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Review

How is Myogenesis Initiated in Chordates?

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Expression of transcriptor factors MyoD, Myf5, myogenin and MRF4 forms the basis of myogenesis. In Acrania, Pisces and Amphibia, as in Aves, myogenesis is initiated by MyoD. In Mammalia expression of Myf5 initiates myogenesis. Signal proteins Wnt and Shh induce the expression of genes encoding for MyoD or Myf5. In fishes and amphibians expression of MyoD starts in non-segmented mesoderm and then in myotomal cells. In birds and mammals expression of MyoD or Myf5 is initiated in the cells of the dermatomyotome. Embryonic myotomes are post-mitotic. Proliferating cells Pax3 and Pax7-positive and mesenchymal cells take part in the growth of myotomal muscles. Cells migrating to the limb bud contain regulatory proteins Six4/Six1, Pax3, Lbx1 and c-met. *Rectus abdominis* develops from cells that contain Pax3 and Lbx1.

Key words: Signaling molecules; regulatory factors; cell interaction; "communication effect"; myotome; dermomyotome; directed migration.

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Axial skeletal muscles originate in the nascent somites – compartments of the paraxial mesoderm that form in rostral-caudal progression during the early development of Chordata. In the embryo, somites differentiate into myotome. The myotome then develops into skeletal muscles.

In vitro studies on myogenesis in the 1950s and 1960s initiated a revision of earlier data and revealed new facts. In the model of myogenesis, after the proliferation period myogenic cells asynchronously withdraw from the cell cycle in phase G1 and pass into phase G0. Post-mitotic myoblasts assume an elongated shape and are arranged along a straight line. Myoblast recognition, adhesion and fusion lead to the formation of polynucleated myotubes. Development of myofibrills starts in the myotube (HOLTZER *et al*. 1958; STREHLER *et al*. 1963; BISCHOFF & HOLTZER 1969). The myotubes grow as a result of post-mitotic myoblasts fusing with them. The cells that do not fuse with the myotube remain under a common basal membrane. These are "dormant" or "spare" myoblasts (satellite cells) (MAURO 1961; ISHIKAWA 1977; MORGAN & PARTRIDGE 2003).

Transcription factors

The discovery of transcription factors at the turn of the 1980s and 1990s stimulated studies into myogenesis at the genetic and molecular levels. Transcription factors form the MRF family (Myogenic Regulatory Factor).

The first studies able to identify myogenic transcription factors exposed non-muscle cells to 5-azacitidin. 5-azacitidin initiates the activation of transcription of gene MyoD, normally inactive in such cells. The products of this gene induced mouse fibroblasts of the C3H10T1/2 lineage to convert into myoblasts (DAVIS *et al*. 1987; WEINTRAUB *et al*. 1989, 1991). Then, a further three transcription factors were discovered: Myf-5 (BRAUN *et al*. 1989), myogenin (WRIGHT *et al*. 1989) and MRF4 (RHODES & KONIECZNY 1989). All the known proteins of the MRF family: MyoD, Myf-5, myogenin and MRF4, have a highly conservative domain bHLH (basic, Helix Loop Helix) (Fig. 1). In the myogenic programme, the basic region b is bound by the "CANNTG" DNA sequence (N is any nucleotide). The sequence is contained in an "E-box" of promoter and/or enhancer, and activates transcription of muscle-specific genes. Alanin and threonin of the basic region of the bHLH

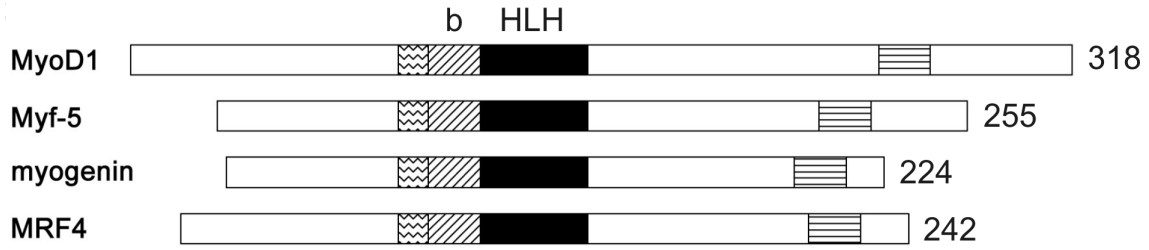

Fig. 1. Diagram showing the structure of transcription factors MyoD1, Myf5, miogenin, and MRF4. The numbers of aminoacids are given. bHLH domain in myogenic transcriptional factors is highly conservative. (after: ARAKI *et al*. 1994, modified).

domain play a particular role in recognition of DNA-binding sites (WEINTRAUB *et al*. 1991). The HLH section of regulatory proteins is responsible for dimerisation with commonly occurring E proteins (Enhancer Binding Factors): E12 and E47, encoded by gene E2A (BLACKWELL & WEINTRAUB 1990). Each member of the MRF family controls its own expression and expression of other factors of the family (OLSON 1992). Expression of the MRF family genes occurs only in myogenic cells of skeletal muscles, but each protein of the family can transactivate muscle-specific genes in non-muscle cells (WEINTRAUB *et al*. 1989).

Expression of the genes of the regulatory factor family shows differences in its pattern and function in consecutive stages of muscle differentiation. In the two-step model transcription factors MyoD and Myf-5 form a pair of equivalent factors whose functions overlap and can replace each other. These proteins determine cells towards myogenesis and initiate myoblast differentiation. Expression of myogenin and MFR4 genes affects the later phase of myogenesis. Myogenin affects myotube differentiation while MRF4 affects the phase of differentiation of muscle fibres (ASTCHLEY *et al*. 1994).

