Muscle-specific Gene Expression in Rhabdomyosarcomas and Stages of Human Fetal Skeletal Muscle Development

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ABSTRACT

Rhabdomyosarcomas (RMS) bear a morphological resemblance to developing striated muscle. It has been reported that two histologically distinct subtypes of RMS, embryonal and alveolar, behave differently in many clinical aspects, such as age distribution, primary site, and prognosis. We have investigated the expression of various genes, which are preferentially expressed in normal muscle tissue or cell culture (actins, myosins, and creatine kinases, and myogenic regulatory genes MyoD, myogenin, MRF4, and Myf5), in embryonal and alveolar subtypes and compared the results to the stages of developing human fetal limb muscle. The data showed that each of the RMS tumors tested, regardless of histological features, expressed MyoD1 and MRF4 transcripts. Expression of the myogenin gene was detectable in all alveolar RMS (n = 8), whereas only 5 of 9 embryonal RMS expressed myogenin transcripts. Trace levels of Myf5 transcripts were visible in all alveolar RMS and 7 of 8 embryonal RMS. The ß- skeletal, ß-cardiac, and ß- and ß-cytoplasmic actin transcripts were detectable in all alveolar RMS. While the ß- and ß-cytoplasmic actin transcripts were evident in all embryonal RMS, only 3 of 8 and 6 of 8 embryonal RMS expressed detectable levels of ß-skeletal and ß-cardiac actin transcripts, respectively. The embryonic form of myosin heavy chain was detectable in 1 of 8 of each type of tumor. Myosin light chain-1/3 transcripts were detectable in 4 of 8 alveolar RMS and 5 of 8 embryonal RMS. Brain creatine kinase transcripts were detectable in all alveolar RMS and 4 of 8 embryonal RMS, whereas none of the RMS samples contained detectable levels of the muscle form of creatine kinase. A comparison of the expression profiles with those of normal developing human fetal limb muscle (from 7.5 to 24 weeks' gestation) suggested that RMS resembled a relatively restricted segment of fetal muscle development. Furthermore, the data also showed a great deal of overlap in the differentiation state achieved by the embryonal and alveolar subtypes of RMS, suggesting that the clinicopathological difference between these two may not be due to malignant transformation of the cells from different positions in the normal pathway of myogenesis.

INTRODUCTION

RMS4 tumors comprise about two-thirds of soft tissue sarcomas in children and about 5% of all pediatric malignancies (1). RMS is a small cell tumor of skeletal muscle phenotype whose differential diagnosis is based on the presence of striated muscle cells at initial stages of differentiation (rhabdomyoblasts). These tumors often display a wide morphological spectrum ranging from poorly differentiated cells that are difficult to distinguish from other tumors composed of cells of similar appearance such as neuroblastoma, Ewing's sarcoma, primitive neuroectodermal tumors, and non-Hodgkin's lymphoma (2–4), to well-differentiated rhabdomyoblasts which characteristically display registered or haphazardly organized sarcomeric components and cross-striations. Diagnosis of poorly differentiated samples remains difficult despite the use of electron microscopy and the development of an increasing number of antibodies suitable for immunohistochemistry. Examples of the latter include reagents that demonstrate desmin, myoglobin, sarcomeric myosins, actin, Z-protein, troponin-T, and isoenzymes of creatine kinases (5–7).

Despite the wide variation in appearance of RMS, they can be roughly grouped into one of four histological categories; embryonal; botryoid; alveolar; and pleomorphic. Among these subtypes, the embryonal and alveolar are the most prevalent (8–10). Embryonal RMS, the most prevalent subtype, consists mainly of spindle-shaped cells with eosinophilic cytoplasm and usually occurs in children between birth and 15 years of age, predominantly in the head and neck, genitourinary tract, and the retroperitoneum, and less often in the limbs. The alveolar subtype, a somewhat less frequent form, is seen in patients of all age groups from younger children to adolescents, and histologically consists of round, oval, or polygonal cells that adhere to fibrous stromal septae forming “alveolar” spaces. It has an anatomical distribution similar to that of the embryonal subtype except for a greater incidence in the upper and lower extremities and trunk. Recently, the two subtypes have been shown to have distinct and exclusive molecular features: a consistent loss of constitutional heterozygosity for chromosome 11p15 occurs in the purely embryonal subtype (11, 12), whereas a translocation involving chromosomes 2 and 13, t(2;13)(q35;q14), has been observed in the classic alveolar subtype (13–15).

