1 (MASA)
		Acid phosphatase 2
		Fumarylacetoacetase (phenylalanine and tyrosine catabolism)
Others	KIAA 0893	Laminin receptor 1 (ribosomal protein SA, 67 kDa)
	KIAA 1109	*KIAA1580 (2 different clones)
		KIAA0601

Note. Genes for which up- or down- regulation were confirmed by Northern blotting, RT-PCR, and/or *in situ* hybridization are labeled as (*).

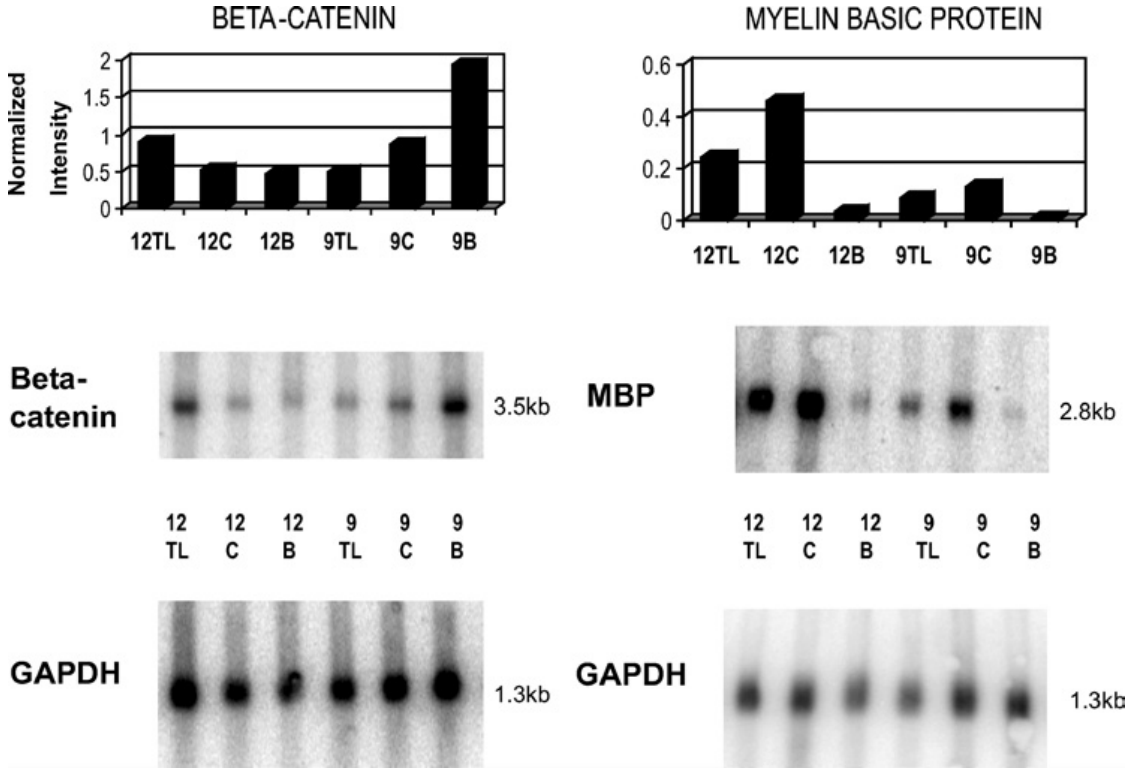

Fig. 3. Northern blotting. The blots show clear up-regulation of the Beta-catenin gene cloned from subtraction 1 (9 minus 12 days), and of Myelin basic protein gene, cloned from subtraction 2 (12 minus 9 days). The results were normalized using GAPDH gene as a standard. Areas from which mRNA was extracted have been labeled as: 'B', brain; 'C', cervical spinal cord; 'TL', thoraco-lumbar spinal cord. '9' and '12' indicate 9- and 12- day old animals.

These results were confirmed by RT-PCR and in the case of NF-kB gene by Real-Time PCR. Fig. 4 shows comparisons of Northern blots and Real-Time PCR, which was performed on RNA samples isolated from single animals (measurements in 2 P8 and 2 P12). Independent evidence for up-regulation of cadherin and NAV3 was provided by *in situ* hybridizations shown in Figs. 5 and 6 which reveal sites of expression in cord and brain. In these sections the label is more intense at 9 days compared to 12. Cells in dorsal as well as ventral regions of cervical cord show staining of the cytoplasm whereas axons as expected are unlabelled.

