Editorial

Stem Cell INVESTIGATION

Harnessing TGF-β and BMP signaling for expansion of p63-positive epithelial stem cells

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p63-expressing stem cells characterize many epithelial cell-lined structures including the epidermis, thymus, mammary gland, lung and prostate. Within these compartments, the p63-postive (p63+) cells proliferate and differentiate to generate a p63-negative population of specialized epithelial cells, while maintaining a basal population of stem cells (1). Research in regional stem cells of epithelial cell origin in human disease, such as breast cancer and genetic lung diseases, has been intensified by recent therapeutic successes and the possibility to personalize therapy by testing individual tissue samples. In a recent report, Mou *et al.* describe a facile method using inhibitors of TGF-β and BMP signaling to expand p63+ cells from multiple different tissues, with the capacity to induce *in vitro* proliferation of even single cells, thus providing a new tool to potentially speed the discovery of new treatments (2).

P63+ stem cell function

Of the two major p63 isoforms encoded by *TP63*, ΔNp63, lacking the N-terminal trans-activating domain, regulates a distinct program of gene expression for stem cell function. This population plays an essential role in development and during homeostasis provides slow-cycling stem cells for self-renewal (transit amplifying cells). Although not well characterized in all tissues, several subpopulations of p63-labeled cells play a role; some p63+ cells are reserved for self-renewal while others undergo differentiation. In the airways, following severe injury such as influenza pneumonia, a subset of p63+ cells rapidly proliferate as first responders for barrier function to cover the basement membrane, while other p63+ cells participate in re-differentiation of the cell types unique to the epithelial structure (3,4). Genetic deficiency of p63 in mice results in a failure of self-renewal, marked defects in the production of cells composing the layers of skin, and subsequent interruption of the epidermal barrier function leading to death (5). TAp63 lacks the tumor suppressor activity of p53, but p63+ cells may also be inappropriately expanded in carcinomas.

Regulation of p63 expansion

Requirements for *in vivo* expansion and maintenance of p63+ cells have been identified in mouse models but not well studied in human diseases. P63+ cells are positioned on extracellular matrix of the basement membrane where underlying mesenchymal fibroblasts can provide instruction through growth and regulatory factors. Transcriptional targets of Δp63 include bone morphogenetic protein (BMP) and WNT/β-catenin, as well as both the Notch receptor and its ligands (1). In the airway epithelium, Notch activation in p63+ cells leads to specialized cell differentiation, and downregulation of p63 as the cells differentiate (6).

BMP signaling pathway

The BMP cytokines are members of the TGF-β superfamily and key factors required for embryonic and pluripotent stem cell development. In normal and malignant mammary epithelial cells, ΔNp63α-mediated activation of BMP7 signaling governs stem cell activity and plasticity (7). In the developing lung, BMP4 is required for branching morphogenesis (8). Tadokoro *et al.* recently showed that the

proliferation of p63+ adult lung tracheospheres cultured on matrigel was inhibited by BMP4, while enhanced by treatment with BMP signaling pathway inhibitors that suppress the phosphorylation of SMADs 1/5/8 (9). BMP4 activation also increases differentiation in esophageal epithelial basal cells (10). Studies of intestine and other epithelial cell structures have shown that BMP signaling through phospho-SMAD 1/5/8 is a negative regulator of stem cell proliferation (11).

The TGF-β signaling pathway

Another member of the TGF superfamily involved with epithelial cell differentiation is TGF-β. In the lung TGF-β function has primarily been linked to the process of epithelial-to-mesenchymal transition and fibrosis, however in other organs TGF-β plays a tissue-context dependent role in stem cell proliferation and differentiation (12,13). TGF-β activation signals to phosphorylate the SMAD2/3 complex, facilitating interaction with SMAD4, and results in nuclear localization of phospho-SMAD complex to activate a transcriptional program. McQualter *et al.* demonstrated that co-culture of airway epithelial cell spheroids in the presence of mesenchymal feeder cells resulted in reduced proliferation following the treatment with recombinant TGF-β, and likewise BMP (14). However, these findings did not demonstrate a clear role for these pathways in airway epithelial cell stemness *versus* differentiation. In the intestinal tract, TGF-β drives intestinal epithelial cell differentiation and halts proliferation. Activation of the TGF-β pathway resulted in stem cell differentiation, whereas inhibition of TGF-β by pharmacologic means induced continued proliferation of the epithelial stem cells (15,16). Still, many questions remain about how TGF-β signaling affects p63+ stem cells.

p63+ cell expansion *in vitro*

Ultimately, the ability to culture stem cells is dependent on *ex vivo* reproduction of the *in vivo* stem cell niche. In some tissues a niche may be transient in the case of development or if there is a need to expand stem cells following injury. In other tissue compartments, stem cell expansion is continuous, most notably for the maintenance of red and white blood cell lineages in the bone marrow and epithelial cells in the intestine. Creating an artificial environment that supports stem cell growth has been achieved successfully in the hematopoietic and gastrointestinal systems by the study of their developmental programs. Use of a series of specialized media that contain growth factors originally identified during specific phases of development, activates programs that enable cells to undergo continuous proliferation. In organ systems that undergo constant renewal, or at least a high rate of turnover of the epithelium (hematopoietic and gastrointestinal), studies of development can be very informative. However, in other organs systems that have a slow rate of turnover (rapidly increased during injury) such as the liver and lung, developmental studies may be less informative for the generation of adult stem cell cultures.