Another important family of transcription factors engaged in positive regulation of muscle differentiation is MEF2 (Myogenin Enhancers Factor 2). Four *MEF2* genes (A-D) code for transcription factors of the MADS-box family (Agamous Deficiens Serum). The MADS domain of the regulatory protein MEF2 is highly conservative (LUDOLPH & KONIECZNY 1995). Proteins MEF2 bind the A/T-rich DNA sequence which occurs in promoters of many muscle-specific genes (GOSSETTI *et al*. 1989; OLSON *et al*. 1995). The regulatory factors MEF2 activate genes through binding with a recognisable DNA site, but they also interact with heterodimers bHLH/E12, which bind E-box sequences of DNA. In contradistinction to the MyoD protein family, the regulatory proteins MEF2 cannot initiate myogenesis in non-muscular cells (YUN & WOLD 1996).

The family of regulatory proteins MyoD/E, with the family MEF2, form a network which initiates the expression of muscle-specific genes, e.g. muscular creatine kinase (MCK), desmin, myosin light chain (MLC) and subunits of acetylocholin receptor (AchR) (YUN & WOLD 1996; MOLKENTIN & OLSON 1996).

Besides the factors engaged in positive regulation of myogenesis, the role of negative regulators is also important. In proliferating cells transcriptional activity of the bHLH protein family is suppressed. Recently, many proteins have been identified as negative regulators. One of them is the Id protein (Inhibitor of DNA Binding) of the protein group HLH (BERKES & TAPSCOTT 2005).

During myogenesis, proliferation and phenotypic differentiation of myoblasts are mutually exclusive (LASSAR *et al*. 1994; OLSON 1992). Differentiation starts in post-mitotic cells. The mechanism leading to the withdrawal of cells from the cell cycle is debatable. It is conjectured that the MyoD protein induces expression of Id, the inhibitor of cyclin-dependent kinase (cdk), engaged in regulation of the course of the cell cycle (HALEVY *et al*. 1995). It has also been suggested that withdrawal of cells from the cell cycle is associated with decreased function of numerous receptors of the growth factor FGF (Fibroblastic Growth Factor), which stimulates cell divisions (GILBERT 2000). The decreased function of the receptors leads to the disappearance of pathways of signals leading from the cell membrane to the nucleus. Myoblasts enter the myogenesis phase *in vitro*, when the FGF concentration decreases below a critical threshold. Following myoblast fusion, myotube nuclei become resistant to the signals stimulating DNA replication (MOORE *et al*. 1991).

Histones

Histones, basic proteins, are components of nucleoproteins which build chromatin and are associated with nuclear DNA. These proteins undergo post-translational, reversible modifications, e.g. acetylation, methylation and phosphorylation. Due

to this, histones have a significant effect on the degree of compacting of DNA in the chromosomes and on the availability of DNA during replication and transcription. HATs (Histone Acetyltransferases) stimulate transcription through histone acetylation. Acetylation leads to a loosening of nucleosome (basic unit of chromatin) while HDACs (Histone Deacetylases) antagonise the process and inhibit transcription (KUO & ALLIS 1998).

Moreover, gene expression requires coordination of function between the transcription factors and chromatic modelling enzymes (MAL *et al*. 2001; SARTORELLI & CARETTI 2005). It has been demonstrated that chromatin associated with muscle genes and regulated by the transcription factors bHLH and MEF2 undergoes acetylation during myogenesis, while deacetylases of class II histones inhibit the process. The HDACs do not interact directly with MyoD but inhibit myogenesis through binding with the bHLH/MEF2 complex. The HDACs bound to active MEF2 inhibit transcriptional acivity. Blocking of myogenesis can be abolished through dissociation of the HDACs-MEF2, an increased MyoD level and the effect of CaMK (Calcium calmodulin dependent protein kinase) and IGF (Insulin-like Growth Factor). The results emphasize the important role of CaMK signalling in the regulation of chromatic modelling which is required for gene activity (LU *et al*. 2000). CaMK stimulates myogenes and also induces the export of HDACs from the nucleus to the cytoplasm through phosphorylation of these enzymes. HDAC5 occurs in the nucleus of proliferating myogenic cells; following initiation of myoblast differentiation HDAC5 is located in the cytoplasm. MEF2 remains in the nucleus in order to fulfill its main transcriptional function. It is thought that the export of chromatin-modelling enzymes from the nucleus to the cytoplasm is associated with control of differentiation of muscle cells (McKINSEY *et al*. 2000).

Trans-membrane proteins

Prior to fusion, myoblasts undergo highly ordered processes which need to be coordinated. An important role in the regulation of these processes is played by adhesion, cell-cell interaction and signal effects (KRAUSS *et al*. 2005). Many adhesion molecules have been identified recently. The family of cadherins and associated proteins CDO and BOC of the Ig superfamily (immunoglobulins) are among the best known (KANG *et al*. 2002, 2003; KRAUSS *et al*. 2005). Cadherins are a family of trans-membrane glycoproteins. Their extra-cellular domain forms adhesive receptors. The cytoplasmic domain binds with actin filaments of cytoskeleton *via* catenins. As a result, the adhesive receptors become bound to the cytoskeleton (HYNES *et al*. 2000). Cadherins play a crucial part in cell interactions. They function not only in establishing strong adhesion between cells, but also determine the properties of cells undergoing adhesion. Moreover, they participate in homotypic intercellular interactions, binding cells which have cadherins of the same type (Fig. 2). Cadherins participate in numerous signal pathways which regulate cell behaviour. The role of cadherins consists of cell recognition, adhesion, selection and signalisation, as well as in determining their shape, polarity and movement (GOICHBERG & GEIGER 1998; HYNES *et al*. 2000; WHEELOCK & JOHNSON 2003).

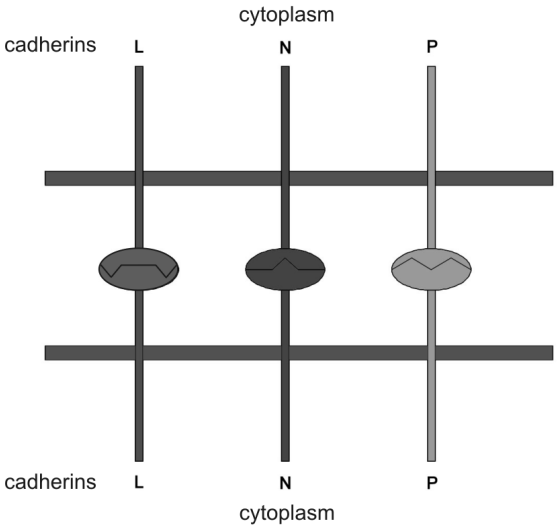

Fig. 2. Diagram showing different trans-membrane proteins, cadherins. Their extra-cellular domains, adhesive receptors, exhibit binding specificity (after: KLEIN, 1998, modified).