Candidate Genes Obtained after Screens of Older Spinal Cords, Incapable of Regenerating: Northern Blots, RT PCR and *in situ* Hybridization

Clear up-regulation at 12 days was established for the following myelin associated genes. Five clones were detected that coded for myelin basic protein (MBP, which is an essential component for structure and compactness) (Mikoshiba *et al.*, 1991; Jones *et al.*, 2004). The enzymes 2',3′-cyclic nucleotide 3′phosphodiesterase (CNP), and autotaxin, both of which play roles in the formation of myelin also stood out after subtraction 2 and cross hybridizations (DiCicco-Bloom *et al.*, 2004; Fox *et al.*, 2003). In Northern blots (see Fig. 3), which were confirmed by RT-PCR (not

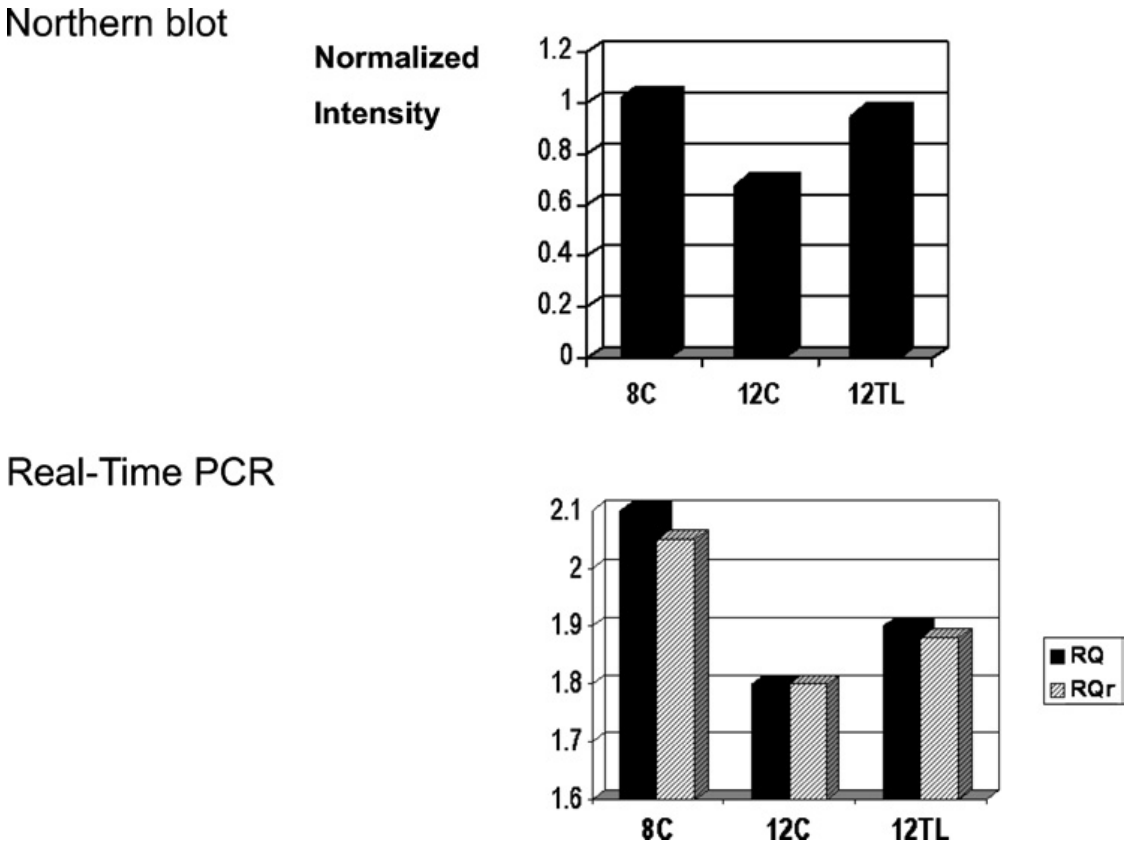

Fig. 4. Comparison of Northern blot and Real-Time PCR for the NF-kB. NF-kB gene expression analyzed by Northern blotting and Real-Time PCR. Northern blotting was performed on RNA samples collected from about 10 animals for each trial; the Real-time PCR was performed on RNA samples isolated from single animals. Up-regulation of NRF-1 is evident in the cervical segments of younger animals (C8) and in thoraco-lumbar segments of older animals (TL 12). RQ: relative quantitation values, RQr: replicates.

