
Forming Embryonic-Like Nervous Tissues and Organs by Muscle-Derived Neuroepithelial Myogenic Progenitors

Zhuqing Qu-Petersen

The Copenhagen Muscle Research Centre, National University Hospital, Copenhagen, Denmark

Email address:

zhuqing.qu.petersen@rh.regionh.dk

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Abstract: Unlike totipotent ES cells, adult-origin multipotent progenitors have limited differentiation. Several CNS carcinomas featuring embryonic nervous and muscle tissues, however, suggest the existence of distinct primitive progenitors. Rhabdomyosarcoma is a soft tissue malignant tumor, and although it displays phenotypical features of neural and muscle elements, its etiology remains largely unaddressed. It has been shown that muscle-derived neuroepithelial myogenic progenitors (NEMPs) differentiate into radial glial-like cells, neurons, and early myoblasts in vitro and generate embryonic/fetal-like myofibers in vivo. The present study reports that NEMPs could also generate chimeric grafts in muscle, which exhibited morphogenetic features of the embryonic brain, developing nerve nuclei/ganglia, and primitive striated muscle. A single NEMP differentiated in vitro into multiple colonies containing neuroepithelial cells, neurons, astroglia, and myoblasts, with embryonic tissue patterns. The data demonstrate that generation of embryonic nervous tissues is NEMP's unique potency, providing translational evidence for a NEMP origin of the different malignant neoplasms.

Keywords: Neuroepithelial Myogenic Progenitors, Chimeric Grafts, Rhabdomyosarcoma, Embryonal Tumors, Brain, Skeletal Muscle

1. Introduction

Totipotent or pluripotent embryonic stem (ES) cells and epiblasts are able to give rise to multi-lineage tissues in the body. Human and mouse ES cells are routinely derived from the inner cell mass of the blastocyst in 4–5-day embryos [1, 2], and epiblasts can be derived from embryonic day 3.5–7.5 mouse embryos [3, 4]. Both ES cells and epiblasts can generate chimeric embryos following blastocyst-injection or form teratomas in grafted immunodeficient mice, which contain derivatives of all three embryonic germ layers [3–5]. Unlike ES cells, multipotent stem and progenitor cells from diverse somatic tissues are generally considered to possess limited differentiation potentials.

It has long been acknowledged that cancer resembles embryonic tissues, which led to the theoretical hypothesis in the mid 19th century that cancer arises from embryonic-like cells in adults [6]. Typically, several malignant embryonal tumors in the central nervous system (CNS), such as medulloblastoma and atypical teratoid/rhabdoid tumor (AT/RT), feature the presence of primitive nervous and

striated muscle tissues [7, 8]. In the early developmental stages, ectodermal cells differentiate into neuroepithelial cells (neuroectoderm) that function as the precursors of neuroblasts and glioblasts. Neuroepithelial cells can proliferate and undergo morphogenetic changes, resulting in the formation of the neural tube, the primitive CNS in embryos [9, 10]. The co-presence of embryonic nervous and muscle tissues in the tumors, therefore, may suggest that distinct primitive progenitors exist in adult tissues and serve as the cell of origin in neoplasms. Notably, tumors recognizable by undifferentiated or very immature cells have been identified in distinct somatic tissues. Rhabdomyosarcoma (RMS), for example, is a malignant soft tissue tumor that contains poorly differentiated round cells and displays striated muscle differentiation [11]. Although RMS can arise in skeletal muscle, exhibition of the phenotypical features of neural and muscle elements is common in the tumor [11], and the etiology of RMS remains largely unaddressed [12].

In the previous investigation, rare primitive progenitors have been isolated from the limb muscles of 5-week normal

mice, which are termed as neuroepithelial myogenic progenitors (NEMPs) based on their capacities of differentiating into early neural and myogenic cells in culture, and generating embryonic/fetal-like muscle fibers after transplantation [13]. It is proposed that NEMPs may represent embryonic-like neural/muscle progenitors from the postnatal tissue. The present study reports an unexpected but natural potency of NEMPs: their clonal populations could spontaneously differentiate into chimeric grafts with embryonic-like nervous and muscle tissues *in vivo*, and generate multiple colonies with embryonic tissue patterns *in vitro*. The findings support the embryonic-like progenitor character of the muscle-derived primitive cells and imply their initial role in tumorigenesis.

2. Materials and Methods

2.1. Cell Populations

NEMP colonies were isolated from the primary muscle cultures of normal mice (C57BL/6 mice, 5 weeks old), and the cells were grown at the density of 2.5×10^3 to 7.5×10^3 cells/cm² in the Dulbecco's modified Eagle's medium supplemented with 20% serum and 0.5% chick embryo extract (growth medium) and expanded for 8–12 passages before transplantation [13].

