Myocyte enhancer factor 2C upregulates MASH-1 expression and induces neurogenesis in P19 cells

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Abstract MEF2C is a transcription factor expressed in neural lineages. After transient transfection, the MEF2 family of factors can act synergistically with the neural-specific transcription factor, MASH-1, and activate exogenous neural-specific promoters. To determine whether MEF2C is capable of modulating endogenous gene expression, P19 cell lines were analyzed that overexpressed MEF2C, termed P19[MEF2C] cells. Here we show that P19[MEF2C] cells differentiate into neurons when aggregated with ME_2SO. MEF2C-induced neurons expressed neurofilament protein, the nuclear antigen NeuN, as well as MASH-1. Our results indicate that MEF2C can directly or indirectly activate the expression of MASH-1, leading to neurogenesis.

Key words: Neurogenesis; Embryonal carcinoma; Transcription; MEF2; Mash-1

1. Introduction

The four vertebrate MEF2 family members, MEF2A–D [1,2], contain a conserved MADS box/MEF2 domain at their N-termini. This domain mediates protein–protein interactions as well as DNA binding to an AT-rich MEF2 binding site. The MEF2 family of transcription factors has been implicated in controlling tissue-specific gene expression in skeletal, cardiac, and smooth muscle cells [2,3] as well as in brain and neuronal cells [4–10].

An essential role for D-MEF2 in the development of cardiac, skeletal, and smooth muscle has been demonstrated by the deficiency of these tissues in *Drosophila* lacking the single *D-mef2* gene [11–13]. During murine somitogenesis, MEF2C is the first MEF2 factor to be expressed in the somite on day 9, just after expression of myogenin [14,15]. In mammalian systems, the analysis of mice lacking MEF2 family members is complicated by possible functional redundancy. For example, mice lacking MEF2C die around embryonic day 10 due to a defect in cardiac looping while mice lacking MEF2B are viable and develop normally.

In order to determine if MEF2 factors are capable of activating endogenous tissue-specific gene expression in mammalian systems, we have analyzed the ability of MEF2C to change the developmental potential of murine P19 embryonal carcinoma cells. These cells have a stable diploid karyotype and are pluripotent. Cellular aggregation in the presence of drugs induces differentiation that emulates the biochemical and morphological events of early embryonic development [16,17]. Aggregation of P19 cells with retinoic acid results in the development of various neuroectodermal derivatives, including neurons, astrocytes, and glia [17,18]. P19-derived neurons express MEF2C [4] and the neurogenic basic helix-loop-helix (bHLH) transcription factor MASH-1 [19]. These two factors can physically interact to synergistically activate transcription. When stably expressed in P19 cells, a dominant-negative MEF2C was able to suppress the expression of the *N*-methyl-D-aspartate receptor subunit 1 in cells treated with retinoic acid, indicating that MEF2C activity is essential for the expression of at least one neuron-specific gene [10]. Furthermore, the transient expression of neural bHLH proteins, including MASH-1, has been shown to convert monolayer cultures of P19 cells into neurons [20].

The approach of stably overexpressing transcription factors in P19 cells has yielded important information about the function and regulation of these factors [21]. For example, P19 cells overexpressing either GATA-4 [22] or MyoD [23] differentiate into cardiac muscle or skeletal muscle, respectively, when aggregated in the absence of ME_2SO. Using this approach, we have shown recently that MEF2C and Nkx2-5 activate each other's expression and induce cardiomyogenesis in P19 cells [24]. Furthermore, MEF2C and myogenin activate each other's expression and induce skeletal myogenesis [25]. Here we show that MEF2C can induce neurogenesis when overexpressed in P19 cells.

2. Materials and methods

2.1. Tissue culture

Routine culturing of P19 cells and the isolation of P19 cells overexpressing MEF2C, termed P19[MEF2C] cells, have been described previously [24]. Each of the three high expressing P19[MEF2C] cell lines behaved similarly and all experiments reported were performed at least twice with at least two of these cell lines, with similar results.

Differentiation was initiated by plating 5×10^5 cells into 60 mm bacterial dishes in the presence of 0.8% ME_2SO. Cells were cultured as aggregates for 4 days and then plated in tissue culture dishes and harvested for RNA or fixed for immunofluorescence at the time indicated.