The resemblance of undifferentiated myxoma-like tissue of RMS to embryonic mesenchyme has led to the proposal (16) that the tumors arise from immature myoblastic tissue and that RMS with embryonal histology most closely resemble muscle of 7 to 10 weeks' gestation, whereas those with alveolar histology resemble muscle of 10 to 12 weeks' gestation (17). A corollary of these intriguing observations is that genes which are differentially expressed during these stages of myogenesis might be expressed coordinately with the relevant histology. Recent evidence suggests that establishment and maintenance of the skeletal muscle lineage may be under the control of a small set of related regulatory genes. These myogenic regulatory factors include MyoD1, myogenin, Myf5, and MRF4 (18–21), proteins which are structurally related, sharing both a common basic region and a proposed helix-loop-helix amino acid domain which are required for the protein oligomerization and DNA-binding properties associated with MyoD1 (22–25). Furthermore, these proteins appear to regulate expression of a number of contractile protein genes (18, 20, 25, 26). Consistent with both the skeletal muscle histogenesis of the tumors and the restriction of MyoD1 expression to myogenic cell lines and murine skeletal muscle, we have found MyoD1 expression to be limited to RMS (19).
Studies with a variety of organisms have demonstrated that under conditions of low mitogenic stimulus in vitro, the differentiation of skeletal muscle myoblasts is characterized by an ordered withdrawal of committed cells from the proliferative cycle followed by fusion and formation of multinucleated myotubes. Upon fusion, there is a coordinate expression of numerous unlinked genes that encode muscle-specific proteins and the accumulation of gene products which are required for the myogenic phenotype (\(\alpha\text{-skeletal}, \beta\text{-cardiac}, \gamma\text{-cytoplasmic}\) actins (31, 32) and \(\beta\text{-CK}\) (40, 41)). The transition to postmitotic myotubes results in the coordinate induction of myosin heavy chain (35), \(\alpha\text{-actin},\) and other contractile proteins (30) when cells initiate fusion as well as switch to \(M\text{-CK}\) (38–42).

A similar sequence of events occurs during myogenesis in vivo, with the superimposition of additional developmental stage-specific isoform differences. For example, in mouse (33) and chick (34) fetal muscle, skeletal and cardiac \(\alpha\text{-actins}\) are both induced at the time of myotube formation and are coexpressed throughout intermediate stages of skeletal muscle ontogeny; the adult isotype of \(\alpha\text{-skeletal actin}\) predominates only in the late stages. The embryonic isoform of MHC is expressed in fetal development of muscle but disappears after birth (43, 44). Postmitotic chick embryonic cells were found to express \(M\text{-CK}\) in vivo (42), correlating with the observation that the number of cells that reacted with anti-\(B\text{-CK}\) antibody decrease with increasing age.

Ultrastructural analysis suggests that the myoblastic process seen in RMS is very similar to that in normal fetal myogenesis (4) prior to innervation (45) but occurs in a disorderly manner (46). Tumors containing cells with structural evidence of myoblastic differentiation have been found to react with antibodies to \(\alpha\text{-actin}\) (47) and the fast and slow myosins (48). Tumor cells with various stages of myoblastic differentiation (i.e., minimal, moderate, and well-differentiated myoblasts) express an embryonic MHC (44, 48). More differentiated tumors express sarcomeric myosins that are undetectable in these poorly differentiated counterparts (48).

