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The generation of olfactory epithelial neurospheres *in vitro* predicts engraftment capacity following transplantation *in vivo*

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ABSTRACT

The stem and progenitor cells of the olfactory epithelium maintain the tissue throughout life and effectuate epithelial reconstitution after injury. We have utilized free-floating olfactory neurosphere cultures to study factors influencing proliferation, differentiation, and transplantation potency of sphere-grown cells as a first step toward using them for therapeutic purposes. Olfactory neurospheres form best and expand most when grown from neonatal epithelium, although methyl bromide-injured or normal adult material is weakly spherogenic. The spheres contain the full range of epithelial cell types as marked by cytokeratins, neuron-specific antigens, E-cadherin, Sox2, and Sox9. Globose basal cells are also prominent constituents. Medium conditioned by growth of phorbol ester-stimulated, immortalized lamina propria-derived cells (LP_{Imm}) significantly increases the percentage of *Neurog1eGFP*(+) progenitors and immature neurons in spheres. Sphere-forming capacity resides within selected populations; FACS-purified, *Neurog1eGFP*(+) cells were poorly spherogenic, while preparations from *ΔSox2eGFP* transgenic mice that are enriched for Sox2(+) basal cells formed spheres very efficiently. Finally, we compared the potency following transplantation of cells grown in spheres *vs.* cells derived from adherent cultures. The sphere-derived cells engrafted and produced colonies with multiple cell types that incorporated into and resembled host epithelium; cells from adherent cultures did not. Furthermore, cells from spheres grown in conditioned media from the phorbol ester-activated LP_{Imm} line gave rise to significantly more neurons after transplantation as compared with control. The current findings demonstrate that sphere formation serves as a biomarker for engraftment capacity and multipotency of olfactory progenitors, which are requirements for their eventual translational use.

Introduction

The olfactory epithelium (OE), the peripheral apparatus for the sense of smell, is renowned for its ability to replace sensory neurons piecemeal during normal life or rapidly and in bulk, along with all the other epithelial cell types, following injury. Regenerative capacity depends upon the life-long retention and activation by injury of neurocompetent stem cells, of which there appear to be multiple kinds. Two types of basal cells – horizontal basal cells (HBCs) and globose basal cells (GBCs) – function as multipotent progenitor and stem cells under a variety of conditions including normal maintenance and regeneration of the OE (Carter et al., 2004; Chen et al., 2004; Huard et al., 1998; Iwai et al., 2008; Leung et al., 2007), and are easily harvested by endoscopically-guided biopsy in the living patient. However, the GBC population is functionally heterogeneous and includes different subtypes that can be discriminated on the basis of transcription factor expression; only a subset of GBCs – most likely the ones that express Sox2 and Pax6 but not the proneurogenic factors Mash1 or Neurog1 – exhibits multipotency (Chen et al., 2004; Guo et al., 2010).

Despite the apparent redundancy provided by multiple types of multipotent progenitors, very severe injury to the epithelium causes the loss of neurogenic potency, via the elimination of both kinds of basal cells, and results in replacement of olfactory by respiratory or stratified squamous epithelium (Holbrook et al., 2005; Naessen, 1971; Nakashima et al., 1984; Schwob et al., 1994). In humans, stem cell depletion/destruction/dysfunction appears to be responsible for many cases of hyposmia and dysosmia, and, at least in part, for the decline in sensory function as a consequence of "normal" aging, which afflicts at

Abbreviations: CK, cytokeratin (used interchangeably with keratin); CM, conditioned media; FACS, fluorescence activated cell sorting; FGF, fibroblast growth factor; GBC, globose basal cell; HBC, horizontal basal cell; IHC, immunohistochemistry; LP, lamina propria; MeBr, methyl bromide; NCAM, neural cell adhesion molecule; OE, olfactory epithelium; OM, olfactory mucosa; ONS, olfactory neurosphere; OSN, olfactory sensory neuron; PECAM, platelet endothelial cell adhesion molecule; PMA, phorbol ester (phorbol-12-myristate-13-acetate, a.k.a. TPA); PND, post-natal day; Sus, sustentacular cell.

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least 25% of the population over 65 years old at a significant cost to overall health (Murphy, 2008).

Since the stem and progenitor cells of the olfactory epithelium are accessible for nearly risk-free harvest yet also susceptible to damage, several potential translational applications for them are apparent, including the use of autologous olfactory stem and progenitor cells to repair damage elsewhere in the nervous system and, perhaps as a shorter-term goal, replacement of those olfactory stem and progenitor cells lost *in vivo* in order to alleviate the consequent dysfunction. Study of the olfactory neurocompetent stem and progenitor cells is also likely to inform our understanding of the biology of tissue stem cells in general because of the numerous technical advantages of the system.

Of the many steps along the way to using them for repair purposes or understanding the cellular events and signals regulating their behavior, one of the most problematic, in the case of the olfactory stem and progenitor cells, has been the shortcomings of conventional tissue culture models. OE cells in adherent, essentially 2-D cultures no longer look or behave like their *in vivo* counterparts. Nonetheless, cultures of this sort have provided several insights into the regulation of olfactory progenitor cells (*e.g.*, by BMPs and follistatin) and have been used to highlight a rare capacity for generating large colonies *in vitro* (Carter et al., 2004; DeHamer et al., 1994; Gordon et al., 1995; Mumm et al., 1996; Shou et al., 2000; Shou et al., 1999). However, it is highly significant that epithelial cells grown in 2-D are incapable of engrafting properly following intranasal transplantation and do not participate in the repair of the OE, in contrast with multipotent olfactory progenitor cells that are isolated directly from the epithelium and transplanted immediately after harvest (Chen et al., 2004; Goldstein et al., 1998; Jang et al., 2008). Indeed, studies of various stem and progenitor systems (hematopoietic, epidermal, and mammary) have used transplantation as an exacting assay of cellular functional capacity (Krause et al., 2001; Purton and Scadden, 2007; Shackleton et al., 2006).

