Role of Skeletal Muscle in the Epigenetic Shaping of Organs, Tissues, and Cell Fate Choices

Boris Kablar

Since the inception of my independent laboratory in July 2000, I have been able to study the role of muscle in the shaping of developing tissues, which is an important example of Waddington epigenetics. This has been the focus of my research program. Muscle tissue is one of the four basic tissue types of which the body consists. There are three types of muscle tissue, and we are interested in one of them, the skeletal or striated muscle. We can study the developmental role of muscle in the whole mouse embryo or fetus because it is enough to knock out two myogenic regulatory factors (MRFs), Myf5 and MyoD, to obtain an embryo without any skeletal musculature. Obviously, such a fetus cannot survive after birth, but it is viable as long as it is in the womb. This experiment was performed for the first time in 1993 by my postdoctoral supervisor, Dr. Michael A. Rudnicki, while he was a postdoctoral fellow in Dr. Rudolph Jaenisch’s laboratory (Rudnicki et al., 1993). Even though it is understandable that the muscle may have numerous functions during development, we think of muscle as either an executor of various movements or a provider of neurotrophic factors. Therefore, I will concentrate on the description of two major research programs.
performed in this laboratory. The first one, also known as “developmental morphodynamics,” deals with studies that examine the ability of muscle to provide mechanical cues for organogenesis. In this program, we are trying to understand mechanical control of tissue morphogenesis during development (Ingber, 2006). In fact, the analysis of Myf5:MyoD compound nulls reveals that several organs have difficulties in fully developing in the absence of the musculature. Organs that depend on continuity between pre- and postnatal motility are the lungs, retina, inner ear, and some parts of the skeleton (e.g., mandible, clavicle, sternum, and palate). The second research program is composed of experiments that test the neurotrophic hypothesis. In this program, we are trying to find out if there is a muscle-provided trigger of motor neuron death ultimately relevant to the motor neuron diseases such as amyotrophic lateral sclerosis (ALS). The reason for this kind of thinking is the fact that a complete absence of lower and upper motor neurons, which is the pathological definition of ALS, is achieved only in the complete absence of the muscle (Kablar and Rudnicki, 1999).

**Myf5:MyoD COMPOUNDS NULLS**

Due to the inability of Myf5 nulls to survive after birth, it is necessary to perform a two-step breeding scheme in order to obtain muscleless embryos. In the first generation of breeding, Myf5 heterozygous mice are crossed with MyoD null mice to obtain Myf5:MyoD double heterozygous mice. In the second generation of breeding, these double heterozygous mice are interbred, resulting in a 1:16 probability of finding a muscleless embryo. Embryos and fetuses without any skeletal myoblasts and musculature are characterized by the existence of unspecified myogenic precursor cells (MPCs) and the coexistence of Myf5- and MyoD-dependent MPCs that erroneously contribute to the bone tissue, undergo apoptosis, become adipocytes, or simply remain as mesenchyme (Kablar et al., 2003, and references therein).

In order to be able to make conclusions pertinent to the absence of the muscle and not to the absence of Myf5 and/or MyoD, a careful analysis of Myf5 and MyoD expression and distribution patterns was necessary. To that end, Myf5lacZ knockin mice, 258/-2.5lacZ transgenic mice (for the –20 kb enhancer of MyoD promoter), MD6.0-lacZ transgenic mice (for the –5 kb enhancer of MyoD promoter), in situ hybridization, and immunohistochemistry were employed. We concluded that neither Myf5 nor MyoD was expressed or contained in any of the tissues of interest. In other words, lungs, bones, and the inner ear neuroepithelial fields did not show any expression or distribution of Myf5 or MyoD, but the central nervous system (CNS) and the neural retina contained ectopic expression of Myf5 and MyoD. However, the function of Myf5 and MyoD was found to be inhibited in the neural tissues, leaving no functional consequences of Myf5 and MyoD in neurogenesis (Tajbakhsh and Buckingham, 1995; Kablar, 2002, 2004).

