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Oral mucosal progenitor cell clones resist *in vitro* myogenic differentiation

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ABSTRACT

Progenitor cells derived from the oral mucosa lamina propria (OMLP-PCs) demonstrate an ability to differentiate into tissue lineages removed from their anatomical origin. This clonally derived population of neural-crest cells have demonstrated potential to differentiate along mesenchymal and neuronal cell lineages.

Objective: Significant efforts are being made to generate functioning muscle constructs for use in research and clinical tissue engineering. In this study we aimed to determine the myogenic properties of clonal populations of expanded OMLP-PCs.

Design: PCs were subject to several *in vitro* culture conditions in an attempt to drive myogenic conversion. Methodologies include use of demethylation gene-modifying reagents, mechanical conditioning of tissue culture substrates, tuneable polyacrylamide gels and a 3-dimensional construct as well as published myogenic media compositions. PCR and immunostaining for the muscle cell markers Desmin and MyoD1 were used to assess muscle differentiation.

Results: The clones tested did not intrinsically express myogenic lineage markers. Despite use of two and 3-dimensional pre-published *in vitro* culture protocols OMLP clones could not be differentiated down a myogenic lineage.

Conclusions: Within the confines of these experimental parameters it was not possible to generate identifiable muscle using the clonal populations. When reviewing the previously successful reports of myogenic conversion, cells utilised have either been derived from tissues that are already 'primed' with the requisite myogenic genetic potential or have undergone specific genetic reprogramming to enhance the myogenic conversion rate. This, along with as yet unidentified stromal interplay, may therefore be required for positive myogenic differentiation to be realised.

1. Introduction

Oral mucosal lamina propria progenitor cells (OMLP-PCs) are a distinct progenitor cell (PC) population that has recently been reported (Davies et al., 2010). These oral PCs are of neural crest origin, express many putative stem cell markers and are potently immunosuppressive (Davies, Lönnies, & Locke, 2012). Furthermore, these OMLP-PCs, like bone marrow-derived mesenchymal stem cells (BM-MSCs), adipose-derived stem cells (ADSCs) and gingiva-derived progenitor cells can be driven down osteoblastic, adipogenic, chondrogenic and neuronal lineages (Davies et al., 2010; Fournier, Ferre, & Couty, 2010; Tang, Li, Xie, & Jin, 2011; Zhang, Shi, & Liu, 2009).

Myogenesis is characterized by a period of myoblast proliferation, followed by the expression of muscle-specific proteins and then fusion to form multinucleated myotubes. It is a developmental cascade that principally involves the regulatory MYOD gene family controlling the transition of multipotential mesodermal stem/PCs into the myogenic lineage (Weintraub et al., 1991).

Both human and animal studies have sought to reproduce the muscle phenotype from embryologically distinct tissue types. Early studies by Guan, Rohwedel, & Wobus (1999) developed protocols for cultivating embryonic stem cells (ESCs) through 'hanging drop/embryoid body' stages prior to their differentiation

Abbreviations: ADSC, adipose derived stem cells; BM-MSC, bone marrow-derived mesenchymal stem cells; ESC, embryonic stem cell; OMLPPCs, oral mucosal lamina propria progenitor cells; 5Aza, 5-azacitidine; MYOD1, myogenic differentiation 1 gene.

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to skeletal muscle. Later animal studies by Zheng, Wang, & Karandikar (2006) demonstrated that human ESC-derived precursors could incorporate into host muscle efficiently and become part of regenerating muscle fibres. Adult MSCs, most commonly BM-MSCs, have been reported to form skeletal muscle under defined *in vitro* conditions (Pittenger et al., 1999; Reyes et al., 2001). *In vivo* studies have demonstrated that BM-MSCs differentiate into myofibres and contribute to the replenishment of the muscle satellite cell compartment and thus to muscle regeneration (LaBarge and Blau, 2002).

Recent work has reported successful derivation of myogenic cell-types with *in vitro* manipulation of cells from a variety of tissue sources. Gang et al. (2004) demonstrated that umbilical cord blood-derived MSCs, when incubated in pro-myogenic conditions expressed myogenic markers in accordance with the myogenic differentiation pattern. Murine ADSCs were reported to convert to the myogenic phenotype when in co-culture with primary myoblasts (Mizuno et al., 2002) and enhanced conversion rates were realised when utilising stiffened 'muscle-mimicking' extracellular matrices that more closely imitate the *in vivo* situation (Choi, Vincent, Lee, Dobke, & Engler, 2011).