2.2. Mice

Dystrophin-deficient mdx mice (C57BL/10ScSn-Dmd^{mdx}/J, 6–16 weeks old) were used as the host, and 1.5×10^5 or 3×10^5 cells were injected into the gastrocnemius muscles [13]. The mice were sacrificed at 40–65 days post-injection, and the grafted muscles were frozen for analysis. All animals were handled according to the institutional guidelines of the Panum Institute Animal Facility and the experimental protocols were approved by the animal care and Ethics Committee in Copenhagen.

2.3. Immunohistochemistry and Histology

Cryosections of injected muscles were fixed with acetone at -20°C for 5–10 min. Sections were incubated with primary antibodies for 90 min (except 30 min for anti-dystrophin), and then species-specific Alexa Fluor 594 or 488 (1:500, Invitrogen A/S) for 60 min. Primary antibodies used in the present study were rabbit anti-dystrophin (1:100, Neo Markers), rabbit anti-Msi1 (1:100, Abcam), rabbit anti-NSE (1:100, Sigma-Aldrich), goat anti-Wnt-1 (1:20, R&D Systems), mouse anti-MyoD (1:80, BD Pharmingen), and mouse anti-Pax7 antibodies (1:50, R&D Systems). The nuclei were revealed by Hoechst 33342 (Sigma-Aldrich). The adult brain and muscle tissues were used as the positive controls and the sections that were labeled with only secondary antibodies were used as the negative control. For histological analysis, the sections were stained with hematoxylin and eosin according to the manufacturer's instructions (Sigma-Aldrich).

2.4. Isolation and Analysis of Single Clones

NEMPs at passage 16 were seeded onto collagen type 1-coated 96-well dishes (25–50 cells/ml growth medium), and the culture wells were checked for single cells one hour after seeding. After the cells were cultured in the growth medium for six days, single clones formed and were picked up. One clone, with cells that were small round and phase-bright, morphologically similar to the primary NEMP clones/colonies and that proliferated well, was used for further colony assay. After the clone was propagated to passage 3, the cells were seeded in 25-cm² flasks or 6-well dishes at a cell density of 10–20 cells/cm², and were grown in the medium for eight days. Cells were then fixed with methanol at -20°C for 5 min for immunocytochemistry.

2.5. Immunocytochemistry

After fixation with methanol, the cells were blocked with 1% BSA or 5% serum in PBS for 30 min. Cells were then incubated with primary antibodies for 90 min at room temperature or overnight at 4°C , and staining was revealed by species-specific Alexa Fluor 594 or FITC. Primary antibodies included rabbit anti-NSE (1:100, Sigma-Aldrich), rabbit anti-Msi1 (1:100, Abcam), rabbit anti-S-100 (1:400, Sigma-Aldrich), rabbit anti-GFAP (1:200, Sigma-Aldrich), rabbit anti-Nanog (1:100, Abcam), goat anti-Wnt-1 (1:20, R&D system), and chicken anti-Sox-1 antibodies (1:200, Millipore). For co-localization of GFAP and MyoD or Msi1 and MyoD, cells were incubated with rabbit anti-GFAP or rabbit anti-Msi1 overnight at 4°C , and the staining was revealed by FITC-labeled sheep anti-rabbit IgG (1:200, Sigma-Aldrich). The cells were then incubated with mouse anti-MyoD (1:80, BD Pharmingen) for 90 min, and the staining was revealed by goat anti-mouse IgG conjugated with Alexa Fluor 594 (1:500, Invitrogen A/S). The nuclei were revealed by Hoechst 33342 (Sigma-Aldrich).

3. Results and Discussion

3.1. NEMP Cells Generated Chimeric Grafts in Skeletal Muscles of Mdx Mice

NEMP cells, used in the present study, were originally derived from three primary colonies (NEMPs) or from a single colony (NEMP-c) [13]. Because technique limitations hindered single-NEMP isolation from primary muscle cultures, the NEMP-c was developed by two times of cloning in the limiting-dilution cultures (Fig. 1A). By the time of grafting, NEMP cells expressed cellular markers for neural progenitors (nestin, Musashi 1/Msi1, Wnt-1 and GFAP) and early myogenic cells (Pax3, MyoD, myogenin and Myf5); they also expressed cell-surface markers for stem cells (ABCG2 and Sca-1), but ES cell markers Oct-4 and Nanog were not detected [13]. A characteristic of the early activated NEMP cells was their co-expression of neural progenitor and myogenic markers Msi1 and MyoD or GFAP and MyoD (Fig. 1B and 1C).