2.2. Immunofluorescence

P19 and P19[MEF2C] cells were plated on day 4 of differentiation onto gelatin-coated coverslips. For identifying the neurofilament 68 kDa protein [26], cells were fixed in methanol at −20°C and reacted with antibody as described [23]. For identifying NeuN and MASH-1, cells were fixed for 1 h at room temperature with Lana's fixative (4% paraformaldehyde, 14% v/v saturated picric acid, 125 mM sodium phosphate) followed by preblocking in 1% goat serum, 0.1% NP40 in phosphate-buffered saline (PBS) for 15 min at room temperature. Cells were then incubated overnight at 4°C with 50 μl of the monoclonal antibody supernatants against NeuN, A60 [27], and MASH-1 [19]. After three 5 min washes in PBS, cells were incubated for 30 min

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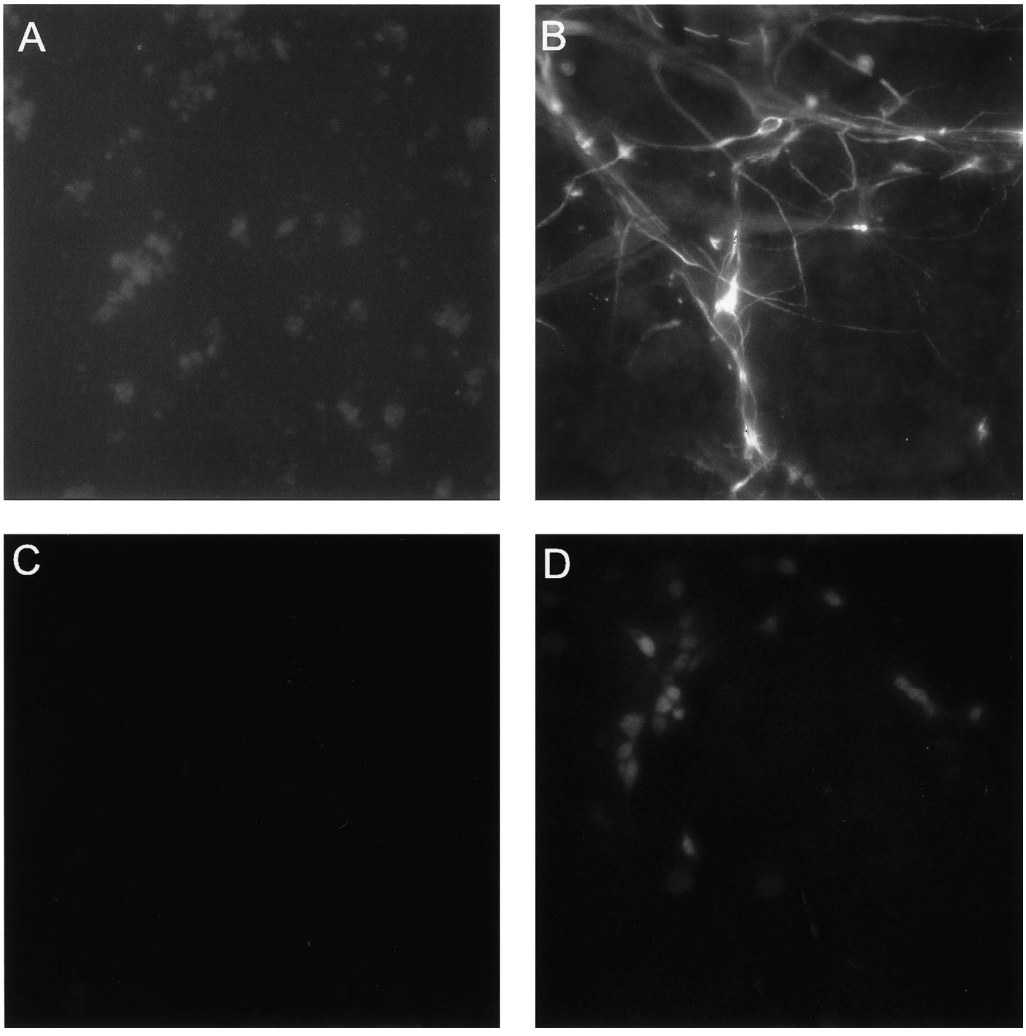

Fig. 1. MEF2C induces the expression of neuron-specific proteins. P19 (A, C) and P19[MEF2C] (B, D) cells were differentiated in the presence of ME_2SO and fixed on day 9. Cells were stained with an anti-neurofilament (NF68) antibody (A, B) [26] or an anti-NeuN antibody (C, D) [27]. Magnification 35×.

in 50 μl of PBS with 1 μl of goat anti-mouse IgG(H+L) Cy3-linked antibody (Jackson Immunoresearch Laboratories, PA). Immunofluorescence was visualized with a Zeiss Axioskop microscope, images were captured with a Sony 3CCD color video camera, processed using Northern Exposure, Adobe Photoshop, and Corel Draw software, and printed with a dye sublimation phaser 450 Tektronic printer.

