

Niklas Pakkasjärvi

# Investigations on molecular aspects of Lethal Congenital Contracture Syndrome

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Department of Molecular Medicine,  
National Public Health Institute Helsinki, Finland  
*and*

Department of Medical Genetics,  
University of Helsinki



# Investigations on molecular aspects of Lethal Congenital Contracture Syndrome

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of Helsinki

Academic Dissertation

To be publicly discussed with the permission of the Medical Faculty of the  
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## **ABSTRACT**

Arthrogryposis is a clinical description of a phenotype caused by fetal immobility. More than 150 conditions presenting with congenital arthrogryposis are known. Prenatally lethal cases of arthrogryposis present a diagnostic challenge although they are more frequently encountered due to improved ultrasound methods. Post-mortem diagnostics is often hampered by fetal mummification. Therefore, accurate diagnosis is at times only descriptive and prognosis is not always possible to predict. The incidence of anterior horn cell disorders associated with arthrogryposis and early fetal demise is poorly known. Thus, more knowledge about the genetics and epidemiology of these disorders is needed. Importantly, identification of the defective pathways in these disorders would most probably expose critical elements in the normal development of human motoneurons.

The Finnish Disease Heritage includes two lethal arthrogryposes. The pathogenetic mechanism of these diseases is still unknown. This thesis sheds light on the molecular mechanisms active during the pathogenesis of Lethal Congenital Contracture Syndrome. LCCS leads to death of the affected fetuses before the 32<sup>nd</sup> gestational week. The hallmark of the syndrome is degeneration of the anterior horn of the spinal cord. We localized the defective gene to chromosome 9q34.1 and provided a scaffold to sequence the genomic region. We then continued by the analysis of the positional candidate genes in the critical DNA-region, but until now the LCCS gene and the corresponding mutation remains unknown.

To obtain further clues of the character of the LCCS gene and involved pathways, we performed DNA microarray experiments on LCCS spinal cords to unravel the molecular pathways that are deranged during the disease process. We observed changes in central developmental themes, including Sonic Hedgehog in addition to an indication of oligodendrocyte dysfunction. To address the implicated oligodendrocyte dysfunction further, neural precursor cells were harvested from post-mortem LCCS CNS and cultured *in vitro*.

Neural precursor cells from both LCCS patients and age-matched controls were successfully enriched in culture. The LCCS neural precursor cells appeared denser and assessment of proliferation indicated increased mitotic

activity. Transcript analysis provided a molecular explanation to the observed phenomenon. The neural precursor cells of LCCS patients seemed to respond normally to differentiating stimuli of morphogens suggesting their normal initial development and implying problems at later stages of differentiation of the cells of spinal cord. However, distinct differences were observed in the transcript profiles of LCCS patients when compared to controls. Aberrant apoptosis during initial differentiation was not found in LCCS neural precursor cells.

LCCS accounts for the majority of prenatally lethal arthrogryposes in Finland. To determine the true number of LCCS cases, we estimated the birth prevalence of LCCS to 1: 25300 births through a register based study during 1987-2002.

The intricate developmental network of the spinal cord is subjective to disturbances at multiple occasions. This thesis shows that neural precursor cells can propagate and differentiate in culture, and thus, most developmental checkpoints are passed normally at least in culture in LCCS patients. Most probably, the disease mechanism is active at post-mitotic stages of the spinal cord cells.

Keywords: LCCS, Arthrogryposis, Development, Motoneurons, Oligodendrocytes, Stem cells, Epidemiology

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## ORIGINAL PUBLICATIONS LISTED

The original publications listed below form the content of this thesis and are referred to in the text by their corresponding Roman numeral. Additional unpublished results are referred to as unpublished.

- I. Päivi Mäkelä-Bengs, **Niklas Järvinen**, Katri Vuopala, Anu Suomalainen, Jaakko Ignatius, Mari Sipilä, Riitta Herva, Aarno Palotie, and Leena Peltonen  
Assignment of the disease locus for lethal congenital contracture syndrome to a restricted region of chromosome 9q34, by genome scan using five affected individuals.  
*Am J Hum Genet.* 1998; 63(2):506-16
  
- II. **Niklas Pakkasjärvi**, Massimiliano Gentile, Juha Saharinen, Jarno Honkanen, Riitta Herva, Leena Peltonen and Marjo Kestilä  
Indicative oligodendrocyte dysfunction in spinal cords of human fetuses suffering from a lethal motoneuron disease  
*J Neurobiol.* 2005; 65(3):269-81
  
- III. **Niklas Pakkasjärvi**, Laura Kerosuo, Heidi Nousiainen, Massimiliano Gentile, Juha Saharinen, Satu Suhonen, Hannu Sariola, Leena Peltonen, Marjo Kestilä and Kirmo Wartiovaara  
Neural Precursor Cells from a fatal human motoneuron disease differentiate despite aberrant gene expression  
*Submitted*

## ABBREVIATIONS

ALS	Amyotrophic Lateral Sclerosis
APECED	Autoimmunepolyendocrinopathy-candidiasis-ectodermal dystrophy
ARF	Cyclin-Dependent Kinase Inhibitor
ARL7	ADP-Ribosylation Factor-Like 7
BAC	Bacterial Artificial Chromosome
BHLH	Basic Helix Loop Helix
BMP	Bone Morphogenetic Protein
bp	Base Pair
BRDU	Bromodeoxyuridine
C.elegans	Caenorhabditis elegans
cM	Centi Morgan
CNS	Central Nervous System
DCTN1	Dynactin
DNA	Deoxyribonucleic Acid
ds	Double Stranded
Dynamin-1	Dynamin-1 Gene
EDG	Endothelial Differentiation Gene
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERBB	Epidermal Growth Factor Receptor
FADS	Fetal Akinesia Deformation Sequence
FCS	Fetal Calf Serum
FDH	Finnish Disease Heritage
FGF	Fibroblast Growth Factor
FISH	Fluorescence In-Situ Hybridisation
FMRP	Fragile-X Mental Retardation Protein
GFAP	Glial Fibrillary Acidic Protein
GLI	Glioma Associated Oncogene Homolog
GTPase	Guanosine 5'phosphate binding protein
HB9	Homeobox Gene HB9
HGP	Human Genome Project
LIF	Leukemia Inhibitory Factor
IBD	Identical By Descent
IGF-1	Insulin-Like Growth Factor I
Isl1	LIM/Homeodomain Transcription factor Isl1
LAAMD	Lethal Arthrogryposis with Anterior Horn Cell Disease
LCCS	Lethal Congenital Contracture Syndrome
LD	Linkage Disequilibrium
Lhx 3	LIM Homeobox Gene 3
Lhx 4	LIM Homeobox Gene 4
LIM	Lin11 and Mec3 domain
LOD	Logarithm of Odds
MAB21L	MAB21 Cell Fate Specification Gene
MHC	Myosin Heavy Chain

miRNA	Micro RNA
MMC	Motor Column Motoneuron
MNR2	MNR2 Homeobox Gene
mRNA	Messenger RNA
NAIP	Neuronal Apoptosis Inhibitory Protein
NEFH	Heavy Neurofilament subunit
Nkx2.2	Nk2 Drosophila Homolog of B Transcription Factor
NPC	Neural Precursor Cell
Olig2	Oligodendrocyte Lineage Transcription Factor 2
PAC	P1-Artificial Chromosome
PAK3	p21-Activated Kinase 3
PAK7	p21-Activated Kinase 7
PAX6	Paired Box Gene 6 Transcription Factor
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PHACTR2	Phosphatase and Actin Regulator 2
PIPOX	L-pipecolate oxidase
pMN	Motoneuron and Oligodendrocyte Precursor Domain
PNS	Peripheral Nervous System
PRPH	Peripherin
RBPMS	RNA-Binding Protein Gene with Multiple Splicing
REST	Repressor Element-1 Silencing Transcription Factor
RNA	Ribonucleic Acid
RPCI	Roswell-Park Cancer Institute
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SD	Standard Deviation
SHH	Sonic Hedgehog
SH3GLB2	Endophilin B2
SMA	Spinal Muscular Atrophy
SMAD	TGFbeta signaling protein 1
SMN	Survival Motor Neuron Gene
SNP	Single Nucleotide Polymorphism
SOD1	Cu/Zn Superoxide Dismutase
SSCP	Single Strand Conformation Polymorphism
STS	Sequence Tagged Site
TGFbeta	Transforming Growth Factor beta Family
URB1	Steroid Sensitive Gene 1
WIF-1	WNT Inhibitory Factor-1
WNT	Wingless-Type MMTV Integration Site Family
YAC	Yeast Artificial Chromosome
ZFHX1B	Zinc Finger Homeobox 1B

## 1. INTRODUCTION

Development is an intricate process during which cells pass different stages and interact to establish an organism. Disturbances in development lead to congenital diseases. Lethal arthrogryposis results from fetal immobility and is manifested by distinct features consisting mainly of joint contractures and facial anomalies. It can be caused by intrinsic or extrinsic reasons. Lethal Congenital Contracture Syndrome (LCCS) is a lethal arthrogryposis part of the Finnish Disease Heritage. LCCS leads invariably to fetal death before the 32<sup>nd</sup> gestational week. The fetuses show the Fetal Akinesia Deformation Sequence (FADS) with severely atrophic muscles. The hallmark lies in the developmental defect of the spinal cord, where degeneration of the anterior horn and descending tracts are observed. The molecular background of LCCS has not been characterized. Here we present investigations on the genetic background of Lethal Congenital Contracture Syndrome, a human disorder with an unknown defect. The characterization of the involved molecular pathways should result in the identification of a critical steps in the development of the spinal cord.

## 2. SURVEY OF THE LITERATURE

### DEVELOPMENT OF THE HUMAN EMBRYO

#### **The early stages**

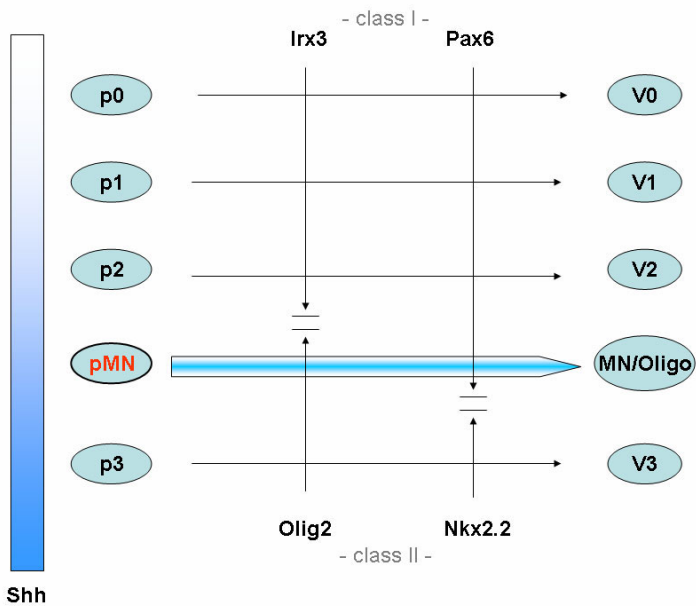
Development relies on the transient stages of cells. An embryo is being built from a single cell, that through continuous symmetric and asymmetric cell division slowly gives rise to a remarkable organism (Gilbert, 1997). The embryo absorbs information from both its genes and the surrounding environment during development, establishing symbiosis of genotype and phenotype. Fertilization is initiated by the combination of the genetic material of the sperm and egg giving rise to a new organism, the zygote. In preparation for fertilization, the germ cells undergo meiosis to reduce the number of chromosomes to haploid. The germ cells also differentiate for proper function. Fertilization occurs in the uterine tube, from where the zygote undergoes a series of rapid mitotic divisions generating a blastula while journeying to the site of implantation in the uterus (Adams et al., 1956; Egarter, 1990). Cleavage is asynchronous after the two-cell stage with one of the two blastomeres dividing to form a three cell embryo. Subsequently, embryogenesis is established, during which the developing embryo is growing and preparing for the life after birth. As the blastula reaches the 16-cell stage it is called a morula deriving from the latin word for mulberry. This period is followed by a decline of the mitotic rate and a displacement of the cells termed gastrulation starting during the second week. Gastrulation gives rise to the three germ layers, ectoderm, mesoderm and endoderm, situated from the outside progressing inside, respectively (Tam and Beddington, 1987). The embryo develops cephalocaudally meaning that gastrulation continues in caudal segments while cranial structures start to differentiate.