NEMPs and NEMP-c were grafted into the gastrocnemius muscles of adult mdx mice. Mdx (x-linked muscular dystrophy) is a mutation in the dystrophin gene and the mdx mice do not express dystrophin protein in the muscle fibers. Because NEMP cells were isolated from the wild type mice, grafting of these cells into mdx mice can form donor-derived myofibers expressing dystrophin, whereby the grafted areas can be identified (Fig. 1D). Unexpectedly, clear chimeric grafts with muscle and non-muscle (ectopic) tissues were detected in one of seven grafts for each cell population by 40–65 days after grafting. Nuclear fluorescent staining revealed many nodular or tubular structures with large numbers of single cells in the NEMP-c graft.

Immunostaining indicated that the number of dystrophin positive myofibers in the grafted area was 5-fold higher than non-grafted area, demonstrating donor-derived myofibers in the graft (Fig. 1E). Unlike the NEMP-c graft, NEMPs generated small ectopic tissue with only several nodular and tubular structures containing mononuclear cells, but large numbers of donor-derived dystrophin positive myofibers (Fig. 1F). Since no ectopic tissue was observed in the other 12 muscles transplanted, the two grafts were possibly the result from clonal propagation of single primitive NEMP cells, which also exhibited differences in their differentiation capacities.

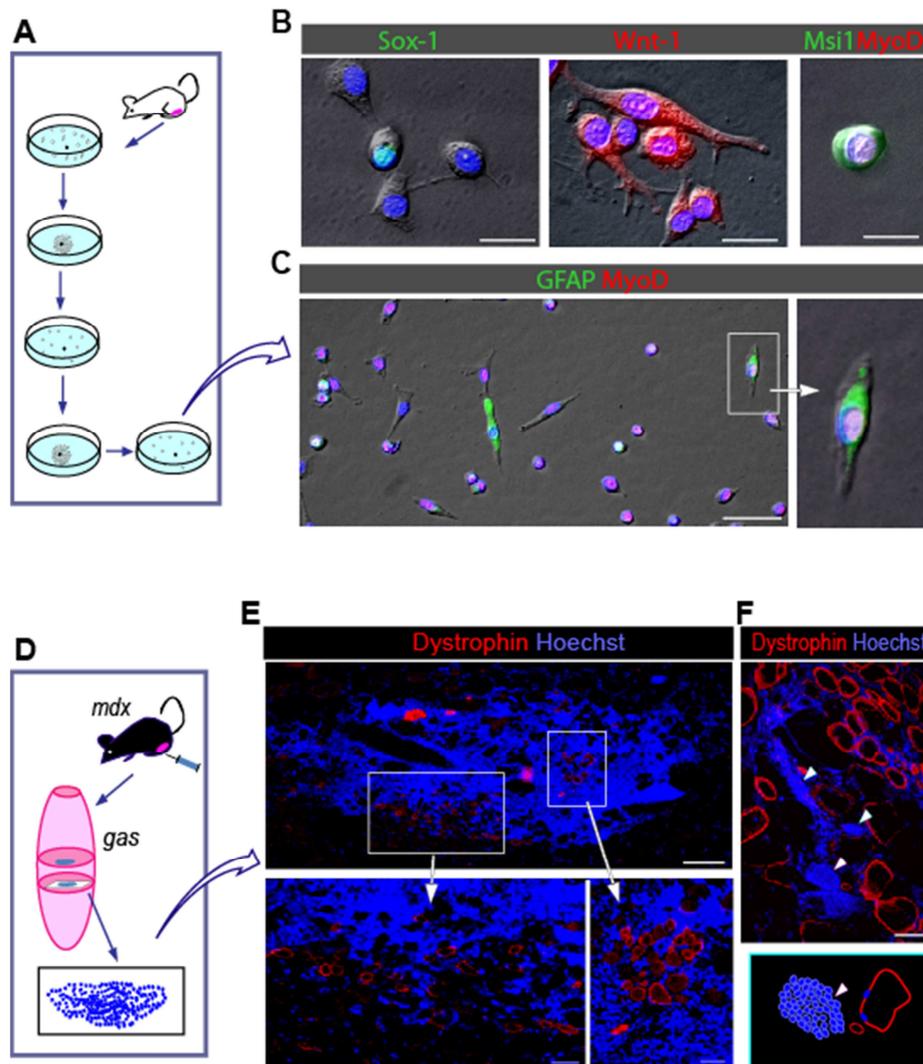


Figure 1. Cells co-expressing neurogenic/myogenic markers produced chimeric grafts.