**LUNG DEVELOPMENT IN THE ABSENCE OF FETAL BREATHING-LIKE MOVEMENTS**

A large body of literature confirms that in the absence of fetal breathing-like movements (i.e., possibly spontaneous neuronal firings in the brain stem, transmitted via peripheral nerves and executed by the striated musculature), the developing lung fails to grow appropriately, resulting in pulmonary (lung) hypoplasia (Inanlou and Kablar, 2005; Inanlou et al., 2005, and references therein). In mice, the histopathological appearance of the hypoplastic lung at term corresponds to the earlier canalicular stage of normal lung development. Moreover, the ratio between body weight and lung weight is less than 4%, due to downregulated cell proliferation and upregulated cell death in the alveolar epithelium and the lung mesenchyme. Several cell cycle molecular players are also downregulated, such as platelet-derived growth factor (PDGF)-β, its receptor (PDGFR-β), and insulin-like growth
factor (IGF)-I. Finally, thyroid transcription factor (TTF)-1 loses its proximal-to-distal expression and distribution gradient.

In addition to the failure in growth, the lungs exhibit failures in cell differentiation. Whereas the conductive system of the lung (i.e., trachea, bronchi, and bronchioli) is unaffected, the respiratory portion, and in particular the alveolar epithelium, fails to differentiate properly (Inanlou and Kablar, 2005). Specifically, type II pneumocytes, responsible for the synthesis of surfactant, in spite of normal distribution of surfactant proteins (SPs) A, B, C, and D, fail to assemble (i.e., do not utilize glycogen adequately), store (i.e., have irregular lamellar bodies), and secrete (i.e., have irregular myelin figures) the surfactant. At the same time, type I pneumocytes, responsible for gas exchange, fail to differentiate from a cuboidal cell type into the squamous cell type (i.e., fail to flatten) in order to become a part of the blood–air barrier, the site of oxygen and carbon dioxide exchange.

The striking normality of various components of the lung tissue in the muscleless fetuses, coupled with the very specific differentiation failures of type II and type I pneumocytes, led us to believe that the systemic subtractive microarray analysis approach (SSMAA) (Figure 15.1) would reveal a profile of genes involved in type I and type II pneumocyte differentiation. In other words, we hypothesized that the difference in gene-expression patterns between the control and the mutant lung would be related to the specific differentiation failures of the alveolar epithelium. Indeed, our Affymetrix GeneChip cDNA microarray analysis revealed nine upregulated and 54 downregulated genes (Baguma-Nibasheka et al., 2007). Out of 54 downregulated genes, the literature and database search detected 24 viable and fertile knockout mice that did not have a lung phenotype as single knockouts. Furthermore, two knockout mice died too early during development to be useful for studies relevant to lung organogenesis. Finally, our analysis revealed four molecules whose knockouts die at birth due to respiratory failure. The knockout mice that die at birth due to pulmonary hypoplasia are connective tissue growth factor (Ctgf), special AT-rich sequence binding protein 1 (Satb1), myeloblastosis oncogene (Myb), and T-cell receptor β, variable 13 (Tcrb-V13). We are currently performing experiments to verify the features of lung hypoplasia in each of the aforementioned mouse knockouts. Our analysis of Ctgf nulls revealed all the criteria for mouse pulmonary hypoplasia and specifically a failure of type II pneumocytes to properly assemble and store the surfactant (Baguma-Nibasheka and Kablar, 2008). In addition, four more mouse knockouts are in the pipeline of the European Conditional Mouse Mutagenesis (EUCOMM) Consortium. Our ultimate goal is to identify new molecular players with precisely attributed functions in lung development and disease, while defining which features of the phenotype are the result of the absence of the gene and which are due to the absence of mechanical forces. We intend to use human fetal hypoplastic lung tissues from fetal akinesia and oligohydramnios to verify if any of the molecules detected via mouse mutagenesis and pathology apply to the human conditions in order to eventually identify a marker for predicting pulmonary hypoplasia. We also intend to apply mechanical factors, such as stretch, to the single alveolar epithelial cell or a layer of epithelial cells to study the role of the identified molecules in the mechanochemical signal-transduction pathways in order to ultimately propose improvements to the protocols for tissue engineering.