Formation of the germ layers is followed by a period relying on active cellular interactions, where tissues and organs are formed. The initiation of gastrulation is a highly sensitive period for extrinsic insult, but the sensitive period does continue during the whole period of organogenesis. After formation of tissues and organs, the embryo is mostly occupied with growth and maturation. These two features will continue well beyond birth and are subjective to surrounding signals.

### **Development of the central nervous system**

The nervous system is one of the earliest organ systems to differentiate from the blastula in the embryo. The central nervous system (CNS) develops from the ectoderm, giving rise to the various cell types of the mature nervous system. The CNS appears during the 3<sup>rd</sup> week as the neural plate, a thickened part of ectoderm located in front of the primitive pit. The lateral edges of the primitive pit elevate, approach and eventually fuse between 18 and 26 days of gestation to form the neural tube (Muller and O'Rahilly, 1987). By the 7<sup>th</sup> week, the cephalic portion of the neural tube has processed dilations from which the brain is developed. The caudal neural tube increases in size parallel to the growth of the embryo and differentiates to form the spinal cord (Cowan, 1979; Herschkowitz, 1988). The peripheral nervous system (PNS) develops from the neural crest cells, which have differentiated from the developing neural tube and migrated to specific regions. Migration is paramount as well in establishing the CNS and during the first 20 weeks, when neurons and glial cells migrate from the germinal sites in the subventricular zones and central canal of the spinal cord to their destinations. The ventral midline portion of the neural tube is called the floor plate and it is central in orchestrating the development of the spinal cord. The floor plate cells of the notochord secrete paracrine factors that pattern the spinal cord to distinct domains.

These domains further continue to specialize into distinct cell types. Sonic Hedgehog (Shh) is central in the patterning of the early spinal cord (Stamatakis et al., 2005). The Shh pathway is relayed by the GLI family of transcription factors (Jessell, 2000). Mutations of Shh in human have been noted as causative of holoprosencephaly (Roessler et al., 1996) and also to impair neural patterning activity (Schell-Apacik et al., 2003). The actions of Shh regulates the expression of a distinct set of homeodomain and basic-helix-loop-helix transcription factors which fall into two categories: class I proteins, that are repressed by Shh and class II proteins that are activated by Shh (Briscoe et al., 2000). These transcription factors promote the development of the pMN domain in the anterior horn of the spinal cord (see figure 1 for specification of the pMN domain by the transcription factors Pax6, Nkx2.2, Olig2 and Irx3), from which both motoneurons and oligodendrocytes develop (Jessell, 2000; Briscoe and Ericson, 2001). Some controversy has been raised on the common origin of motoneurons and oligodendrocytes and it remains unclear whether or not these two cell types share a common progenitor cell (Rowitch et al., 2002; Noble et al., 2004). The formation of both cell types is dependent on the action of Olig2, a bHLH transcription factor first identified in oligodendrocyte development (Zhou et al., 2000; Takebayashi et al., 2002). Olig2 functions in concert with other transcription factors. When Olig2 is coexpressed with Neurogenin2, motoneurons are developed from the pMN domain (Takebayashi et al., 2002). As development proceeds a little further, the expression of Neurogenin2 is downregulated and Olig2 is coexpressed with Nkx2.2, oligodendrocytes are generated from the same pMN domain (Zhou et al., 2001).



**Figure 1.** The formation of the motoneuron precursor domain and the postmitotic cells determined by the expression of specific transcription factors. P represents for precursor domains, V for postmitotic cells.

In addition to hedgehog-related signaling, other components play specific roles during the development of the spinal cord. Wnt signaling is essential for the maintenance of neural progenitor cell proliferation, for controlling the size of the progenitor population, and to influence the decision of progenitor cells to differentiate or proliferate (Zechner et al., 2003). Further, Wnt signaling has been identified as dorsal factors that directly inhibit oligodendrocyte development. The addition of a Wnt antagonist rmFz-8/Fc, increases the number of immature oligodendrocytes in vitro spinal cord explants, demonstrating that endogenous Wnt signaling controls oligodendrocyte development (Shimizu et al., 2005). FGF signaling is involved in motoneuron development by controlling Hox expression (Dasen et al., 2003).

### **The identity of motoneurons**

As motoneurons exit the cell cycle, they acquire columnar subtypes revealed by the position of the cell soma in the spinal cord and by the pattern of peripheral projections (Landmesser, 1978; Tosney et al., 1995). There are five columnar groups of motoneurons; two are found in the median motor column supplying to axial muscles, one set is the pre-ganglionic autonomic motoneurons and the remaining two groups are found within the lateral motor column at limb levels of the spinal cord (Landmesser, 1978; Prasad and Hollyday, 1991). The medial lateral motor column motoneurons supply ventrally derived limb muscles whereas lateral motor column motoneurons project axons to dorsally derived limb muscles (Landmesser, 1978; Tosney et al., 1995). The columnar identity of motoneurons can be distinguished by the expression profile of LIM homeodomain transcription factors. Islet-1 expression is required for the generation of all spinal motoneurons (Pfaff et al., 1996), Lhx 3 and Lhx4 denote medial MMC identity (Sharma et al., 1998). The expression of the homeodomain protein MNR2 persists in motoneurons of the medial MMC, whereas HB9 is expressed more widely in somatic motoneurons. The downregulation of MNR2 and HB9 expression is needed for the generation of preganglionic autonomic motoneurons (William et al., 2003).

### **Glial cell origin**

Glial cell maturation partly parallels and overlaps neuronal differentiation. The precursor cells mature in the specific domains and aggregate with neurons to form the specific brain regions. The number of glial cells is regulated such, that the proportion of neurons to glia is maintained at 1:10 (Sommer and Rao, 2002). The two main classes of glial cells, the oligodendrocytes and astrocytes, serve different functions in the nervous

system. Astrocytes are more versatile and they can be distinguished by the expression of Glial Fibrillary Acidic Protein (GFAP) (Eng et al., 1971; Bignami et al., 1972). Other glial cell types also exist including radial glia, pituitary glia and olfactory ensheathing cells.

As the precursor cells in the specific domains differentiate, they generate more restricted precursors that undergo progressive maturation. According to this model at least three types of restricted precursors exist: the neuronal precursors, the glial precursors and the neural crest precursors (Rao, 1999). In reality, additional precursors are likely to exist as several other precursors have also been identified (Rowitch et al., 2002). These include oligodendrocyte and type-2 astrocyte precursors (Raff et al., 1983), glial restricted precursors (Rao et al., 1998), polydendrocytes (Nishiyama et al., 1997), motoneuron-oligodendrocyte precursors (Lu et al., 2002; Sun et al., 2003), astrocyte precursor cells (Liu and Rao, 2004), and white matter precursors (Roy et al., 1999). How these precursors relate to others remains unclear. Multiple developmental pathways may exist, however, revision to current models is needed.

### **Programmed Cell Death during development**

As motoneurons mature, they start sending axons out to the periphery during the 5<sup>th</sup> week (Sariola et al., 2003). When they reach their target muscles, a period of programmed cell death (PCD) ensues, during which superfluous or nonfunctional motoneurons are eliminated. Approximately 50% of the developed motoneurons undergo PCD. Most cell types of the CNS encounter a similar destiny and it has been shown that PCD is an essential component of development (Oppenheim, 1991; Sendtner et al., 2000; Sommer and Rao, 2002). Much of developmental PCD is executed via apoptosis (Vaux and Korsmeyer, 1999). Central components in the

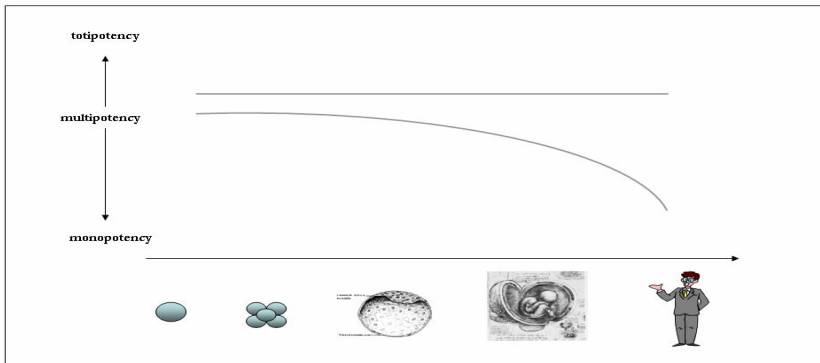
apoptosis cascade are the caspase family of proteases (Troy and Salvesen, 2002). The regulation of caspases is complex and is achieved by interactions of pro- and antiapoptotic regulators (Danial and Korsmeyer, 2004). Shh has been implicated as a regulator of apoptosis, where too much or too little Shh expression results in an increase of apoptosis (Oppenheim et al., 1999; Charrier et al., 2001; Paganelli et al., 2001).

Survival of motoneurons through the period of PCD requires activity in the form of both afferent input and efferent response, trophic factors and cell interaction between the neurons and adjacent Schwann cells (Hamburger, 1934; Levi-Montalcini and Levi, 1942; Riethmacher et al., 1997). In addition, motoneuron PCD can be experimentally reduced by various factors including steroids (Nakamizo et al., 2000), growth factors (Ang et al., 1992), neurotrophic factors (Zhao et al., 2004) and neuromuscular blocking agents (Oppenheim, 1991).

## **Stem Cells**

As cells differentiate, they reach a state of full function that is considered irreversible. The next step of differentiation is apoptosis, which eliminates the cell. These cells, if needed, are compensated for by less differentiated cells that are capable of self-renewal and retain the potential to differentiate depending on their specific environments. These cells are called stem cells. In the early embryo, the stem cells are uncommitted and before the blastocyst stage they are totipotent. The potency of cells describes the amount of options the cell has to differentiate. As development proceeds, the fate of the cells becomes somewhat more restricted (see figure 2 for schematics of cell potency as a function of time). According to current knowledge, adult stem cells have a pluripotent capacity and their fate is dependent on regional and developmental

contexts. The definition of stem cells is based on their capacity to self renew and differentiate under the appropriate stimuli. It has however been suggested that stemness is a state in the life cycle of the cell (Zipori, 2004). What actually determines the outcome is the environment in which the cell resides. Stem cells are dependent on their niche, which keeps them undifferentiated (Doetsch, 2003). Creating of these niches for stem cells in vitro is a challenge to be solved.



**Figure 2.** Potency of progenitor cells as a function of developmental stage. Straight line indicates ES cells, curved line adult stem cells.

Each tissue is constructed from tissue specific stem cells which respond to activating signals when needed. As differentiation proceeds, the number of stem cells is reduced. In regions of ongoing development, small numbers of stem cells are still encountered in the adult. Adult tissues also contain cells that display pluripotent capacity in vitro cultures. Multipotential adult progenitor cells capable of differentiating into mesodermal derivatives, ectodermal cells and to endodermal products have been found from bone marrow (Jiang et al., 2002). Further, skin progenitor cells have been shown to differentiate into mesodermal derivatives and neuroectoderm (Toma et al., 2001). Thus, pluripotent stem cells prevail in the adult organism.