CDO (CAM-related/Down-regulated by Oncogenes) is a trans-membrane protein composed of an external domain, a trans-membrane section and a long cytoplasmic tail (KANG *et al*. 1998). It has been suggested that there exists a positive feedback between CDO and the nuclear regulatory proteins bHLH, and that CDO may be a new integral component of the myogenic regulatory network of the MyoD/E family with MEF2. Furthermore, it is suspected that CDO transfers signals which stimulate the activity of the bHLH domain, transcription factors and which reinforce heterodimerisation of HLH with E proteins, most probably through hyperphosphorylation of E proteins (KANG *et al*. 1998, 2002; KRAUSS *et al*. 2005; COLE *et al*. 2004). BOC proteins have an ectodomain similar to that of CDO, a trans-membrane section and a long cytoplasmic tail. BOC is an additional participant of the positive feedback between CDO and

the regulatory proteins. Simultaneous expression of CDO and BOC in myoblasts of the C2C12 lineage suggests that BOC and CDO may function together. A very attractive hypothesis assumes that BOC and CDO form a complex receptor which responds to a still unidentified ligand probably engaged in the regulation of the muscle differentiation programme (KANG *et al*. 2002). CDO and BOC proteins do not show active adhesion but their association with cadherins indicates localisation of adhesive functions of the cadherins. It has been suggested that the CDO-BOC association may modify adhesive properties of the cadherins, reinforcing their adhesive potential (KANG *et al*. 2002, 2003).

Myogenesis requires interaction of myogenic cells with the extracellular matrix (ECM). This condition is met by integrins which are the main receptors for many ECM ligands. Integrins are trans-membrane heterodimers composed of two chains: α and ß. It has been found that the complex of subunits α3/ß1 combined with ADAM12 proteins is associated with myoblast fusion, which leads to interruption of cell membrane continuity and cytoplasm fusion of the fusing myoblasts. Following fusion, the integrin subunits ß1 participate in the building of the cytoskeleton and in arranging myofilaments into sarcomeres (SCHWANDER *et al*. 2003; BRZÓSKA *et al*. 2006).

Results from mainly in *in vitro* studies, described above, have made it possible to define many essential factors engaged in myogenesis. However, these studies have failed to demonstrate a fully genetic or morphogenetic control of myogenesis during embryogenesis. The dynamic nature of these processes requires signallisation factors, new transcription factors and new functions of trans-membrane proteins.

Initiation of myogenesis in embryonic development

1. Tunicata

Tunicate tail muscles, compared to chordate muscles, show no metamerisation and are built of unicellular, transversely striated muscle cells.

In *Halocynthia roretzi*, the eight-cell embryo is composed of four pairs of blastomeres: B41, b42, A41 and a42. The "determinants" of yellow ooplasm (myoplasm) of the fertilised egg discovered by CONKLIN (1905) are segregated to blastomeres of the B41 lineage during cleavage. The determinants lead to autonomous development of primary muscle cells. The cells derived from blastomeres of the b and A lineages differentiate into secondary muscle cells as a result of cell interactions, probably during gastrulation (NISHIDA 1990). Almost a hundred years after Conklin's discovery, it was shown that in *H. roretzi* a component of the determinants was mRNA, a transcript of the gene *Macho-1*. The gene *Macho-1* codes for a nuclear regulatory protein with a domain of zinc fingers (NISHIDA & SAWADA 2001). These authors point out that the "maternal macho-1 mRNA may be both required and sufficient for specification of muscle fate during embryogenesis".

Furthermore, *H. roretzi* has one *MyoD* gene (*AMD1*), like invertebrates. The gene *AMD1* codes for a protein whose bHLH domain is homologous with the bHLH domain of vertebrate MyoD proteins (SATOH *et al*. 1996). Transcripts of the *AMD1* gene appear in cells of the B41 lineage beginning at the stage of 64 cells. The level of expression of *AMD1* decreases at the neurula stage. In secondary muscle cells the expression of *ADM1* is strong at the stage of the neural plate (ARAKI *et al*. 1994). Transcripts of the gene of muscle isoform of actin occur in the embryos of *H. roretzi* earlier than those of *ADM1*, since they appear already at the stage of 32 cells (SATOH *et al*. 1996; KUSAKABE *et al*. 1991). Likewise, transcripts of the myosin heavy chain appear at this stage (MAKABE *et al*. 1990). The expression of the *AMD1* gene appears in tunicate development later and thus cannot have any effect on the determination of muscle cells (MEEDEL *et al*. 1997), but may be responsible for maintaining their differentiated state and reinforcing the expression of structural muscular genes (SATOH *et al*. 1996).

2. Acrania

The lancelet is a living precursor of vertebrates. In its development the paraxial mesoderm undergoes segmentation, as in vertebrates. The lancelet's myotomal cells differentiate into thin (1 μm) mononucleate muscular lamellae, transversely striated and remaining in this form throughout life. *Branchiostoma belcheri tsingtauense* has only one myogenic gene *AmphiMDF* (Amphioxus Myogenic Determination Factor) in its genome; it codes for the bHLH protein only. The aminoacid sequence of the bHLH domain and its phylogenetic analysis indicate great similarity to the bHLH domain of regulatory proteins of the MyoD family in vertebrates, and the "basic" domain shows 100% identity. It has been suggested that the gene *AmphiMDF* is not only a sister gene but also the ancestor of the four myogenic genes of vertebrates, and that its duplication may have been the main mechanism of the origin of the *MyoD* gene family in vertebrates (YUAN *et al*. 2003). The generally accepted model of evolution of vertebrate genes is "one-two-four" (MEYER & SCHART 1999; YUAN *et al*. 2003). According to this model, the gene underwent double duplication, resulting in two and then four genes of the *MyoD* family.