3. Results and discussion

P19 cells differentiate into neuroectodermal lineages when aggregated with retinoic acid but not when aggregated with ME_2SO or in the absence of retinoic acid [16]. To examine whether MEF2C can activate the expression of endogenous genes in the neuronal pathway, P19[MEF2C] cells were aggregated in the presence and absence of ME_2SO. Immunofluorescence was performed with an anti-neurofilament antibody, NF68 [17,23], and with an anti-NeuN antibody [27]. Neurons were observed in P19[MEF2C] cultures that were aggregated with ME_2SO, as shown by the expression of neurofilament protein (Fig. 1B) and by the nuclear expression of NeuN (Fig. 1D). As expected, control P19 cells did not express neuron-specific proteins when aggregated with ME_2SO (Fig. 1A,C). The neurons appeared to have a similar morphology when compared to neurons induced by retinoic acid [17].

In order to quantify MEF2C-induced neurogenesis, the numbers of neurons were counted in P19 and P19[MEF2C] cultures treated with and without ME_2SO. P19[MEF2C] cells

Table 1
Comparison of the requirements and extent of differentiation for various P19 cell lines

	P19[MEF2C]			P19[MyoD]	P19		
Cell types produced	skeletal	cardiac	neurons	skeletal	skeletal	cardiac	neurons
Drug requirement	DMSO	None	DMSO	None	DMSO	DMSO	retinoic acid
Extent of differentiation (%)	∼5	∼2	5–9	∼30	<5	∼15	∼75
Time course (days)	9	6	6	6	9	6	6
Reference	[25]	[24]	this work	[23]	[17]	[17]	[16]

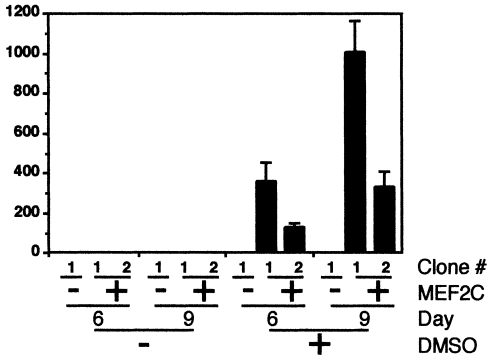

Fig. 2. MEF2C induces neuronal development by day 6 after cellular aggregation in the presence of ME_2SO. The total numbers of cells expressing NeuN were counted in cultures of P19 and P19[MEF2C] cells which had been aggregated in the presence or absence of DMSO and fixed on day 6 or day 9. These numbers were averaged and depicted graphically. Error bars represent S.E.M. ($n = 3$).

differentiated into neurons as early as day 6 in the presence of ME_2SO (Fig. 2) and they represented 5–9% of total cells. No neurons were detected in cultures treated without ME_2SO. In addition to the presence of neurons on day 6, P19[MEF2C] cultures treated with dimethyl sulfoxide (DMSO) also contained ~15% of DMSO-induced cardiac muscle [25], ~5% skeletal muscle precursors [25], ~10% smooth muscle (smooth muscle actin-positive; data not shown), and ~5% endoderm (cytokeratin-positive; data not shown). Cardiac muscle, smooth muscle, and endoderm are present in control cultures treated with DMSO.

We have previously reported that MEF2C induces cardiomyogenesis [24] and skeletal myogenesis [25] in aggregated P19 cells. It seems that the initiation of skeletal myogenesis, neurogenesis, and cardiomyogenesis by MEF2C is an all-or-none event and requires specific aggregation conditions (summarized in Table 1). There was no indication of a mixed phenotype between the three cell types produced. For example, neurofilament protein was not found in cells with a skeletal or cardiac myocyte morphology and myogenin was not found in cells with a neuronal morphology. Therefore, MEF2C activity must be carefully regulated by lineage-restricted factors to ensure that the correct combination of genes is activated in order to define a specific developmental pathway.

Since MASH-1 is known to act cooperatively with MEF2 factors, to be expressed during P19 cell differentiation into neurons [4,7,19], and to convert P19 cells into neurons [20], we examined aggregated P19[MEF2C] cultures for the presence of MASH-1. P19[MEF2C] cells expressed MASH-1 after differentiation in the presence of ME_2SO (Fig. 3A), whereas P19 control-transfected cells did not (Fig. 3B).