(A) Derivation of NEMP-c from primary muscle cultures by two times of cloning.

(B and C) Early cultured NEMP cells exhibited morphology and marker profiles for neural or neural/muscle progenitors, and co-expressed GFAP/MyoD or Msi1/MyoD. Nuclei were revealed by Hoechst.

(D) Detection of chimeric grafts in the gastrocnemius muscles (gas) of mdx mice.

(E) In NEMP-c grafted muscle, large ectopic tissues were revealed by Hoechst nuclear staining, and the grafted areas were recognized by dystrophin positive myofibers.

(F) In the muscle grafted with NEMPs, many dystrophin positive myofibers were present, with Hoechst-stained small cell-clusters in nodular and tubular structures (arrowheads, upper panel). Diagram in the lower panel shows the nodule (red arrowheads) with many single cells.

White scale bars: (B) 20 μ m; (C) 50 μ m; (E) 200 μ m; (F) 50 μ m. Blue scale bars: (E) 50 μ m.

3.2. Chimeric Grafts Exhibited Histological Features of Embryonic Nervous Tissues and Organs

To investigate the nature of ectopic tissues with nodularity, the histological features of the NEMP-c graft were first examined by H&E staining. It is interesting that similar to the neuroepithelial cells capable of generating the neural tube in the early development (Fig. 2A), the NEMP-c-resulted ectopic tissues bear a close morphogenetic resemblance to the embryonic brain, including the three primary brain vesicles (the prosencephalon, mesencephalon and rhombencephalon), vesicle cavities, and developing nerve nuclei/ganglia (Fig. 2B and 2C). The majority of cells in the brain vesicle- and nucleus-like structures were small round or elongated (Fig. 2B-a), morphologically similar to undifferentiated tumor cells or neuroepithelial cells that can

be seen in medulloblastomas and CNS embryonal tumors (previously designated as primitive neuroectodermal tumors) [8, 14]. Other cell types, including ependymal-like cells and astrocyte-like cells, were also present in the graft at a low frequency (Fig. 2B-b and 2B-c), and the latter was frequently localized close to vascular structures. Very few of cells in the area were large round cells (Fig. 2B-d), resembling rhabdomyoblasts in RMS [11] or rhabdoid cells in AT/RT [14, 15]. Outside the neural tube- and nucleus-like structures, many small round cells (Fig. 2D), and spindle-shaped myofibers with embryonic/fetal-like muscle fibers (Fig. 2E) were present. The spindle-shaped myofibers likely represent a primitive muscle element that can be seen in NEMP's early cultures [13].

