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# Gene expression studies of pregastrulation development:

the basement membrane is essential for  
cell differentiation

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*It is sometimes important for science to know how to forget the things she is surest of.*

**Jean Rostand**

# List of papers

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## Paper I

**Akerlund, M., Carmignac, V., Scheele, S. and Durbeej, M.** (2009). Laminin alpha1 domains LG4-5 are essential for the complete differentiation of visceral endoderm. *Cell Tissue Res*. DOI 10.1007/s00441-009-0845-3

## Paper II

**Meszaros, R., Akerlund, M., Hjalt, T., Durbeej, M. and Ekblom, P.** (2007). Gene expression profiling of differentiating embryonic stem cells expressing dominant negative fibroblast growth factor receptor 2. *Matrix Biol* **26**, 197-205.

## Paper III

**Meszaros, R.\*, Akerlund, M.\* and Durbeej, M.** (2008). Global gene expression analysis during early mouse embryonic stem cell differentiation. *Manuscript*.  
\*These authors contributed equally to this work

## Paper IV

**Gawlik, K. I., Carmignac, V.\*, Akerlund, M.\*, Elamaa, H.\* and Durbeej, M.** (2009). Distinct roles for laminin  $\alpha$ 1 globular domains in laminin  $\alpha$ 1 chain mediated rescue of laminin  $\alpha$ 2 chain deficiency. *Manuscript*. \*These authors contributed equally to this work

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# Abbreviations

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$\alpha$ 1LG4-5	Globular domains LG4-5 in the laminin $\alpha$ 1 chain
BM	Basement membrane
DGC	Dystrophin-glycoprotein complex
E	Embryonic day
EB	Embryoid body
EC	Embryonal carcinoma
ECM	Extracellular matrix
EHS	Engelbreth-Holm-Swarm
ES	Embryonic stem
FC	Fold change
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
ICM	Inner cell mass
MDC1A	Congenital muscular dystrophy type 1A
NMJ	Neuromuscular junction
PNS	Peripheral nervous system
VE	Visceral endoderm

# Abstract

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Basement membranes (BMs) are sheet-like structures of extracellular matrix. They act as a supporting structure but can also significantly influence cellular behavior in development, tissue homeostasis and disease. Laminins, a major BM component, are multidomain proteins, consisting of three polypeptide chains ( $\alpha$ ,  $\beta$  and  $\gamma$ ). During pregastrulation development, stem cells convert and epithelial tissues are formed. This process is faithfully mimicked in vitro by embryoid body (EB) cultures. Fibroblast growth factor (FGF) signaling is crucial when the step-like process of EB development is initiated with the formation of an endoderm. A subendodermal BM is formed, in which the globular domains LG4-5 of the laminin  $\alpha 1$  chain ( $\alpha 1$ LG4-5) are responsible for the induction of the epiblast

EBs derived from embryonic stem (ES) cells, modified to repress FGF receptor signaling, have been described before. However, a full-scale analysis of the transcriptome was missing. We therefore analysed these EBs at four time points during differentiation by the use of microarray technique. An extensive catalogue of affected genes was reported. A majority of the genes directed by FGF signalling were encoding BM and endodermal proteins. In addition, we also analysed the expression profile of wild type EBs. In both these studies, we found interesting genes not previously described in early development or identified as FGF targets. Hopefully, our gene catalogue will be a valuable source for the scientific community interested in FGF signaling, developmental biology and stem cell research. Furthermore, a gene expression study was set up to get a better insight of epiblast induction by  $\alpha 1$ LG4-5. EBs derived from ES cells with a targeted deletion of the  $\alpha 1$ LG4-5 domains were analysed. To our surprise, we found several indications of an incomplete differentiation of the visceral endoderm. We therefore hypothesize a novel autocrine mechanism for  $\alpha 1$ LG4-5 in regulating the developing endoderm.

We also suggest novel roles for laminin LG4-5 in the neuromuscular system. Using laminin  $\alpha 2$  chain deficient mice overexpressing laminin  $\alpha 1$  chain lacking the LG4-5 domains, we show that these domains, and consequently binding to the receptor dystroglycan are not crucial in diaphragm and heart, but essential in the peripheral nervous system.

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# Background

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## Introduction

Your body is made up of trillions of cells. There are at least two hundred different cell types performing such diverse functions as producing insulin, conducting nerve signals, contracting muscles and attacking invading bacteria. All these different cell types are then combined and organized into complex systems where they act together, making up organs. Then consider the fact that we all, from the very beginning, started out as just one tiny cell; the fertilized egg. It is quite a journey! Usually, the life span is considered to be the period between birth and death. However, the nine months before birth are a critical part of your existence. This is when your body is formed and most of us might consider this process a true wonder. Thus, as you ponder the intricate mechanisms that must be involved to make individual cells communicate to make all this happen, you are bound to study developmental biology. Besides its major role in basic biological research, developmental biology is also clinically relevant. Understanding the molecular basis of many developmental events may facilitate the development of therapies for several diseases (e.g cancer, diabetes and muscular dystrophy).

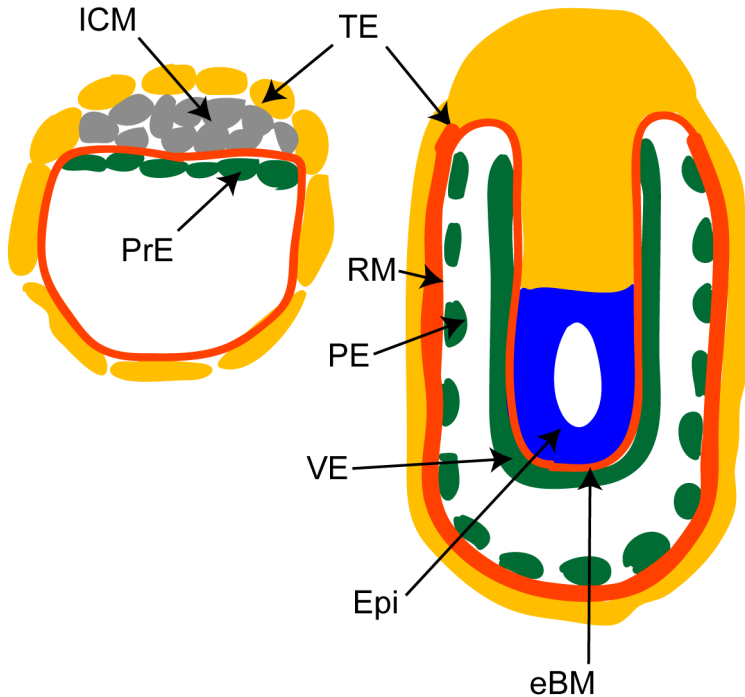
## The mouse as a model organism

Why is the mouse such a popular model organism? In many situations other models like flies, worms and yeast are most suitable but the mouse is often favoured for its physiological similarities to humans and the similarities of their genomes. For most human genes there is a mouse counterpart and importantly, mutations that cause diseases in humans often cause similar diseases in mice. Besides being a mammalian and a close relative to human, the mouse is also easy to maintain and have a short breeding cycle which makes it suitable for genetic studies. Powerful genetic technologies are available to produce custom-made mouse models for a wide array of specific diseases, as well as to study the function of specific genes. Gene transfer is one technique often utilized. Then transgenic mice can be created, in which any foreign gene of interest is inserted into the animal's germline. Another, maybe even more powerful technique is the creation of knockout-mice. In these mice, pre-selected endogenous genes can be mutated in a precise manner, generating non-functional gene products as well as altered or truncated proteins. This means that it is possible to create exact replicas of the genetic defects that cause diseases in humans. However, when studying early developmental events the major drawback with the mouse model is the inaccessibility of the early embryo after implantation into the uterine wall of the mother.

The mother has to be scarified and larger quantities are not possible to obtain. Luckily, an in vitro model has emerged where so called embryoid bodies (EBs) can be cultured from embryonic stem cells. This technique will be described later.

## Pregastrulation development in mouse

Development begins with the fertilized egg. This is followed by the early cleavage phase when the embryo divides slowly without any increase in mass. When the embryo reaches the 16-cell stage, the first differentiation event is initiated. This process starts with compaction that is when the cells (called blastomeres at this stage) form a tightly packed mass. Cells that end up on the surface differentiate to trophectoderm, whereas the cells on the inside give rise to the inner cell mass (ICM). The trophectoderm is an epithelium forming a seal against the outside environment. At embryonic day (E) 3.5, the blastocyst stage is reached and a fluid-filled cavity (called blastocoel) has formed inside the embryo. Now, the next cell fate decision takes place. The ICM is localised at the apical pole of the blastocyst and the ICM cells that face the cavity transform into an epithelium, the primitive endoderm. All cells derived from the trophectoderm and the primitive endoderm will exclusively form extraembryonic tissues (e.g. the placenta), while the remaining cells of the ICM will form the fetus. At the blastocyst stage, two basement membranes (BM) can be visualized; one on the basal side of the trophectoderm and one that separates the primitive endoderm from the ICM (Salamat et al., 1995)(Figure 1). Subsequently, the ICM becomes the epiblast and the primitive endoderm separates into cell lineages; the parietal endoderm and the visceral endoderm (VE)(Gardner, 1982). Parietal endoderm cells start to migrate over the trophectodermal BM (Enders et al., 1978), adding to it extra layers of BMs thus forming the Reichert's membrane (Salamat et al., 1995). Reichert's membrane is however only found in rodents. Subsequently, the VE differentiates from the primitive endoderm cells that remain attached to the BM facing the epiblast (embryonic BM). After the embryo has implanted into the uterus, the epiblast and polar trophectoderm proliferate and extend into the blastocoel, forming a conical structure, the so called egg-cylinder.