RETINAL DIFFERENTIATION IN THE ABSENCE OF FETAL OCULAR MOVEMENTS

Another project carried out in the laboratory deals with the fact that the neural retina of muscleless fetuses does not contain any cholinergic amacrine cells (CACs) (Kablar, 2003, and references therein). CACs are apparently responsible for motion vision and directional selectivity (Moran and Schwartz, 1999; Yoshida et al., 2001, and references therein). Whether this is an example of cell differentiation being
Figure 15.1 Lung model for the systematic subtractive microarray analysis approach (SSMAA). In the absence of the respiratory musculature, and therefore in the absence of the executors of the fetal breathing-like movements, the growth of the lung is severely affected, to the point that the seven criteria for pulmonary hypoplasia in mice can be met. Importantly, in spite of the correct expression of molecular markers such as surfactant proteins for type II pneumocytes and Gp38, a controversial marker for type I pneumocytes, transmission electron microscopy elucidated that further differentiation steps of the alveolar epithelium were affected in the hypoplastic lung. The cuboidal type II pneumocytes, currently thought to be the source of type I pneumocytes, had failures in assembly, storage, and secretion of surfactant. Meanwhile, the type I pneumocytes could not flatten to become squamous epithelial cells and function in the blood–air barrier for gas exchange. To discover new molecular players with precisely attributed functions in lung development and disease, we performed Affymetrix Gene Chip cDNA microarray analysis, followed by the analysis of mouse mutants. In fact, Ctgf is an example of the SSMAA that worked because out of approximately 25,000 genes we did identify one whose knockout mouse had specific differentiation failures as “predicted” by the original phenotype (i.e., the phenotype described in Myf5:MyoD nulls). Moreover, defining which features of the phenotype were the result of the absence of the individual genes (e.g., Ctgf) and which were due to the general absence of mechanical forces (i.e., Myf5:MyoD nulls) was another advantage of this approach. This approach could be successful for the analysis of the molecular basis of hair-cell differentiation in the cristae amplaria since it has some analogies with the lung example. Importantly, this approach systematically addressed various tissues and organs of the fetal or embryonic body that were affected by the absence of the skeletal musculature (e.g., retina, inner ear, skeleton) or Myf5 and MyoD (e.g., limb and back myogenic precursor cells). Finally, the approach benefited from the fact that the mutant tissues and cells had particular differentiation failures (e.g., alveolar epithelium, hair cells in the crista, myogenic precursor cells) or were completely absent (e.g., cholinergic amacrine cells, palate, secondary cartilage), and therefore, the analysis potentially provided a profile of genes involved in that particular differentiation failure by subtraction from the normal control.
dependent on its function and vice versa (i.e., form–function mutual interaction) or just a coincidence we do not know at this point, and we cannot address it more directly than we already have. In fact, another way of addressing the mechanisms of this phenotype would be to identify another mouse mutant without extraocular musculature and examine its neural retina. For instance, Pitx2 nulls apparently specifically lack the extraocular muscle and are viable at term (Diehl et al., 2006), therefore representing an ideal occasion for testing our hypothesis. In the meantime, we used the opportunity provided by the retinas from muscleless fetuses to assess the differences in gene-expression pattern between the control and the mutant retinas, employing 15K mouse cDNA microarray slides from Ontario Cancer Institute (Baguma-Nibasheka et al., 2006). In this way, otherwise inaccessible CACs have been assessed for differences in gene expression, and the profile of genes obtained after SSMAA revealed some molecular players relevant to the differentiation of CACs. Our analysis discovered two molecules whose mouse mutants lack CACs: adapter-related protein complex 3, δ1 subunit (Ap3δ1) and β-transducin repeat containing (Btrc) (Baguma-Nibasheka and Kablar, 2009a, 2009b). While Ap3δ1 mutants are not going to be very useful for further studies of CAC function, because they become blind soon after birth (Qiao et al., 2003), the Btrc nulls are viable and not blind and, therefore, represent a model for further motion vision and directional selectivity studies. Additionally, two more genes have been identified, and their knockout mice are in the EUCOMM pipeline (protein sorting nexin 17, or Snx17, and WD repeat domain 5, or Wdr5). Finally, the identified molecules could also serve as specific markers for CACs (Figure 15.2).