Continuous expansion of stem cell populations *in vitro* has been successfully accomplished for some tissue types but remains a major goal for others. Culture systems for short-term expansion were based on recipes originally determined from known developmental cell requirements gleaned from knock out mice and embryonic studies. These have resulted in a limited number of cell doublings and passage numbers. For example, in the human proximal airways, standard culture methods of primary epithelial cells typically achieve 5 or 6 passages before cell crisis, likewise in the mouse 2–3 passages are possible. A marked improvement in the ability to expand epithelial stem cells from many tissue sources has been achieved by using feeder cells from the 3T3-J2 fibroblast cell line (or conditioned media) in combination with the Rho activated kinase (ROCK) inhibitor (Y27632). This approach is based on the pioneering work by Richard Schlegel of Georgetown University and expands cells through conditional reprogramming (17,18). Using this method, multiple publications have demonstrated expansion of epithelial cells from diverse tissue sources such as airway, cervix, breast and ear hair cells (19,20). One potential downside of this approach is that differentiation may be limited at later passages. How this protocol alters key signaling pathways to achieve p63+ stem cell expansion has also not yet been identified. Rather than use adult epithelial stem cells, another approach has been to expand large number of iPS cells. These technically demanding protocols are still evolving and may yet hold promise, but currently have been stymied by a limited capacity to differentiate stem cells to all highly specialized epithelial cell types.

Dual SMAD inhibition for p63+ cell expansion *in vitro*

Leveraging observations related to the roles of TGF-β and

BMP as essential regulators for stem cell expansion and differentiation, Jay Rajagopal's laboratory recently reported dramatic successes for *in vitro* expansion of p63+ cells using inhibition of these growth factors (2). The approach was also based on spying a shift in SMAD 2/3 and 1/5/8 phosphorylation as tracheal airway epithelial cells move from undifferentiated regional stem cells expressing the basal cell marker p63 (and Krt5), to well-differentiated cells. This was observed both in humans and mice, and both *in vitro* and *in vivo*. The authors then depleted the TGF-β and BMP pathways genetically and by using small molecule inhibitors showed that these pathways were critical for halting stem cell expansion and promoting differentiation. The importance of this pathway was also demonstrated in p63+ cells isolated from all three germ layers of epithelium (endoderm, ectoderm, and mesoderm), albeit with differing levels of success. However, this does suggest that the role of SMAD signaling is generalizable across epithelial cell types.

In particular, the authors focused on the tracheal epithelial basal cells known to be Krt5+p63+. Using a system of dual BMP and TGF-β chemical inhibition the authors clearly show a dramatic improvement in long-term stem cell expansion (up to passage 20–25) creating billions of cells from a small sample biopsy without the need for feeder cells. These cells retained the expression of stem cell markers over time and also the ability to differentiate into mature cells at air-liquid interface. Eventually the cells ceased to expand, which was attributed to telomere shortening and cell senescence, independent of SMAD signaling. This suggests that airway stem cells, unlike some other organs systems, have an intrinsically limited lifespan and capacity for expansion. The implication is that the much shorter passage numbers that were traditionally achievable with standard culture techniques over the past 2 decades was due to SMAD-mediated cell differentiation.

There are several advantages to expansion of stem cell populations using this method. Simple methods to obtain large numbers of purified stem cells is an attractive aspect. Separating stem cells from feeder cells can be achieved with some technical maneuvers, however feeder cell contamination is a realistic concern. The method described by Mou *et al.* avoids this issue. The method is also powerful enough to expand small numbers of basal epithelial cells isolated from human bronchoalveolar lavage samples from subjects with cystic fibrosis, making it a powerful tool for expanding cells for therapeutic studies of individuals with rare genotypes without a biopsy. It could also be applied to the expansion of cells from routine small biopsy samples. Mou *et al.* also reported that their system of SMAD inhibition is capable of expanding single cells. This may make it possible to use gene editing technology to generate pure clones.

The new findings by Mou *et al.* demonstrate the critical role of phospho-SMAD in balancing epithelial cell differentiation with stem cell proliferation. By manipulating the two major SMAD pathways, the authors have successfully developed a new technique that allows the long-term expansion of p63+ epithelial basal cells from diverse organs. The method is simple enough to allow immediate adoption by many laboratories for a number of applications that have been previously difficult to achieve.

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