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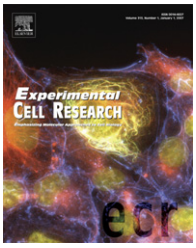

Research Article

The oncogenic transcription factor PAX3-FKHR can convert fibroblasts into contractile myotubes

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ABSTRACT

PAX3-FKHR, the product of a rearrangement of PAX3 with FKHR is the pathogenetic marker for alveolar rhabdomyosarcoma, an aggressive form of childhood cancer. In this work we show that PAX3-FKHR, which is a stronger transcriptional activator relative to PAX3, can lead to two apparently irreconcilable outcomes: transformation and terminal myogenic differentiation. Fibroblasts (10T1/2, NIH3T3, and a newly established murine line named 'Plus') transduced by PAX3-FKHR acquire transformed features such as anchorage independence and loss of contact inhibition and concomitantly undergo various degrees of myogenic conversion depending on the host cells, including, in the case of the Plus line, terminal differentiation into contractile myotubes. This work highlights the potential of PAX3-FKHR to functionally operate as a deregulated Pangene and may have implications with regard to the identity of the precursor cell giving rise to alveolar rhabdomyosarcoma.

Introduction

Pax genes comprise a nine member gene family that encodes a set of transcription factors named for the common DNA binding domain (*Paired Box*) [1]. In the embryo, Pax genes guide development by promoting cell-lineage specification, as well as cell survival, proliferation and migration. When development is complete their expression is usually attenuated. However, in the adult, expression of some of these genes persists in self-renewing stem cells responsible for organ regeneration [2].

Pax3 and Pax7 are closely related members representing a subfamily [1]. During embryogenesis, Pax3 initiates the myogenic program by acting upstream of the muscle regulatory factors (MRFs) MyoD and Myf-5 [3,4]. Splotch mice, where Pax3 is mutated, lack muscles of the limb, tongue and diaphragm. These muscles originate from progenitor cells located at the ventrolateral tip of the dermomyotome. Pax3 is

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essential for their survival and it is also required for their delamination and migration [5–9].

Pax7 is also expressed in somitic muscle precursors, albeit with a slight temporal delay with respect to Pax3 [10]. Pax7 mutants, however, do not display muscle defects during embryogenesis, but rather show postnatally a progressive loss of satellite cells by cell death, leading to a defect of muscle growth and to severe impairment in muscle regeneration [11].

PAX3 and PAX7 are overexpressed in a variety of cancers, among which sarcomas and melanomas [1]. A causal link with a human malignancy has been demonstrated for their translocations with FKHR [t(2;13)(q35;q14) or t(1;13)(p36;q14)], which are found in the vast majority of alveolar rhabdomyosarcoma (ARMS) [1]. The translocations juxtapose the 5'portion of the PAX3 gene on chromosome 2 [t(2;13)] or of the PAX7 gene on chromosome 1 [t(1;13)] with the 3'region of the FKHR gene on chromosome 13 [12]. Fkhr (also known as Foxo1a) is a member of a family of genes coding for proteins that have been linked to diverse roles in embryonic and adult tissues [13]. The chimeric molecules generated by these fusions retain the DNA binding domain of the PAX proteins while acquiring the transactivation domain of FKHR. As a consequence, they gain a more powerful transcriptional activity [14,15]. Although Pax3 and PAX3-FKHR by and large regulate an overlapping set of genes it has been shown that the latter has a relaxed DNA target specificity [16–18]. PAX3-FKHR is capable of transforming both chicken embryo (CEF) and mouse fibroblasts [19,20]. Its tumorigenic activity has been confirmed in vivo in conditional PAX3-FKHR knock-in mice where transcription of the chimeric gene was triggered by Cre in terminally differentiating myofibers. In these animals PAX3-FKHR homozygosity (with accompanying Ink4a/Arf loss of function) resulted in development of alveolar rhabdomyosarcoma [21]. A number of targets potentially responsible for PAX3-FKHR-mediated transformation have been identified, among which Bcl-xl, Skp2 and Met [22–24]. Recently we have provided genetic evidence that absence of a functional Met receptor abrogates PAX3-FKHR-mediated transformation and that downregulation of the Met receptor via RNAi in RMS cell lines results in a pro-apoptotic effect [25].