Embryonic stem cells can in contrast to adult stem cells be amplified for long periods in vitro. An embryonic stem cell stems from the blastocyst and hence can generate all functional adult cell types. Much anticipation

has therefore been laid on tissue replacement therapies for various conditions, including myocardial disorders, diabetes and neurological disorders (Mayhall et al., 2004; Davani et al., 2005).

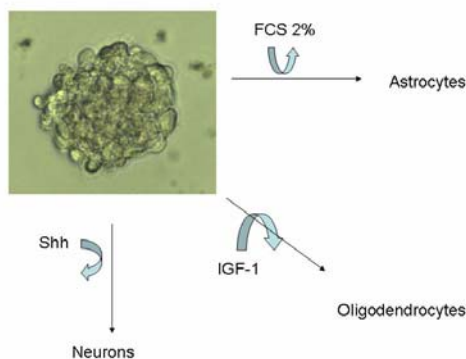
The dark side of stem cells is their close resemblance to cancer cells. Cancer cells are defined by their properties to reproduce beyond normal restraints and to invade regions of other cells. In these terms, stem cells bear close resemblance to cancer cells, and indeed several solid cancer forms have been found to originate from stem cells (Reya et al., 2001; Pardal et al., 2003; Singh et al., 2004). Thus, for therapeutics to be safe, we need to understand how stem cells are controlled before interventions become daily practice. To turn the coin around, by understanding stem cell characteristics, we gain much in the knowledge of cancer cells.

### **Neural stem cells**

Neural stem cells or neural precursor cells (NPC) contain the blueprint for constructing the individual cells of the CNS (Gage, 2000). The hardwiring of the connections between the network of neural cells is influenced by activity on the other hand. Early studies led to the isolation of progenitor cells with stem cell like capacities from the embryonic CNS and PNS (Reynolds et al., 1992). Thereafter, NPCs have been identified from various regions of the nervous system (Mignone et al., 2004). The isolation of NPCs from adult tissues opened up new avenues for the utilization of these pluripotent cells. Adult NPCs are found to reside in at least the hippocampus and the subventricular zone and in the spinal cord (Emsley et al., 2005). Much hope has been set on NPCs in the therapeutic field, and time will tell how successful they will be (Lindvall et al., 2004). Neural precursor cells have already shown some of their potential in modeling neurological disease (Jakel et al., 2004). Early studies reported the capacity of transdifferentiation of adult stem cells, including neural stem cells, but

recent studies have not succeeded in proving these claims (Galli et al., 2000). However, stemness has also been described as a certain state of cells and the state would thus govern what the outcome of the cells is at later stages (Zipori, 2004). The creation of an appropriate niche remains precarious despite recent progress in the field (Shen et al., 2004). Thus, the true nature of stem cells is still warrants further delineation.

Neural precursor cells can be isolated directly from embryonic or adult mammalian CNS by selecting cells responsive to mitogens. NPCs enrich in the presence of Epidermal Growth Factor (EGF) and Fibroblast Growth Factor (FGF) (Vescovi et al., 1999). These mitogens keep most of the NPCs undifferentiated. To increase the amount of undifferentiated human NPCs, Leukemia Inhibitory Factor (LIF) can be added to the culture (Wright et al., 2003), but is not obligatory for the enrichment of NPCs. Once the mitogens are withdrawn, the NPCs initiate differentiation. Differentiation can be expedited and instructed by various signals (see figure 3 for instructed differentiation of NPCs). Fetal Calf Serum (FCS) can be used for the generation of neurons and astrocytes from NPCs and IGF-1 instructs them to generate oligodendrocytes (Hsieh et al., 2004).



**Figure 3.** Differentiation of neurosphere cells by instruction of external factors.

Differentiation of NPCs isolated from the subventricular zone of postnatal day 7 mice has been studied in detail by transcript analysis, where distinct genetic programs of NPC differentiation were found (Gurok et al., 2004). Interestingly, that study found almost no overlap between genes identified from previous studies performed by different methods using rat derived NPCs (Zhou et al., 2001; Wen et al., 2002). Similar studies using fetal human samples have not been performed.

Neural precursor cells have been utilized in the study of Down syndrome, where the gene expression of neuronal target genes was analyzed with neurosphere derived material (Bahn et al., 2002). It was hypothesized that the disease mechanism of Down syndrome is present in the neural stem cells, where the authors describe a set of genes whose mRNA content was extremely low in the case neurospheres. This set of genes included transcription factors, adhesion molecules and synaptic components, all of which are concerned with neuronal maturation in some stage. All of the genes are also under the control of neuron-restrictive silencing factor REST (repressor element-1 silencing transcription factor). To test the validity of these results, the authors differentiated the neural stem cells, where only a diminutive proportion of the Down syndrome derived cells differentiated into neurons as compared to the controls. This study demonstrated the effect of neural stem cells in the investigation of developmental neurologic diseases. Fragile X syndrome has also been studied by the use of neural progenitor cells from an adult male with fragile X syndrome (Schwartz et al., 2005). The fragile X derived progenitor cells were cultured and differentiated into neuronal and astrocytic lineages. The expression of FMRP was reduced in neural cell culture in the patient as compared to the unaffected control, demonstrating the use of neural stem cells in the study of neurologic

diseases. Further studies using human neural stem cells are still under way and the true potential of neural stem cells in these are awaiting discovery.

### **Regulation of gene expression**

Cell and tissue interaction during development relies on signaling. Hormones act over long distances, whereas paracrine factors influence the fate of their vicinity. The coordination of a group of cells changing the behavior of adjacent cells making them change in some way (eg differentiate) is termed induction. The proximate interactors are comprised of a limited set of proteins that the body uses for numerous processes. These proteins can be grouped into four major families based on their structures: (1) the fibroblast growth factor family (FGF)(Wilkie et al., 1995), (2) the Hedgehog family (Ingham and McMahon, 2001), (3) the Wntless (Wnt) family (McMahon et al., 1992), and (4) the transforming growth factor beta superfamily (TGFbeta) (Kingsley, 1994). Tissue interactions have been intensely studied especially during tooth and kidney formation, where epithelial-mesenchymal interaction is central (Thesleff et al., 1989; Sariola et al., 1991). The response of a group of cells depends on its genome.

The regulation of gene expression is accomplished on several levels. Genes can be transcribed differentially, nuclear RNAs can be regulated by selection of which get into the cytoplasm and become mRNA, mRNAs can be selected for translation into proteins and proteins can be modified differentially (Derman et al., 1981; Darnell, 1982). Differential gene transcription can be regulated by transcription factors and regulative elements.

Transcription of genes is a highly regulated process controlling the interaction of transcription factors with cis-regulatory elements of DNA

and additional co-factors (Kadonaga, 2004). Trans-acting proteins bind cis-regulatory elements and control the rate of transcription of individual genes. Transcription factors have proven to be central during development and it has been estimated that one transcription factor exists for ten genes in human (Levine and Tjian, 2003).

Non-coding small endogenous RNAs have been discovered as important regulators of gene expression. The first small non-coding RNAs found were the small temporal RNAs *lin-4* and *let-7*, identified in *Caenorhabditis elegans* as key regulators of developmental timing (Lee et al., 1993; Reinhart et al., 2000). According to current knowledge microRNA (miRNA) genes constitute about 1-2% of the known genes in human and miRNA genes are known to be important regulators of translation and stability of target mRNAs and it has been estimated that they regulate protein production for 10% of all human genes (John et al., 2004). Neuronal differentiation has been reported to be influenced by small, noncoding double-stranded RNAs where the mechanism appeared to be mediated by a dsRNA/protein interaction (Kuwabara et al., 2004). The involvement of miRNAs have also been implicated in diseases such as Spinal Muscular Atrophy and Fragile X syndrome (Caudy et al., 2002; Ishizuka et al., 2002; Dostie et al., 2003).

Regulation of gene expression is a context dependent dynamic process that requires the interaction of several proteins. Transcription factors can both activate and repress the transcription of any given gene determined by the surroundings and its own concentration and physical form (Ma, 2005). The expression of a large number of genes can be studied by microarray methods. RNA microarray studies have proven their validity in global transcript analysis in many different fields from study of

pathogenesis to classification of malignancies and diseases (DeRisi et al., 1996; Pomeroy et al., 2002).

## **HUMAN GENETICS**

The human haploid genome contains 3 billion base pairs of DNA harbored in 22 autosomal and one sex chromosome. DNA is also present in mitochondria in addition to the chromosomes. Variations in DNA may contribute to disease and based on the role of genetic factors the diseases can be roughly divided into categories: (i) monogenic disorders, (ii) multifactorial disorders, (iii) mitochondrial disorders, (iv) chromosomal aberrations and (v) acquired somatic genetic disorders. Our present understanding of genetics lies on the foundations of work by Gregor Mendel, who studied the inheritance of genes in the garden pea, establishing the basic laws of inheritance (Mendel, 1866). Experiments by Oscar Avery showed that a nucleic acid was the chemical basis for specific heritable transformations in bacteria (Avery et al., 1943). Avery's discovery sparked research into the nature of DNA and almost a decade passed before Francis Crick and James Watson discovered the chemical structure of DNA which by the order of its bases, encodes the genes (Watson and Crick, 1953). Watson and Crick based their proposal of the DNA structure on the work of Rosalind Franklin who had used X-rays to discover that DNA had phosphate groups on the outside and that DNA existed in two forms (Maddox, 2003).

## **The Human Genome Project**

The Human Genome Project (HGP) was initially launched in 1990 with the goal to obtain the euchromatic sequence of the human genome. The initial approach was biphasic, with mapping of the human genome to provide a scaffold for genome assembly and subsequent sequencing (Bentley et al., 1998; Deloukas et al., 1998). To facilitate and confirm the mapping approach, both human and mouse genomes were mapped and to gain knowledge before the actual sequencing effort, smaller and less complicated genomes were sequenced (Goffeau et al., 1996; Adams et al., 2000; Waterston et al., 2002). While the approaches gained success, the ultimate goal seemed within reach and finally, in February 2001, the International Human Genome Sequencing Consortium and Celera Genomics both provided a draft sequence as a first overall view of the human genome (Lander et al., 2001; Venter et al., 2001).

Both reported draft sequences were far from complete and the finished sequence was published three and a half years later (Consortium, 2004). Although this sequence was published as complete, covering 99% of the euchromatic genome, it still contained gaps and requires further complementation. Most of the gaps are associated with segmental duplications requiring novel methods for solving them.

The HGP opened the door to a new era in biomedical research, where a collaborative effort generated and characterized systematically a vast domain of biological knowledge. However, as most knowledge paves way for further questions, does the sequence lay several challenges ahead. All genetic polymorphisms need to be systematically identified to enhance their role in the study of diseases. Every functional element, including genes, proteins, regulatory controls and structural element needs systematic characterization and annotation. And finally, the functional

modules for genes and proteins need systematic identification. These issues require the comprehensive studies of numerous human genomes, comparative analyses with additional genomes and improved comprehension of expression, localization and interaction of functional modules.

### **Positional cloning to find disease genes**

Identification of disease genes based on their location in the genome is called positional cloning (Collins, 1992). The approach consists of several steps, starting off with definition of the disease phenotype and collection of an appropriate family material, followed by a genome wide scan and the identification of the disease susceptibility locus by linkage methods, advancing to linkage disequilibrium and haplotype mapping to further define the disease locus. The first human gene behind a genetic disease identified by a positional cloning effort was chronic granulomatous disease in 1986 (Royer-Pokora et al., 1986; Royer-Pokora et al., 1986), but positional cloning became a standard procedure only several years later (Collins, 1995). The late 1980s witnessed the evolution of the needed technologies e.g. polymerase chain reaction and the HGP for high throughput positional cloning (Saiki et al., 1985; Mullis et al., 1986; Collins and Galas, 1993).