**Figure 1.** Pregastrulation development. To the left a schematic drawing of a mouse embryo at the blastocyst stage and to the right just before gastrulation. Inner cell mass (ICM), trophoctoderm (TE), primitive endoderm (PrE), parietal endoderm (PE), visceral endoderm (VE), epiblast (Epi), embryonic basement membrane (eBM) and Reichert's membrane (RM).

Before the gastrulation begins, a cavity is formed inside the epiblast and the cells surrounding it form columnar epiblast epithelium (Coucouvani and Martin, 1995)(Figure 1), a process governed by the embryonic BM (it is actually a specific domain in the BM component laminin-111 that is the inducer but that will be described later in the laminin chapter). The columnar epiblast epithelium, the first cell layer of the fetus, is covered by the VE, a tight epithelial tissue that provides a barrier for protection and maternofetal exchange of nutrients. The environment within the developing embryo is thus initially regulated by the VE, later on the placenta takes over this function (Bielinska et al., 1999). In addition, it is an essential signal provider during the following gastrulation.

## Embryoid bodies

The embryoid body (EB) model represents a unique in vitro tool to investigate early developmental processes. EBs are formed in culture dishes when pluripotent

stem cells are allowed to aggregate and, under the right conditions, begin to differentiate towards an embryo-like structure. They thereby to some extent recapitulate early development.

This field originates from studies of teratomas in the 50's and the 60's. Teratomas are benign tumours that can occur in the testes in males and ovaries in females. They then originate from germ cells and contain a complex mixture of tissue types and appear to recapitulate many of the events that occur during early embryonic development but in a more disorganized manner. Teratomas were found to arise spontaneously in the testes of male mice of the 129 strain or they could be induced by implantation of an early embryo into the adult testis (Stevens, 1964; Stevens, 1970). In testis, the teratomas often are malignant and are then referred to as teratocarcinomas. The teratocarcinomas differ from the teratomas in that they, besides the mix of differentiated tissues, also contain some undifferentiated cells.

In the early 70's some labs started to isolate clonal pluripotent stem cell lines that they were able to grow in tissue culture (Evans, 1972; Kahan and Ephrussi, 1970; Rosenthal et al., 1970). These cells were so called embryonal carcinoma (EC) cells and originated from the undifferentiated cells within teratocarcinomas. When injected into a host, the EC cells could, like the cells of the early embryo, differentiate to form derivatives of all three primary germ layers. EC cells had previously been propagated as tumours *in vivo* but now when the cells could be maintained *in vitro* the possibility to grow cells in relatively large numbers and in a controlled environment emerged. In addition, this opened up for various biological and biochemical manipulations of the EC cells. For the EC cells to keep their pluripotent stem cell character it was found that they had to be maintained on a layer of feeder cells contributing some factors essential for self renewal of the EC cells.

A structure which resembles the 5- to 6-day-old mouse embryo had earlier been found in ascites fluid of animals bearing intraperitoneal teratocarcinomas (Teresky et al., 1974). These structures, called embryoid bodies, were found to have an inner core of EC cells surrounded by a single layer of endodermal cells and a basement membrane separating the two cell types. Then, in 1975, a major step was taken when Martin and Evans managed to cultured EBs *in vitro* (Martin and Evans, 1975). They found that upon withdrawal of the feeder cells the EC cells could differentiate, forming EBs, and that some detached from the dish surface floating in the medium. When the EBs were allowed to re-attach to a substratum they showed the potential to form a variety of cell types like fibroblasts, cartilage, adipose tissue, beating cardiac muscles and neural cells. Now, an *in vitro* model system, suitable for studying early developmental processes and addressing questions related to lineage commitment, was at hands.

A big breakthrough came in 1981 when two papers were published describing the establishment of pluripotent embryo stem (ES) cells, isolated from the ICM of mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). Some years later, in 1985, Doetschman and colleagues presented for the first time an *in vitro* model of mouse embryogenesis based on differentiating ES cells (Doetschman et al., 1985). It was shown that, within the EB, organized development similar to that of the embryo occurred and that derivatives from all three germ layers were formed in the cultured EBs. Yet another source of pluripotent cells that could form EBs came in 1992 when primordial germ cells were isolated (Resnick et al., 1992). Much of the early EB research employing EC cell lines (e.g. F9) gave useful information on developmental processes but when ES cells were made available they would become the preferable cells in later years.

Mouse ES cells can be maintained and self-renew on mouse embryonic fibroblast feeder cell layers or in media containing leukemia inhibitory factor. When ES cells are transferred to non-adhesive culture dishes, in media without leukemia inhibitory factor and no feeder cell layers, they form floating aggregates (spheres roughly 50–100  $\mu\text{m}$  in diameter). This method, called liquid suspension, has been used in all of our studies but other techniques, like hanging drops and cultivation in methylcellulose semisolid media, can also be used to initiate EB differentiation (Kurosawa, 2007). After two to three days, primitive endoderm cells appear at the surface of the EBs. During further development, the primitive endoderm cells mature into a VE epithelium, occurring as an orderly progression of multiple events (Grover et al., 1983b). This multi-step maturation includes an early expression of the transcription factors Gata-4/6, production of a subendodermal BM and ends with a mature VE that synthesizes and secretes several serum proteins e.g. alpha-fetoprotein. In fact, targeted disruption of Gata-4 disrupts the development and maturation of VE (Soudais et al., 1995). The assembly of a BM has been found to be necessary for the subsequent events: cavitation and epiblast polarization (Li et al., 2004; Li et al., 2001; Murray and Edgar, 2000; Scheele et al., 2005). Cavity formation is the result of programmed cell death and only those epiblast cells in contact with the BM will survive and become polarized (Coucovanis and Martin, 1995). This process is completed at day 6-7 of EB culture and at this stage, the EBs resemble the pregastrulation embryo. Notably, the trophectoderm and Reichert's membrane are not part of the EB model.

EBs culture is an ideal model for investigating several biological processes. First, and most obviously, it is an excellent tool to study early embryonic development. In particular, it is well suited to study homozygous null alleles causing early embryonic lethality. Second, it can be used for unraveling the cascade of molecular events that lead to the development of a certain somatic cell type, which in the end could be useful for therapeutic applications. Third, the EBs offer a model to

investigate the process of epithelialization and how the BM regulates that process. In paper I-III, we have made use of the EB model to study the early developmental steps and the process of epithelialization at the molecular level. Paper III focuses on wild type EB differentiation, paper I on the role of a cell binding domain in laminin  $\alpha 1$  (LG4-5) and paper II on FGF-signaling targets.

## Laminins and the basement membrane

### **The basement membrane**

All tissues are composed of a mixture of cells and, by the cells, secreted proteins and carbohydrates making up a complex network; the extracellular matrix (ECM). The matrix components vary between different tissues. Also, within a tissue the ECM composition varies depending on developmental state, during disease or when injured. So, the ECM is not to be seen as a static structure, it is continually produced and remodeled. The ECM can take on different forms with two major ones being the connective tissue matrix and the basement membrane (BM). While the connective tissue matrix is a porous framework, the BM is a dense and thin sheet-like structure. Although having quite different structures, both consist of the same types of protein, including collagens, adhesive glycoproteins and carbohydrate-rich proteoglycans.

Transmission electron microscopy typically reveals a BM of 30-100 nm thickness (Osawa et al., 2003). It can be divided into three cross-sectional structures: lamina lucida, densa and fibroreticularis. Variations of both morphology and thickness can be found. Some thick BM like Reichert's membrane (embryonic membrane in rodents) and Descemet's membrane (found in the cornea) can be greater than 2  $\mu\text{m}$  (Grant and Leblond, 1988). Epithelia and endothelia are polarized and tightly packed cells having one apical part facing a "free" side and one basal part that most often overlays a connective tissue. BMs can be found throughout the body. They localize to: a) the basal side of all epithelia and endothelia; b) cover the surface of muscle and fat cells; c) separate nerve fibers and they enclose Schwann cells from connective tissue (Inoue, 1989). In addition, BMs are the first ECM seen during embryonic development and they can be found in nearly all multicellular animal species.

The BM was first discovered by William Bowman in the 19<sup>th</sup> century and described as a barely distinguishable membrane underlying epithelia. It was also described as generally not stained by routine stains but in 1940s it was noted that periodic acid-Schiff stained basement membranes. Basement membrane research gradually increased during the 1970s and 1980s when considerable progress took place in

the biochemical knowledge of the BM. The major breakthrough at this time was the identification of a basement membrane producing tumor; the Engelbreth-Holm-Swarm (EHS) mouse tumor (Orkin et al., 1977). Collagen IV was the first BM protein to be analyzed followed by the glycoprotein laminin, first isolated in 1979 (Timpl et al., 1979). In the 1990s to present, many new protein isoforms were discovered, particular in terms of laminin, and the utilization of targeted mutations for generating knockout-mice resulted in new knowledge to the field (table). Some other important techniques have been used like the production of recombinant BM modules, to resolve their three-dimensional structures, and organ cultures to study developmental processes e.g. the mesenchymal-epithelial interactions.

The typical BM components are laminin, collagen IV, nidogen, perlecan, agrin, fibulin, osteonectin, collagen XV and XVIII. These basic proteins are conserved from *Caenorhabditis elegans* throughout evolution (Hutter et al., 2000), though vertebrates having more isoforms. The backbone of the BM consists of two polymeric networks, formed by collagen IV and laminin (Timpl and Brown, 1996; Yurchenco et al., 1992). The collagen network is highly cross-linked, providing mechanical stability, while the laminin network is mainly of a noncovalent nature. The two networks are interconnected via crosslinkers such as nidogen (Yurchenco and O'Rear, 1994). If one looks more carefully into the BM composition it is clear that the BM is not just “one type of membrane” reused throughout the body. In fact, the ratios and isoforms vary from different tissues and also within the same tissue. It is a typical reciprocal relationship between the cells and the BM. The cells, in a specific location or at a specific time in development, produce a BM with a unique composition and thereby unique properties. In turn, the BM serves as positional information to the attached cells.