Interestingly, it has recently emerged that PAX3-FKHR, when expressed at low level transforms NIH3T3 fibroblasts, while when expressed at higher level it inhibits growth [26]. It has also been shown that in NIH3T3 fibroblasts PAX3-FKHR can trigger a myogenic transcription program [27], while in the context of myogenic cells it inhibits differentiation [28].

To further investigate these apparently conflicting effects, we re-examined the ability of PAX3-FKHR to promote transformation and to confer myogenic identity in a panel of fibroblast lines. These included the widely used 10T1/2 and NIH3T3 cells, as well as a newly established murine fibroblast line ('Plus'), immortalized in our laboratory by sequential passaging. We found that fibroblasts transduced by PAX3-FKHR, while acquiring transformed features such as anchorage independence and loss of contact inhibition, underwent various degrees of myogenic conversion depending on the host cells, including, in the case of the Plus line, terminal differentiation into contracting myotubes.

Materials and methods

Cell lines and culture conditions

Plus fibroblasts were derived by standard procedures [29]. Briefly, at E14.5 mothers were sacrificed, the head and the red organs of embryos were removed, then the torso was minced and dispersed in 0.1% trypsin for 45 min at 37 °C with gentle agitation. Cells were passaged by plating 3×10^6 cells in 10 cm dishes every 3 days until immortalization occurred. Plus, NIH3T3 and 10T1/2 murine fibroblasts were cultured in standard DMEM (Invitrogen, USA) supplemented with 20 units/ml penicillin, 100 μg/ml streptomycin and 10% Foetal Bovine Serum (Euroclone). To establish differentiating condition, switch to DMEM containing 2% Horse Serum (Euroclone) was applied.

Growth curves

To measure growth rates, 1500 cells/well were seeded in 96-well plates and MTT assay (Roche) was performed according to manufacturer's instructions. Each time point was determined in triplicate and each experiment was repeated three times.

Plasmids and retroviral-mediated gene transfer

To produce ecotropic virus Phoenix cells (5×10^6) were plated in 10 cm dishes, incubated for 24 h and then transfected by calcium-phosphate precipitation with 15 μg of pBabe-Pax3-Fkhr, pBabe-MyoD (Addgene, USA), pBabe-Egfp or pBabe-RasV12 for 15 h at 37 °C. Virus containing supernatant was collected twice at 24 h and 48 h after transfection and 0.45 μm filtered. Viruses titer was established by determining the number of puromycin resistant colonies in a clonogenic assays on NIH3T3 at limiting dilutions. For infections, target cells were plated at 5×10^4 cells in 6-well plates, incubated with virus-containing medium, and supplemented with 8 μg/ml polybrene (Sigma). After 4 h, 1 ml of fresh medium was added to the infected cells. After reaching sub-confluent conditions, cells were trypsinized and re-plated in 10 cm dishes where puromycin selection was applied. The appropriate concentration of puromycin was established for each cell line by survival curves. The following concentrations were used: for 10T1/2 1.5 μg/ml, for NIH3T3 1.5 μg/ml and 2 μg/ml for the Plus line. Selection was applied for four days and puromycin-containing medium was replaced every 2 days. For each experiment at least two independent pools for each genotype were generated.

Focus forming and soft agar assay

To evidence transforming colonies over the untrasformed populations, PAX3-FKHR and GFP-transduced cell lines were plated in 10 cm dishes and let grow to confluence in 10% medium. At confluence serum concentration was lowered to 2% FCS and dishes were observed for 15 additional days. Anchorage-independent growth was determined in 0.4% type VII Agarose (Sigma) with a 0.8% agarose underlay in six wells plates. Fifteen thousands cells per well were plated in a 10%