However, in *Brachiostoma floridae*, two genes – members of the *MyoD* family: *BMD1* and *BMD2* – are of different origin. Aminoacid sequences of the bHLH domains of the BMD1 and BMD2 proteins are not similar to each other or to sequences of any member of the vertebrate MyoD family. It has been suggested that an independent duplication and reorganisation of the genes took place, leading to the four myogenic genes of the vertebrate *MyoD* family (ARAKI *et al*. 1996). The expression of the genes *AmphiMRF1* and *AmphiMRF2* in this species occurs independently, at different developmental stages, in the unsegmented paraxial mesoderm and in the myotomes. Moreover, the expression of these genes partly overlaps (SCHUBERT *et al*. 2003).

Results of various studies raise the question when and how in chordate evolution the single gene inherited from invertebrates was duplicated and when the four genes of the MyoD family, present already in fishes, came into existence (ASTCHLEY *et al*. 1994; RESCAN 2001).

3. Pisces

In *Brachydanio rerio*, the model fish species, myogenesis starts before mesoderm segmentation, in adaxial cells. The notochord produces signalling proteins Hh (Hedgehog), inducing expression of the *MyoD* and *Myf5* genes in adaxial cells (COUTELLE *et al*. 2001). Studies on other fish species show that the notochord induces expression of the *MyHCs* (slow myosin heavy chain) in *Acipenser ruthenus* (STEINBACHER *et al*. 2006), *MyHCs* and *MyoD* in *Coregonus lavaretus* (KACPERCZYK *et al*. 2009) and *MEF2* in *Salmo trutta lacustris* (STEINBACHER *et al*. 2008).

Adaxial cells in *B. rerio* migrate onto the lateral surface of the embryonic myotome. There they differentiate into mononucleate slow-twitch muscle fibres (DEVOTO *et al*. 1996; BLAGDEN *et al*. 1997; BARRESI *et al*. 2000; COUTELLE *et al*. 2001). Molecular studies have shown that their migration is associated with N and M cadherins (CORTES *et al*. 2003). Non-migrating cells of the embryonic myotome differentiate into fast-twitch muscle fibres. Hh signals induce morphogenetic processes of myotomal cells *via* migrating cells (HENRY & AMACHER 2004). In many fish species synchronously differentiating cells of embryonic myotome result in the formation of a post-mitotic myotome (DACZEWSKA 2006; KACPERCZYK & DACZEWSKA 2006).

Formation of dermomyotome in *B. rerio* is associated with somite rotation by 90 degrees. Signalling by a ligand of Sdf (stromal derived factor) is crucial for the rotation. Expression of cxcr4a and cxcr4b receptors, Sdf ligand (CHONG *et al*. 2001) has been identified in the proximal domain of the somite. Expression of the Sdf ligand occurs in the distal lateral edge of the somite. It has been suggested that the stimulus of the Sdf ligand induces and directs cells which contain cxcr4 receptors to undertake somite rotation. Following rotation, cells of dermomyotomal characters are located on the lateral surface of the somite (HOLLWAY *et al*. 2007; STELLABOTTE *et al*. 2007).

The dermomyotome cells, the "external cell layer", in *B. rerio* are characterised by the presence of transcription factors Pax3 and Pax7. These cells, after immigrating into the myotome, become MRFs-positive (DEVOTO *et al*. 2006; HOLLWAY *et al*. 2007). The expression of Pax3 has also been detected in the dermomyotome cells of *C. lavaretus*. These cells, together with Pax7 cells, are engaged in hypertrophic and hyperplastic growth of myotomal muscles (KACPERCZYK *et al*. 2009). In *Salmo trutta* immunolabelling indicates that almost all dermomyotomal cells are Pax7-positive. Only a few nuclei stained for markers such as myogenin and MEF2. De-epithelialization of these cells from dermomyotome is the main mechanism promoting fast muscle growth (STEINBACHER *et al*. 2008). Pax7-positive cells have also been found in post-larval myotome of *B. rerio* (STELLABOTTE *et al*. 2007). However, their very small number suggests that cells of different origin may also take part in the growth of myotomal muscles. Perhaps they include cells of mesenchymal origin; such cells have been found in many fish species, they have high myogenic potential and participate in hyperplasy and hypertrophy of myotomal muscle fibres (DACZEWSKA 2006).

The Hh signallisation in *B. rerio*, irrespective of its earlier function inducing adaxial cells into slow-twitch fibres, in later developmental stages induces Pax3 and Pax7–positive cells in the dermomyotome which differentiate into fast-twitch fibres. Genetic studies have shown that the Hh signals are directly received by dermomyotome cells (FENG *et al*. 2006).

Pectoral fin muscle cells in *B. rerio* originate from the ventral part of the somite. They are migrating cells characterised by the expression of the gene encoding the regulatory protein *Lbx1*. Cell migration control is the role of this factor. Differentiation of fin muscle cells starts after their immigration into the fin bud. MyoD is the first to appear in myoblasts, followed by MyHC (myosin heavy chain) (NEYT *et al*. 2000).

4. Amphibia

In *Xenopus laevis* initiation of myogenesis is associated with mesoderm induction. Accumulation of *MyoD* transcripts in paraxial mesoderm cells at

the stage of mid gastrula is a molecular response to inducing factors FGF and TGF-ß from the newly formed endoderm; two hours later actin transcripts accumulate. Mesodermal induction in *X. laevis* leads to activation of myogenic determination. However, these cells while expressing of MyoD do not complete the differentiation programme (BUCKINGHAM 2002). Paraxial mesoderm cells in early and mid gastrula, translocated singly into new positions, to the gastrocoel, do not differentiate. They enter myogenesis only after having formed a large group. It has been shown that the expression of the MyoD gene requires communication of more than 100 cells. This is the "communication effect" in which the MyoD expression occurs independently of mesoderm induction. At the stages of late gastrula and neurula the cells lose their communication dependence and can resume differentiation without their original neighborhood (HOPWOOD *et al*. 1989, 1992; GURDON *et al*. 1993). A diffusion substance, which needs to reach a required concentration threshold in order to be effective, mediates cell communication (GURDON *et al*. 1993). The condition is met by a great number of cells. The substance is most probably endogenous eFGF (embryonic Fibroblastic Growth Factor) (STANDLEY *et al*. 2001; FISHER *et al*. 2002). Another factor which participates in the "communication effect" is cadherin which has a signallisation function and is able to coordinate the activity of *MyoD* genes in adaxial mesodern cells (HOLT *et al*. 1994).