shown), marked increases of myelin basic protein were apparent between 9 and 12 days, greater in cervical than in lumbar regions. A similar pattern and selective distribution of these genes in ventral spinal cord regions at which myelin first appears was revealed by *in situ* hybridization, with as expected greater expression in cervical than in lumbar segments (Figs. 7 and 8; see also Varga *et al.*, 1995, for antibody staining of myelin at 12 days).

DISCUSSION

Validity of the Techniques Used for Detecting Genes

The approach that we have used entails a comprehensive search for genes and molecules that promote and impair regeneration of mammalian spinal cord. In contrast to studies in which single molecules are selected to test for their effectiveness (Schwab, 2004), our experiments are based on the idea that combinations of a number of factors might be responsible for determining the success or failure of repair after injury. And that some of the players might not yet have been detected. Inevitably at

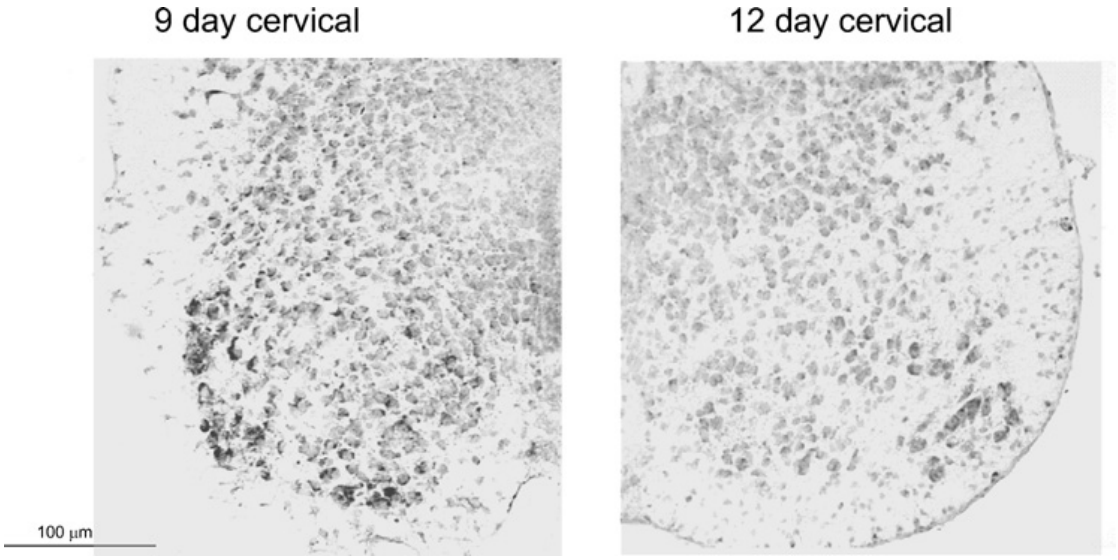

Fig. 5. *In situ* hybridization: protocadherin alpha, a cell adhesion molecule. Enhanced expression of protocadherin alpha gene in ventral cervical spinal cord at 9 days (left) compared to 12 days (right). Nonradioactive *in situ* hybridization.