In this study, a molecular investigation has been conducted in RMS and in normal myogenesis of skeletal muscle with the hypothesis that the phenotypic variance within the tumor class could be explained on the basis of malignant transformation of cells at different stages of the myogenic pathway. We have determined the expression of genes whose products are essential for the myogenic phenotype (\(\alpha\text{-skeletal}, \alpha\text{-cardiac}, \beta\text{-cytoplasmic}, \gamma\text{-cytoplasmic}\) actins, myosins (embryonic MHC and MLC-1/3), and creatine kinases (B-CK and M-CK), as well as of the myogenic regulatory genes (\(MyoD1,\) myogenin, \(MRF4,\) and \(Myf5\)) during human fetal limb muscle development and in a set of embryonal and alveolar RMS. The data suggest that RMS as a group resemble a relatively restricted segment of fetal muscle development at the molecular level, bounded on one end by the commitment of cells to the myogenic pathway and on the other by the earliest overt stages of myogenesis. Based on the considerable overlap in expression between embryonal and alveolar RMS it is speculated that the difference between these two subtypes within the same tumor class may result from factors other than myogenic determination.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Site</th>
<th>Histology</th>
<th>LOH* (t(2;13))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11</td>
<td>M</td>
<td>Hand</td>
<td>Embryonal</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>M</td>
<td>Calf</td>
<td>Embryonal</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>F</td>
<td>Thigh</td>
<td>Embryonal</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>F</td>
<td>Middle ear</td>
<td>Embryonal</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>M</td>
<td>Pelvis and retroperitoneum</td>
<td>Embryonal</td>
<td>ND</td>
</tr>
</tbody>
</table>

* LOH, loss of heterozygosity on chromosome 11p15; ND, not done.

**MATERIALS AND METHODS**

Human fetal limb tissue was obtained by the Central Laboratory for Human Embryology, University of Washington (Seattle, WA). Fetal age was determined from the date of conception. Rhabdomyosarcoma tumor tissue was either from primary tumor or nude mouse xenografts as previously reported (11, 12); the relevant clinical characteristics of the affected patients and the cytogenetic analysis and molecular details of t(2;13) and 11p15 zygoty, where known, are given in Table 1. Northern blots were prepared from total RNA that was extracted by guanidinium-isothiocyanate homogenization, electrophoresed, and transferred to Hybond-N (Amersham) membrane as described previously (12).

Table 2 lists the probes used in the Northern blot analysis of tumor and fetal tissue RNA. The human myogenin homologue was isolated from a cDNA library made from RNA isolated from a rhabdomyosarcoma tumor sample and cloned into \(\lambda gt11\). The identity of the myogenin clone was confirmed by comparison of restriction fragments in human genomic DNA and by DNA sequence analysis. Since the myogenic regulatory factors share extensive nucleotide sequence homology among their putative DNA-binding and helix-loop-helix domains, probes were prepared consisting of the 3' ends of the cDNAs, regions that share little or no nucleotide sequence homology. Hybridization probes were labeled by the random primer method (49). Northern blots were hybridized as described previously (12). The blots hybridized to the actin, creatine kinase, MyoD, MRF4, or myogenin probes were washed at high stringency (0.1× standard saline citrate/0.1% SDS, twice for 30 min at 50°C), whereas the blots hybridized to Myf5 and embryonic MHC were washed at low stringency (0.2× standard saline citrate).
citrate/0.1% SDS, twice for 30 min at 50°C) and subjected to autoradiography using Fuji X-ray film.