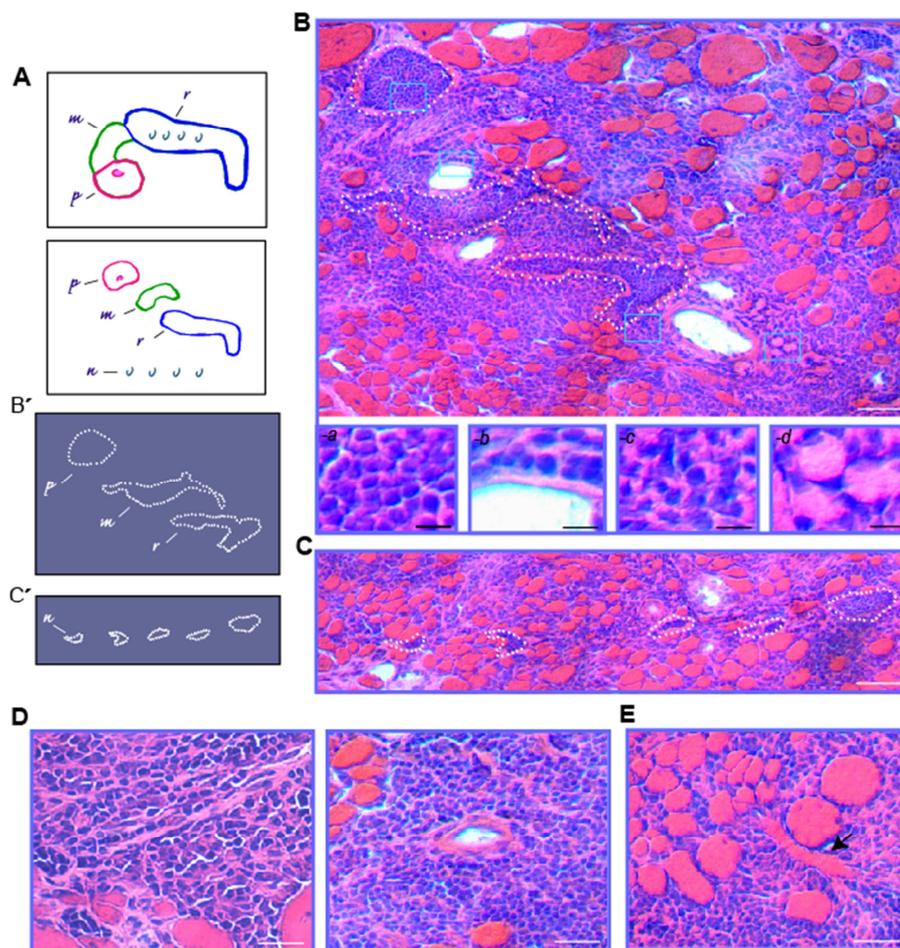


Figure 2. The NEMP-c graft recapitulated morphogenetic features of the embryonic brain, nerve nuclei and muscle tissues.

(A) Diagram shows that the early embryonic brain consists of three primary brain vesicles of the forebrain (prosencephalon-p), midbrain (mesencephalon-m), and hindbrain (rhombencephalon-r), and cranial nerve nuclei (n).

(B) Ectopic tissues consisted of many single cells within nodular, tubular, and lobular structures (dashed lines). (B'): Dashed lines from (B) show the resemblance between the different structures and the three parts of the developing brain, as seen in (A). The boxed areas in (B) were magnified in the lower panels showing multiple types of cells morphologically similar to (-a) neuroepithelial cells, (-b) ependymal cells, (-c) astrocytes, and (-d) large rhabdomyoblasts.

(C) Five clusters of cells (dashed lines) were present in the graft. (C'): The dashed lines from (C) show that the cell clusters grew like embryonic nerve nuclei/ganglia, as seen in (A).

(D and E) Representative areas show large numbers of small and round cells (D), and a spindle-shaped primitive myofiber (arrow) with a cluster of embryonic/fetal-like muscle fibers (E).

White scale bars: (B and C) 50 μ m; (D and E) 25 μ m. Black scale bars: (B) 10 μ m.

Notably, the NEMP-resulted ectopic tissues were mainly related to the primitive neuroectodermal and early muscle tissues in the present grafting model. No clear other derivatives of the mesoderm and endoderm were detected, such as fat, cartilage, bone and gut epithelium, which have been reported in the teratomas formed by injection of ES cells [2] or epiblasts from both preimplantation and post-implantation mouse embryos [3, 4]. This could be explained by the differences in differentiation potentials between the adult-origin NEMPs and ES cells or epiblasts. In addition, the microenvironments for cell growth and grafting may influence or limit the developmental capacity of pluripotent cells. Nevertheless, the generation of embryonic-like nervous tissues and organs in the grafted muscles suggests that the primitive NEMP cells share many similarities with ES cells and epiblasts, rather than adult muscle progenitor satellite cells. In our laboratory, multipotent myogenic progenitors have also been isolated from the bone marrow [13] and no ectopic nervous tissues were detected in 12 muscles injected with these cells in the same animal model.

3.3. Ectopic Tissues in the Chimeric Graft Exhibited a Neuroepithelial Cell Phenotype

Next, the chimeric grafts were analyzed by immunohistochemistry to explore the molecular phenotypes of the ectopic tissues. Since only limited markers of neural progenitors are available for use in the muscle, three markers including Msi1 and Wnt-1 for neuroepithelial cells and NSE (neuron-specific enolase) for more committed neurons were examined. Msi1 is a RNA binding protein, expressed in the neuroepithelial cells and brain cancers [16, 17], and Wnt-1 is a secreted protein that may play a role in the early development of the midbrain and cerebellum [18]. Both proteins were detected in more than 90% of the early cultured NEMP cells [13]. In NEMP-c graft, the majority of small round cells in the neural tube- and nucleus-like tissues expressed Msi1 at a higher intensity; outside these tissues, the mononuclear cells expressed Msi1 at various levels and exhibited rosette-like cell growth (Fig. 3A and 3B). Very few mononuclear cells in the graft were positive for Wnt-1 and NSE (Fig. 3C and 3D). The detection of Msi1 but not the lineage more specific marker NSE in the majority of cells suggests that the ectopic tissues exhibited a primitive neuroepithelial cell phenotype, which is correlated with their histological features revealed by H&E staining. Because the presence of embryonic nervous tissue and rhabdoid components is the characteristics of several embryonal tumors in the CNS [7, 8], and also due to the muscle and neural phenotypes of RMS [11], the graft was further analyzed by myogenic markers MyoD and Pax7. Whereas myogenic markers can be detected in the subtypes of RMS [11, 19], neither Pax7 (Fig. 3E), nor MyoD (not shown) was detected in the single cells of muscle and non-muscle tissues in the chimeric grafts. However, the detection of donor-derived dystrophin positive myofibers in the grafts, as shown above, demonstrated that the myogenic cells had contributed

to myofiber formation in the areas. It is likely that the majority of the rest mononuclear cells were non-myogenic or their myogenic potential was restricted at this stage.