In genetic mapping, loci in the genome are studied in relation to each other. Two genetic loci are linked if they are inherited together more often than expected by chance. Linkage analysis tests two assumptions; two loci are linked with a given recombination fraction or they are not linked. The result of linkage analysis is expressed as a lod score, meaning the logarithm to the 10 base of the ratio of the two assumptions tested in linkage analysis. Linkage disequilibrium denotes the non-random distribution of alleles at linked loci. In linkage disequilibrium, the region adjacent to a disease marker will be overrepresented by a specific allele. In the disease

chromosomes, the length of the chromosomal region in linkage disequilibrium depends on both the age of the mutation and the recombination frequency of the region of interest (Peltonen et al., 1995). Linkage disequilibrium offers shortcuts in the localization of disease genes in consanguineous families and in the search for chromosomal segments in isolated founder populations (Nikali et al., 1995). When the disease locus has been mapped, positional candidate genes are identified from the genomic sequence and positional cloning ends in the identification of the disease associated mutation. Prior to the availability of genomic sequences, an intermediate step of physical mapping was needed to establish a frame for identification of positional candidate genes.

The HGP initiated with the mapping of the human and mouse genomes to facilitate the study of inherited diseases in addition to providing the scaffold for sequencing. The building of physical maps in the late 1990s utilized the isolation of artificial chromosome clones in the form of Yeast Artificial Chromosomes (YAC) (Burke et al., 1987), P1-Artificial Chromosomes (PAC) (Ioannou et al., 1994) and Bacterial Artificial Chromosomes (BAC) (Shizuya et al., 1992). The process begun with physically mapping these clones to the desired chromosomal region and thereafter organizing the clones relative to each other. A well utilized technique was Fluorescence In-Situ Hybridization, where the discovery of the use of extended DNA fibers paved way for increased resolution of the physical maps (Heiskanen et al., 1995). The ends of the clones were sequenced, which aided in the positioning of first candidate genes to the critical DNA region. As the HGP accelerated and made progress, preliminary sequences became available for gene prediction. Ab initio gene prediction software were set to high sensitivity and thus initial estimates of gene number in the genome may have been overly optimistic. In addition to gene prediction, the obtained genomic sequences were used for

construction of novel polymorphic genetic markers, which then might help in the further restriction of the targeted genetic region. As the physical map of the critical DNA region was finished, positional candidate genes were analyzed for disease associated mutations by way of e.g. motility assays, sequencing and expressional analyses.

Nowadays positional cloning could be denoted a positional candidate gene approach, as the regional candidate genes are well listed in the databases and can be directly analyzed based on their position under the linkage peak.

At times, the disease gene identification by positional cloning is not as straightforward as expected. Consequences of the mutations in non-coding regions are more difficult to detect than those found in coding regions of the DNA. A good example of such a mutation was the Finnish founder mutation in diastrophic dysplasia, a GT-to-GC transition in the donor splice site of the 5-prime untranslated exon of the disease gene. The mutation acts by severely reducing mRNA levels (Hastbacka et al., 1994; Hastbacka et al., 1999). Duplications and larger inversions may lead to the disruption of a gene or its regulatory element (Chen et al., 2004). Large chromosomal rearrangements are well documented to be associated to monogenic chromosome abnormalities and malignant transformation (Kolomietz et al., 2002). Hence, caution should be exercised when excluding mutations by traditional methods as they may not expose these alternative mechanisms behind the disease.

### **The Finnish Disease Heritage**

In the light of current knowledge, the ancestors of the modern Finns have arrived both from eastern Ural some 4000 years ago and from a more

stable immigration of groups arriving from the South and West during some thousand years (Kittles et al., 1998; Norio, 2003). During centuries, the forefathers inhabited the coast lines, and only during the 16<sup>th</sup> century did the migration reach the northern parts of Finland as the Swedish king Gustavus Wasa ordered migration to populate especially the Russian border (Cornell et al., 1966). Internal immigration was also propelled by the need for new land and the avoidance of taxation by the Crown. The slow migratory movements in a proportionally vast country lead to the emergence of small regional isolates and village societies. Thus, the relatively small gene pool of the ancestors was spread unevenly, and lead finally to the regional clustering of heterozygote disease gene carriers and the incidence of autosomal recessive diseases in their offspring. Random drift was powerful in small founder populations, while some mutations got enriched, the incidence of others declined markedly.

In a genetic isolate, one mutation can be assumed behind each disease that has enriched in the population. In other words, the genetic region of a distinct disease is derived most often from one early Finn carrying the gene defect in his or her genome. This leads to a definitive technical advantage in the identification of mutations behind rare diseases using the Finnish genome. The peculiar enrichment of mostly autosomal recessive diseases (carrying one major mutation in the Finnish genome) became known as the Finnish Disease Heritage. Early reports deviated from today's criteria of the FDH. Poverty and socioeconomic factors leading to inadequate hygienic conditions in prewar Finland in the 19<sup>th</sup> and 20<sup>th</sup> century were reasons for high prenatal mortality. Thus, diseases leading to early childhood death were not recognized as own entities. Still, diseases that belong to the modern FDH-definition, such as Diastrophic Dysplasia were described (Lahdensuu, 1939). However, some of the diseases thought to have enriched in Finland (Gripenberg, 1953), were in reality prevalent

due to non-genetic causes. The first publications containing the FDH steps, the so called Perheentupas steps, appeared in the 1970s (Norio et al., 1973).

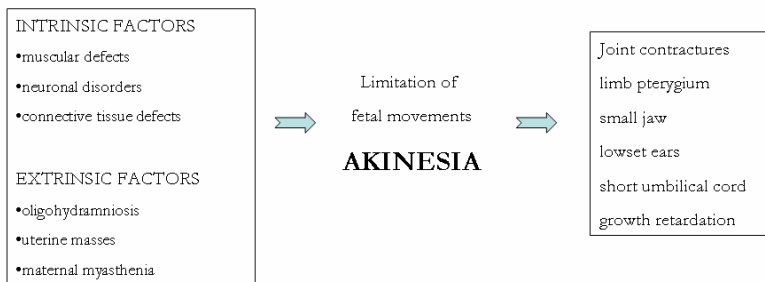
Examples of success stories where the Finnish genome has been utilized to study the pathomechanism of disease include Polycystic lipomembraneous osteodysplasia with sclerosing leucoencephalopathy (Paloneva et al., 2002; Klunemann et al., 2005) and variant late neuronal ceroid lipofuscinosis (Savukoski et al., 1998; Holmberg et al., 2004). PLO-SL is good model for frontal lobe dementia and is intriguing due to mutations in two genes part of the same signaling cascade. VLINCL on the other hand represents a disease with a fairly young mutation where most families originate from a small village society in southern ostrobotnia.

Nowadays the Finnish Disease Heritage is composed of 36 disorders (Norio, 2003). The genetic loci has been established for 34 of these and the gene has been characterized for 33 ([www.findis.org](http://www.findis.org)). The FDH contains two lethal arthrogryposes, Lethal Congenital Contracture Syndrome (LCCS) and Lethal Arthrogryposis with Anterior Horn Cell Disease (LAAHD), which have not been so far found elsewhere.

## **LETHAL ARTHROGRYPOSIS**

Arthrogryposis is derived from the Greek words arthron for joint and gryposis for crooked and is used to describe the phenotype of joint contractures. Lethal arthrogryposis is a clinical description of a phenotype of heterogeneous etiology. It represents the end-point of akinesia during pregnancy and can be due to neurological, connective tissue or extrinsic reasons schematically presented in figure 4 (Moessinger, 1983). The

descriptive findings consist of distinct facial features, joint contractures and a short umbilical cord. The facial features include an expressionless face, a petite mouth, a small receding chin and lowset poorly lobulated ears. Autopsy reveals pulmonary hypoplasia. At times, the joint contractures are accompanied by pterygia (Hall, 1986). The severity of the features depends on the time of onset of the fetal akinesia during pregnancy (Moerman et al., 1990).



**Figure 4.** Development of the Fetal Akinesia Deformation Sequence according to Spranger et al 1982

## Epidemiology

Estimations of the frequency of arthrogyrosis in different populations have been variable depending on the definition of the condition and the type of study. Multiple congenital contractures (arthrogyrosis) has been estimated to occur with a frequency of about 1:3000 live births and about 1 in every 200 newborns is estimated to be born with some form of contractures (Hall, 1997; Hall, 2002). Silberstein and co-workers reported a birth prevalence of 1 in 12000 for arthrogyrosis multiplex congenita in Western Australia from a cohort born between 1980-1993 (Silberstein and Kakulas, 1998). Darin and co-workers reported the birth prevalence to be 1 in 5100 in a cohort from western Sweden born between 1979 and 1994 (Darin et al., 2002). However, none of these studies encountered very early onset forms associated with lethality in utero.

The incidence of anterior horn cell disorders associated with arthrogryposis and early fetal demise is poorly known. In one study from Finland, the incidence of LCCS only was estimated to be 1:12 700-1:19 000 births (Vuopala and Herva, 1994). In another study on LAAHD, a rough estimate of the incidence to be at least 1:50 000 was given (Vuopala et al., 1994). SMN-gene analyses were not done. The figures for all types of anterior horn cell diseases with arthrogryposis are lacking.

### **Pathogenesis**

The reasons behind intrauterine fetal akinesia can be both intrinsic and extrinsic. The clinical picture remains fairly uniform despite the great number of conditions causing the disorder. Multiple explanations for this exists. Normal joint and adjacent tissue development is dependent on active fetal mobility (Drachman and Coulombre, 1962). As the joints become immobile, extra connective tissue develops around the joint, further limiting joint movement (Hall, 1997). The length of the umbilical cord is dependent on the proportion to the tractile tension which varies according to fetal size and mobility (Katsumata et al., 1991). Both fetal breathing and the volume of amniotic fluid are critical to the development and growth of the lung (Wigglesworth, 1997). The facial features are thought to be caused by disturbed fetal swallowing that accounts for the hypoplastic jaw (Moessinger, 1983). The ear abnormality stems from defective muscle function in the small ear muscle (Zerin et al., 1982).

Fetal causes to akinesia can be grouped into anatomical locations: (I) muscular defects, (II) neuronal disorders and (III) connective tissue defects (Spranger et al., 1982; Moerman et al., 1990). The most common

cause to fetal akinesia and lethal arthrogryposis is neuronal dysfunction (Banker, 1986; Hageman et al., 1987; Hall, 1997).

External causes of arthrogryposis can be divided into: space limitations, intrauterine circulation and maternal disorders (Hall, 1997).