RESULTS

Gene Expression during Myogenesis of the Human Fetal Limb. To determine the molecular markers of myogenesis during the development of the human limb, we analyzed the expression of the actins, myosins, creatine kinases, and various myogenic regulatory genes in the limb musculature of human fetuses ranging in age from 7.5 to 24 weeks' gestation. Representative Northern blots of total RNA hybridized to probes representative of these genes are shown in Fig. 1A and diagrammatically represented in Fig. 1B. Transcripts that hybridized to 3' end-specific probes for MyoD and myogenin were detectable at all ages of skeletal muscle development analyzed. However, the expression of the myogenic regulatory factors, Myf5 and MRF4, were not detectable in our analysis (data not shown). Isotype-specific probes were used to detect transcripts specific for α-skeletal, β- and γ-cytoplasmic, and α-cardiac actins. The actin transcripts were detectable at all stages of fetal muscle limb development. However, as the level of α-skeletal actin RNA increased, the levels of the ubiquitous β- and γ-cytoplasmic actin RNAs decreased. Both isoforms of myosin were detectable at all stages of fetal muscle development. However, the level of expression of the embryonic MHC gene peaked between 7.5 and 17 weeks. The abundance of the MLC-1/3 transcripts increased with age of the fetus and peaked between 17 and 24 weeks, coincident with both the highest levels of expression of α-skeletal actin and the lowest level of expression of β- and γ-cytoplasmic actins. Transcripts for both the brain and muscle isoforms of creatine kinase were detected at all stages of fetal muscle development. The level of B-CK transcripts remained at comparable levels between 7.5 and 12 weeks and then declined between 17 and 24 weeks. The level of M-CK transcripts remained at low levels from 7.5 to 12 weeks and increased at 17 and 24 weeks, coincident with the lowest level of expression of the B-CK form.

Expression of Myogenesis-associated Genes in Rhabdomyosarcomas. We compared the expression of myogenic regulatory genes and muscle-specific markers (the actins, myosins, and creatine kinases) in 8 alveolar and 8 embryonal RMS tumors. Representative Northern blots of total RNA hybridized to probes representative of these genes are shown in Fig. 2, and the expression profiles are summarized in Table 3.

All 16 RMS tumor samples expressed transcripts that hybridized to the 3' end of the MyoD cDNA probe (Fig. 2). These results are consistent with a recent analysis of the same tumor samples using the entire human homologue of the mouse MyoD cDNA (12). These tumors also expressed comparable levels of expression of the B-CK form. However, as the level of α-skeletal actin RNA increased, the levels of the ubiquitous β- and γ-cytoplasmic actin RNAs decreased. Both isoforms of myosin were detectable at all stages of fetal muscle development. The level of B-CK transcripts remained at comparable levels between 7.5 and 12 weeks and then declined between 17 and 24 weeks. The level of M-CK transcripts remained at low levels from 7.5 to 12 weeks and increased at 17 and 24 weeks, coincident with the lowest level of expression of the B-CK form.

Expression of myogenic regulatory genes in the limb musculature of human fetuses ranging in age from 7.5 to 24 weeks' gestation were hybridized to probes representative of the myogenic regulatory genes (MyoD and myogenin), the actins (α-skeletal, α-cardiac, β-cytoplasmic, and γ-cytoplasmic), myosins (embryonic myosin heavy chain and myosin light chain 1/3), and the creatine kinases (brain and muscle forms). Different blots were used with probes representing brain creatine kinase, α-skeletal actin, and γ-cytoplasmic actin. The same blot was used with all remaining probes. A, position of the 28S ribosomal band; B, diagrammatic representation of the expression of various myogenic regulatory genes, the actins, myosins, and the creatine kinases.
GENE EXPRESSION IN RHABDOMYOSARCOMAS AND FETAL SKELETAL MUSCLE

Fig. 2. Northern blot analysis of rhabdomyosarcoma tumors. Northern blots containing 30 μg of total RNA from 8 embryonal (E) and 8 alveolar (A) tumor samples were hybridized to probes representative of the myogenic regulatory genes (MyoD, MRF4, myogenin, and Myf5), the actins (α-skeletal, α-cardiac, β-cytoplasmic, and γ-cytoplasmic), myosins (embryonic myosin heavy chain and myosin light chain-1/3), and brain creatine kinase. Different blots were used with probes representing β-cytoplasmic-, α-skeletal-, and α-cardiac-actin. The same blot was used with probes representing γ-cytoplasmic actin and embryonic myosin heavy chain. The same blot was used with all the remaining probes. △, position of the 28S ribosomal band; ▲, position of the 18S ribosomal band.