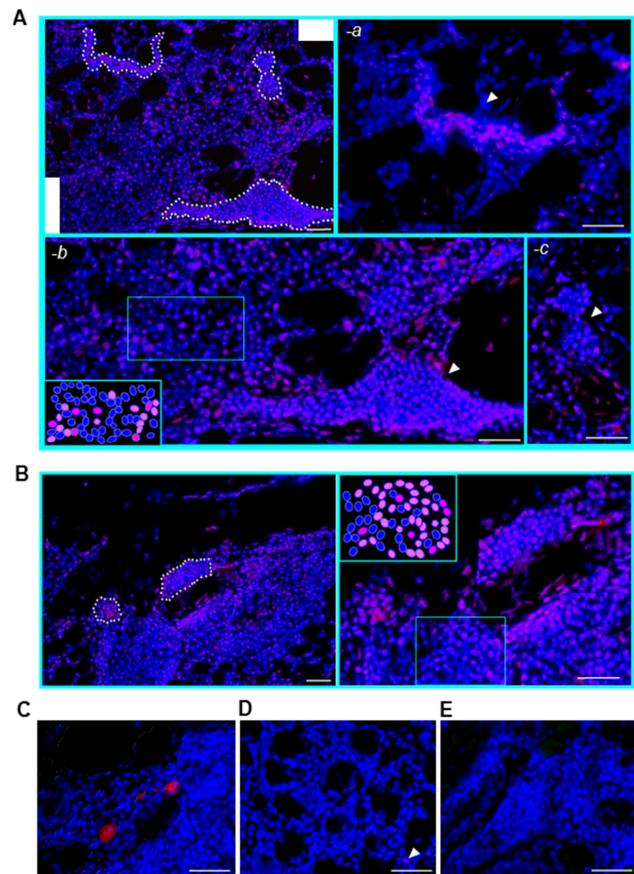


Figure 3. Ectopic tissues displayed a molecular phenotype for neuroepithelial cells but not for lineage-specialized neuronal and myogenic cells.

(A) Immunohistochemistry shows large numbers of Msi1+ cells in ectopic nervous tissues (dashed lines). These embryonic-like tissues enclosed in the dashed lines are magnified (arrowheads) in the panels (-a) the neural plate, (-b) rhombencephalon, and (-c) developing nerve nuclei/ganglia. Inset in (-b) is a diagram of the boxed area showing rosette-like cell growth. (B) Cells in nodular structures (arrows) expressed higher levels of Msi1, and many single cells in non-nodular tissues were also expressed Msi1 to various intensities. Inset in the left panel is a diagram of the boxed area showing rosette-like cell growth. (C-E) Signaling protein Wnt-1 (C) and neuronal marker NSE (D) were expressed in a small number of cells in the chimeric grafts and myogenic marker Pax7 (E) was not detected. Nuclei were revealed by Hoechst. Scale bars: (A-E) 25 μ m.

3.4. Clonal NEMPs Generated Multiple Colonies in Vitro, with Embryonic Nervous Tissue Patterns

The possibility that a primitive NEMP might colonize the chimeric graft in vivo raised the question whether a single NEMP in culture could function as a similar manner. To address this issue, single clones were developed from cultured NEMP cells, and one clone was chosen for colony assay (Fig. 4A). In culture, a small number of cells (4.88–

9.76%) developed into large colonies, with cells exhibiting the morphology of neural progenitors or neuroblasts (Fig. 4B). Like the ectopic tissues *in vivo*, some of the colonies exhibited the tissue patterns of the developing brain vesicles, in which rosette-like cell growth was present (Fig. 4C). The majority of cells in the colonies expressed neural progenitor markers Msi1 and GFAP (Fig. 4C and 4D), and neuronal marker NSE or ependymal cell marker S-100 was heterogeneously expressed in the colonies (Fig. 4D). Nearly all the large colonies analyzed were positive for Msi1 (35/35, 100%) or GFAP (40/42, 95.24%), but none of the colonies expressed Nanog (0/34, 0%). Colonies with a lower cell density were also present in cultures, frequently growing as 4–5 colonies in an area, similar to the developing ganglia (Fig. 4E).