DIFFERENTIATION OF THE CRISTA AMPULLARIS IN THE ABSENCE OF FETAL ANGULAR ACCELERATION

As previously mentioned, extrinsic mechanical stimuli have been shown to affect differentiation of specific cell subpopulations in the lungs and retina of mouse fetuses when devoid of mechanical stimulation from skeletal musculature during embryonic development. In this section, we focus on the link between mechanical stimuli, both acoustic and static, on the embryonic development of inner ear neuroepithelial fields. As usual, we employed double mutant Myf5−/−: MyoD−/− mouse fetuses that completely lacked skeletal musculature and analyzed the development of sensory fields in both the vestibular and the auditory components of the inner ear. Embryos and fetuses that lack skeletal muscles are deficient in the ability to move the chain of three middle ear ossicles and consequently are unable to properly transfer sound vibrations into the inner ear. They also cannot tilt their head due to the fusion of cervical vertebrae and the lack of musculature (Rot-Nikcevic et al., 2006), which prevents the perception of angular acceleration. Our results show that the development
of crista ampullaris, vestibular sensory fields sensitive to the angular acceleration, was the most affected (e.g., the size of the mutant haircell area was significantly smaller in comparison to the control by 43%). A somewhat lesser effect was observed in the vestibular macula sacculi, responsible for the perception of lateral acceleration and gravity (i.e., the supporting cells were affected but not the hair cells). Cochlear sensory field, the spiral organ of Corti, which serves as a receptor for acoustic signals, displayed normal development in mutant embryos (Rot and Kablar, 2010). This initial analysis of the complex relationship between the inner ear neuroepithelial fields’ cell differentiation and the mechanical stimuli they perceive during the last stages of development will be followed by the next step of our SSMAA in order to compare gene expression in crista ampullaris of \textit{Myf5}^{+/−}:\textit{MyoD}^{+/−} term fetuses to the analogous normal neuroepithelial field of wild-type term fetuses. By employing this approach, it will be possible to identify genes and to measure the expression of genes associated with the detected phenotype and specific cell subpopulations that may reside within the inner ear’s neuroepithelial fields.

DEVELOPMENT OF THE SKELETON IN THE COMPLETE ABSENCE OF THE MUSCULATURE

Both the vertebrate skeleton and the musculature function as one system in which the muscles execute the movements and the skeleton serves as the support. Importantly, the connecting feature between the two systems appears to be their ability to react to mechanical stimuli (Herring, 1994, and references therein). Mutant mice that completely lack skeletal myogenesis and the musculature, as obtained in \textit{Myf5}^{−/−}:\textit{MyoD}^{−/−} embryos and fetuses, are providing us with an opportunity to explore the mechanical aspects of a complex developmental relationship between the muscles and the bones. In this section, I will concentrate on the role of mechanical cues provided by the skeletal muscle (i.e., the static loading from the skeletal muscle) in determining the timing and the character of morphogenetic events during skeletogenesis (e.g., the formation of the secondary cartilage) and comment on the role of skeletal muscle in bone mergence, fusion, and the determination of size and shape of bones and joints.