At the tail bud stage in *X. laevis*, a high level of the transcription factor MyoD has been detected in the nuclei of the youngest myotome; MyoD disappears gradually in increasingly older myotomes in a cephalic direction. The event is associated with progressing differentiation of myotomal muscles in a rostro-caudal direction. It is emphasised that MyoD only initiates myogenesis and does not maintain cell differentiation (HARVEY 1992). Parallel with the effect of MyoD, Myf5 participates in the initiation of myogenesis (HOPWOOD *et al*. 1991). The combined action of genes *MyoD* and *Myf5* emphasizes their simultaneous function as myogenesis promoters (HOPWOOD *et al*. 1992). In *Hymenochirus boettgeri*, the presence of MyoD has been detected in the nuclei of unsegmented mesoderm cells, in myotomal cells during their rotation and in the nuclei of myoblasts during differentiation into mononucleate myotubes. The gradual disappearance of the reaction in increasingly older myotomes confirms the initiating role of MyoD and suggests that other regulatory factors take control over myogenesis (DACZEWSKA 2001).

During somitogenesis in *X. laevis* and *H. boettgeri*, myotomal cells undergo rotation by 90 degrees (KIEŁBÓWNA 1981; DACZEWSKA 2001). Based on morphological studies on rotating cells, it has been hypothesised that the first cell initiating rotation induces other cells in a coordinated way (YOUN & MALACIŃSKI 1981; AFONIN *et al*. 2006). At the molecular level, the process requires intercellular signallisation. The main role in this event is played by type I, Ca-dependent cadherins (GIACOMELLO *et al*. 2002).

In *X. laevis*, *H. boettgeri* and *Bombina variegata* (closely related species), myogenesis starts in mononucleate myoblasts, omitting cell fusion; the cells differentiate into mononucleate myotubes. Myotube nuclei in *B. variegata* contain 4cDNA and do not incorporate tritium-labelled timidin. These facts indicate withdrawal of myoblasts from the cell cycle in the G2 phase (KIEŁBÓWNA & KOŚCIELSKI 1979). Nuclei of differentiating myotubes of *X. laevis* also do not incorporate tritium-labelled timidin, as in *B. variegata* (KIEŁBÓWNA & DACZEWSKA 2005). Synchronous myoblast differentiation in the studied amphibians leads to the formation of post-mitotic myotomes.

In *Xenopus*, after the initiation of the myogenic programme by the expression of MyoD and Myf5, different muscle-specific genes are sequentially expressed during primary myogenesis, including members of the actin, tropomyosin and myosin gene families. *Xenopus* myogenesis is unique in that myogenin is not expressed in the primary myotome. This observation supports the hypothesis that MyoD and/or Myf 5 could play the role of myogenin during *Xenopus* primary myogenesis (CHANOINE & HARDY 2003). MRF4 mRNA is detected only at stage 18 and increses until around stage 22-23, remaining approximately constant thereafter (JENNINGS 1992).

At later developmental stages (around stage 37), proliferating bipotential mesenchymal cells (of unknown origin), capable of differentiating into myoblasts and fibroblasts, migrate into the myotomes. In *B. variegata*, myogenic cells of mesenchymal origin, prior to fusion with the mononucleate myotube, withdraw from the cell cycle at the G1 phase (their nuclei contain 2cDNA) (KIEŁBÓWNA & KOŚCIELSKI 1979). Cells of mesenchymal origin fusing with the myotube in *H. boettgeri* are MyoD-positive which confirms their myogenic character (DACZEWSKA 2001). TEM and autoradiographic analyses confirm that multinucleated myotubes in *Xenopus* arise through fusion of secondary myoblasts (of mesenchymal origin) with mononucleted myotubes (KIEŁBÓWNA & DACZEWSKA 2005).

During muscle development of *Xenopus*, myogenin mRNA accumulation is limited strictly to secondary myogenesis (stage 52) (NICOLAS *et al*. 1998). The secondary multinucleated myofibres arise as a result of fusion of migrated myoblasts

during metamorphosis (NISHIKAWA & HAYASHI 1994). The dermomyotome (previously called dermatomyotome) has been recently identified in amphibians. In *X. laevis* it has many features which are characteristic of the dermomyotome in *Amniota.* Similarities include the epithelial structure of the dermomyotome, its location on the lateral surface of the myotome and the expression of *Pax3*, as well as – in the dorsal and ventral margins of the dermomyotome – the expression of *MyoD* and *Myf5*. Following delamination, cells showing expression of *MyoD* and *Myf5* probably differentiate into myotomal muscles (GRIMALDI *et al*. 2004).

Recently, very early development of limb muscles has been observed in *Eleutherodactylus coqui* (which has no tadpole stage). The development of limb muscles starts just after closing of the neural tube; it starts with expression of *lbx1* in the cells of the ventral part of trunk somites. Then the cells are observed at the base of developing limb bud. At later stages, cells with *lbx1* expression are present among the mesenchymal cells in the limb bud (SABO *et al*. 2009). Development of the abdominal muscle has been analysed in *B. bombina* and *X. laevis*. In *B. bombina musculus rectus abdominis* develops from the ventral part of the somite. Sclerotome and dermatome cells migrate in a ventral direction without participation of myotomal cells, contrary to earlier ideas. Muscle development starts at the contact of migrating cells with somatopleura and proceeds towards the medioventral line, with a clearly marked gradient of fibre development. Myogenesis of the muscle proceeds according to the classical model of myogenesis, with fusion of myoblasts at the G1/G0 phase (KIEŁBÓWNA 1993). During the development of *X. laevis*, in cells of the ventro-lateral part of the somite, the presence of transcription factors Pax3, Lbx1 and Myf5 has been found. Pax3- and Lbx1-positive cells migrate in a ventral direction and differentiate into *rectus abdominis*. The role of Lbx1 is promotion of the migrating cells. The front of migrating cells is Pax3-positive. Close to the migrating cells, dorsally, myoblasts show expression of the gene *Myf5* which initiates myogenesis. The MyoD-positive cells become located just posterior to the Myf5-positive cells (MARTIN and HARLAND 2001, 2006). The presence of differentiating myoblasts in the neighborhood of migrating cells in *X. laevis* is explained by histological studies on the development of *musculus rectus abdominis* in *B. bombina*. In the gradient of fibre development, undifferentiated myogenic cells precede the youngest myotubes (KIEŁBÓWNA 1993).