The frequency of high- or low-cell-density colonies (hcd- and lcd-colonies) is 29.41% and 70.59% respectively (Fig. 4F), and the average cell density is 1.7×10^5 cells/cm² for hcd-colonies, and 3.8×10^4 /cm² for lcd-colonies. Analysis of

myogenic marker MyoD revealed that the majority of hcd-colonies were MyoD⁺ (23/25), but only about half of the lcd-colonies were MyoD⁺ (31/60, Fig. 4G). Co-localization of myogenic and neural progenitor markers indicated that all MyoD⁺ colonies were positive for Msi1 and GFAP (Fig. 4H and 4I), thereby being bi-potential. The MyoD negative colonies were considered for the neural lineage (Msi1+GFAP+). In part of the hcd-colonies (22/75, 29.33%), myotubes were frequently detected, frequently correlated with the high percentage of MyoD⁺ cells, while no myotubes were found in the brain vesicle-like colonies. The results indicate the heterogeneity in myogenic potential of the hcd- and lcd-colonies, some of which may reflect their intrinsic connection to the sensory or motor nuclei. Collectively, the *in vitro* results demonstrate that a single NEMP contributes to both neural and myogenic cells, with embryonic nervous tissue patterns. This supports the clonality of the chimeric graft observed *in vivo*.

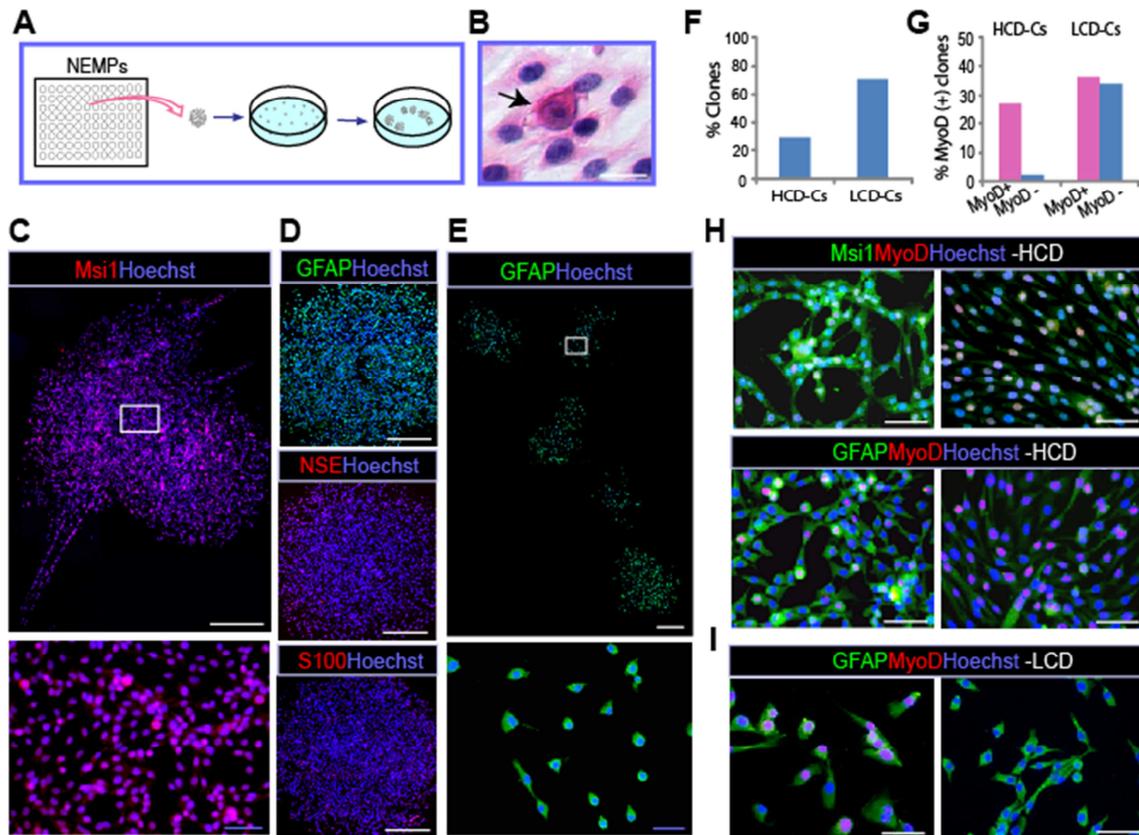


Figure 4. NEMP colonized embryonic nervous and muscle tissues *in vitro*.