Today, we consider the BM to fulfill three major functions:

- 1) Tissue support and compartmentalization
- 2) Molecular sieving
- 3) Regulation of cell function

Originally the BM was believed to provide a scaffold to which cells could adhere and serve as a selective barrier. The function as a selective barrier is perhaps most prominent in the kidneys glomerular basement membrane which takes part in blood filtration. Compartmentalization is the concept of the BM to prevent passage of cells. Overriding this function is one of the hallmarks of metastatic tumor cells. Another example of cells traversing the BM is when inflammatory cells migrate out of the blood vessels. In the last decades it has become evident that the BM also regulates biological activities like cell growth, survival, differentiation and migration. This regulatory activity can be either indirect, as the BM functions as a



reservoir for growth factors, or direct when BM components in them self serves as ligands to cell signaling receptors. One such ligand is laminin.

### **Laminin structure and isoforms**

Laminins are a family of heterotrimeric proteins assembled from three different classes of polypeptides chains,  $\alpha$ ,  $\beta$  and  $\gamma$ . In vertebrates, 11 genes have been found coding for laminin chains. There are five  $\alpha$  chains (named  $\alpha 1$  to  $\alpha 5$ ), three  $\beta$  chains ( $\beta 1$  to  $\beta 3$ ) and three  $\gamma$  chains ( $\gamma 1$  to  $\gamma 3$ ) which in theory could combine into 45 ( $5 \times 3 \times 3$ ) different heterotrimers. In addition, the  $\alpha 3$  chain has two alternative splice variants ( $\alpha 3A$  and  $\alpha 3B$ ) making even more combinations possible. To date, 18 isoforms have been described, although some of these still awaits some further confirmation of existence (Table 1). This quite limited set of isoforms (when compared to all possible combinations) can have two explanations. First, certain chain combinations may not form a stable trimer and second, the spatial and temporal expression pattern of individual chains may limit the possible combinations. In 2005 a new naming convention of the laminins was established (Aumailley et al., 2005). Previously, the laminin trimers had been numbered in the order of their discovery (laminin-1 to -15) but now the naming is based on chain composition. For example, the former laminin-2, composed of  $\alpha 2$ ,  $\beta 1$  and  $\gamma 1$ , is now termed laminin-211.

**Table 1.** Laminin isoforms

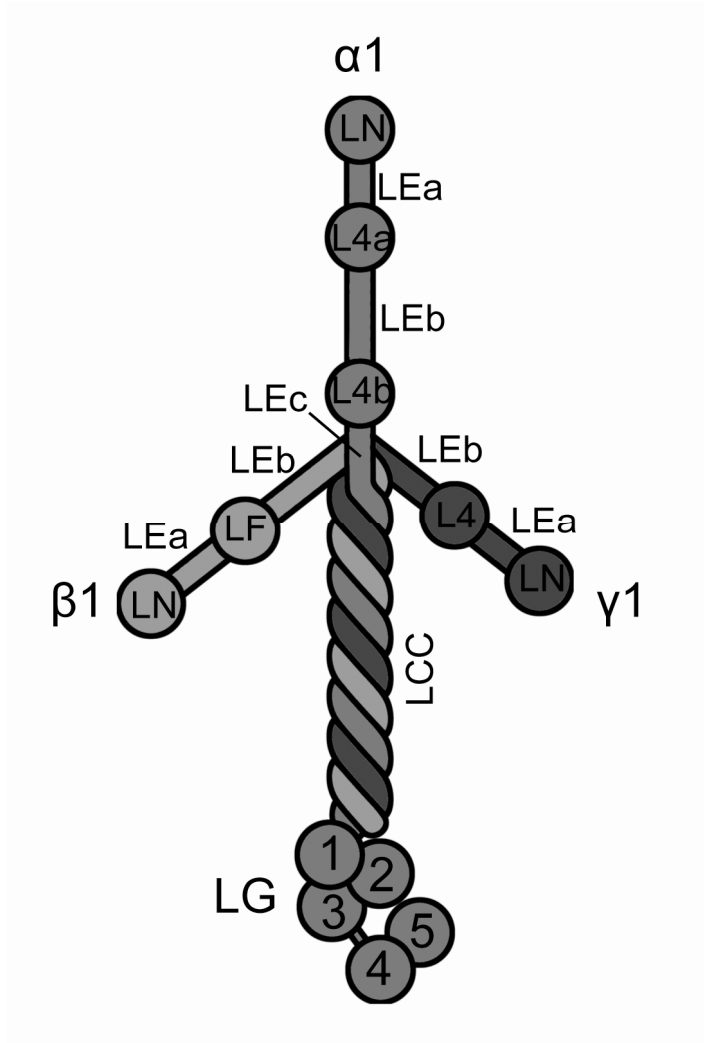
Laminin isoform	Previous names	Reference
111	Laminin-1, EHS laminin	(Timpl et al., 1979)
211	Laminin-2, Merosin	(Ehrig et al., 1990)
121	Laminin-3, S-laminin	(Hunter et al., 1989)
221	Laminin-4, Merosin/S-laminin	(Sanes et al., 1990)
3A32	Laminin-5, Kalinin/Nicein	(Rousselle et al., 1991)
3B32	Laminin-5B	(Garbe et al., 2002)
3A11	Laminin-6, K-laminin	(Marinkovich et al., 1992)
3A21	Laminin-7, KS-laminin	(Champlaud et al., 1996)
411	Laminin-8	(Miner et al., 1997)
421	Laminin-9	(Miner et al., 1997)
511	Laminin-10	(Miner et al., 1997)
521	Laminin-11	(Miner et al., 1997)
213	Laminin-12	(Koch et al., 1999)
423	Laminin-14	(Libby et al., 2000)
523	Laminin-15	(Libby et al., 2000)
212/222*		(Gawlik et al., 2006a)
3A33*		(Yan and Cheng, 2006)
522*		(Siler et al., 2002)

\* Awaits some further confirmation of existence

Laminin-111 was the first isoform to be discovered and, since the EHS mouse tumor produces large quantities of laminin-111, it has also been available for extensive studies (Timpl et al., 1979). Therefore, laminin-111 has often served as a laminin prototype when describing structure and domains. Since this thesis mainly addresses laminin-111 and laminin-211, and these are highly similar in structure, the laminin-111 will be the model hereafter. The new laminin naming convention discussed above also applies to naming the different laminin domains. By electron microscopy, it has been shown that laminin-111 has the shape of a cross, i.e. three short arms (34 and 48 nm) and one long arm (77 nm), showing both globular and rod-like domains (Engel et al., 1981) (Figure 2). Most of these domains are also shared by several other extracellular proteins such as the proteoglycans perlecan and agrin. In the long arm a coiled-coil domain (LCC) can be found. Here, the C-terminal parts of all three chains are joined and interact covalently (Paulsson et al., 1985). The assembly of the laminin molecule takes place through several steps inside the cell. First, the formation of a  $\beta$  and  $\gamma$  chain dimer takes place and then an  $\alpha$  chain is incorporated, which also drives secretion of the trimers (Yurchenco

et al., 1997). At the distal part of the long arm there are five globular domains, exclusively formed from the most C-terminal segment of the  $\alpha$  chain. These are named LG1 to LG5 and are the major cell interacting parts of the laminin molecule. They will be discussed in more detail below. The three short arms are formed from the individual N-terminal segments of the  $\alpha$ ,  $\beta$  and  $\gamma$  chain, respectively. Each of the short arms is terminated by globular N-terminal domains (LN). The LN domains are significantly conserved between the chains and can interact with each other to enable the polymerization of laminin molecules into large networks. Additional globular domains are found on the short arms (L4 and LF) separated by rod-like structures consisting of repeated epidermal growth factor like domains (LE).

All the members in the laminin family share the structure of the long arm with its coiled-coil and LG1-5 domains. However, the short arms show variability in their length. The  $\alpha$ 3A and  $\alpha$ 4 chains are truncated at the short arm giving the molecule more of a T-shape. The  $\beta$ 3 and  $\gamma$ 2 are also slightly truncated. Laminins are both co- and post-translationally modified, intracellularly as well as extracellularly. All laminins are glycosylated but different isoforms are glycosylated in varying amounts (Champlaud et al., 2000). Proteolytic cleavage has been well described for the  $\alpha$ 3A chain in laminin-332. Notably, this cleavage between the LG3 and LG4 domains changes the biological function of the laminin from promoting motility to adhesiveness (Goldfinger et al., 1998).



**Figure 2.** Structure of laminin-111. Laminin globular domain (LG), N-terminal domain (LN), laminin 4 domain (L4), laminin four domain (LF) and laminin epidermal-growth-factor domain (LE).

### The laminin $\alpha$ chains

Laminin chain expression is regulated, leading to tissue and developmental stage-specific localisation of isoforms. Of the three laminin subunits, the  $\alpha$  chain is most important in determining the tissue-specific distribution and specific biological

activity of the laminin isoforms (Miner et al., 1997). The  $\alpha 1$  chain appears in laminin-111 and laminin-121 and was the first  $\alpha$ -chain to be described. Laminin-111 is primarily expressed during embryogenesis and it is the first laminin isoform to be expressed. The  $\alpha 1$  chain is detected already at the blastocyst stage when the very first BM is formed. Reichert's membrane is an extraembryonic BM, found in rodents in early development, in which laminin-111 is the major isoform. The biological relevance for laminin-111 in the early embryogenesis has been confirmed by genetic approaches. Mice deleted of either the  $\beta 1$  or the  $\gamma 1$  gene do not survive past embryonic day 5.5 (E5.5). It was found that no BMs were formed in these embryos and that the endoderm differentiation was defective (Miner et al., 2004; Smyth et al., 1999). When the  $\alpha 1$  gene is targeted, embryo development is extended slightly and embryos die by E7 (Miner et al., 2004). The embryonic BM forms, presumably by the fact that also the  $\alpha 5$  chain (and thus the laminin-511 isoform) is expressed in this BM. However, this compensation is not seen in Reichert's membrane. In another study, the  $\alpha 1$  gene was targeted in a more delicate approach by restricting the deletion to the cell binding LG4-5 domains (Scheele et al., 2005). By this way the  $\alpha 1$  chain is still expressed and incorporated into the BM, although truncated. This makes it possible to exclusively elucidate the role of these domains. The phenotype observed was more severe compared to  $\alpha 1$  deleted mice. From immunofluorescence staining it was concluded that the embryonic BM formed, and probably also Reichert's membrane. The embryos died by E6.5 and failed to cavitate and form a columnar epiblast epithelium. This indicates that when a truncated  $\alpha 1$  is expressed and incorporated into the BM, there is no compensation by the  $\alpha 5$  chain.