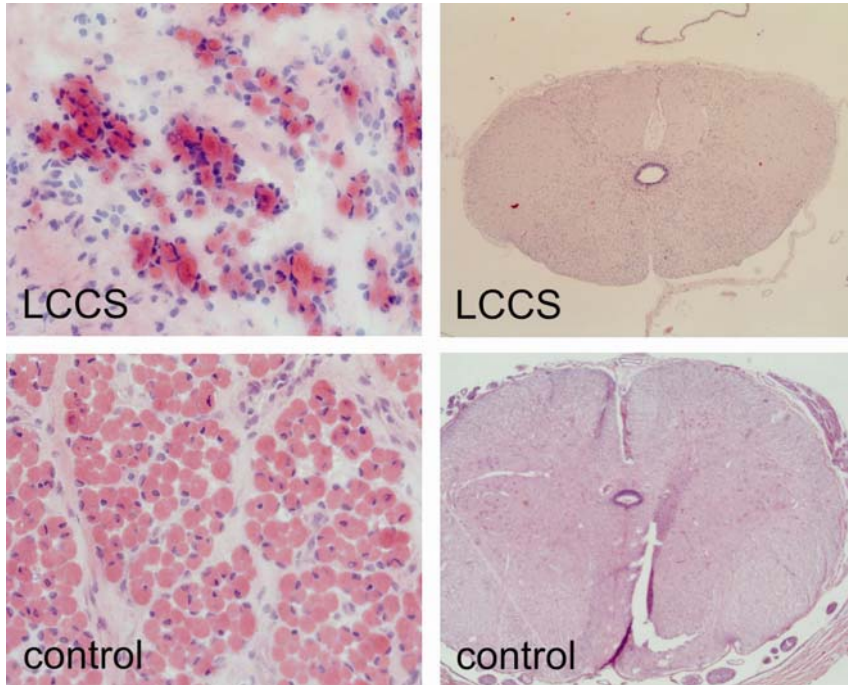
### **Lethal Congenital Contracture Syndrome**

Lethal Congenital Contracture Syndrome is a fetal motoneuron disease characterized by the Fetal Akinesia Deformation Sequence (Spranger et al., 1982). The fetuses are small for gestational age (<2SD) and present with a typical arthrogryposis phenotype (fig 5 displays a 23 week old LCCS fetus with all phenotypic features) consisting of multiple joint contractures, skeletal muscle atrophy with stick-like extremities, distinct facial features and hydrops (Herva et al., 1985). The facial features consist of ocular hypertelorism, lowset and posteriorly angulated ears and a hypoplastic jaw. Tissue pathology is characterized by severe muscle atrophy, lung hypoplasia and degeneration of the anterior horn of the spinal cord as the hallmark of the syndrome (see fig 6). The CNS is normal above the decussatio pyramidalis as compared to age-matched controls (Herva et al., 1988). The skeletal muscle atrophy is considered neurogenic as embryonic and neonatal myosin heavy chain (MHC) isoforms are expressed, but the intrafusal muscle spindles are lacking. Motor innervation is needed for differentiation of intrafusal fibers (Soukup et al., 1990; Soukup et al., 1993) and the lack of intrafusal fibers support the idea of defective motoneuron innervation in LCCS (Vuopala et al., 1996). Fetal akinesia and hydrops can be observed in early sonography during pregnancy, and LCCS invariably leads to death of the affected fetuses before the 32<sup>nd</sup> gestational week. The incidence of LCCS has priorly been estimated to 1:19000 in Finland. LCCS is inherited in the autosomes, recessively.

The changes in LCCS spinal cord have been interpreted as degenerative, with the demise of motoneurons partly overlapping the period of natural spinal PCD. Indeed, LCCS is most often initially recognized due to fetal immobility during ultrasound examinations. The reason for prenatal death of LCCS fetuses remains unknown, but may depend on evolutionary factors.



**Figure 5.** LCCS fetus displaying all the characteristic features including distinct facial features, multiple contractures and atrophic muscles.



**Figure 6.** Histological sections of LCCS and age-matched controls of quadriceps femoris muscle and lumbar spinal cord. In the upper row are sections from a 23+4 week LCCS fetus shown, the lower row displays identical sections from the age-matched control. LCCS muscle shows scattered myotubes without clear organization. The spinal cord shows marked reduction at the anterior part with degeneration of the ventral and lateral columns (Pakkasjärvi et al, 2005).

### **Lethal Arthrogryposis with Anterior Horn Cell Disease**

Lethal Arthrogryposis with Anterior Horn Cell Disease (LAAHD) shares many similarities to LCCS. The fetuses may survive delivery, but die within one month. The neuropathological findings closely resemble those of LCCS. The locus for the LAAHD gene is currently unknown, but it is also inherited in the autosomes recessively (Vuopala et al., 1996). The incidence of LAAHD is at least be 1:50 000 in Finland (Vuopala et al., 1995).

## **Spinal Muscular Atrophy type I**

The neuropathological findings of LCCS resemble those of type I Spinal Muscular Atrophy (Hoffmann, 1893; Werdnig, 1894; Emery, 1971; Pearn, 1980). SMA type I is the most common autosomal recessive neurodegenerative disorder of childhood. It is at times encountered with arthrogyposis. SMA is characterized by degeneration of motoneurons associated with muscle paralysis and atrophy. The most common form of SMA involves defects in the SMN-gene on chromosome 5q, where the severity of the disease depends on the defects in the neighboring NAIP-gene (Lefebvre et al., 1995; Mahadevan et al., 1995; Roy et al., 1995) and is at times associated with fetal arthrogyposis. Deletion of both copies of the SMN genes and the NAIP gene leads to most severe form of SMA designated SMA-0 (Burglen et al., 1996). The SMA-disease spectrum has proven to be heterogenic, and distinct entities can be found in SMA with pontocerebellar hypoplasia, and SMA caused by defects in the IGHMBP2-gene leading to SMA with early diaphragmatic affision called SMARD1 (MIM 604320), which usually is encountered with arthrogyposis (Rudnik-Schoneborn et al., 2003). One x-chromosomal form is also known that presents with arthrogyposis (Greenberg et al., 1988).

The incidence of SMA in Finland has been estimated to 1:15 000 (Ignatius, 1992).

## **MOTONEURON DISEASES**

Amyotrophic Lateral Sclerosis (ALS) is the most common motoneuron disease. The hallmark is the dysfunction and death of neurons in the motor pathways leading to spasticity, hyperreflexia, generalized weakness, muscle atrophy and paralysis (Mulder et al., 1986). ALS is clinically and genetically heterogeneous. Linkage has been found to several genetic loci

for familial ALS, which constitute about 5-10% of cases (Hand and Rouleau, 2002). Several predisposing genes have also been identified to the most common sporadic form of ALS. Among the familial cases, some 20% are associated with mutations in the Cu/Zn superoxide dismutase (SOD1) on chromosome 21q22.1 (Rosen et al., 1993). Sporadic cases are occasionally also encountered with novel mutations in SOD1 (Jones et al., 1993). Susceptibility is further associated with deletions or insertions in the gene encoding the heavy neurofilament subunit NEFH (Figlewicz et al., 1994), by deletions in the gene encoding peripherin PRPH (Gros-Louis et al., 2004) or by mutations in the dynactin DCTN1 (Munch et al., 2004). Several other ALS loci have also been identified. The gene mutated in ALS2, resides on chromosome 2q33 (Hadano et al., 2001; Yang et al., 2001), ALS3 on chromosome 18q21 (Hand et al., 2002), juvenile ALS or ALS4 on chromosome 9q34 in relative proximity to the LCCS region (Chen et al., 2004). ALS5 is linked to chromosome 15q15-q21 (Hentati et al., 1998), ALS6 to chromosome 16q12 (Abalkhail et al., 2003; Ruddy et al., 2003), ALS7 to chromosome 20q13.33 (Sapp et al., 2003) and ALS8 to chromosome 20q13.33 (Nishimura et al., 2004; Nishimura et al., 2004).

Motoneuron diseases have sometimes been described as systemic diseases with primary manifestation in the spinal cord. What then makes motoneurons so vulnerable to extrinsic noci? Motoneurons are large post-mitotic cells that can extend the axons in length over 1m. They have a high metabolic rate and their cytoskeletons face large demands to sustain stresses encountered (Cookson and Shaw, 1999). Motoneurons possess large amounts of glutamate receptors and they lack calcium binding proteins and they express high levels of SOD1 rendering them vulnerable to oxidative stress (Shaw and Ince, 1997). So in addition to the complex and delicate development of motoneurons, they contain special features which makes them vulnerable to cell death.

### **3. AIMS OF THE STUDY**

- I. Localize the LCCS gene in well characterized Finnish families to a chromosomal region
- II. Characterize the critical DNA-region and analyze the candidate genes for the disease mutation
- III. Decipher the molecular pathways disturbed in the pathogenesis of LCCS
- IV. Study the development of neurons and cells of glial lineage and the role of neural stem cells during pathogenesis of LCCS

## 4. MATERIALS & METHODS

All methods and materials applied to this thesis are listed below and cited according to the original publications in which they appear.

### **Ethical considerations**

All samples were collected after written informed consent from the parents. The project has been approved by the ethical committee of Helsinki University Central Hospital, Hospital District of Helsinki and Uusimaa, and all work has been done in accordance with the Helsinki declaration.

### **Patients**

Initially, the family material consisted of 15 Finnish LCCS families, a total of 68 individuals, of whom 26 were affected. During the course of the study 13 new cases were included. The diagnosis of patients with LCCS has been confirmed in autopsy and histologic examination of the spinal cord. The material used in each publication is presented in the respective materials section of the corresponding work.

The LAAHD family material consisted of 6 Finnish families, a total of 14 individuals, of whom 6 were affected. No new cases were included during the study. All of the LAAHD patient DNA was isolated from paraffin embedded sections. The diagnosis of LAAHD patients has been confirmed in autopsy and histologic examination of the spinal cord.

<b>Method</b>	<b>Original Publication</b>
DNA marker analysis	I
Linkage analysis	I
Linkage Disequilibrium Analyses	I
Fluorescence In Situ Hybridization	I
DNA Sequencing	I
RNA Isolation	II, III
Genechip Processing	II, III
Gene Expression Data Analysis	II, III
cDNA Synthesis	II
Reverse Transcriptase-PCR	II, III
Real Time Reverse Transcriptase- PCR	II
Transcription Factor Analysis	II
Neural Precursor Cell Culture	III
Cryopreservation	III
In Vitro Differentiation of Precursor Cells	III
Immunocytochemistry	III
Apoptosis assay	III
Proliferation assay	III

**Methods not described in the individual publications are presented below:**

**Bacterial clone contig**

The PAC (Pieter de Jong, Roswell Park Cancer Institute, Buffalo, NY) and BAC (Genome Systems Inc.) libraries were screened by PCR using primers for known polymorphisms and STSs in the region. New markers were generated by chromosomal walking. Positive clones were colony purified

and grown overnight in TB in the presence of the appropriate antibiotic. DNA was extracted by alkaline lysis.

### **Radiation Hybrid mapping**

A whole genome radiation hybrid (rh) panel was analyzed (Research Genetics). Markers were amplified by PCR from each rh well. PCR was performed according to standard procedure and products were visualized on 1-2% agarose gels. The screening results for the panel were analyzed by the application of the rh server at stanford human genome center.

### **Computational analyses**

The genomic sequence for the critical region was analyzed in sections by accessing individual programs Genscan (Burge and Karlin, 1997), Genie (Kulp et al., 1996), MZEF (Zhang, 1997) and Grail (Xu et al., 1994) in addition to the Genotator workbench (Harris, 1997), which represented the initial version of the current Genomebrowser (Karolchik et al., 2003) and Ensembl-platforms (Hubbard et al., 2002). MiRNA prediction was performed by accessing the mirSeeker server (Lai et al., 2003). In addition, sequences were compared using the basic local alignment search tool BLAST (Altschul et al., 1990). Sequences were also aligned locally using the Sequencher software (Gene codes, Inc.).

### **Northern blot analysis**

PolyA<sup>+</sup> RNA was isolated from skin fibroblasts, CNS biopsies, liver biopsies, lung biopsies, kidney biopsies or muscle biopsies of post-mortem LCCS fetuses and compared to RNA from similar tissues of age-matched control fetuses aborted for social reasons. 5ug of polyA<sup>+</sup> RNA was electrophoretically separated in a 0.8% agarose gel in the presence of formaldehyde and blotted onto a nylon membrane. Northern hybridizations were performed at 65C utilizing Express Hyb hybridization

solution (Clontech). RT-PCR products were processed to be used as probes according to standard protocols.

## **Epidemiology**

To attempt a complete ascertainment, we collected information from multiple independent sources to identify all fetuses and infants affected with arthrogyposis in Finland between years 1987-2002. Information was collected from all live births, stillbirths and terminated pregnancies having the diagnosis or cause of death 3350A-3359X and 7558A (International Classification of Diseases-9), and G12.0-G12.9 and Q74.3 (ICD-10), respectively, corresponding to the diagnoses of spinal muscular atrophy or anterior horn cell disease or arthrogyposis.