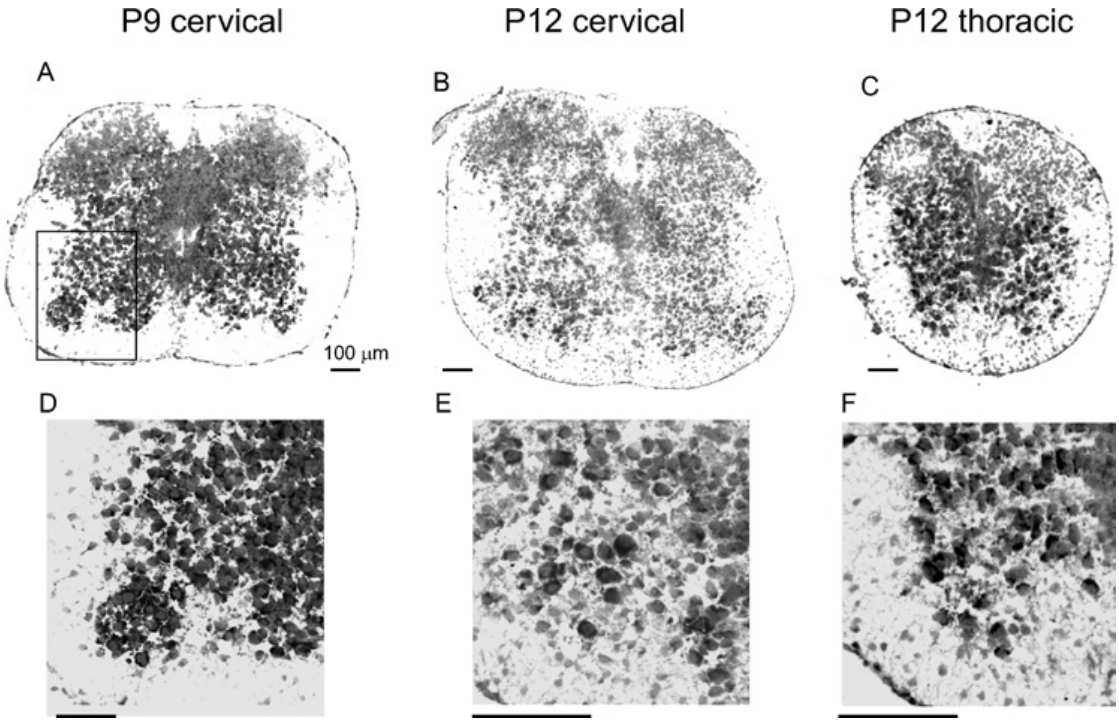

Fig. 6. *In situ* hybridization: NAV 3, a guidance molecule. Enhanced expression of NAV 3 gene in ventral cervical spinal cord at 9 days (A, D) compared to 12 days (B, E). At 12 days up-regulation is still seen as expected in thoraco-lumbar spinal cord (C, F). Nonradioactive *in situ* hybridization.