Throughout embryogenesis, the hallmark of  $\alpha 1$  expression is its localization in epithelial BMs (Ekblom et al., 1998). And even more specific, were new epithelia are formed. This process, termed epithelial morphogenesis, is a main feature in the development of organs such as kidney, lung, liver and many glandular organs. In some of these, the  $\alpha 1$  expression is retained further on during embryogenesis, and even to adult, but not in all. The kidney is an example when the  $\alpha 1$  chain continues to be expressed into the adult and the lung is an example when it is lost (Pierce et al., 2000). Overall, its expression is more prominent during embryogenesis and more restricted in the adult (Falk et al., 1999; Virtanen et al., 2000). But quantitative measurements of adult organs has demonstrated that  $\alpha 1$  is a major  $\alpha$ -chain in some of these (kidney, liver, male and female reproductive organs and eye)(Sasaki et al., 2002). The main expression in epithelial BM is certainly true, but there are some exceptions when the  $\alpha 1$  chain can be found not adjacent to an epithelium. Examples of this is in the central nervous system were it is expressed in the pial BM and around blood vessels. Conflicting data regarding  $\alpha 1$  expression was resolved when the antibody 4C7 was shown to react with  $\alpha 5$  and not  $\alpha 1$  as first believed (Tiger et al., 1997).

A number of in vitro studies have been set up to assess the role of  $\alpha 1$  chain in epithelial morphogenesis. In organ cultures with antibodies blocking the interaction between  $\alpha 1$  and its cell receptors, perturbed tubule formation was noted (Durbeej et al., 2001; Kadoya et al., 1995; Klein et al., 1988; Sorokin et al., 1992). Since new epithelia are formed during tubulogenesis, these experiments suggested that the  $\alpha 1$  chain was required for epithelial morphogenesis. In vivo data on this function is missing due to the early lethal phenotype in  $\alpha 1$ -deficient mice. However, columnar epiblast epithelium is not formed in embryos lacking the cell binding LG4-5 domains of the  $\alpha 1$  chain, indicating a potential role for laminin  $\alpha 1$  chain in epithelial morphogenesis.

A paralogue of the  $\alpha 1$  chain, originally called merosin but now named  $\alpha 2$ , was discovered in 1988 (Leivo and Engvall, 1988). It was first described as a protein localized to the trophoblastic BM in placenta and to BMs surrounding skeletal and heart muscle cells and Schwann cells. Two years later it was recognized as being a new subunit of laminin and that it associated with the  $\beta 1$  and  $\gamma 1$  chains forming the characteristic cross-like shape molecule (Ehrig et al., 1990). Later phylogenetic analysis of laminin  $\alpha$ -chains has shown that the closest relative to  $\alpha 1$  is  $\alpha 2$  (Zinkevich et al., 2006). The  $\alpha 2$ -chain has been found to form laminin-211, -221 and 213. A complex of  $\alpha 2$ ,  $\beta 1$  and/or  $\beta 2$ , and the  $\gamma 2$  chains is also likely in the PNS (Gawlik et al., 2006a). In contrast to the early embryonic expression of  $\alpha 1$ ,  $\alpha 2$  chain expression is predominantly found in the adult and during later stages of embryogenesis. Laminin-211 is the major laminin isoform in the adult striated muscle (skeletal and cardiac) BM. Developing muscle fibers, called myotubes, start to form about E11 in the mouse embryo. Patches of BM can then be seen on the myotube surface and by E15, most of these BMs have become continuous. This BM is rich in laminin chains  $\alpha 2$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\beta 1$  and  $\gamma 1$  making three isoforms possible; laminin-211, -411 and -511 (Patton et al., 1997). As the myotubes mature into myofibers, the chain composition changes and both  $\alpha 4$  and  $\alpha 5$  are lost. The  $\alpha 2$  expression pattern suggests an important role in muscle and indeed, knock-out mice deficient in the  $\alpha 2$  chain ( $dy^{3k}/dy^{3k}$ -mice) develop a severe muscular dystrophy (Miyagoe et al., 1997). Furthermore, mutations in the human laminin  $\alpha 2$  gene are a common cause for congenital muscular dystrophy. As mentioned above, the  $\alpha 2$  chain is also strongly expressed in Schwann cell BM. In the peripheral nervous system, Schwann cells form the myelin sheath around axons. Consequently,  $dy^{3k}/dy^{3k}$ -mice display myelination abnormalities (Gawlik et al., 2006a).

A neuromuscular junction (NMJ) is the synapse where a motor neuron meets and interacts with a muscle fiber. The composition of laminin chains is some different in the NMJ compared to the extrasynaptic muscle BM. The  $\alpha 2$  and  $\gamma 1$  chains can be found both extrasynaptically and in the NMJ. The  $\beta 1$  chain however, is forming

the laminin-211 trimer in the muscle BM but is excluded in the NMJ, where instead the  $\beta 2$  chain is important. Mice lacking  $\beta 2$  have seriously defective NMJ and die by 3 weeks of age. In addition,  $\alpha 4$  and  $\alpha 5$  chains can also be found in the NMJ, thus three isoforms are possible: laminin-221, -421 and 521. Expression of  $\alpha 2$  can also be found outside the neuromuscular system. The laminin-213 has been identified in human placenta and quantitative measurements of protein levels in tissue extracts have revealed  $\alpha 2$  expression in several adult organs, including intestine, stomach, lung and brain (Sasaki et al., 2002).

The  $\alpha 3$  is expressed in two variants: the shorter  $\alpha 3A$  (or just  $\alpha 3$ ) and full-length  $\alpha 3B$ . The main expression characteristic of  $\alpha 3$ , and the 332 trimer, is its localization in stratified epithelium, contributing to the hemidesmosomes (anchoring site between cell membranes and BM). Stratified epithelium can be found in the skin and mutations in  $\alpha 3$ ,  $\beta 3$  or  $\gamma 2$  chains have been found in patients suffering from junctional epidermolysis bullosa. In this disease the skin becomes very fragile. Expression of  $\alpha 3A/B$  can also be found in lung and intestine.

The  $\alpha 4$  chain is, like  $\alpha 3A$ , truncated in the N-terminal part and has a wide expression. As mentioned above, it can be found in developing muscles and in the NMJ, but also in heart, nerve and adipose cells. However, it is predominantly found in the endothelial basement membranes and the laminin-411 is expressed by all endothelial cells regardless of their stage of development (Hallmann et al., 2005). Mice lacking the  $\alpha 4$  chain are viable and fertile although suffering from a minor blood vessel phenotype. In addition, they have abnormalities in NMJ, peripheral nervous system and heart

The most widely distributed  $\alpha$ -chain in the adult is the  $\alpha 5$  chain. In epithelia, the  $\alpha 5$ -chain appears to be the major  $\alpha$ -chain occurring in later embryonic development and it is often retained in the adult (Ekblom et al., 1998). Interestingly, expression of the  $\alpha 5$  chain appears to succeed that of the  $\alpha 1$  chain in diverse mature epithelia (Durbeej et al., 1996). The  $\alpha 5$  can also be found at non-epithelial sites, such as in some endothelial BMs as well as in the BM of smooth muscle cells and developing skeletal muscle. In adult skeletal muscle, the  $\alpha 5$ -chain expression is lost except for the NMJ where it is retained. Mice deficient of the  $\alpha 5$  gene display a complex phenotype and die late in embryogenesis (Miner et al., 1998).

In summary, all five  $\alpha$ -chains are expressed both in the adult and during embryogenesis, but each is expressed in a distinct pattern. The main patterns of  $\alpha$ -chain expression are established embryonically, but some individual BMs switch  $\alpha$ -chains as development proceeds.

## Receptors

In 1980 it was discovered that EHS laminin (laminin-111) could promote attachment of epithelial cells to type IV collagen substrate (Terranova et al., 1980). Since then, a huge amount of research has focused on elucidating the biological relevance of cell interactions as well as identifying the receptors. The use of proteolytic laminin fragments and of recombinant laminin domains has led to the mapping of several cell binding sites. It has been found that the major cell binding part of the laminin molecule is the globular domains LG1-5 of the  $\alpha$ -chain. Integrins,  $\alpha$ -dystroglycan and syndecans are major laminin receptors (table). The laminin-cell interactions can functionally be divided into three categories: a) promoting polymerization of the laminin network; b) linking the intracellular cytoskeleton to the BM; c) triggering receptor signaling.