The following sources were used:

- 1) The death certificates of children who died before 12 months of age in Finland during 1987-2002
- 2) The National Registry for Congenital Malformations in Finland which also receives data on all terminated pregnancies (ref. [www.stakes.fi](http://www.stakes.fi))
- 3) All seven Departments of clinical genetics in Finland (five university hospitals (Helsinki, Turku, Tampere, Kuopio and Oulu), The Finnish Family Federation (Väestöliitto) and The Folkhälsan Department of Medical Genetics)
- 4) The five University hospital residential and outpatient registers
- 5) All those cases that had been analyzed for SMN-gene deletions at the department of medical genetics, University of Turku were listed. SMN-gene testing became available in Finland in 1996 and the laboratory of Turku has been the only diagnostic laboratory for SMA in Finland.

In addition, further cases were looked for at the registers of the perinatology units as well as at the autopsy and muscle biopsy registers of the departments of paediatric pathology of the five university hospitals.

The National Research and Development Centre for Welfare and Health (STAKES) maintains a nationwide register with data on all malformations from live births and stillbirths in Finland that are diagnosed or suspected in children under 12 months of age, or in terminated pregnancies. The information on malformations is continually updated nationwide.

For all cases found, the clinical, laboratory, autopsy and ultrasonic data were re-evaluated. All cases with death in utero or before 12 months of age were included in the original cohort. Arthrogyriposis was defined as joint contractures present in at least two regions of the body as seen at clinical examination or autopsy.

For incidence calculations, numbers of livebirths and stillbirths for years 1987-2002 were obtained from the Central Statistical Office of Finland.

Birth prevalence was defined as number of cases (live births or stillbirths) per total number of births (including live births and stillbirths).

Incidence was defined as total number of cases (live births, stillbirths, intrauterine deaths and terminated pregnancies) per total number of births.

Lethal arthrogyriposis was defined as arthrogyriposis with survival under 12 months of age.

This study was approved by the ethical committee of Helsinki University Central Hospital, Hospital District of Helsinki and Uusimaa, and all work has been done in accordance with the Helsinki declaration. Approval for re-evaluation of patient records and analysis of the registries from STAKES (the National Research and Development Centre for Welfare and Health), and the death certificate records from the Central Statistical Office of Finland was given by the Ministry of Social Affairs and Health.

## 5. RESULTS AND DISCUSSION

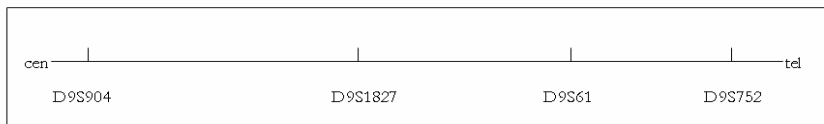
### **Linkage studies – Search for shared chromosomal regions and assignment of the LCCS locus to chromosome 9q34.1 (Publication I + unpublished)**

LCCS shows enrichment in the genetically isolated subpopulation of the late settlement area of Finland (Vuopala and Herva, 1994). Therefore, the disease can be assumed to be caused by a single major mutation and linkage disequilibrium can be utilized in the final positioning of the gene (Hastbacka et al., 1992). Thus, the search for a shared chromosomal segment was used as the method to find the disease locus. We genotyped five affected individuals from two families, but found no evidence for a shared haplotype on any chromosomal region. The haplotypes of the two affected siblings from family nr 2 (shown in publication I) used in this study were, however, identical by descent (IBD) for a total of 18 chromosomal regions and the siblings in family nr 3 were IBD for eight regions. (Two alleles are identical by descent when the identical allele is found in an earlier generation without any intervening mutations) Four of the identified IBD-regions were located in the same chromosomal regions. Linkage analysis using dense marker maps on those regions with the complete family material revealed evidence for linkage between LCCS and chromosome 9 markers. The maximum pairwise LOD score of 4.7 was obtained with marker D9S61. Obligatory recombinations restricted the LCCS locus to a 4cM interval between markers D9S1825 and D9S1830. The LAAHD family material proved to be insufficient for linkage studies or exclusion of this chromosomal locus.

## Haplotype mapping to fine map the critical DNA region (Publication I, unpublished)

Population isolates are beneficial for fine mapping of disease loci and the applicability of the haplotype mapping in the process aiming at the restriction of the critical DNA region. Limited numbers of recombinations have occurred in the original founder chromosome rendering the founder haplotype conserved in modern chromosomes. In the case of LCCS, haplotype mapping was utilised to significantly restrict the disease locus.

Initial proof of linkage restricted the critical DNA-region to a vast stretch of DNA on chromosome 9q33-34. Extended haplotypes were built in the region to further restrict the critical region. The ancient recombinations observable on the core haplotype defined the critical region between markers D9S904 and D9S61, which was subsequently analysed in details for candidate genes. However, new markers derived from the accumulating sequences produced by the HGP and finer mapping and re-evaluation of the diagnoses eventually lead to the restriction of the LCCS locus to the adjacent region between markers D9S1827 and D9S752 (the positional relations of the critical region markers are depicted in figure 7). Sequencing of candidate genes was expected to yield at least novel polymorphisms to be utilized for haplotype mapping, but thus far, only a small amount of single nucleotide polymorphisms (SNP) were identified from the coding sequences of the analyzed candidate genes.



**Figure 7.** Physical relationships of the critical region markers

### **Physical mapping (Publication I + unpublished)**

Linkage-based mapping of LCCS was concluded in 1998, during which the genome project was underway. The physical maps for the 9q34 region were non-existent and, thus initially a physical map for the region between markers D9S904 and D9S61 was constructed by PAC and BAC-maps. The clones were ordered mainly by FISH-methods as described previously. For certain markers, radiation hybrid-mapping was applied. The coverage for the PAC-library and a commercial BAC-library was not sufficient for the region and a collaboration with the Sanger Centre was established to obtain clones for the region from the RPCI-11 BAC library (Osoegawa et al., 2001). This collaboration yielded a complete physical map for the critical LCCS region and subsequently also the genomic sequence. The orientation of the individual genomic clones in relation to each other was still confirmed by STS-mapping (sequence tagged site) and a scaffold for candidate gene identification was established. At the time, an identical physical map was constructed by the HGP, but it did not increase the resolution or information content for the LCCS locus.

### **Candidate genes (Publication I + unpublished)**

Initial positional candidate genes (NGAL, Notch1) were mapped by metaphase-FISH. By the construction of the physical map, further candidate genes were located in the critical region and analysed as candidate genes. As the genomic sequences were finished, a detailed transcript map for the region was obtained and candidate genes (see table 1) were sequenced through systematically to detect the causative mutation for LCCS. The structure of the candidate genes were based on published

cDNA or genomic sequences, which are continually updated online. The region contained several interesting positional and functional candidate genes including Dynamin-1. Dynamin-1 was considered the perfect candidate gene as it was functionally in line with the clinical picture of LCCS (van der Blik et al., 1993). Namely, a temperature sensitive *Drosophila* mutant of Dynamin-1 designated Shibire showed paralysis at permissive temperatures (Clark et al., 1997). Homozygous mammalian mutations were suspected to be embryonically lethal (van der Blik, personal communication). Dynamin-1 was involved in the recycling of endocytic vesicles in the synapse, where the role of the proteins is thought to be to cut off the throat of the budding vesicle (Sweitzer and Hinshaw, 1998).

Another good regional and functional candidate gene was Syntaxin binding protein 1 (STXBP1), which was localized to the critical region by sequencing the ends of physical clones in the region (Swanson et al., 1998). STXBP1 resided reasonably close to Dynamin-1. It is involved in the localization of synaptic vesicles in the synapse (Verhage et al., 2000). It is similar to Endophilin B2 (SH3GLB2) located more telomeric in the critical region (Pierrat et al., 2001). The mouse model with a defective STXBP1 gene dies at the time of birth, it is paralyzed, but the brain is normally developed (Verhage et al., 2000). Hence, close resemblance to the LCCS phenotype can be observed. The other positional candidate genes did not evidently present as functional candidates, but were sequence analyzed in due order. However, none of the coding sequences of the positional candidates presented with mutations causative for LCCS.

To provide further evidence for the exclusion of the positional candidate genes, we verified their expression levels and transcript sizes by reverse transcriptase PCR and/or Northern blotting. RNA from different tissues

and patients of different ages were selected to exclude possible developmental stage dependent variations. No differences between patients and controls could be detected in the steady state transcript level of the regional genes.

As previously noted, non-coding small endogenous RNAs have been proposed as key regulators of developmental timing. We sought to identify potential miRNA genes in the critical LCCS region by the application of the mirSeeker software (Lai et al., 2003). Multiple putative miRNA clusters were identified, which we subsequently analyzed for mutations. Unfortunately however, no disease associated mutations were revealed by this approach. This does not exclude the role of miRNA genes in the molecular pathogenesis of LCCS, as the identification of miRNA genes is still developing. Further, regulatory regions in the noncoding regions also demand additional studies. Thus, the next task is to analyze the whole critical region genomic DNA. The mutation can be in the regulatory regions of a gene, which can reside relatively far away from the coding region (Kondo and Duboule, 1999). Hence, the identification of the LCCS mutation will provide interesting times for future work.

**Table 1:**

Full genes excluded by sequencing and expression analysis as causative of LCCS

Gene	Full name
LCN2	Lipocalin2
STXBP1	Syntaxin binding protein 1
ZNF-X	Zinc Finger X transcription factor
DNM-1	Dynamamin-1
KIAA1069	Novel gene
CCBL	Cysteine Conjugate Beta-Lyase
SPTAN	brain Spectrin alpha/Fodrin
ZYG	ZYG-11 Homolog B
Endog	Endonuclease G
VSN	Novel gene
SH3GLB2	Endophilin B2

CEECAM	cerebral endothelial cell adhesion molecule 1
DOLPP1	dolichyl pyrophosphate phosphatase 1
CRAT	carnitine acetyltransferase
LRRC8	LEUCINE-RICH REPEAT-CONTAINING PROTEIN 8
PHYHD1	phytanoyl-CoA dioxygenase domain containing 1
IER5L	Immediate early response gene 5L
PPP2R4	Phosphotyrosyl phosphatase activator 2A

### **Revised diagnostic criteria and incidence figures for LCCS (unpublished)**

Since no mutations in the critical 9q34.1 region genes were verified, a re-evaluation (clinical features, gestational anamnesis, family history, genetic mapping) of all patients was performed. Combining clinical data with the extended haplotypes identified two fetuses that differed from the majority of patients. These two cases survived beyond the 32<sup>nd</sup> gestational week and also restricted the critical DNA region differently from the other patients. When these two cases were omitted from the haplotype mapping, the region could be pinpointed more accurately between markers D9S1827 and D9S752. This process generated new diagnostic criteria for LCCS: (i) total immobility during pregnancy, (ii) death before 32<sup>nd</sup> gestational week, (iii) typical verified spinal cord neuropathological findings and (iv) muscle atrophy in addition to the typical phenotype. Special emphasis should be laid on the fetal akinesia and prenatal death, as both excluded cases survived beyond this period and had recorded fetal movements in the 13week ultrasound examination.