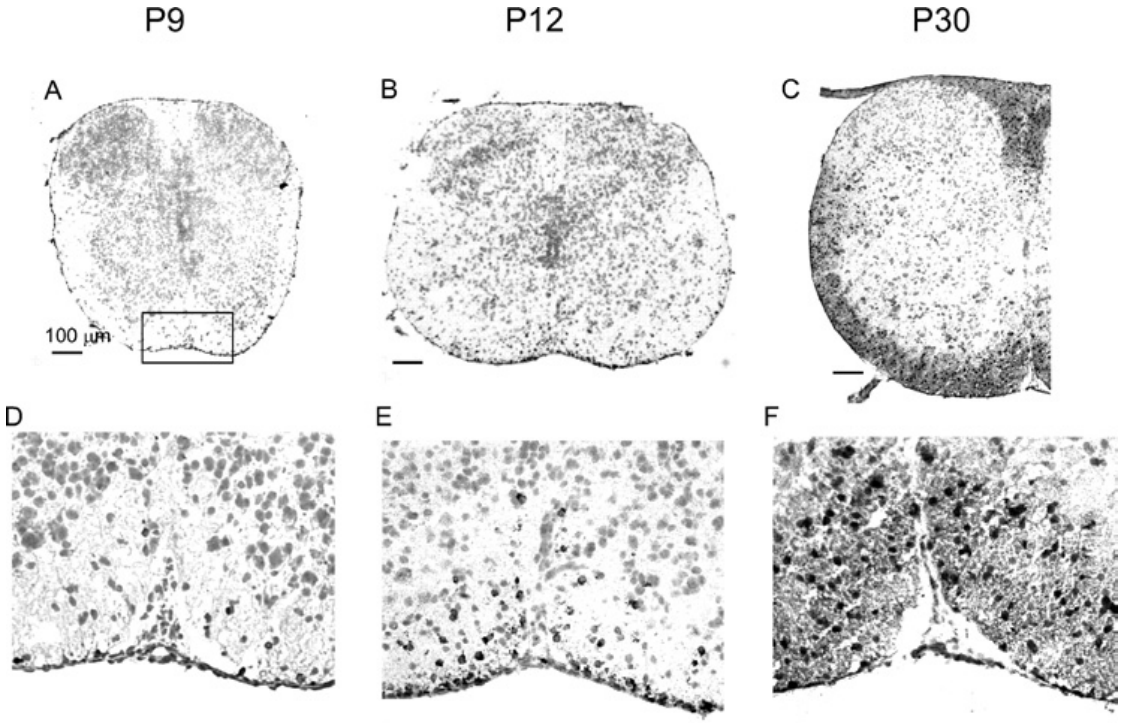

Fig. 7. Non radioactive *in situ* hybridization: Myelin basic protein. Nonradioactive *in situ* hybridization for Myelin basic protein mRNA in cervical spinal cords of P 9 (A, D), P 12 (B, E) and P 30 (C, F) animals showing clear up-regulation at day 12. In panels (D), (E) and (F) the ventral region has been enlarged to show the appearance of the oligodendrocytes in white matter.

this stage our experiments involve large screens. This type of search is like trying to find a needle in a haystack. Except that one does not know the shape of the needle or even how many needles there are.

A first question concerns our methods for identifying those genes that are up and down regulated at critical periods. In many respects microarrays would have simplified our task. We could have rapidly screened differences at 9 and 12 days. But genes with very low levels of expression would tend to be lost, unknown genes would

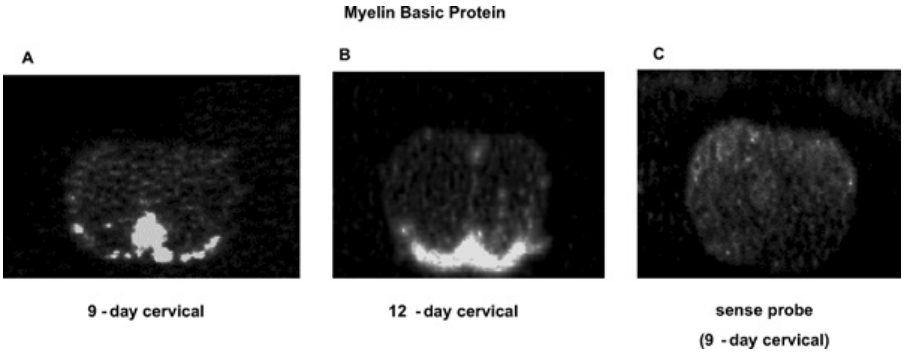

Fig. 8. Radioactive *in situ* hybridization: Myelin basic protein. Radioactive *in situ* hybridization for Myelin basic protein mRNA in cervical spinal cord of (A) P9 and (B) P12 animals showing clear up-regulation at day 12. Note absence of signal with sense probe (C).

not be characterized by the chips and the opportunity for comparing preparation after preparation would be seriously limited by great expense of chips with human or mouse sequences (the effectiveness of which we did not know when we began).

By contrast, differential subtraction allows genes of low abundance and unknown genes to be detected and amplified (Blackshaw *et al.*, 2004). Nevertheless, the procedure is subject to pitfalls. Contaminants may be amplified, since abundant genes are not excluded and the normalization process precludes information about levels of expression. Consequently, confirmation with Northern blots is essential, before one can conclude that the gene is up-regulated. The CNS of a 9 or 12 day opossum weighs approximately 120–150 mg and for a successful blot one requires about 15 pups. Fortunately, RT-PCR provides an independent validation of the presence of the candidate gene in the original tissue and an estimate of quantity with far less material (approximately 1–2 pups for a single test). Moreover, for one gene, NRF-1, up-regulation at early stages was confirmed by Real-Time PCR.

In addition, to validate the candidacy of a gene, the sites and times of expression are important. Where is it expressed? Is it expressed only in connective tissue and blood vessels, or in neurons, glial cells or both? Is there a shift in position between 9 and 12 days? Where is the protein expressed? Such tests are now shown to be practicable and informative in our preparations.