Further investigations have utilised more invasive methods of cellular and genetic manipulation in order to achieve differentiation. Work by Taylor and Jones (1979) (replicated by Wakitani, Saito, & Caplan, 1995, on use of the DNA methylation inhibitor 5-azacitidine (5-Aza), reported that subpopulations of mouse and rat cell lines undergo transformation to the myotube muscle phenotype when treated. Nakatsuka et al. (2010) demonstrated 5-Aza demethylation resulted in skeletal muscle differentiation by mouse dental pulp stem cells. Authors have also demonstrated similar *in vitro* muscle conversion on utilisation of the ß-galactoside-binding lectin Galectin-1. Goldring, Jones, Sewry, & Watt (2002) reported that human dermal fibroblasts expressed the myogenic marker, Desmin, and Chan et al. (2006) presented data that suggest human foetal-MSCs readily undergo muscle differentiation in response to Galectin-1. The purpose of this study was specifically not to undertake such invasive molecular manipulation techniques but rather to investigate any innate myogenic potential in order to be able to exploit this via pre-published myogenic maintenance/differentiation tissue culture protocols which may lend themselves towards future translatability.

In addition, there are reports that suggest conversion rates for functioning muscle may be exaggerated. Di Castro, Bonci, Musumeci, & Grassi (2008) cited conflicting evidence of myogenic differentiation from human haematopoietic-derived stem cells. They demonstrated that human peripheral blood-derived stem cells labelled with Green Fluorescent Protein (GFP) and co-cultured with mouse C2C12 myoblast cell line demonstrated transference of GFP markers to muscle cells but without the signs of myogenic stem cell differentiation. It appears that the method of stem cell and muscle fibre fusion is not clear and it is debated as to whether this represents true cellular fusion, transdifferentiation or the effects of circulating tissue-specific precursors.

Given the multipotent nature of OMLP-PCs and the ease with which these can be isolated from individuals (via a simple non-scarring buccal mucosal biopsy) the aim of this investigation was to determine their potential to form identifiable muscle subunits when subjected to previously published myogenic tissue culture approaches.

2. Materials and methods

2.1. Isolation and expansion of the OMLP-PCs

Unless otherwise stated all laboratory reagents were obtained from Invitrogen, UK. Normal, disease-free buccal mucosa biopsies were obtained from written consented patients undergoing dental procedures at the School of Dentistry, Cardiff University. Local ethical committee approval had been previously obtained (South East Wales Research Ethics Committee; 09/WSE03/18). OMLP PCs were obtained by differential adhesion to fibronectin as previously reported (Davies et al., 2010). In brief, biopsy tissue was separated into epithelial and lamina propria component. LP tissue was then disaggregated and PCs were separated by subsequent differential adhesion to fibronectin. Briefly, OMLP single-cell suspensions in basal culture media (Dulbecco's modified Eagle's medium [DMEM]

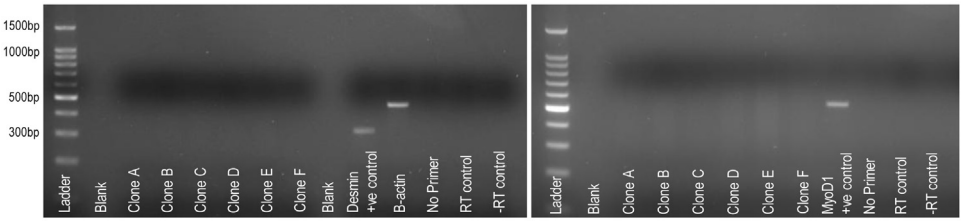

Fig. 1. Myogenic polymerase chain reaction.
RT-PCR analysis demonstrating positive identification of myogenic markers Desmin and MYOD1 within positive control C2C12 muscle cell line but a lack of expression in the OMLP-PC clones.

Table 1
Myogenic polymerase chain reaction results. Undifferentiated OMLP-PCs did not express myogenic markers nor could they be stimulated to do so by alteration of the culture conditions. The positive control C2C12 muscle cell line demonstrated expression of myogenic markers.

PCR Product		OMLP-PC Clones (combined cell data)				
		Untreated Cells	Myogenic Media Exposure	5-Aza Exposure	+ve Control	-ve Control
Muscle	Desmin (335 bp)	–	–	–	+C2C12 myoblast RNA	$-H_2O$
	MyoD1 (515 bp)	–	–	–	+C2C12 myoblast RNA	$-H_2O$
Control	Actin (480 bp)	+	+	+	+total Human Skeletal Muscle RNA	$-H_2O$