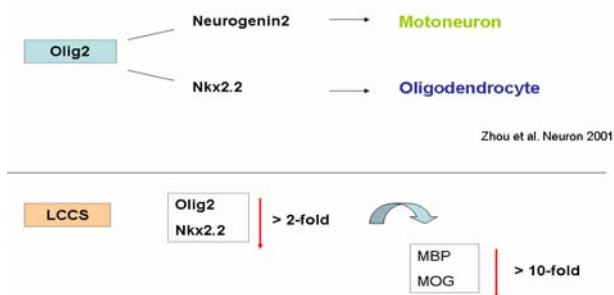
To clarify the true number of LCCS cases in Finland and to derive revised incidence figures, a register based study was initiated. Information on all fetuses and infants affected with arthrogyriposis in Finland between 1987-2002 were collected from multiple independent sources. During this time

period, a total of 214 cases, live births, stillbirths, intrauterine deaths or terminated pregnancies having the diagnosis of arthrogryposis were found. Of these, 74 were found due to arthrogryposis with prenatal death, either as intrauterine death or terminated pregnancy. Among this group, a distinct subset of 39 cases consisted of LCCS fetuses. During 1987-2002, the total number of recorded births was 984 743. Hence, the new incidence for LCCS was estimated at 1: 25 250 births (39 cases to 984743 births) as opposed to the prior estimate of 1:19 000 births.

Previously, 8% of affected newborns have suffered death within the first year of life in a Canadian series of 350 cases (Hall, 1985). In our study, the observed frequency of lethal arthrogryposis was markedly higher, presumably due to the enrichment of LCCS and LAAHD in the Finnish gene pool.

### **Transcript analysis (Publication II)**

To determine which genes and underlying molecular mechanisms are disturbed in LCCS, and thus essential for normal development of anterior motoneurons, we compared gene expression profiles of LCCS spinal cords against their respective age-matched controls. By the application of contemporary bioinformatics during data analysis, changes in pathways central in the development of the CNS were observed. Particularly, Olig2 and Nkx2.2, two transcription factors crucial in the development of oligodendrocytes from the pMN domain, were observed to be downregulated in LCCS spinal cord. Further, several myelin related components were heavily downregulated fitting the hypothesis of an oligodendrocyte dysfunction shown in figure 8.



**Figure 8.** Hypothesized pathogenesis of myelin downregulation in LCCS where the downregulation of Olig2 and Nkx2.2 leads to an oligodendrocyte dysfunction

### Data Analysis to identify deviating transcripts in LCCS spinal cord

Global linear scaling was performed on the intensity values from the scanned images of the microarray chips. The normalizing of data for each individual gene to its median value across all probe arrays removed the occurrence of intensity-dependent bias in further analyses. After filtering out data of lesser quality and various data points pertaining to quality control probes, the initial 22 283 transcripts were reduced to 9 286. Within- and between group distribution of signal ratios, as well as their variance, were evaluated through analysis of log-log scatter plots of all possible pair-wise comparisons between probe arrays. This information, giving us an estimate of the variability caused by biological and technical noise, was used to determine cut-off levels to discriminate significant changes from non-significant ones in expression profiles of LCCS samples and controls. The cut-offs were applied individually to each sample-control comparison, thus resulting in three sets of upregulated and three sets of downregulated genes. To further increase stringency, the finalized result lists contained only genes found to share the direction of regulation in all three comparisons. This conservative approach identified a total of 34 genes as being significantly downregulated, the negative expression change between LCCS samples and controls ranging from two up to 15-

fold. Only three genes were found to be significantly upregulated, expression levels changing from two up to over 5-fold.

### **Genetic networks**

The upregulated genes in the spinal cord samples of the LCCS fetuses consisted of only three transcripts: WIF1, MAB21L1 and ARL7. Both WIF1 and MAB21L1 represent crucial genes for early fetal development and ARL7 is involved in cellular traffic. WIF1 is an inhibitor of Wnt signaling (Hsieh et al., 1999). Wnts are secreted molecules assigned to a vast amount of developmental processes, and they have been implicated in pre- and postsynaptic differentiation during *Drosophila* (*Drosophila melanogaster*) development (Packard et al., 2002). MAB21L is a homolog of the *C. elegans* (*Caenorhabditis elegans*) *mab-21* cell fate specification gene (Mariani et al., 1999). *Mab-21* has been implicated as a downstream target of TGFbeta (Morita et al., 1999). ARL7 represents a subgroup of the ARF family together with ARL4 and ARL6. The function of these genes is still unclear, and it has been speculated that they may be involved with protein transport between cell organelles (Jacobs et al., 1999).

ZHFX1B, which shows a significant downregulation of expression in LCCS spinal cords, can be linked to the same *TGFbeta* pathway as MAB21L. ZHFX1B is a zinc finger homeobox 1b transcription factor that binds SMAD and represses SMAD-mediated transcriptional activation (Verschueren et al., 1999). SMAD transcription factors are mediators of the TGFbeta-family (Derynck and Zhang, 2003) and activation of SMAD is thought to lead to inhibition of MAB-21 (Morita et al., 1999). ZHFX1B is activated by Churchill, a zinc finger transcriptional activator that switches between different functions of Fibroblast Growth Factor (FGF) signaling (Sheng et al., 2003). ZHFX1B associates with SMAD1 only when the latter is phosphorylated, therefore acting as a putative sensor of Bone Morphogenetic Protein (BMP) activity in the cell (Postigo et al., 2003;

Sheng et al., 2003). FGF signaling on the other hand has been shown to be involved in motoneuron development by controlling Hox expression (Dasen et al., 2003). These data suggest links between different developmental pathways operating in the development of human spinal cord.

### **Spinal cord development**

Motoneurons develop from the pMN domain of the anterior spinal cord under the influence of a set of transcription factors. Especially SHH has been implicated as central in the process of determining the identity of the progenitor cells of the spinal cord. Mutations of SHH in human have been noted as causative of holoprosencephaly (Roessler et al., 1996) and also to impair neural patterning activity (Schell-Apacik et al., 2003). The intensity levels of SHH were below the detection limit on the genechips, and thus we were not able to deduce any changes in expression between the LCCS-patients and controls. However, we could observe changes in the expression in factors linked to the SHH-signaling pathway, especially of a few involved in the specification of the pMN domain. In our experiments, the expression of PAX6 was marginally upregulated, and the expression of OLIG2 shows downregulation in the LCCS spinal cord. NKX2.2 also shows marginal downregulation of expression. PAX6 has been shown to repress the expression of NKX2.2 (Ericson et al., 1997; Jessell, 2000). From the phylogenetic footprinting and transcription factor analysis we identified PAX6 as a putative regulator of the downregulated genes. Furthermore, a member of the TGF $\beta$ -signaling pathway, MAB21L1, and a factor capable of binding Smad, ZHFX1B, show altered expression in LCCS spinal cord. There is some evidence pointing towards a genetic interaction between mab-21 and mab-18, which is a PAX6 homolog, adding to the complexity of networks (Ericson et al., 1992). We could not see ISL1 expression in the microarray experiments, but at least

conventional RT-PCR reveals expression of ISL1 from LCCS spinal cords, thus making it possible that motoneurons are formed in LCCS-patients. We also looked at GLI-signaling to see if there were any significant changes in that signaling pathway, but we could only detect marginal upregulation of GLI2. By summarizing the findings above, we found alterations in several components in the pathways leading to mature motoneurons. These changes seem to be of a relatively high magnitude, since the cellular populations expressing such components define only a subset of the spinal cord and hence the signal may get diluted as we did not specifically dissect subregions of the spinal cord for this analysis.

### **Identifying PAX6 as a regulatory transcription factor**

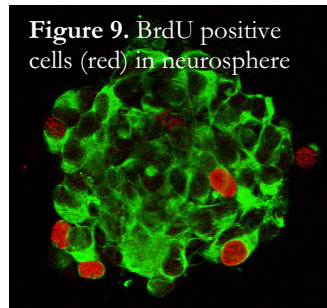
To identify common transcriptional motifs and transcription factors resulting in the observed expression patterns, phylogenetic footprinting methods were utilized. Amongst the set of 34 transcripts being downregulated in all LCCS patients, Paired-box gene 6 (PAX6) was found as a transcription factor with statistical significance in both tests compared to the control dataset and being overrepresented in the set of genes downregulated in LCCS. PAX6 plays an important role in central nervous system development and PAX6-null mice exhibit anophthalmia with central nervous system defects and lethality (Kioussi et al., 1999). PAX6 is conserved from invertebrates to vertebrates and functions as a regulator in central developmental processes of several organs (Chi and Epstein, 2002).

Transcript analysis was also performed with one LAAHD spinal cord sample. The result was in line with the LCCS samples, indicating close similarities in the disease molecular pathways. However, detailed conclusions are not possible due to the analysis of only one sample. In addition, we looked at the transcript profiles of fetal diaphragmatic muscle from one LCCS patient as compared to an age-matched control. Severe

downregulation of myosin heavy chain expression could be observed as well as other findings. These data remain unpublished until additional samples are obtained for analysis.

### **Neural precursor cells (Publication III)**

Since the global transcript analysis with LCCS fetuses suggested defects in the development of motoneurons and oligodendrocytes (Pakkasjarvi et al., 2005), we addressed the question of differentiation experimentally. We isolated neural precursor cells from fetal central nervous system by growing homogenized biopsies in non-adherent conditions where they form neurospheres and can be cultured in the presence of mitogens (EGF, FGF and LIF) for extended passages. No macroscopic differences between LCCS- and control neurospheres could be observed when the cells were maintained undifferentiated and both cell lines were passaged for extended periods (>P20). However, the LCCS neurospheres appeared to grow slightly denser as compared to the controls. To study the proportion of dividing cells in culture, neural precursor cells were subjected to BrdU to stain for mitotic cells. On average, 7% of LCCS NPCs stained positive for BrdU-incorporation, whereas only 3% of control NPCs were positive, indicating an increase in proliferation in LCCS NPCs. A neurosphere stained with anti-BrdU to detect mitotic cells is shown in figure 9, where the amount of dividing cells can be visually observed.



To determine the molecular background of the observed increase in mitotic activity, the expression profiles of LCCS undifferentiated neural

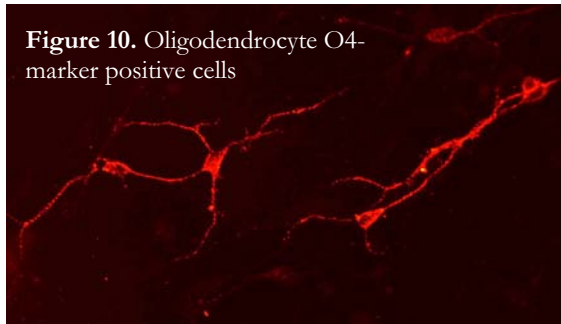
precursor cells were compared to their respective controls. This conservative approach identified a total of 49 genes as being significantly upregulated, the expression change between LCCS samples and controls ranging from two up to 27-fold. 63 genes were found to be significantly downregulated, the negative expression levels changing from two up to over 15-fold. The lists of significantly up-/down-regulated genes were examined for biologically relevant associations using the *Web-based Gene Set Analysis Toolkit* available at <http://genereg.ornl.gov/webgestalt/>. Annotation information defining the biological processes to which each gene could be ascribed to was retrieved from the classifications provided by the *gene ontology* (GO) consortium (Ashburner et al., 2000), the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) and BioCarta. Statistical evaluation of enrichment of categories represented in each gene list, compared to the proportion observed in the total population of genes on the probe array, was performed using the hypergeometric test and p-values less than 0.01 were considered significant.