The integrins constitute a large family of cell-surface adhesion molecules involved in cell-extracellular matrix and cell-cell interactions. They are transmembrane heterodimeric proteins composed of one  $\alpha$  and one  $\beta$  chain. There are 18  $\alpha$  and 8  $\beta$  subunits that have been found to assemble into 24 integrin isoforms (Takada et al., 2007). The integrins have a bidirectional signaling capacity. That is, they can convey signaling from the inside to the outside of the cell, but also from the outside to the inside (Hynes, 2002). Both the  $\alpha$  and  $\beta$  subunit are involved in ligand-binding, with the  $\alpha$  subunit being central in determining ligand specificity. The list of ligands bound by the integrin family is long (Barczyk et al., 2009), but laminin-111, which is in focus in this thesis, is one major ligand. At least five integrins ( $\alpha1\beta1$ ,  $\alpha2\beta1$ ,  $\alpha6\beta1$ ,  $\alpha7\beta1$  and  $\alpha9\beta1$ ) have been shown to be major laminin-111 binding integrins (Belkin and Stepp, 2000; Nishiuchi et al., 2006). While the  $\alpha$  chains of integrins have a more restricted distribution pattern, the  $\beta1$  subunit is extremely widely expressed; all adherent vertebrate cells produce the  $\beta1$  subunit. Integrin  $\beta1$  deficient mice die during very early development, shortly after implantation (Fassler and Meyer, 1995). In  $\beta1$  null EBs it was found that the laminin  $\alpha1$  chain synthesis was turned off and thus no BM was formed (Aumailley et al., 2000). The  $\alpha6$  and  $\alpha7$  integrin subunits are very closely related integrin  $\alpha$  subunits and both have two splice variants located in their cytoplasmic domain, termed A and B. In addition, both these subunits also have several extracellular splice variants ( $\alpha6$  X1 and X2;  $\alpha7$  X1 and X1X2) (Delwel et al., 1995; Ziober et al., 1993). Integrin  $\alpha6$  deficient mice die at birth displaying skin blisters, reminiscent of the human disorder epidermolysis bullosa (Georges-Labouesse et al., 1996). The integrin  $\alpha7$  subunit is expressed in the neuromuscular system and  $\alpha7$  null mice develop muscular dystrophy (Mayer et al., 1997).

Dystroglycan is a distinct type laminin receptor first isolated from skeletal muscle as a component of the dystrophin-glycoprotein complex (DGC), thus linking the



muscle cell cytoskeleton to the ECM (Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya et al., 1992). It consists of two non-covalently attached subunits, the extracellular  $\alpha$ -dystroglycan and the transmembrane  $\beta$ -dystroglycan, both of which are encoded by a single gene (*Dag1*) and generated by posttranslational cleavage and processing of a precursor molecule (Michele and Campbell, 2003). The laminin  $\alpha 1$  chain binds  $\alpha$ -dystroglycan through its LG4 domain (Andac et al., 1999) whereas both LG1-3 and LG4-5 in laminin  $\alpha 2$  can act as dystroglycan ligands (Talts et al., 1999). Other laminin  $\alpha$  chains have moderate or poor binding affinities to  $\alpha$ -dystroglycan. In addition to laminins,  $\alpha$ -dystroglycan also binds other BM proteins containing laminin globular domains such as perlecan (Talts et al., 1999), agrin (Gee et al., 1994), pikachurin (Sato et al., 2008) and neurexin (Sugita et al., 2001). At the muscle sarcolemma,  $\beta$ -dystroglycan associates with the sarcoglycan-sarcospan complex and binds intracellularly to dystrophin, which in turn is connected to the actin cytoskeleton. The  $\alpha$ -dystroglycan subunit is heavily glycosylated, a process that is required for its ligand binding properties. There are tissue-specific differences in both the extent and nature of this glycosylation, resulting in diversely molecular weight: 120 kDa in the brain and peripheral nerve, 140 kDa in cardiac muscle and 156 kDa in skeletal muscle. Importantly, incomplete glycosylation can lead to a variety of clinical symptoms including muscular dystrophy and abnormal central nervous system development and function. Although originally found in the skeletal muscle, dystroglycan is also expressed in a variety of other tissues including heart and smooth muscle, central and peripheral nervous system and epithelial cells (Durbeej et al., 1998a; Durbeej et al., 1998b).

Despite the central role of dystroglycan in the DGC and its wide tissue distribution, no primary mutations in dystroglycan have been identified in any human disease. Targeted disruption of the dystroglycan gene in mice results in an early lethal phenotype (Williamson et al., 1997). The embryos die at approximately E6.5, most probably due to disruption of the Reichert's membrane. In a more recent study, dystroglycan was conditionally targeted in epiblast derived cells (forming the fetus), while still expressing it in extraembryonic tissues (Satz et al., 2008). Null mice were born at a 12% frequency (about half of the predicted Mendelian ratio) and developed brain and eye defects resembling Walker-Warburg Syndrome. That result also strengthens the hypothesis that the disrupted Reichert's membrane causes the early lethality in mice with constitutive null mutation. Dystroglycan also play important roles in skeletal muscle, brain and peripheral nervous system (Cohn et al., 2002; Moore et al., 2002; Saito et al., 2003). Furthermore, dystroglycan has been ascribed an important role in epithelial morphogenesis throughout embryo development (Durbeej and Ekblom, 1997). This was exemplified by perturbed epithelial branching morphogenesis in kidney, salivary gland and lung in organ cultures with antibodies blocking the laminin  $\alpha 1$  chain-

dystroglycan interaction (Durbeej et al., 1995; Durbeej et al., 2001). Also, dystroglycan has been shown to be important for epithelial cell development in drosophila embryos (Deng et al., 2003). Yet, mice lacking dystroglycan in the embryo have not been reported to display defects in epithelial organs (Satz et al., 2008). However, it should be noted that some of these mice die already during embryogenesis indicating important roles for dystroglycan in embryonic tissues.

## Muscular dystrophy

Muscular dystrophy is a general term that describes a group of inherited and gradually debilitating myogenic disorders, for which there is no treatment. The genetic defects underlying many muscular dystrophies have been elucidated and to date, mutations in more than 30 different genes have been identified to cause different forms of muscular dystrophy. These genes encode proteins located both outside and inside the muscle cell (e.g. interstitial extracellular matrix, BM, sarcolemma, cytoplasm, rough endoplasmic reticulum, Golgi apparatus, sarcomere and nucleus). Duchenne muscular dystrophy, with X-linked recessive inheritance pattern, is the most common form of muscular dystrophy and it affects roughly 1/3500 boys. This disease is caused by mutations in the large intracellular protein dystrophin, which is linked to the sarcolemma through interactions with the DGC.

Congenital muscular dystrophy type 1A (MDC1A) is the most common form of congenital muscular dystrophy, at least among the European population. The general clinical hallmarks of MDC1A are neonatal onset of muscle weakness, hypotonia often associated with joint contractures, severe muscle atrophy, inability to stand and walk and defects in central and peripheral nervous systems. Thirty percent of patients die in the first decade of life (Durbeej and Campbell, 2002; Sciandra et al., 2007). It is therefore urgent to understand the pathology of MDC1A at the molecular level and find an effective treatment for patients. Therapeutic approaches that include the introduction of a functionally related gene seem to be a promising method (so called paralogous gene therapy). Accordingly, we have demonstrated that laminin  $\alpha 1$  chain, which structurally is the most similar to laminin  $\alpha 2$  chain and binds to the same cell surface receptors (integrin  $\alpha 7\beta 1$  and dystroglycan) as laminin  $\alpha 2$  subunit, indeed can compensate for complete laminin  $\alpha 2$  chain deficiency in muscle, testis, and peripheral nerve (Gawlik et al., 2004; Gawlik et al., 2006a; Hager et al., 2005). However, the molecular mechanisms of this phenomenon remain elusive. Most probably, the presence of transgenic laminin  $\alpha 1$  chain in laminin  $\alpha 2$  chain deficient muscle drives the correction of dystrophic symptoms via interaction with either dystroglycan, and/or integrin  $\alpha 7\beta 1$  or, alternatively, other unknown receptors. Our previous data suggest that binding to integrin  $\alpha 7\beta 1$  is more important for the maintenance of normal muscle

function in laminin  $\alpha 2$  chain deficiency - integrin  $\alpha 7$  is lost from sarcolemma in laminin  $\alpha 2$  chain deficient muscles and reconstituted upon overexpression of laminin  $\alpha 1$  chain, whereas the expression of dystroglycan is not that dramatically changed (Gawlik et al., 2006b). In paper IV, we elucidate the roles of dystroglycan and integrin  $\alpha 7\beta 1$  in MDC1A pathogenesis and laminin  $\alpha 1$  chain mediated rescue.

## The Fibroblast Growth Factor family

The fibroblast growth factor (FGF) protein family are known to act as signals between cells and were originally identified because they were essential for the survival and proliferation of mammalian cells in tissue culture. There are 22 members of the mammalian FGF family, differentially expressed in most tissues. Although FGFs can often substitute for one another, the expression patterns of the FGFs and their receptors give them separate functions. The FGF receptors (FGFRs) belong to the receptor tyrosine kinase family and four have been identified so far (FGFR1-4) but their functional diversity is increased by alternative splicing (Powers et al., 2000). This process regulates the number of extracellular Ig domains (two or three) and specifies the sequence of the C-terminal half of Ig domain III, resulting in either the b or the c isoform (Miki et al., 1992). The ligand-binding specificity and tissue-specific expression patterns of the b and c isoforms are different.

During embryonic development, FGFs have important roles in regulating cell proliferation, migration and differentiation and they often signal directionally and reciprocally across BMs in epithelial-mesenchymal boundaries (Hogan, 1999; Lonai, 2003). FGFRs of isoform b are mostly expressed in epithelia whereas isoform c tends to be expressed in mesenchymes (Orr-Urtreger et al., 1993). Ligands specific for these respective isoforms are expressed in adjacent tissues, resulting in directional epithelial-mesenchymal signaling. For example, FGFR2b is expressed in epithelia and can be activated by ligands produced in mesenchymes, such as FGF7 and FGF10 (Igarashi et al., 1998; Ornitz et al., 1996), whereas FGF8 is expressed in epithelia and activates mesenchymally expressed FGFR2c (MacArthur et al., 1995; Ornitz et al., 1996).