We propose a working model for the regulation of MEF2C activity in P19 cells that extends previous models of MEF2C function [2,28]. In this model, different lineage-restricted factors are expressed in a subset of P19 cells during cellular aggregation, with a specific time course and drug requirement. These factors regulate the activity of MEF2C to initiate the expression of tissue-restricted factors, such as MASH-1 in the neuronal development pathway, MyoD and myogenin in the skeletal muscle development pathway [25], and Nkx2-5 and GATA-4 in the cardiac muscle development pathway [24]. Finally, as proposed previously, these tissue-restricted factors may act cooperatively with MEF2C in activating the expression of neuron-specific [4,7], skeletal muscle-specific [29–31], and cardiac muscle-specific genes [32].

The putative lineage-restricted factors may be other transcription factors, kinases, or phosphatases. MEF2C can be regulated by phosphorylation events due to interactions with casein kinase II [33], mitogen-activated protein kinase p38 [34], and Erk5/BMK [35]. These factors are candidates for regulating MEF2C-induced skeletal myogenesis, neurogenesis, and cardiogenesis.

The ability of MEF2C to initiate the development of multiple cell types in differentiating P19 cells is distinct from the regulation of gene expression observed with other transcription factors in P19 cells, such as Nkx2-5 [24], MyoD [23], myogenin [25], and GATA-4 [22]. Nkx2-5, GATA-4, MyoD, and myogenin trigger the development of one cell type, representing a large proportion ($> 10\%$) of the culture, and exhibit no ME_2SO requirement. In comparison, MEF2C induces the development of multiple cell types, representing small percentages ($< 10\%$) of the cells, and requires ME_2SO for myogenesis and neurogenesis. Consequently, it would appear that Nkx2-5, GATA-4, MyoD, and myogenin are regulated by factors present in a large proportion of cells in the culture and are expressed in a ME_2SO-independent fashion during

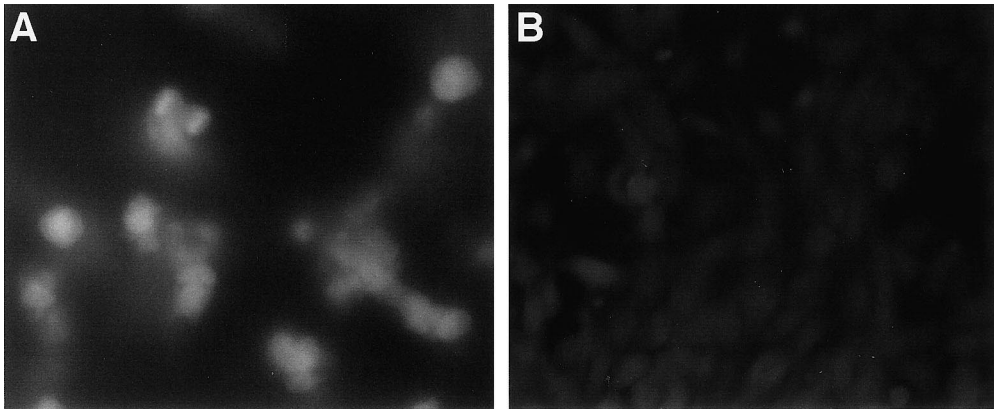

Fig. 3. MEF2C activates the expression of MASH-1. P19[MEF2C] (A) and P19 (B) cells were differentiated in the presence of ME_2SO and fixed on day 9. Cells were stained with an anti-MASH-1 antibody. Magnification 70×.

cellular aggregation. In comparison, factors that regulate MEF2C activity would be present in a small proportion of the cells and their expression would be dependent upon the presence or absence of ME_2SO during the aggregation step.

Since MEF2C is known to bind to neuron-specific promoters, it is logical to assume that MEF2C is functioning in this manner in P19 cells. However, it is possible that the observed neurogenesis is due to an indirect effect of overexpression of MEF2C, without any specific effect. For example, MEF2C could be functioning by a 'squelching' mechanism by binding a cofactor or by derepressing gene expression. Alternatively, MEF2C could be enhancing the spontaneous differentiation of P19 cells.

In summary, MEF2C initiates neurogenesis in P19 cells aggregated with ME_2SO. This finding is consistent with the location of MEF2C during the development of the embryo since MEF2C is expressed in the developing brain [2]. These results are also consistent with the known activities of MEF2C since, in transient transfection experiments, MEF2C activates the promoters of neuronal-specific genes [10]. Our findings extend these observations to show that MEF2C expression can result in the activation of endogenous genes including the neuronal transcription factor MASH-1, and neuron-specific proteins such as NeuN and neurofilament, resulting in neurogenesis. Therefore, the P19 cell system provides a useful tool for examining the ability of transcription factors to initiate cellular differentiation and for subsequent analysis of the mechanism(s) involved. Aggregated P19 cells provide the transfected gene of interest with a heterogeneous population of cells, which in turn provides the appropriate regulatory proteins and/or chromatin structure to allow for the activation of function of the gene of interest.

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