Actin probes were detectable at comparable levels in all RMS samples (Fig. 2). The cardiac and skeletal α-actins were detectable in all 8 alveolar RMS samples (Fig. 2). Only 3 of 8 embryonal RMS tumors (Fig. 2, Samples 6, 31, and 33) expressed detectable levels of α-skeletal actin. (The slightly higher molecular weight species represents cross-hybridization to β-actin RNA.) Cardiac α-actin was detectable in 6 of 8 embryonal RMS tumors (Fig. 2, Samples 5, 7, 10, 31, and 33). Embryonic MHC transcripts were detectable in only 1 of 8 alveolar RMS tumors (Sample 4) and 1 of 8 embryonal RMS tumors (Sample 31). Trace levels of MLC-1/3 transcripts were visible in 4 of 8 alveolar RMS tumors (Fig. 2, Samples 3, 4, 27, and 34). Five of 8 embryonal RMS tumors (Fig. 2, Samples 5, 6, 7, 31, and 33) expressed MLC-1/3 (Fig. 2). B-CK transcripts were detectable in all alveolar RMS tumors and 4 of 8 embryonal RMS tumors (Samples 5, 6, 7, 31, and 33). None of the tumors expressed detectable levels of the muscle form of creatine kinase (data not shown).

DISCUSSION

We have determined the expression of muscle-specific markers such as the actins, myosins, and creatine kinases, together with the known myogenic regulatory genes in embryonal and
alveolar RMS as well as in stages of developing human fetal limb muscle. Our studies were predicated on the observation that, histologically and immunocytochemically, RMS resemble fetal striated skeletal muscle (16) and that the histological variance within this group more closely resembles stages in the development of this tissue (17). We restricted our analysis to the actins, myosins, and creatine kinases because changes in the expression of these markers have been correlated with myoblastic differentiation in the in vivo analysis of developing limb (33, 34) and in the in vitro analysis of differentiation of myoblasts by mitogen deprivation (31, 33).

The expression of the myogenic regulatory factors in all RMS samples is consistent with both the skeletal muscle histogenesis of the tumors and the restriction of expression to myogenic cell lines and skeletal muscle (18–21). Although all of the RMS samples expressed detectable levels of MyoD (Table 3), the level of expression was variable. Samples 25, 26, and 10, for example, expressed the lowest levels of MyoD and represented the tumor samples that were the least differentiated with respect to the myogenic phenotype. These samples also expressed the lowest levels of MRF4, no myogenin, and little or no Myf5 transcript (Table 3). In order to test the possibility that these tumors consist of an admixture of primitive cells only some of which are committed to the myogenic phenotype, we are currently preparing antibodies specific to human MyoD to be used in immunocytochemical analyses. The establishment of muscle during vertebrate embryogenesis involves induction of primary ectoderm to mesoderm, a portion of which segregates to somites (Ref. 54 and references therein). Subsequently, myogenic precursor cells migrate into the developing limb bud. Myogenin transcripts have been detected at high levels in the somite myotome, during murine development, 2 days before the appearance of MyoD1 (55, 56). Paradoxically, cells that invade the limb bud and ultimately differentiate into muscle initially show no expression of myogenin or MyoD but later express both coordinately. In contrast to the early expression of myogenin and MyoD, MRF4 is not expressed until after birth, when it increases to the highest level of the myogenic factors (21). The apparent restriction of MRF4 expression to adult skeletal muscle is consistent with our analysis of developing fetal human limb, where MRF4 transcripts were undetected at any stage of development.