Secondary cartilage is the tissue that provides growth sites and articulations during the subsequent steps of bone and joint morphogenesis and, therefore, is critical for the later growth and shaping of bones and joints. In mice, secondary cartilage is seen in the mandible (Fang and Hall, 1997, and references therein) and in the clavicles (Hall, 2001, and references therein). Formation of the secondary cartilage is apparently dependent on mechanical stimulation from the skeletal muscle (Herring, 1994). Indeed, we investigated the development of the mandibles and the clavicles with special attention to the formation of secondary cartilage in muscleless fetuses in comparison to normal controls. We also paid particular attention to the initiation versus maintenance of the secondary cartilage in the two bones of interest in the absence of mechanical stimulation from the muscles. Our findings conclude that in most cases secondary cartilage formation can be initiated in the absence of the striated muscle but the amount of tissue is reduced (with the exception of the angular process of the mandible that cannot be initiated and the condylar process that is unaffected). On the other hand, maintenance of the secondary cartilage is impossible in the absence of the musculature since all of the processes are either reduced or completely absent (Rot-Nikcevic et al., 2007).

An important example of skeletal muscle requirement for a morphogenetic event is the process of secondary palate fusion in mammals (Herring, 1994, and references therein). Earlier in development, the palatal shelves extending from the maxilla into the oral cavity are widely separated by the tongue. At a later stage, the tongue is moved out, clearing the way for the palatal shelves to merge and fuse in the midline. The current belief is that the tongue is moved
away by the contraction of the tongue muscles, which flattens the tongue; the opening of the jaw to pull the tongue away; and the growth of the lower jaw to accommodate the flatter tongue. Strikingly, although the tongue muscle is completely absent from the earliest stages of \textit{Myf5}^+/-:\textit{MyoD}^+/- embryo development, the lack of fusion of the palatal shelves—\textit{palatoschisis} or cleft palate—is clearly visible in double-mutant term embryos (Rot-Nikcevic et al., 2006, and unpublished data). Therefore, we suggest that the cleft palate is not a consequence of the tongue obstruction but of a complete absence of any mechanical help from the facial musculature during the process of mergence and fusion of the palatal shelves. In support to this hypothesis are the findings in paralyzing conditions that result in a high frequency of cleft palate (Herring and Lakars, 1981). We will further test this hypothesis by examining the process of palatal shelf fusion at different embryonic days in the absence of the tongue and all facial skeletal muscles.

Similarly, fusion of the sternum is also arrested in paralyzed chicken embryos, representing a major failure in morphogenesis that allows the viscera to herniate out of the incomplete body wall (Hall and Herring, 1990). One possible explanation of these findings is that in the paralyzed embryo the pectoral muscles attached to the paired sternal primordia prevent (instead of helping, as occurs during normal development) their fusion in the midline (i.e., analogous to the palatal shelves fusion). We tested this hypothesis by examining the features of sternal fusion at term and found that the sternum of muscleless fetuses is cleft (Rot-Nikcevic et al., 2006). However, we will further test this hypothesis by employing embryos and fetuses at different days of development in the absence of the pectoral muscles.