(A) Strategy for colony assay of a single clone derived from the cultured NEMPs.

(B) H&E staining shows a neural progenitor or neuroblast-like cell (arrow) in a colony.

(C) Large colonies exhibited the brain-like tissue pattern, with cells purified for Msi1 immunostaining. The box area was magnified in the lower panel, showing rosette-like cell growth.

(D) Three large colonies expressed GFAP (upper), and heterogeneous NSE (middle) and S-100 (lower).

(E) Five colonies grew as a fashion of developing nerve nuclei/ganglia, and were positive for GFAP. The box area was magnified in the lower panel.

(F and G) Histograms show the percentage of high- and low-cell-density colonies (hcd- and lcd-colonies), and their connection to MyoD expression ($n = 85$ colonies, from three individual cultures; an average of 34.93% of MyoD⁺ cells was counted in the MyoD⁺ colonies).

(H) Immunostaining of four hcd-colonies shows their co-expression of neural progenitor (Msi1 or GFAP) and myogenic (MyoD) markers. Note rosette-like cell growth in the left panels and myotubes in right panels.

(I) Two lcd-colonies were GFAP+MyoD⁺ or GFAP+MyoD⁻.

White scale bars: (B) 15 μ m; (C–E) 500 μ m; (H) 50 μ m. Blue scale bars: (C and E) 50 μ m.

A previous study has demonstrated NEMP's spontaneous differentiation into embryonic and fetal-like myogenic lineages [13]. The most important new finding in this report is that besides the evidence for myogenic differentiation, non-modified NEMP cells generated embryonic-like nervous tissues and organs both in vivo and in vitro. In comparison with the totipotency of ES cells, the NEMP's potency was mainly related to primitive neural and muscle components. In addition, unlike the more differentiated neuroectodermal tissues from ES cells, the ectopic nervous tissues from NEMP-c were more immature based on the presence of large numbers of undifferentiated small round cells. Although cells expressing markers for neuronal and glial lineages were present in NEMP colonies in vitro, the majority of mononuclear cells in the grafts exhibited marker profiles for neuroepithelial cells but not lineage specific neurons. It is possible that the muscle environment inhibited neuronal differentiation of NEMP cells in vivo. On the other hand, more differentiated cell types were detected by histological analysis only in small focal regions, suggesting that the generation of large numbers of immature or undifferentiated neuroepithelial cells likely represents an intrinsic biological property of the primitive NEMP in vivo.

Embryonal tumors with undifferentiated or poorly differentiated tumor cells in nodules or clumps include medulloblastoma in the cerebellum and CNS embryonal tumor in the extracerebellar sites in the CNS, both of which may have primitive muscle tissue [7, 8]. AT/RT is also an embryonal tumor in the CNS, and histologically, all the tumors feature a population of rhabdoid cells and 70% may contain primitive neuroectodermal components [15]. In soft tissues, RMS displays characteristic histological features of ill-defined, poorly differentiated round cells in large numbers of lobular or nest structures, as so-called alveolar RMS, commonly arising in the limb muscles [11]. It is interesting that histopathologically, the ectopic tissues from NEMP-c do exhibit the characteristic features of the embryonal tumors in the CNS and also in RMS, being large numbers of small round blue cells in primitive nervous tissues with nodularity, and exhibition of striated muscle elements. Thus, the NEMP's recapitulation of the developing brain and nuclei/ganglia in the grafted muscle not only reveals the potential link between a primitive NEMP and RMS but also indicates that RMS may in fact share the cell of origin of several CNS carcinomas.

Notably, unlike a disorganized tissue pattern of the advanced neoplasms, the NEMP-related embryonic-like nervous tissues were better organized, morphologically. This feature, in line with in vitro results, leads to the proposal that the ectopic tissues share anatomical presentations of the developing CNS. This might be explained by the fact that NEMP cells were isolated from the normal muscle tissue, and is in agreement with the critical role of neuroepithelial cells in temporal regulation and spatial organization of the primary and secondary brain vesicles [9, 10]. More importantly, however, because many documented malignant tumors are presented at their advanced stages, it is conceived in this regard that the NEMP-related ectopic nervous tissue most likely represents

one of the earliest cancerous tissues from skeletal muscles.

4. Conclusion

This investigation demonstrates that apart from generating immature muscle elements, NEMP cells could generate embryonic-like nervous tissues, which resembled the developing CNS. These primitive nervous and muscle tissues recapitulated characteristic morphological features of several embryonal tumors in the CNS, and also RMS in soft tissues. The findings thus provide new insights into the cell of origin in the different types of malignant neoplasms.

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