More extensive studies have been done with established muscle cell lines which have shown that only subsets of the myogenic regulatory factors are expressed. For example, either MyoD or Myf5 (but not both) is expressed constitutively in many muscle cell lines (18, 19, 57). These data are consistent with our analysis of developing human fetal limb (where no Myf5 transcripts were visible at any stage of development, although high levels of MyoD transcripts were clearly visible) and with our analysis of RMS tumors, where trace levels of Myf5 transcripts were visible in the majority of RMS samples (Table 3). In contrast to the in vitro expression of Myf5, myogenin is repressed until myoblasts are triggered to differentiate and then is rapidly up-regulated in every muscle cell line that has been examined (20, 56). MRF4 is not expressed in most established cell lines, consistent with its possible role later in development (21). The mechanism that accounts for differential expression of members of the myogenic regulatory family in some cell types remains to be elucidated.

Our analysis of the expression of the actins, myosins, and creatine kinases in developing fetal human limb is consistent with the expression of similar genes in myoblast differentiation in vitro. During skeletal muscle development both α-cardiac and α-skeletal actins are coexpressed at proportional levels, whereas transcripts were detectable at low levels as early as 7.5 weeks of gestation and transcript accumulation occurred up to 24 weeks (Fig. 1). In contrast, the β- and γ-cytoplasmic actins were present at high levels early in myogenesis but declined during later stages in development. During myoblast fusion and the formation of myotubes, the α-skeletal and α-cardiac actins are induced and the cytoplasmic actins are down-regulated (30–34). During this transition, the brain form of creatine kinase is replaced by the muscle form (38–42). Although both isoforms were detectable in the earliest tested stage of fetal limb development, higher levels of B-CK expression were evident in the earliest stages of muscle development coincident with the lowest levels of expression of M-CK. By 24 weeks, the B-CK expression decreased, whereas M-CK increased. The transition from myoblast fusion and the formation of postmitotic myotubes is also accompanied by an accumulation of MLC 1/3 (34–38). Our analysis is also consistent with this pattern of transcript accumulation during in vivo myogenesis, in that although trace levels of MLC transcripts were detectable at 7.5 weeks, the highest levels were evident at later stages in development. The level of expression of various transcripts during the in vivo development of fetal human limb thus appears to mirror the differential expression of actin, myosin, and creatine kinase genes observed in the transition of myoblasts to the formation of postmitotic myotubes during in vitro differentiation.

Comparison of the expression profile of muscle-specific markers in RMS with the developing human fetal limb muscle suggests that as a group, RMS resemble a relatively restricted segment of fetal muscle development, bounded on one end by the commitment of cells to the myogenic pathway and on the other by the earliest overt stages of myogenesis. As a subgroup of rhabdomyosarcoma, the alveolar subtype phenotypically represented a restricted group, where variability was observed in the expression of embryonic MHC and MLC-1/3, consistent with the histological classification of this type (8). In contrast, the embryonal subtype exhibited a broader range of phenotypes showing a considerable overlap with the alveolar subtype, suggesting that the two subtypes of RMS may not represent malig-

Table 3 Composite expression profile in rhabdomyosarcomas

<table>
<thead>
<tr>
<th>Gene</th>
<th>Embryonal</th>
<th>Alveolar</th>
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<tbody>
<tr>
<td>E-MHC</td>
<td>++</td>
<td>+</td>
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<tr>
<td>MLC-1/3</td>
<td>+</td>
<td>+</td>
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<tr>
<td>B-CK</td>
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<td>+</td>
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<tr>
<td>M-CK</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Actins</td>
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<tr>
<td>α-Skeletal</td>
<td>++</td>
<td>+</td>
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<tr>
<td>α-Cardiac</td>
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<td>β-Cytoplasmic</td>
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<tr>
<td>γ-Cytoplasmic</td>
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<td>MyoD</td>
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<tr>
<td>MRF4</td>
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</tr>
<tr>
<td>Myogenin</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Myf5</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Representative Northern blots were scored for the presence (+) or absence (−) of hybridizing signal.
* Increasing phenotypic differentiation (arrows from left to right) based on gene expression.
* E-MHC, embryonic MHC; ND, not done.
trand transformation of normal cells of different positions in myogenesis.

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REFERENCES


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