FGFR signaling gets activated when FGF ligands, connected by heparan sulfate proteoglycan, bind to the extracellular Ig domains II and III of the receptor leading to its dimerization. (Schlessinger et al., 2000). Upon dimerization, the receptors get activated and transmit their signal through different pathways like PLC- $\gamma$ , PI3K/AKT or Ras/MAP kinase. The important role of FGF signaling in early embryogenesis is exemplified by disrupted development in early blastocyst stage when expressing a dominant negative FGFR4 (Chai et al., 1998). Furthermore, in

EBs, the expression of dominant negative FGFR2 inhibits the differentiation of both endoderm and epiblast (Chen et al., 2000). The effect of this was investigated by gene expression profiling in paper II



# Summary and discussion of papers

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## Paper I, II and III

At the cellular level, embryogenesis can be broken down into four basic cell activities: a) cell fate decisions; b) differentiation/maturation; c) growth/proliferation and d) cell migration. For these events to take place, cells often need to be triggered by external signals. Many of these signals come from soluble factors, but inductive events can also be governed by cell-cell and cell-matrix interactions. The way a cell responds to signals varies, but in a cell fate decision, the ultimate result is a change in DNA transcription. Genes are switched on and off leading to a change in the cell's character. In developmental biology research, one major objective has been, and still is, to identify factors that promote cell fate decisions. But we also need to understand these processes at the molecular level. For instance, what are the changes in the transcriptome? Microarray-based gene expression profiling is a powerful technique that can be used to assess changes in the whole transcriptome simultaneously.

Some of the earliest cell fate decisions in mammalian development are when the ICM is committed to form the extra-embryonic endoderm and the epiblast, both being epithelial tissues. While mouse embryos at this early stage are small and hard to isolate, ES cell derived EBs are considered to be faithful models of these tissues and they can be grown in large quantities. Briefly, FGFs and their receptors are crucial for the formation of the endoderm and the subendodermal BM. The conversion of ES cells to columnar epiblast is subsequently induced by the BM, or more specifically, by the LG4-5 domains in laminin-111.

In paper I-III, the aim was to characterize these early developmental steps at a molecular level. Therefore, we used ES cells, mutated and wild type, to culture EBs and analyzed changes in their transcriptome by microarray-based gene expression profiling.

## Paper I

There are several EBs studies describing an active role for laminin-111 in epiblast differentiation into a polarized epithelium and the process of cavitation (Li et al., 2002; Li et al., 2001). By domain specific knockout, it has further been demonstrated that this signal is facilitated by the globular domains LG4-5 in the laminin  $\alpha 1$  chain ( $\alpha 1$ LG4-5, also known as E3)(Scheele et al., 2005). In this study the aim was to investigate the process of ES cell differentiation into endoderm and epiblast

epithelium, and to elucidate the molecular changes in the transcriptome directed by  $\alpha 1\text{LG4-5}$ . For this purpose, EBs derived from ES cells lacking  $\alpha 1\text{LG4-5}$  ( $\alpha 1\text{LG4-5}^{-/-}$  EBs) were cultured until day 6, a time point when a BM has formed and both endoderm and polarized epiblast epithelia can be seen. RNA expression levels were measured by microarrays and, when compared to control EBs, two lists of differentially expressed genes could be reported: down-regulated and up-regulated. In the analysis of microarray raw data, we decided to use a slightly different approach compared to the one we used in paper II. First, we did not set a fold change (FC) cutoff value and second, we corrected the P-values for multiple testing, thus being more stringent when considering the statistics. In addition, we also made the decision to use four independent replicates (as opposed to the so often used minimum of three) and in that way increase the validity. We also ranked our lists with differentially expressed genes according to fold change.

In the list of down-regulated genes we noticed a common theme among the top ranked ones (i.e. those with highest fold change), eight of the top 20 were encoding secreted plasma proteins (five different apolipoproteins, Apo; alpha fetoprotein, Afp; retinol-binding protein 4, Rbp4 and transthyretin, Ttr), known to be expressed by the VE (Duncan et al., 1997; Meehan et al., 1984). Histological analysis of  $\alpha 1\text{LG4-5}^{-/-}$  EBs reveals a morphologically distinguishable VE, therefore this finding was surprising. We raised the question if differentiation of the VE in some way could be dependent on the BM and  $\alpha 1\text{LG4-5}$ . Yet another observation was that other well known VE markers were not found among down-regulated genes (e.g. Gata4, Gata6, Bmp2 and collagen type IV). The formation and organization of the VE is a multi-step process involving: aggregation, proliferation, differentiation, BM synthesis, maturation and secretion (Grover et al., 1983a; Grover et al., 1983b). Hence, the hypothesis that arose was that  $\alpha 1\text{LG4-5}$  acts in the late stage of VE differentiation, giving the already morphologically formed VE a clearance signal and subsequently the VE completes the final differentiation step to become a functional tissue.

We also found other genes down-regulated that could support the hypothesis of a non-functional VE. One of those was disabled-2 (Dab2), a well used marker for VE and known as a mediator of endocytosis. Other genes encoding proteins participating in endocytosis, like amnionless (Amn) cubilin (Cubn), were down-regulated as well. A major function of the VE is to supply the growing embryo with nutrients (Bielinska et al., 1999). For this, the secreted plasma proteins facilitate transport by acting as carriers and endocytosis is part of the transcellular transport of nutrients (Assemat et al., 2005). Reduced Dab2, Amn and Cubn expression therefore strengthens the idea of a not fully differentiated and functional VE.

One weakness of this study was that we only had access to one  $\alpha$ 1LG4-5<sup>-/-</sup> cell clone, since previous attempts to generate a second clone failed (data not shown). There is always a potential risk that the observed phenotype is not an effect of the mutation itself, but instead is caused by some other mutation that has arisen in the clone. It is therefore good practice to repeat the experiments using another clone. To compensate for the lack of a separate cell clone, we instead verified some of the in vitro results in our mouse model. By crossing heterozygous mice lacking  $\alpha$ 1LG4-5 and examine null embryos we could confirm an abrogated Dab2 expression in the VE.

Our proposed hypothesis also implies another interesting feature of the laminin  $\alpha$ 1 chain. Since the BM, and the laminin  $\alpha$ 1 chain, is synthesized and secreted by the VE in itself, this would mean that the laminin  $\alpha$ 1 chain acts as an autocrine signal. Also, laminin  $\alpha$ 4 and  $\alpha$ 5 chains promote postsynaptic maturation by an autocrine mechanism at the neuromuscular junction. (Nishimune et al., 2008).

## **Paper II**

In this study, the aim was to identify essentially all genes affected by FGF-signaling during EB development. For this purpose, we used ES cells stably expressing a truncated form of FGFR2 (1c6 or dnFGFR2), thereby exerting a dominant negative effect on most FGF receptors (Chen et al., 2000). EBs derived from 1c6 cells form aggregates but then the differentiation ceases. None of the characteristic epithelia are formed and consequently there is no BM. At four time points during EB differentiation we collected RNA for the microarray analysis: 2h (day 0), 24 h (day 1), 48 h (day 2) and 96 h (day 4) after initiation. We compared 1c6 with wild type EBs and listed those genes that were differentially expressed at each time point.

There are several ways to conduct the analysis of microarray data and the recommended choices of methods have changed over time. Well defined guidelines and some framework for the analysis have not yet been defined, although a first step has been taken in setting up The MicroArray Quality Control (MAQC) project (Shi et al., 2006). In paper I, we used the t-test as the statistical test and a P-value of 0.05 was chosen to indicate significance. When doing multiple testing, like in a microarray study, this P-value is often considered too sloppy, generating many false positives. But in our analysis we combined it with the additional cutoff value of 2.0 for the FC and thereby, hopefully, increasing the specificity. Still, this generated an extensive amount of data. To deal with this, we decided to apply two additional approaches, with the first being an EASE analysis.



In an EASE analysis, one looks at a list of genes and try to find if there are some significant “themes” within it. The method is based upon the Gene Ontology (GO) classification of genes into predefined categories under three main topics: a) biological process, b) cellular component and c) molecular function. A significant theme in a gene list could then be some category that is overrepresented, when comparing it to a randomly generated list of the same size. Using the EASE method, we found that the cellular component category of basement membrane was highly overrepresented. This confirms, and strengthens, the view that FGF-signaling is a main regulator of BM formation (Li et al., 2001). Another interesting theme was the biological process of imprinting. Imprinting of genes has previously been shown to take place during early development, in both the ICM and endoderm (Kunath et al., 2005; Okamoto et al., 2004). Our finding raises the possibility that gene imprinting is under the control of FGF-signaling.