5. Aves and Mammalia – Amniota

The amniote somite is a short-lasting structure which undergoes early morphological changes. Its dorsal part retains epithelial structure and transforms into dermomyotome. The ventral part of the somite and somatocoel cells develop into mesenchymal tissue – the sclerotome.

In avian and mammalian embryonic development, axial organs (neural tube, notochord) and surface ectoderm emit the signal proteins Shh (Sonic Hedgehog) and Wnt as positive signals, acting jointly, to initiate myogenesis while lateral mesoderm emits signal proteins BMP4 (Bone Morphogenetic Protein), which probably have an inhibitory effect on myogenesis (Fig. 3) (MÜNSTERBERG & LASSAR 1995; COSSU *et al*. 1996; CURRIE & INGHAM 1998).

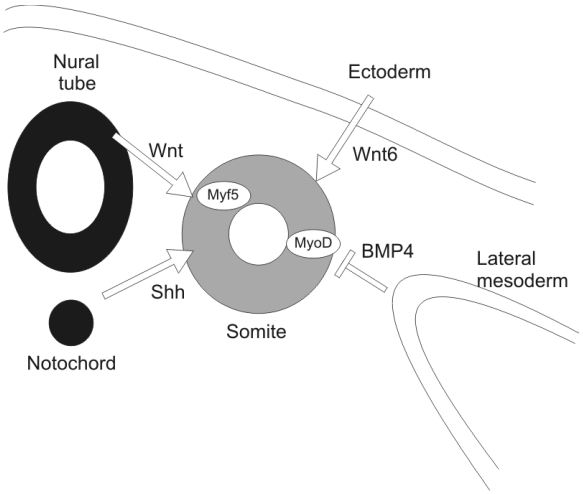

Fig. 3. A model showing influences of Wnt and Shh on the paraxial mesoderm, resulting in activation of myogenesis through a Myf5 and MyoD dependent pathway in mouse. The possible inhibitory effect of lateral structures in also indicated (after: MOLKENTIN & OLSON 1996, modified).

Receptors of Wnt and Shh and their post-receptor pathways lead to the activation of transcription factor genes *Myf5*, *MyoD* and *Pax3* (COSSU & BORELLO 1999). Most attention in the post-receptor pathway of Shh is directed towards *Gli* genes. The Gli proteins are transcription factors which are activated in coordination with the forming somite. The Gli protein activated by Shh can directly bind to the promoter of the genes *MyoD* and *Myf5* (BORYCKI *et al*. 1998; GUSTAFSSON *et al*. 2002). The pathway dependent on Wnt signals is correlated with expression of the *Frizzled* genes coding for the receptor protein of Wnt. Eight *Frizzled* genes have been implicated in the formation of mouse somites. Many of them are expressed in epithelial somite and newly formed myotome. Since various regulatory factors bHLH are expressed in different domains of the somite, it is conjectured that there is a correlation between members of the bHLH family and the expression of *Frizzled*. Regulation of post-receptor pathways

is very complex and occurs at various stages (BORELLO *et al*. 1999).

The signals sent from axial tissues initiate and maintain expression of the genes of bHLH proteins in newly formed somites, whereas in older somites gene expression is autonomous (CURRIE & INGHAM 1998). Independent from the myo-genesis-inducing external signals, the myogenesis programme can be initiated autonomously. In birds, at the pre-somitic stage, MyoD expression is induced by the Wnt signal sent by neighboring cells of presomitic mesoderm. Once the somites have been formed, the effect of surrounding axial tissues is still necessary to maintain the subsequent myogenesis programme (LINKER *et al*. 2003).

The "communication effect" discovered in *Xenopus laevis* occurs also in mammals. Isolated myogenic cells of unsegmented mesoderm and cells of the youngest somites, from I to V, require communication among 30-40 similar cells in their vicinity in order to enter the differentiation phase. At that stage the inductive effect of axial tissues is not sufficient. Cells of older, cranial somites are no longer dependent on the "communication effect". The factor mediating cell communication in mammals is unknown (COSSU *et al*. 1995).

The bird myotome is built of cells which originate from the median part of the somite. They are post-mitotic, pioneer cells with expression of *MyoD* and *Myf5* genes (KAHANE *et al*. 2002). The dermomyotome cells are the next to be translocated into the myotome; they are also post-mitotic with the *MyoD* gene expression. These cells fuse with the pioneering cells (KAHANE *et al*. 1998, 2002). At this developmental stage the myotome is post-mitotic. Then mitotically active Pax3/7-positive cells, originating mainly from the central part of dermomyotome, appear in the myotome (KAHANE *et al*. 2001).

The dermomyotome cells differentiate not only into myoblasts but also into other cells of mesodermal origin, e.g. fibroblasts and endothelial cells (BEN-YAIR & KALCHEIM 2005). In a young, growing myotome N-cadherins maintain epithelial integrity of the dermomyotome. In the central part of the desintegrating dermomyotome, in the assymetrically mitotically dividing cells, both sister cells contain N-cadherins. Subsequently the N-cadherins remain only in the apical cell directed towards the myotome. The sister cell which has lost N-cadherins differentiates into a fibroblast. The N-cadherin-positive cell translocates into the N-cadherin-positive myotome as a result of a homophilic reaction. The N-cadherin-positive cells populating the myotome contain the regulatory proteins Pax3 and Pax7 (CINNAMON *et al*. 2006). They are the only mitotically active cells in the myotome. Some of them, at the late stage of muscle development, can also differentiate into muscular fibroblasts and endothelial cells while Pax7-positive cells can remain as satellite cells (CINNAMON *et al*. 2006).