Accordingly, the development and characterization of a culture system that retains the potency of olfactory stem and progenitor cells for reintroduction *in vivo* would have multiple benefits. As a model that retains important similarities to the cells *in situ*, it could be used as a more rapid and more valid means of identifying and characterizing the nature of the signals that regulate progenitor cell behavior. Moreover, the effect of such signals can be assayed with reference to the consequences on engraftment and repair. Likewise, the culture model should be informative as an assay for the differentiative capacity of specific progenitor cell types *in vitro* and *in vivo* after transplantation and engraftment. Ultimately, to be maximally useful, a culture system has to support the expansion of the population of stem and progenitor cells to accumulate sufficient numbers for translational purposes.

We and others have begun to study culture models of the OE that maintain what is increasingly appreciated as a critical aspect of stem and progenitor biology: complex three-dimensional interactions of cultured cells designed to mimic *in vivo* architecture. Of course, data from other neurogenic and epithelial tissues have shown that free-floating spheroids that form from isolated cells (*e.g.*, neurospheres from the subventricular zone and mammospheres from the mammary epithelium) illuminate various characteristics of stem and progenitor cells (Dontu et al., 2003; Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Woodward et al., 2005) and can engraft following transplantation. We have shown that 3-D epithelial-like structures, which we term spheres, form in air–media interface cultures from olfactory progenitor cells harvested following damage to the adult OE (Jang et al., 2008). The OE-derived spheres recapitulate numerous features of epithelial regeneration *in vivo*, and also preserve differentiative potency, allowing for engraftment and differentiation into multiple cells types following transplantation, in contrast to adherent cultures (Jang et al., 2008). Although olfactory epithelial spheres from the air-interface cultures will passage, they cannot be used for large-scale expansion or a throughput assessment of which factor(s) support passaging of olfactory stem cells. More recently, free-floating cultures has been adapted to the olfactory system, and recent data suggest that cells present in these olfactory neurospheres (ONSs) express markers of various progenitor cells and may have multi-lineage differentiation capacity (Barraud et al., 2007; Pixley et al., 1994; Tome et al., 2009) (Mona Lisa Khan, 2002, personal communication). These studies demonstrated that ONSs can be generated from OE harvested at different developmental time-points, but the work did not explore the features of the spheres with regard to a number of key issues. These include, of course, the ability of the floating sphere-derived cells to engraft following transplantation. Another has to do with the effect on the ONSs of signals that emerge from the lamina propria (LP), a kind of stroma that is deep to the OE and is capable of driving sphere formation in the air–media interface cultures; in other epithelia, such as breast, stromal cells analogous to the LP cells release molecular signals that drive complex tissue-like assembly *in vitro*. In particular, activation of LP-derived cells has the potential to reveal aspects of the functional regulation of olfactory stem and progenitor cells by modeling the signaling from the lamina propria during development or after injury, in a controlled *in vitro* environment. To that end, phorbol 12-myristate 13-acetate (PMA) is a potent activator of PKC and has been shown to stimulate the release of numerous growth factors or activate growth factor receptor pathways for a variety of cell types, including fibroblasts (Amos et al., 2005; Montero et al., 2000).

We present here the results of a set of foundational studies designed to investigate the potential usefulness of ONSs as a model for the complexities of assembling/reconstituting the OE and as an assay for the influence on the OE of specific growth factors and of a more complex, but biologically relevant melange of molecules that derives from the cells of the LP. We describe experiments that relate the formation of ONSs to the proliferative status of the epithelium and identify the cells responsible for this proliferation and ONS-forming capacity. We investigate the effects of conditioned media from LP-derived cells on cell fate within ONSs. We also describe experiments that seek to correlate findings in the ONS system with outcome in a transplantation assay as an especially stringent assay for the biological relevance of the *in vitro* model.

Material and methods

Animals

B6.129F1 mice obtained from the Jackson Laboratory were used as the source for normal and lesioned cells put in sphere culture and as transplantation hosts. *ΔSox2eGFP* mice are an ES-cell knock-in line and have been described previously (Ellis et al., 2004). These mice produce GFP in all Sox2-expressing cells of the olfactory epithelium — GBCs, HBCs, and sus cells. *Neurog1eGFP* is a BAC transgenic line generated by the GENSAT project (Gong et al., 2003). Constitutive GFP-expressing mice (C57BL/6-Tg(CAG-EGFP)1Osb/S) have been utilized previously for transplantation assays and have been described (Chen et al., 2004; Okabe et al., 1997). For all cases involving GFP reporter mice, heterozygotes were used exclusively. All animals were housed in a heat- and humidity-controlled, AALAC-accredited vivarium operating under a 12:12-hour light–dark cycle. All protocols for the use of vertebrate animals were approved by the Committee for the Humane Use of Animals at Tufts University School of Medicine, where the animals were housed and the experiments were conducted.

Isolation of cells for olfactory neurosphere (ONS) cultures

Cells isolated from neonatal, normal adult, and methyl bromide-lesioned olfactory epithelia were dissociated according to a standard