The second approach in our data analysis was to limit the gene lists of differentially expressed genes. We therefore ranked the genes according to their FC and continued to analyze only the ones in the top (highest FC). This approach has later been highly recommended in a comprehensive study aiming at giving practical guidance to obtain reproducibility in microarray experiments (Shi et al., 2008). Their recommendation was to use FC-ranking plus a non-stringent P-value cutoff. When we looked at our top ranked genes, the first observation was that the data fitted very well with results published before. Two previous papers have described the step-like process of EB differentiation, beginning with FGF-signaling that induces endoderm development and BM synthesis (Li et al., 2004; Li et al., 2001). Many of our top ranked genes that were dependent on FGF-signaling were of endoderm and BM character (Gata6, Foxa2, Sox17, collagen type IV and laminin  $\alpha 1$  and  $\beta 1$ ). Remaining genes could be categorized into two groups: a) genes associated with FGF-signaling but not described in early embryogenesis and b) genes not before associated with FGF. If these genes are truly regulated by FGF-signaling or just affected due to secondary effects remains to be investigated. On highly down-regulated and top-ranked gene was Flrt3, later shown to be required in the VE for suppressing mesoderm differentiation and regulating BM integrity (Egea et al., 2008)

### **Paper III**

Since ES cells have the potential to differentiate into cells of all three germ layers, they could potentially provide a source for gene therapy treatment of a variety of inherited or acquired diseases. Greater understanding of stem cell differentiation will increase the ability to direct the differentiation of stem cells in a more precise direction. But it will also widen our understanding of early embryonic development. In paper III, we used a subset of the microarray data obtained in paper II to

investigate the gene expression changes in wild type EBs during the first four days of differentiation. By statistical analysis we obtained a list of 429 genes significantly different in at least one of the time points. To get an overview of representative patterns of expression, we used a clustering algorithm that generated six groups with similar expression profile. Stem cell markers like *Rex1* (*Zfp42*), *Nr5a2* and *Esrrb* are important factors for maintaining ES cells in a pluripotent state. When ES cells are induced to differentiate and form EBs, these markers get down-regulated. In agreement with that, we found these three genes down-regulated. In contrast, genes representative for differentiated cell lineages were found up-regulated over time (e.g. *Ilk* for the polarized epiblast and *Afp* for the VE). We believe that this gene expression profiling of differentiating EBs will be a valuable source of information and inspire to further experiments.

## Paper IV

Congenital muscular dystrophy type 1A (MDC1A) is a severe form of muscular dystrophy for which there is no cure. Most patients die as teenager although some can survive into adulthood. The disease is caused by mutations in the gene encoding laminin  $\alpha 2$  chain. Several MDC1A mouse models exist but only *dy<sup>3K</sup>/dy<sup>3K</sup>* mice are completely deficient in laminin  $\alpha 2$  chain. They develop severe muscle degeneration, fibrosis and peripheral neuropathy (Gawlik et al., 2004; Miyagoe et al., 1997). Using this mouse model, our lab has successfully demonstrated that laminin  $\alpha 2$  deficiency can be compensated by expressing laminin  $\alpha 1$  chain in muscle and nerve (Gawlik et al., 2004; Gawlik et al., 2006a).

Lm-211 is the major laminin isoform in muscle and peripheral nerve. It interacts with cell surface receptors through the globular domains LG1-5 of the  $\alpha$ -chain. LG1-3 binds integrins, preferably integrin  $\alpha 7\beta 1$  (in muscle and PNS) and integrin  $\alpha 6\beta 1$  (in PNS). Dystroglycan is bound by both LG1-3 and LG4-5 (in muscle and PNS). The binding pattern is slightly different for the laminin  $\alpha 1$  chain. Integrins and dystroglycan are bound by separate domains with no overlap. LG1-3 binds integrins and LG4-5 binds dystroglycan and importantly,  $\alpha 1$  chain also binds integrin  $\alpha 7\beta 1$ ,  $\alpha 6\beta 1$  and dystroglycan.

The aim of this study was to gain a better insight into the pathogenesis of the disease. Specifically, we wanted to reveal the division of roles between integrin and dystroglycan interactions in the neuromuscular system. For this purpose we decided to express a truncated laminin  $\alpha 1$  chain lacking the LG4-5 domains in *dy<sup>3K</sup>/dy<sup>3K</sup>* mice. The question was whether these domains, and thus the interaction with dystroglycan, were required to rescue the laminin  $\alpha 2$  deficiency.

Two transgenic lines of mice overexpressing  $\delta E3L\alpha 1$  in skeletal muscle, heart and peripheral nerve were obtained. These mice had no observable pathological phenotype and were further mated with mice carrying the mutated laminin  $\alpha 2$  gene to create the rescue mice ( $dy^{3K}/\delta E3L\alpha 1$ ) for this study.  $Dy^{3K}/dy^{3K}$  mice develop a severe phenotype, outwardly recognized by growth retardation and die approximately 5 weeks after birth. In contrast, the overall health and survival of  $\delta E3L\alpha 1$  mice was substantially improved. About 75% were still alive after three months. They gained weight and up to 5 weeks of age they were much alike their wild type littermates. However, beginning from this time point, a severe peripheral nerve phenotype became apparent. Not all animals were affected, but many displayed temporary hindleg paralysis and motor dysfunction. From these observations we could draw two conclusions. First, the laminin  $\alpha 1$  chain rescue is not dependent on the LG4-5 domains when it comes to improve the survival and overall health during the first months of life. Second, the LG4-5 domains do play an active role since the full length laminin  $\alpha 1$  chain compensates the peripheral nerve phenotype better than the truncated  $\alpha 1$  chain. This prompted us to go further in our investigation.

We continued with histological analysis of skeletal muscle by examining both limb muscles and the diaphragm. In the limb muscles we noticed a degeneration/regeneration process evidenced by the presence of centrally located nuclei, small muscle fibers and apoptotic muscle cells. Interstitial fibrosis, a typical hallmark of  $dy^{3K}/dy^{3K}$  muscle, was noted but it was quite modest. To our surprise, neither of these features were noted in the diaphragm. This indicates a different response to the LG4-5 domains within different skeletal muscles. We further hypothesized that the improved diaphragm could be the explanation for the prolonged lifespan in the rescue mice. However, the improved heart might also contribute to increased survival.  $Dy^{3K}/dy^{3K}$  mice die too early to develop heart fibrosis but other MDC1A mouse models with longer lifespan do. Therefore, we examined the heart in  $dy^{3K}/\delta E3L\alpha 1$  mice. There was no sign of fibrosis. This suggests that the LG4-5 domains are needless in correcting both the diaphragm and heart phenotypes. On the other hand, in limb muscles, the LG4-5 domains seem to protect muscle fibers from apoptosis.

The whole process of muscle regeneration can be examined in more detail by intramuscular injection of cardiotoxin. This causes muscle damage and a subsequent regeneration of the muscle fibers. We performed histological analysis and immunofluorescence stainings for regeneration markers at 4 days and 11 days after injection and found the  $dy^{3K}/\delta E3L\alpha 1$  muscles to regenerate as good as the control muscles. After 11 days the muscles were fully restored.  $Dy^{3K}/dy^{3K}$  mice did however show a delay in the regeneration process. This indicates that the regeneration is not dependent on LG4-5 domains but might instead involve the

integrin binding LG1-3 domains. This is further supported by the fact that  $\alpha 7$  integrin-null mice also display delayed muscle regeneration (Rooney et al., 2009).

Next, we went on to investigate the PNS. Spinal roots of  $dy^{3K}/dy^{3K}$  mice display extensive areas with unmyelinated axons. In the full-length rescue this was fully prevented (Gawlik et al., 2006a). The truncated rescue however, failed in that and wide-spread unmyelinated axons were present in spinal roots. In the sciatic nerve, representing the distal part of the PNS, myelin had formed but electron microscopy analysis revealed abnormal myelin sheets. Therefore, the presence of LG4-5 domains in the BM surrounding Schwann cells seems to be of high importance. The question is which is the LG4-5 receptor in the PNS? The major laminin receptors in Schwann cells (dystroglycan,  $\beta 1$  and  $\beta 4$  integrin) have all been targeted in conditional knockout mice and none of them display the same phenotype as  $dy^{3K}/dy^{3K}$  and  $dy^{3K}/\delta E3L\alpha 1$  mice in the PNS (Feltri et al., 2002; Nodari et al., 2008; Previtali et al., 2003; Saito et al., 2003). Therefore, we suspect that the LG4-5 domains interact with some other receptor in the PNS and that this interaction is crucial for myelination.

Muscle and Schwann cell BMs are disrupted in  $dy^{3K}/dy^{3K}$  mice. These were to some extent restored in  $dy^{3K}/\delta E3L\alpha 1$  rescue mice. Electron microscopy analysis revealed patchy BMs. Interestingly, this patchy appearance could also be seen in the diaphragm and in the heart. Two conclusions can be drawn from this. First, the LG4-5 domains are required to assemble fully continuous BMs in the neuromuscular system. However, the BMs in truncated rescue were certainly improved as compared to ones in the  $dy^{3K}/dy^{3K}$  mice. This indicates that LG1-3 and LG4-5 may act together in the assembly of BMs. Second, a fully continuous BM is not a prerequisite for correcting the dystrophic phenotype in the diaphragm and in the heart.

In paper IV, we present data that clearly demonstrate distinct roles for integrins and dystroglycan in the neuromuscular system. In addition, the overall health improvement and substantially prolonged lifespan in  $dy^{3K}/\delta E3L\alpha 1$  mice suggest a more critical role for LG1-3 domains regarding the severity in the course of the disease.



# Conclusion and future perspectives

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In paper I, we suggest a novel function for  $\alpha 1$ LG4-5 in regulating the late stage of VE differentiation. Since laminin  $\alpha 1$  chain is synthesised by the VE itself, this signal would act through an autocrine mechanism. Two important functions for a functional VE are endocytosis and the synthesis of secreted plasma proteins. Our study indicates that both of these functions could be dependent on  $\alpha 1$ LG4-5. In a wider perspective, our result is yet another example of the important role for laminin  $\alpha 1$  chain in epithelia development. Following issues could be addressed in the future:

- A more detailed functional study of the VE could be conducted by an endocytosis assay. Proteins bound for uptake by endocytosis of the VE can be labeled and added to EB culture medium. Differences in endocytosis efficiency can thereby be addressed.
- A genetic approach to elucidate the role of  $\alpha 1$ LG4-5 in epithelial morphogenesis could be addressed by generating tissue specific knockouts. Kidney organogenesis would be especially interesting to study.

In paper II and III, we performed an extensive gene expression profiling of developing EBs, measured at four time points. The time frame is representative for pregastrulation development in vivo. We could conclude that FGF-signaling has an apparent effect on activating VE and BM related genes. Also, genes important for the stemness of ES cells were found to be down-regulated over time. In addition, the catalogue of genes, found to be regulated in developing EBs, also present many genes not previously described to have a functional role in ES cell differentiation. Therefore a future follow-up could be:

- Selecting some of these uncharacterized genes for further studies, starting with a detailed analysis of their expression pattern. One such follow-up has already been published by our lab (Meszaros et al., 2008).