Reducing the Numbers of Candidate Genes

With more than 1000 genes appearing after each subtraction there is no practical way to assay all of them for their effects on regeneration. The selection procedure that we used allowed every clone to be tested for its presence in each of the preparations: cervical and lumbar 9 and 12 day preparations. We have now also begun to compare the expression of genes in injured and uninjured cords. In principle, one might expect genes to be expressed in common in tissues that do and do not regenerate. By multiple cross hybridizations between different regions of spinal cords at different stages of development, the numbers were reduced by a factor of ten. There remains the danger that one might discard or miss important genes. In addition the blotting technique would have failed to detect differences of the order less than 30%.

A problem arises in relation to genes that appear at 9 days and also at 12 days in cervical and lumbar segments of spinal cord; does that imply that their functions are *not* related to regeneration? A possibility would be that a gene and its product remain constant in total amount, but that its distribution in the spinal cord has shifted. This speculation reveals a further aspect of the subtraction procedure. Subtractions 1 and 2 (old from young and young from old) are not necessarily symmetrical. Thus between 9 and 12 days, myelin and a host of myelin associated molecules appear, which were not present or were in very low abundance earlier (Fernández and Nicholls, 1998). Such molecules should stand out from subtraction 2. But as already indicated above, a gene could play a role in development both at 9 days and at 12 days with only a minor shift in *total* concentration, albeit one that could be essential for regeneration to occur. We are currently testing the differential expression of interesting transcripts listed in Table II with semiquantitative and Real-time RT.PCR.

The Search for and Testing of Relevant Molecules

From those genes that appeared to show up- or down-regulation a further selection was made. Our selection was based on roles attributed to specific genes in other regenerating systems, such as peripheral nerves, liver or the CNS in lower animals such as leeches or *C. elegans* (Properzi and Fawcett, 2004; Blackshaw *et al.*, 2004).

Once a molecule had been selected, further tests are required to assay quantitatively its effectiveness in promoting or preventing spinal cord regeneration (Kawaguchi *et al.*, 2004). Approaches in isolated opossum CNS that are underway at present include alterations in the levels of gene expression by transfection of sense and antisense, as well as short chains of double stranded inhibitory RNA, using lipofectin, the gene gun (Sambrook and Russell, 2001) and electroporation (Calegari *et al.*, 2004). A direct test for function is to use the protein itself. For candidates such as cadherin this is a practicable and relatively simple procedure. Other extracellular matrix molecules, growth promoting and inhibitory molecules would be obvious choices for this approach. Similarly, antibodies can be made and applied to test for reversal of effects.

For measurements of the effectiveness of molecules in enhancing or inhibiting regeneration, the opossum is well suited: spinal cord regeneration is reliable and rapid, both in culture and in the animal still attached to the mother (Nicholls and Saunders, 1996; Lepre *et al.*, 1998). Moreover, it is known already that cells can be transfected around the central canal, as shown by labeling with BrdU (5-bromodeoxiuridine) in culture (Nicholls *et al.*, 1990) and in the animal (unpublished). The definition of a narrow critical period between 9 and 12 days constitutes a further advantage. Although dorsal root fibers continue to grow through lesions for up to 19 days, in the assays that we use, 12 days does represent the cut off (Varga *et al.*, 1995).

CONCLUDING REMARKS

What are the prospects for characterizing the genes that play key roles in regeneration? The large number of genes shown in Table II represents a challenge. The table lists genes already supposed to play a role in regeneration, such as transcription factors and regulators, RNA splicing factors, and genes affecting cell signaling, proliferation, differentiation, motility, adhesion, cytoskeleton and extracellular matrix. How many of these genes act in concert and what tests could be used to identify combinatorial messages with multiple levels of protein?

In spite of the complexity of the task, it is possible to hope that a broad screen such as that shown in Table II will lead to discovery of novel molecular mechanisms of spinal cord repair after injury. The results presented here are satisfying since in spite of the difficulties, clear candidates of prime interest have emerged. Moreover the testing procedures seem practicable and should allow multiple genes and their proteins to be screened for their efficacy in promoting regeneration in a well-defined preparation at the cellular and molecular level.

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