Finally, we analyzed all of the skeletal elements in the absence of muscles as some skeletal parts were suggested not to grow normally (resulting in alterations in shape and size) in the absence of skeletal musculature and its mechanical support. An important morphogenetic consequence of skeletal muscle presence is the formation of joints (Herring, 1994). The maturation of synovial joints requires embryonic movements. Similarly, the intra-articular structures, such as discs, cannot form without embryonic movements (Herring and Lakars, 1981). In addition, in paralyzed chickens, overall growth of several bones, such as appendicular bones (i.e., long bones in the limbs: tibia, fibula, femur, radius, ulna, humerus) and bones that develop secondary cartilage (i.e., clavicle and mandible), and of the external ear is affected. The bone growth is most reduced in the clavicle, followed by the tibia, other long bones, and finally the mandible, which is least affected (Herring, 1994). In both the paralyzed chicken embryos and the genetically paralyzed mouse embryos, the abnormal curvatures of the bones were shown to be the result of gross body distortion secondary to the paralysis. Additionally, the static loading from the striated muscle is often sufficient for normal bone formation (Herring, 1994). Therefore, our approach represents an opportunity to reexamine some of the acquired knowledge on the mechanical shaping of the embryonic mouse skeleton because, unlike previously employed models, in the muscleless fetuses an absence of various structures is obtained and with it a part of static loading is eliminated. For instance, various influences from the vasculature within the muscle, the electric currents from the muscle, the inputs to and from the CNS (Kablar and Rudnicki, 1999), and the actual muscle weight (i.e., instead of muscle, muscleless term fetuses contain loose connective tissue, adipose tissue, or edema, which all have a significantly lower specific weight) are abolished or lessened (Kablar et al., 2003). Consequently, we examined how the bone growth and the joint development were affected in \textit{Myf5}^+/-:\textit{MyoD}^+/- embryos that have no skeletal musculature, by examining the whole skeletons at different embryonic days in the absence of all of the muscles and comparing the bone length and bone and joint shape to the control embryos. As previously reported (Rot-Nikcevic et al., 2006), the phenotype includes
enlarged and fused cervical vertebrae and postural anomalies, some viscerocranial anomalies, long bone truncation and fusion, absent deltoid tuberosity of the humerus, scapular and clavicular hypoplasia, cleft palate, and cleft sternum. While the magnitude of individual effects varied throughout the skeletal system, the results are consistent with skeletal development depending on functional muscles. In conclusion, our findings imply that there are at least two major points that require further analysis, one being the examination of the events of fusion (e.g., cleft and sternum) and the other being the examination of the secondary cartilage maintenance. We anticipate that it would be of value to apply the SSMAA, previously explained in the examples of the lung and retina development, and molecularly describe the maxillary complex in mouse fetuses that suffer from cleft palate or the mandibular complex in fetuses that do not have an adequate temporomandibular joint due to the lack of maintenance of the secondary cartilage. Through this approach it would be possible to gain insight into the molecular players that may be responsible for the process of fusion and secondary cartilage maintenance that seem to depend heavily on the mechanical cues from the skeletal musculature.

SURVIVAL AND MAINTENANCE OF MUSCLE-DEPENDENT NEURONS

Muscleless embryos initially do not have a motor neuron phenotype, but from embryonic day 13.5 (E13.5) they gradually lose all somatic motor neurons from the spinal cord to the brain, including the giant pyramidal cells in the motor cortex (Kablar and Rudnicki, 1999), in spite of the fact that only a very small number of these cells directly form synapses with the motor neurons. Considering that the pathological definition of motor neuron diseases (MNDs) describes these conditions as the death of not only lower but also upper motor neurons, we propose that the muscle must have a more active role in the etiology and pathogenesis of MNDs. In fact, it is only natural to consider not only that the intrinsic properties of the motor neurons can lead to the death of neurons, as occurs for instance in superoxide dismutase 1 or SOD1+/− mice (Ripps et al., 1995) but also that the environment found in the physical proximity of motor neurons, such as the surrounding CNS, Schwann cells, synapses, and muscle, can contribute to the process of triggering motor neuron programmed cell death. To that end, we studied the relationship between the (level of) commitment of MPCs and their ability to provide the neurotrophic support to the motor neurons. In other words, while Myf5:MyoD nulls survive until birth without any muscle specification and their MPCs are completely unable to support motor neurons, MPCs from single Myf5 or MyoD nulls do specify, showing different levels of commitment to the myogenic lineage, and consequently lack some neurotrophic factors but can fully support motor neuronal survival (Kablar and Belliveau, 2005). We see this fact as a window of opportunity which literally lasts for about 3 days (precisely until E13.5, which is the ideal day because it coincides with the onset of neuronal programmed cell death) and that potentially can tell us what neurotrophic factors are required by the lateral motor column (LMC) motor neurons, innervating MyoD-dependent MPCs, and what neurotrophic factors are required by the median motor column (MMC) motor neurons innervating Myf5-dependent MPCs (Kablar et al., 1997). In fact, after our initial analysis, where we discovered that, for instance, brain-derived neurotrophic factor (BDNF) was not essential for the survival of LMC neurons, glial cell–derived neurotrophic factor (GDNF) was not essential for the survival of MMC neurons, and neurotrophin-4/5 (NT-4/5) was not essential for either of the two neuronal groups, we assessed a large number of known and unknown muscle-expressed factors employing SSMAA. In our opinion, the information from this analysis is potentially important for various reasons: (1) ALS-affected neurons usually innervate MyoD-dependent musculature (limbs, diaphragm, head), (2) MyoD is downregulated in presymptomatic...
superoxide dismutase 1 or SOD1−/− mice (Park and Vincent, personal communication), and (3) Mdx:MyoD−/− 9th fetuses have normal-appearing limb and back musculature (Inanlou et al., 2003) and normal MMC neurons but their LMC neurons are 50% reduced (Kablar, unpublished data), indicating that a muscle-expressed factor (or a group of factors), potentially related to MyoD and situated within the MyoD-dependent MPCs (and not Myf5-dependent MPCs), triggers this phenotype. In addition, a series of in utero treatments with neurotrophic factors indicated that the ability of a particular neurotrophic factor to support the survival of motor neurons is dependent on and modified by the presence of the skeletal musculature (Geddes et al., 2006; Angka and Kablar, 2007; Angka et al., 2008; Angka and Kablar, 2009).