In mammals the first cells forming the myotome originate from the dorso-medial lip of the dermomyotome. These are pioneering cells with *Myf5* gene expression. In the myotome they elongate symmetrically. Next to the pioneering cells which are large with large nuclei, numerous small myoblasts appear which will probably fuse with the pioneering cells. In mammals, Myf5 is the first transcription factor to initiate myogenesis. In myotomal cells *MyoD* gene expression follows the expression of *Myf5*. Desmin appears in cells of the central part of the myotome while sMHC (heavy chain of slow myosin) – in lateral myoblasts (VENTERS *et al*. 1999). Following the formation of the embryonic post-mitotic myotome, further myogenesis is associated with the Pax3- and Pax7-positive mitotic cells which originate from the central part of the dermomyotome (KASSAR-DUCHOSSOY *et al*. 2005; RELAIX *et al*. 2005). In the myotome these cells differentiate into muscle cells with *Myf5* and *MyoD* gene activity (BUCKINGHAM 2006).

The mechanism determining entrance into myogenesis or persistance in the pool of proliferating cells is poorly understood. The process involves an FGF signallisation pathway. In mouse myotomal myogenesis, FGF signalling promotes both proliferation and differentiation of cells (BUCKINGHAM 2006). In the bird myotome co-localisation of *Fgfr4* transcripts and Pax7 proteins has been detected (BEN-YAIR & KALCHEIM 2005). Recently it has been shown that the mouse *FGFr4* gene is directly activated by Pax3. It has been suggested that Pax3, through regulation of FGF signalling, controls the balance between proliferating and differentiating myogenic cells (LAGHA *et al*. 2010).

In birds and mammals, Shh and Wnt produced by axial organs induce expression of the *MyoD* or *Myf5* genes in cells of epaxial myotomes. Hypaxial myotome cells do not require the inducing action of axial organs (TAJBAKHSH *et al*. 1996; BORYCKI *et al*. 1999; CHRIST & BRAND-SABERI 2002). Hypaxial myotome develops from the lateral part of the dermomyotome. It is thought that determination of hypaxial dermomyotome requires signals from the surface ectoderm (Fig. 4) (SCHMIDT *et al*. 2001). In chicken development Wnt6 produced by the ectoderm induces expression of the *Pax3* and *Myf5* genes, whereas other ectodermal signals induce expression of *MyoD* (GEETHA-LOGANATHAN *et al*. 2005). Genetic studies have shown that Pax3 activates expression of the *Myf5* genes in cells of hypaxial myotome

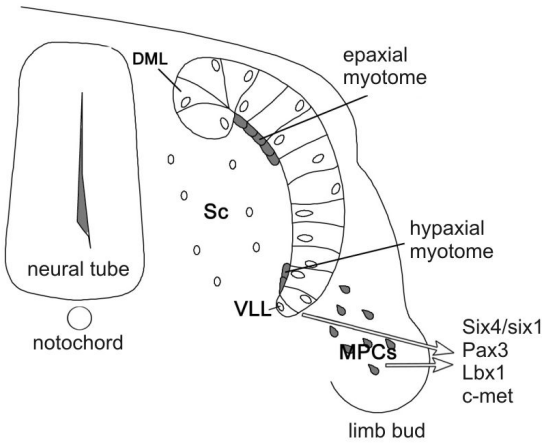

Fig. 4. A schematic view of the dermomyotome that gives rise to epaxial and hypaxial myotome muscles and muscle progenitor cells (MPCs). The latter (specified by Six4/Six1, Pax3, Lbx1, c-met) migrate to the limb bud. DML – dorsomedial lip of dermomyotome; VLL – ventrolateral lip of dermomyotome; SC – sclerotome (after: TAJBAKHSH 2003, modified).

and its derivatives and that the action of Pax3 is direct (BAJARD *et al*. 2009).

Other muscles, e.g. those of limbs, develop from cells of the ventro-lateral part of the dermomyotome. They are determined muscle cells under a repression effect of BMP4 (Bone Morphogenetic Protein) signals sent from the lateral mesodermal plate. The released cells migrate into the limb bud retaining their myogenic potential (CHRIST & BRAND-SABERI 2002). They regain their differentiation capacity once they have reached their destination (CURRIE & INGHAM 1998).

Six4 (Sine oculis homeobox homolog) proteins acting jointly with Six1 are important myogenesis regulators. At the limb level, Six4/Six1 have an effect on delamination of myogenic cells of the dermomyotome and on their migration, through control of expression of *Pax3* genes. Moreover, Six4 and Six1 coordinate the expression of *Pax3* and *Met* genes, required for cell migration. These facts suggest that *Met* is under direct control of the Six proteins, independent from *Pax3*. The regulatory proteins Six4 and Six1 are also required for expression of the *Lbx1* gene in the cells of the ventral part of the dermomyotome (GRIFONE *et al*. 2005). Expression of the *Lbx1* gene also occurs in the migrating cells and the cells which populate the limb bud (Fig. 4). It has been suggested that this gene determines the specific properties of migrating cells and is essential for recognition of signals which direct migration and maintain migrating potential (JAGLA *et al*. 1995; BROHMANN *et al*. 2000). Signals leading to de-epithelialisation and migration of myogenic cells are sent from the somatopleura of the limb bud. Expression of the tyrosine kinase receptor gene, *c-met,* is essential for migration of myogenic cells of the ventral part of the dermomyotome. The specific ligand binding *c-met* and regulating its activity is the ligand SF/HGF (Scater Factor/Hepatocyte Growth Factor) which is active in the limb bud. Ligands, recognised by the receptor, send signals informing cells that they should undertake migration (BLADT *et al*. 1995; DIETRICH *et al*. 1999). Despite being potential myoblasts, the migrating cells do not show the presence of MRF transcription factors (DIETRICH *et al*. 1999). Only after reaching their destination in the limb bud is myogenesis initiated with *MyoD* expression in chicken (CHRIST & BRAND-SABERI 2002). Six proteins regulate the activation of *Myf-5* expression in embryonic mouse limbs (GIORDANI *et al*. 2007). Myogenic cells in the developing limb form two clusters which will develop into dorsal and ventral muscle primordia. The muscle pattern is determined by the limb bud somatopleura but it is unclear which somatopleura component is the source of this information (CHRIST & BRAND-SABERI 2002).