In paper IV, we elucidated the role of  $\alpha 1$ LG4-5 in MDC1A using a mouse model deficient in laminin  $\alpha 2$  chain but instead overexpressing truncated laminin  $\alpha 1$  chain, lacking the LG4-5 domains. The phenotype of mice lacking laminin  $\alpha 2$  chain is severe while a rescue model with overexpressed full-length laminin  $\alpha 1$  chain reduces the dystrophy symptoms to a large extent. In the rescue model with truncated laminin  $\alpha 1$  chain, presented in paper IV, we could report a partial rescue. The  $\alpha 1$ LG4-5 domains were found needless in correcting the diaphragm and heart phenotype but required to some extent in limb skeletal muscle. The discrepancy between limb muscle and diaphragm, both being skeletal muscles, was notable. Furthermore, a muscle regeneration assay clearly showed that the  $\alpha 1$ LG4-5

domains were not involved herein. In the peripheral nervous system, we found myelin abnormalities indicating a crucial role for  $\alpha$ 1LG4-5 in both the myelination process and in myelin maintenance. Finally, by ultrastructure analysis, the  $\alpha$ 1LG4-5 domains were shown to be essential for a proper formation of a continuous BM. Following issues are suggested to be addressed in future studies:

- Elucidate the differences in response between the diaphragm and limb muscle. A microarray analysis could be a first step to characterize the main differences in gene expression.
- Further elucidate the myelination process utilising in vitro myelination assays to investigate the mechanisms of  $\alpha$ 1LG4-5 signal transduction that regulate myelination by Schwann cells.
- Expand the characterization of the mouse model by a detailed analysis of the neuromuscular junction and possible abnormalities.

# Populärvetenskaplig sammanfattning på svenska

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Denna avhandling handlar till stor del om en proteinstruktur som kallas basalmembraner. Dessa finns i stort sett överallt i vår kropp, t.ex. under vår hud finns ett basalmembran vilket betyder att hela vår kropp är omslutet av ett basalmembran. Men även inuti kroppen finns det basalmembraner på många ställen. Nästan överallt där det finns ett hålrum, en gång eller en kanal har vi en vävnad som kallas epitel och alla epitel sitter förankrade på ett basalmembran. Epitel bildas av hårt sammansatta celler som tillsammans med basalmembranet bildar ett tätt skikt som avgränsar hålrummet från den underliggande bindväven. Lungorna och magtarmkanalen är exempel på ställen där ett epitel med basalmembran utgör det yttersta skiktet mot ett hålrum. Förutom i anslutning till epitel så finns det dessutom basalmembraner som klär in enskilda celler, som muskelfibrer, fettceller och nervceller.

Basalmembraner utgör ett stöd för de intilliggande cellerna men är också en källa för att påverka och styra hur våra celler utvecklas och fungerar. Ett av de viktigare proteinerna som bygger upp ett basalmembran kallas laminin. Det finns inte bara ett laminin utan det är en familj som består av ett femtontal varianter varav det som kallas laminin-111 är det jag mest studerat i denna avhandling. Laminin-111 har visat sig kunna påverka framförallt våra epitelceller, och då speciellt när ett nytt epitel skall bildas. Jag har arbetat med att studera betydelsen av basalmembraner och laminin-111 i två skilda system: 1) i väldigt tidig embryoutveckling och 2) i muskler och nerver på en musmodell som efterliknar en kongenital muskeldystrofi.

Vad är en gen? I alla celler (nästan) finns det en cellkärna med DNA-molekyler. Utspritt i detta DNA finns det specifika delar som beskriver hur ett protein skall tillverkas, en slags mall. Dessa proteinmallar i DNA:t är gener. DNA:t och generna är de samma i alla kroppens celler. Detta betyder att alla proteiner som är möjliga för kroppen att tillverka finns beskrivna i alla celler. Men, alla proteiner tillverkas inte i alla celler utan detta är reglerat. Substanser som nervceller tillverkar för att sända ut signaler tillverkas bara i nervceller och insulin tillverkas av cellerna i bukspottskörteln osv. I en viss celltyp är alltså alla gener tillgängliga, men vissa är påslagna och andra är avslagna. Om en gen är aktiverad så tillverkas det protein som den beskriver (för det mest i varje fall). Detta kallas då att gene är uttryckt. Inte nog med det, det finns också en gradskillnad. En gen kan vara mer eller mindre uttryckt, vilket då leder till mer eller mindre av proteinet. Att studera



geners uttryck är mycket vanligt inom medicinsk och biologisk forskning. Vi har i några av våra studier använt en teknik kallad microarray. Med microarray:s är det möjligt att, med en avläsning, kontrollera alla geners uttryck i ett prov man tagit. De blir en slags genprofil på ens prov.

En del av denna avhandling är en studie av vilka gener som är viktiga vid väldigt tidig embryoutveckling. Vi har till stor del gjort studier med hjälp av musstamceller som med en speciell odlingsteknik kan fås att efterlikna samma händelseförlopp som sker när en riktig mus börjar utvecklas. Den stora frågan är hur olika slags celltyper kan bildas utifrån stamceller. När en stamcell ombildas så förändras alltid också dess genuttryck. Detta har vi studerat med microarray:s. Vi har kunnat konstatera att basalmembraner är med och styr denna process. I en av våra studier tittade vi extra noga på laminin-111:s roll i detta. Vi hade tillgång till musstamceller som tillverkade en muterad variant av laminin-111. En viktig cellbindande del av laminin-111 var borttaget. Frågan var: vad händer med genuttrycket när dessa celler utvecklas? Vi mätte genuttrycket i dessa och jämförde med det genuttryck vi fick när musstamceller utan denna mutation fick utvecklas. Skillnaderna gav oss ett svar. En av celltyperna som bildas, kunde inte fullfölja sin utveckling hela vägen om laminin-111 var muterat. Alltså, har vi kunnat tilldela denna cellbindande del en viktig biologisk funktion då musstamceller ombildas till andra celltyper.

När musstamcellerna skall börja utvecklas så är de beroende av en signal från en molekyl som kallas FGF. Vi har i en andra studie gjort en katalog över alla de förändringar i genuttryck som denna FGF-signal ger upphov till. Detta gjorde vi genom att låta både vanliga stamceller och stamceller med en förhindrad FGF-signal utvecklas och sedan jämföra skillnader i deras genuttryck. Det visade sig att, de gener som ser till att det bildas ett basalmembran var väldigt framträdande. I en andra studie gjorde vi katalog över skillnader i genuttryck då vanliga stamceller utvecklas under fyra dagar. Vi kunde konstatera att gener som är viktiga för att stamceller förblir en stamcell stängdes av under utvecklingen. Sådana gener som styr bildandet av nya celltyper blev däremot påslagna. I framtiden har man en förhoppning om att kunna använda stamceller från människor till att i laboratoriet producera olika celltyper. Dessa skulle sedan kunna bli en källa till att behandla sjukdomar, som till exempel diabetes typ 1. För att kunna styra stamceller är det viktigt att först förstå vad det är för processer som är viktiga när de utvecklas. I våra tre första studier har vi katalogiserat förändringar i genuttryck när stamceller utvecklas. Vår förhoppning är att dessa skall komma till användning i fortsatt stamcellsforskning.

Kongenital muskeldystrofi typ 1A (förkortat MDC1A) är en nedärvd sjukdom som drabbar barn i tidig ålder. Barnen lider av muskelsvaghet och muskelförtvining, men även centrala och perifera nervsystem drabbas. Det finns idag ingen bot mot

sjukdomen och många dör innan de blir vuxna. Patienter med MDC1A har någon form av mutation i den gen som beskriver ett laminin kallat laminin-211. Detta laminin är en byggsten i de basalmembraner som omger muskelfibrer, men även i basalmembraner som omger nerver. För att bättre studera denna sjukdom har det tagits fram musmodeller som till stor del utvecklar samma symptom. Vi har arbetat med en musmodell där genen för laminin-211 är muterad så att inget protein kan uttryckas. Dessa möss blir så sjuka att de dör inom fem veckor.

För att ha bättre möjligheter att finna någon bot mot denna sjukdom måste grundforskning först tydliggöra sjukdomens underliggande mekanismer. Vi vet att grundproblemet är ett icke fungerande laminin-211, men vi vill veta mer i detalj vad detta laminin har för viktig funktion. Lamininer är ganska stora proteiner med flera delar som har lite olika funktion. En del av lamininer binder till celler via så kallade receptorer på cellens utsida. Laminin-211 binder till framför allt två receptorer, kallade integrin och dystroglykan. Vi ville undersöka vilken roll respektive receptor har i sjukdomen.

I en ett antal tidigare studier har vi visat att den musmodellen som saknar ett uttryck av laminin-211 kan "räddas" (mössen blir i stort sett friska och lever upp till två år) genom att man låter laminin-111 kompensera. Laminin-111 binder till samma receptorer som laminin-211 och detta utnyttjade vi. Vi kompensterade musmodellen med ett laminin-111 som saknade den delen som binder till dystroglykan, men behåller den delen som binder integrin. Det visade sig att dessa möss också "räddades", men inte riktigt lika bra. Mössen levde längre och hade relativt bättre muskelförmåga men det var framförallt störningar i det perifera nervsystemet som inte blev förbättrade. Slutsatsen är att den interaktion som sker med dystroglykan är viktig i det perifera nervsystemet men inte lika viktig i muskler. Denna upptäckt ett steg i riktning mot en bättre förståelse för de mekanismer som orsakar sjukdomsbilden i kongenital muskeldystrofi.



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