This analysis indicated the Epidermal Growth Factor Receptor processing as being enriched in the upregulated undifferentiated NPC samples. The EGFR is a tyrosine kinase and signaling involves small GTPases of the Rho family (Wong and Guillaud, 2004). The oncogene ERBB is implicated to derive from EGFR and mutations in EGFR have been associated with small-cell lung cancer (Downward et al., 1984; Kobayashi et al., 1995). EGF is essential to the maintenance of neural precursor cells and EGFR levels are known to determine progenitor cell proliferation and differentiation (Lillien, 1995). These findings support the observed proliferative activity of LCCS NPCs. However, further experiments are warranted before definitive conclusions, as the biology of NPCs in neurosphereculture is still not completely understood. The *in vitro* setting for the creation of the appropriate niche remain precarious despite recent progress in the field (Shen et al., 2004). It is known that more than one

kind of precursor cells are present in the nervous system and that the different precursor cells exist at different times during development (Rao, 2004), which may limit our study with the human samples. Further, not only true precursor cells display self-renewal capacity (Trentin et al., 2004). Still, the transcriptome analysis revealed the molecules associated with proliferative activity and supports the notion that EGF-responsive cells are present at later stages of development (Svendsen et al., 1996). Indeed, a derangement of developmental timing may be critical for the survival of motoneurons (Hausmanowa-Petrusewicz and Vrbova, 2005) and the observed proliferation change may cause developmental delays that render the LCCS motoneurons vulnerable at later stages.

To determine the potential to differentiate, the neural precursor cells were analysed during withdrawal of mitogens. Both LCCS and control derived neural precursor cells differentiated into neurons and cells of glial lineage. We stained cells for neuronal (neuronal class III  $\beta$ -tubulin), astrocyte (GFAP) and motoneuron (Islet-1, Hb9) markers as the cells were challenged by fetal calf serum addition after mitogen withdrawal. No macroscopic differences were observed between LCCS patients and controls in the number or appearance of the differentiated cells. Wu et al. have previously shown that by priming NPCs, many will acquire a cholinergic phenotype upon grafting into spinal cord (Wu et al., 2002). Gao et al. also showed how this approach was successfully used for innervation of peripheral muscle with primed NPCs in rats suffering from motoneuron degeneration (Gao et al., 2005). In our hands, priming of neural precursor cells in vitro did not increase the percentages of cells positive for motoneuron markers. To address the question of oligodendrocyte development, the precursor cells were supplied with IGF-1 and stained after differentiation for the oligodendrocyte marker O4. Again, no differences between patients and controls could be observed.

Figure 10 shows oligodendrocyte marker positive cells from differentiated LCCS NPCs. To further study the differentiation of NPCs into motoneurons, the cells were stained with choline acetyltransferase (Chat), a marker found in mature cholinergic motoneurons. We could not identify truly Chat-positive cells in our cultures. It is possible that our culture conditions do not favor the final maturation of motoneurons into cholinergic cells in the absence of further environmental clues. However, we are confident that our experiments show clear induction of differentiation and immunocytochemical evidence of cell fates that are affected in LCCS.



To investigate whether excessive apoptosis occurs during the differentiation of LCCS derived neurospheres, we differentiated both LCCS and control neurospheres and measured apoptosis by fluorescence-activated cell sorting. Apoptosis was studied by detecting the translocation of phosphatidylserine to the extracellular side of the cytoplasmic membrane, an early indicator of apoptosis (Vermes et al., 1995). Necrotic cells were detected by DNA staining with propidium iodide to distinguish apoptotic and necrotic cells. Of the LCCS patients, 25% ( $n=5$ ,  $SD=6.3$ ) of the differentiated cells were apoptotic, whereas the number for controls was 17% ( $n=3$ ,  $SD=6.8$ ). Although the mean number of apoptotic cells was higher in LCCS derived progenitors, statistically significant differences could not be detected (student t-test p-value 0,29). The proportion of

necrotic cells was also similar in LCCS and control cultures (19%, SD=5.2 and 18%, SD=4.6, student t-test p-value 0,53). An exaggeration of naturally occurring PCD of motoneurons has been proposed as a part of the pathogenesis of spinal muscular atrophy (Fidzianska and Rafalowska, 2002; Soler-Botija et al., 2002). The pathogenesis behind the lack of anterior horn tissue in LCCS has remained unknown and speculations on an inability to produce the appropriate cell types or whether excessive PCD eliminates cells after differentiation have occurred. We compared PCD during initial differentiation of NPCs, but statistically significant differences in PCD could not be observed. To further address the question of motoneuronal cell death, we would have to enrich for motoneurons and then pursue the apoptosis assays.

To address any molecular disturbances during differentiation of neural precursor cells, RNA was collected from neurospheres and differentiated precursor cells from both LCCS-patients and the controls. The RNA was utilized in transcriptome analyses with commercial microarray chips. Aberrant gene expression in LCCS patients could be detected. Combining the gene lists of abberantly expressed transcripts in both neurospheres and differentiated cells yielded a set of 8 genes putatively active during initiation of the disease process. The transcripts that were upregulated in both conditions consisted of: (1) RBPMS, coding for an RNA binding protein; (2) URB, a steroid sensitive gene (Aoki et al., 2002); (3) GLT25D2, a gene containing a glycosyltransferase domain; (4) XM\_371647, a transcript with a putative glycosyltransferase domain; (5) PIPOX, a gene with oxidase activity (Dodt et al., 2000). The downregulated transcripts common to both conditions consisted of: (1) PHACTR2 and (2) PHACTR3, two genes with phosphatase and actin regulator activity (Allen et al., 2004); and (3) PAK7, a brain specific gene encoding for a protein with serine/threonine kinase activity (Waterston et

al., 2002). Another member of the PAK-family was also observed to be downregulated in the undifferentiated NPCs, namely PAK3. In addition, EDG4, a lysophosphatidic acid G-protein-coupled receptor was observed to be downregulated. Lysophosphatidic acid has been recognized as an activator of the Rho family of GTPases, which use PAK7 as a downstream effector.

In this study, we isolated and cultured NPCs from fetal LCCS brains. We demonstrate that despite the genetic defect leading to the absence of motoneurons in the patients, the NPCs retain the potential to differentiate into distinct cell types of the CNS. We also provide evidence that after differentiation the cells do not undergo excessive apoptosis. The gene expression changes support and explain the observation of an increased growth rate of NPCs. Thus, the disease may be due to a non-cell autonomous mechanism at the stem cell level meaning defective niche circumstances. It may as well be due to defects in interaction of cells at later stages.

We also isolated neural precursor cells from the spinal cord of one LCCS fetus successfully. The growth kinetics of spinal cord derived NPCs are however too slow for further analysis with current in vitro niche conditions and thus, any true conclusions cannot be drawn based on these experiments. It is possible that the pool of spinal cord precursor cells is small and the culture conditions are not optimal for the necessary propagation of these cells to enable similar studies as was done for the brain NPCs.

Of interest to note is, that priorly it has been unclear whether cells of spinal cord origin can be generated from brain derived neural precursor cells and only a small number of publications providing definitive evidence

are currently found (Wu et al., 2002; Gao et al., 2005). We show that cells expressing motoneuron markers can be differentiated from the human neural precursor cells in vitro. Their functional capacity awaits analysis. These CNS NPCs do not represent a developmentally relevant pool of motoneuron precursors, however, the results obtained provide further evidence to previous studies with CNS NPCs and motoneurons.

## Synopsis

The incidence of anterior horn cell disorders associated with arthrogryposis and early fetal demise is poorly known. Although obviously rare, early onset lethal arthrogryposes constitute a significant problem to the affected families due to the risk of recurrence. They are a diagnostic challenge to obstetricians performing ultrasonographic investigations as well as to geneticists giving genetic counseling. Thus, more knowledge about the genetics and epidemiology of these disorders are needed. Importantly, identification of the defective pathways in these disorders would most probably expose critical elements in the normal development of human motoneurons.

This thesis describes the localization of the LCCS disease gene to chromosome 9q34.1 by linkage mapping. Despite high hopes and intense investigations, the true molecular background of LCCS remains unknown. The physical mapping of the region advanced paralleled to the progress of the Human Genome Project and provided at later stages novel information to the sequencing effort. Candidate genes were localized to the region, several of which had not previously been assigned to a specific genomic region. Sequencing of these genes witnessed the evolution of Sangers dideoxy chain termination reactions by manual propagation to the era of automated sequencers. However, none of the obvious candidate genes contained disease causing mutations, neither did their expression levels indicate any association to LCCS. To try an alternative approach, microRNA genes were predicted from the critical 9q34.1 region, but none of the identified microRNA genes were mutated in LCCS. It may well be that the mutation lies in a regulatory element in 9q34.1, but the gene regulated is further away from this region. Therefore, interesting times are ahead once the gene defect is verified.

As another alternative avenue to identify involved genes and pathways and to gain further insight into the molecular pathways that are disturbed during the pathogenesis of LCCS, a global transcript analysis was performed. RNA from fresh LCCS spinal cord and age matched controls was isolated and analyzed with commercial microarray methods. An indicative oligodendrocyte dysfunction was recognized with specific changes in the expression of distinct transcription factors central in the development of the spinal cord. In detail, Olig2 and Nkx2.2 showed downregulation in expression in LCCS; two transcription factors of importance during both motoneuron and oligodendrocyte development. Further, downregulation in components of the oligodendrocyte product myelin were observed. The microarray study provided a still shot of a distinct phase in the pathogenesis of LCCS and many questions evolved.

To analyze the differentiation of neurons and cells of glial origin during the LCCS pathogenesis further, neural precursor cells were harvested from post-mortem LCCS CNS. In vitro studies showed an increase in the proliferative activity of LCCS NPCs and the neurospheres also grew denser as compared to the controls. When differentiated, no morphological differences between patients and controls could be observed, and both lines readily differentiated into neurons, astrocytes, motoneuron-like cells and oligodendrocytes upon instruction. However, molecular changes could already be observed in microarray analyses from differentiating NPC cells. The true impact of these changes claim extensive further studies, but provide a glimpse into the molecular details of progenitor cell differentiation.

## 6. CONCLUDING REMARKS

More than 150 conditions presenting with arthrogryposis multiplex congenita are known (Hall, 1985; Hall, 2002). Most of these conditions, however, can accurately be diagnosed only after birth and in living patients. Prenatally lethal cases of arthrogryposis still remain a challenge although they are more and more frequently encountered due to improved ultrasound methods. Post-mortem diagnostics is often hampered by fetal mummification as a function of intrauterine retention time. Therefore, accurate diagnosis is at times only descriptive and prognosis is not always possible to predict. When lethal arthrogryposis is suspected in ultrasound due to immobility, a regular and intense follow-up is recommended.

The estimated birth prevalence of arthrogryposis varies greatly depending on the definition of the condition and the type of study. Multiple congenital contractures (arthrogryposis) has been estimated to occur with a frequency of about 1:3000 live births and about 1 in every 200 newborns is estimated to be born with some form of contractures (Hall, 1997; Hall, 2002). In this study, we estimated the birth prevalence of LCCS to 1:25300 births through a register based study. The epidemiology of early onset lethal arthrogryposis is poorly known, but unpublished results indicate a relatively high frequency of the perinatally lethal cases which usually have been omitted in previous studies. This puts emphasis on the burden caused by these entities for the parents. Clearly more studies of the very early onset and prenatally diagnosed cases are needed to enable proper genetic counseling and prenatal diagnosis.

This thesis work has used of different approaches towards the understanding of disease mechanisms. The first publication shows how linkage studies have been carried out using only a few individuals.

Previously unpublished results present the inherent difficulties in identifying disease affecting mutations. The second publication utilizes global transcript analysis and contemporary bioinformatics in deciphering clues to the molecular pathways deranged during the disease process. In the third publication human neural precursor cells have been utilized in disease mechanisms. It provides an innovative approach to study a developmental neurologic disease and shows that cells expressing motoneuron markers can be generated directly from neural precursor cells *in vitro*.

The intricate developmental network is subjective to disturbances at multiple occasions. This thesis shows that most of the developmental checkpoints of the spinal cord are passed normally at least in culture in LCCS patients. Most probably, the disease mechanism is active at post-mitotic stages of the spinal cord cells. The revelation of the genetic defect ensures interesting times ahead.

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