More than 15 factors from different gene families are now known to enhance motor neuron survival during development and to be expressed in a manner consistent with a role in regulating motor neuron numbers. Different factors may act on different subpopulations of motor neurons, and these factors may act in synergy on a given motor neuron (reviewed by Henderson et al., 1998). In our experiment, we are comparing gene expression, first, between control and MyoD null limbs and, second, between control and Myf5 null back musculature. In the first generation of subtraction, we will obtain lists of down- and upregulated genes for each of the two experiments, in comparison to the normal control. Subsequently, we will be able to perform another subtraction, comparing the MyoD to the Myf5 lists of genes (e.g., a downregulated gene from the MyoD experiment may be upregulated in the Myf5 experiment). Using various databases, we are currently gaining insight into the information so far known about the identified genes, and we are making priority lists for further actions. In other words, either we will find the information we are looking for in the literature (e.g., a mouse mutant of the gene of interest has a decreased number of LMC neurons, the expression pattern of the gene is consistent with its role in regulation of the motor neuron numbers, the protein is retrogradely transported to motor neuron cell bodies), we will have to analyze the mouse mutant and the expression pattern ourselves (e.g., a mouse mutant of the gene of interest has a lung phenotype, but its neuronal phenotype has not been examined; the gene is expressed in the spinal cord, but it is not known if its protein is contained within the motor neurons), a mouse mutant will have to be generated (e.g., a mouse mutant of the gene of interest is in the EUCOMM or another pipeline), or we will perform in utero treatments with the protein of interest. In conclusion, I believe that by this systematic approach we will generate a large amount of very useful comprehensive information that will be testable by us and others in various ways.

Fortunately, we can already share some of our findings. For example, a well-known candidate BDNF is normally present in the developing limb musculature at E13.5 (Kablar and Belliveau, 2005), it is retrogradely transported by the motor neurons (Koliatsos et al., 1993), and it is known to be a survival factor for motor neurons (Oppenheim et al., 1992); but it is completely absent in MyoD−/− E13.5 limb muscle, indicating that it may not be essential for the survival of LMC neurons because LMC is completely normal in MyoD−/− nulls (Kablar and Belliveau, 2005). Consistently, BDNF−/− mice also have normal LMC neurons (Conover et al., 1995), and BDNF preferentially rescues MMC neurons (Geddes et al., 2006). Together, these data suggest that BDNF is a preferred survival factor for MMC, and not LMC, neurons, potentially excluding BDNF as a candidate for the etiology of ALS with limb symptoms (see Table 15.1 for more examples).