Concluding Remarks

In development, organisation of myogenic cells and their entrance into the myogenesis programme are controlled by inducing proteins, transcription factors and adhesion molecules. Expression of transcription factors, MyoD, Myf5, myogenin and MRF4 is the basis of myogenesis. Myotomal myogenesis is initiated by transcription factors MyoD or Myf5. In lower chordates, *Acrania*, *Pisces* and *Amphibia,* myogenesis is initiated by MyoD; in *Aves* and *Mammalia,* myogenesis is initiated by Myf5. Expression of *MyoD* or *Myf5* genes is induced by the proteins Wnt and Shh produced by axial organs (neural tube and dorsal chord), and Wnt6, produced by surface ectoderm. The signal pathway leads from the surface receptors of the cell membrane to the nucleus. In *Brachydanio rerio* Hh signals sent from the dorsal chord induce expression of *MyoD* in adaxial cells and of Pax3/Pax7 in dermomyotome cells. Moreover, Hh signals direct the fate of cells of dermomyotome which differentiate into fast-twitch muscles.

Initiation of myogenesis in development can be autonomous. In tunicates, the transcript of gene *Macho-1* which codes for a transcription factor with a zinc fingers domain is a component of oocyte myoplasm determination. Blastomeres containing this information develop into muscle cells. Transcription factor MyoD, present in later developmental stages, does not initiate myogenesis. In *Xenopus*, autonomous initiation of expression of

the gene *MyoD* in cells of non-segmented mesoderm (mid gastrula stage) is the "effect of communication" of about 100 cells. The mediating factors are eFGF and signalling cadherins. In mammals autonomous entrance into myogenesis by cells of non-segmented mesoderm and cells of the youngest somites is associated with communication of about 40 cells. In mammals the mediating factor is unknown.

In fishes and amphibians expression of *MyoD* starts in cells of non-segmented mesoderm and then in myotomal, post-mitotic cells. The embryonic myotome is post-mitotic. In birds and mammals expression of *MyoD* or *Myf5* is initiated in cells of the dermomyotome, in its medial and lateral lips. Post-mitotic MyoD- or Myf5-positive cells become located under the dermomyotome. The developing embryonic myotome is also post-mitotic.

In a young dermomyotome, N-cadherins ensure integrity of the structure; in an older disintegrating dermomyotome, N-cadherins determine muscle cells. In an asymmetrical mitotic division the cadherin-containing cell differentiates into a myoblast, its sister cell, devoid of cadherins, becomes a fibroblast. Pax3 and Pax7–positive cells occurring in the central part of the dermomyotome are proliferating cells. In the myotome these cells enter the myogenesis programme or remain as proliferating cells. In mammals the process depends on the growth factor FGF. A dermomyotome-like structure has been identified also in fishes and amphibians. In *Brachydanio rerio* it contains Pax-positive cells which take part in the development of myotomal muscles. In amphibians participation of dermomyotome cells with expression of MyoD, Myf5 and Pax3 in the development of myotomal muscles is poorly known. Earlier studies have shown that in many fish and amphibian species further development of myotomal muscles is associated with mesenchymal cells which migrate into the myotomes. These are proliferating, bipotential cells which differentiate into myoblasts and fibroblasts. The probable source of such cells is the dermomyotome.

Cells of the ventral part of the dermomyotome are the source of chordate limb muscles. In birds they consist of determined muscle cells blocked by BMP signals from the mesodermal plate. Cells migrating to the limb bud contain regulatory proteins Pax3 and Lbx1, which promote cell migration. It has been suggested that the gene *Lbx1* receives signals which direct cell migration and maintain the cells' migration potential. Regulatory proteins Six4 and Six1, which are present in the dermomyotome cells, lead to cell delamination and in migrating cells control expression of *Pax3*, *Lbx1* and *Met.* Migrating cells, having reached their destination, enter the myogenesis programme under the effect of Six4/Six1. In amphibians *musculus rectus abdominis* develops from cells of the ventral part of the somite. In *Xenopus,* migrating cells contain Pax3 and Lbx1. Its development is characterised by a fibre developmental gradient. The oldest stages of fibre development occur below the myotomes, the younger stages occur towards the ventral median line. The front of migrating cells is Pax3-positive, cells with expression of *Myf5* and *MyoD* are located just behind them dorsally.

Limb and abdominal muscles develop independently from myotomal muscles; their myogenesis is also different. While myotomal myogenesis is much varied among chordates, limb and abdominal muscle myogenesis follows the classical pattern.

Future perspectives

Data presented in this review indicate the complexity of the initial steps of myogenesis. Although many essential factors engaged in myogenesis have been defined, important issues remain that should be addressed. Further findings of new transcription, signallisation and cell communication factors as well as trans-membrane proteins, are required to fully understand the mechanisms that govern the process of myogenesis.

Future studies should also reveal when and how the single gene inherited from invertebrates was duplicated in chordate evolution and when the four genes of the MyoD family, present already in fishes, came into existence.

It should be emphasized that some aspects of myogenesis are much less known. One of the main subjects worthy of comprehensive studies is the process of myoblast fusion focusing on the molecular mechanisms responsible for myogenic cell shape alterations preceding myoblast adhesion and fusion, myoblast membrane fusion and cytoskeleton reorganisation before and during fusion. Another very interesting issue demanding further investigation is the genetic mechanism of myotomal myogenesis without myoblast fusion.

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