Another interesting finding from our current muscle microarrays (Baguma-Nibasheka and Kablar, unpublished data) is that E13.5 MyoD−/− limb muscle has downregulated the amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 13 (human) gene (Alsl2c13); but unfortunately the molecular and biological functions of this gene have not been
specified yet. Additionally, two other downregulated genes with existing knockouts have been identified to have decreased motor neuron numbers: kinesin family member 5C (Kif5c), whose molecular function is structural and cytoskeletal (Kanai et al., 2000), and syntaxin binding protein 1 (Stxbp1), whose molecular function is in transport and carrier activity and whose knockouts have massive motor neuronal apoptosis, which causes death at birth due to respiratory failure (Verhage et al., 2000). Meanwhile, among upregulated genes, we detected the polymerase (DNA-directed), beta gene (Polβ), whose molecular function is metabolic and housekeeping but whose knockout also has excessive neuronal apoptosis (Sugo et al., 2000).

On the other hand, our current muscle microarrays (Baguma-Nibasheka and Kablar, unpublished data) with E13.5 Myf5–/– back muscles have identified downregulated genes whose knockout mice also have motor abnormalities: peroxisome proliferator–activated receptor, gamma, coactivator 1 alpha (Ppargc1α), with molecular function in receptor and signal-transduction activity (Cui et al., 2006), and glycine receptor, beta subunit regulator of G-protein signaling 7 binding (Glrb), with molecular function in receptor and signal-transduction activity, whose knockouts have extensive motor neuron loss (Karaplis et al., 1994). Meanwhile, among upregulated genes, we detected homeobox D10 (Hoxd10) transcription factor, whose knockout mouse spinal cord had motor neuron column shifts (Carpenter et al., 1997).

**TABLE 15.1**
The Lack of MMC Data Incorrectly Suggests That LMC and MMC Neurons Share the Same Neurotrophic Requirements

<table>
<thead>
<tr>
<th>NEUROTROPHIC FACTOR KOs</th>
<th>MMC</th>
<th>LMC</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF–/–</td>
<td>?</td>
<td>N.S.</td>
<td>Liebl et al. (1997)</td>
</tr>
<tr>
<td>NT-3–/–</td>
<td>?</td>
<td>28%*</td>
<td>Woolley et al. (1999)</td>
</tr>
<tr>
<td>NT-4/5–/–</td>
<td>?</td>
<td>N.S.</td>
<td>Conover et al. (1995)</td>
</tr>
<tr>
<td>GDNF–/–</td>
<td>?</td>
<td>22%*</td>
<td>Moore et al. (1996)</td>
</tr>
</tbody>
</table>

NOTE: Data from muscle developmental biology only recently indicated the existence of important differences between the limb and the back musculature (Kablar et al., 1997), while our work, based on myogenic diversities, has been furthering our knowledge on differences in neurotrophic requirements that exist between the respective muscle innervating neurons. For instance, according to our data (Kablar and Belliveau, 2005) and the published knockout (KO) data shown in the table, BDNF and NT-4/5 seem not to have an effect on LMC neurons, while NT-3 and GDNF have an effect on LMC neurons. We find it essential to reexamine the existing KOs to determine the MMC neuronal numbers and, with them, their neurotrophic requirements. In fact, the first contribution so far has been a recent report in which it seems that the MMC neurons react to the absence of GDNF in a fashion dissimilar to that of LMC neurons (MMC vs. LMC = 22% vs. 37%) (Oppenheim et al., 2000).

*, not studied before 2000; N.S., no significant difference in neuron numbers when compared to the control; *, reported as a significant decrease in the literature.

CONCLUSION

Mutual embryonic inductive interactions between different tissue types and organs, between individual cell types belonging to the same or different lineages, and between various kinds of molecular players are only some examples of the complex machinery that operates to connect genotype and phenotype. Our studies so far indicate that some aspects of this interplay can indeed be studied as proposed, confirming the role of skeletal muscle contractile and secretory activity in the epigenetic shaping of organs, tissues, and cell fate choices. We will therefore continue this analysis as outlined to gain insight into the nature of the epigenetic events that lead to the emergent properties of a phenotype.

**ACKNOWLEDGMENTS**

This chapter represents a summary of the work performed since the inception of my independent laboratory in July 2000. I would like to thank some exceptional individuals who performed various parts of this


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revealed in Myf5Δ/Δ:MyoDΔ/Δ embryos. Dev Dyn 232:772–82.