Embryonic Stem Cells as a Source of Pulmonary Epithelium *In Vitro* and *In Vivo*

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Embryonic stem cells (ESCs) derived from the preimplantation blastocyst are pluripotent and capable of indefinite expansion *in vitro*. As such, they present a cell source to derive a potentially inexhaustible supply of pulmonary cells and tissue. ESC-derived pulmonary epithelium could be used for *in vitro* cell or tissue models or, in the future, implanted into the damaged or diseased lung to effect repair. Efforts to date have largely focused on obtaining distal lung epithelial phenotypes from ESCs, notably alveolar epithelium. Several disparate methods have been developed to enhance differentiation of ESCs into pulmonary epithelial lineages; these are broadly based on recapitulating developmental signaling events, mimicking the physical environment, or forcibly reprogramming the ESC nucleus. Early findings of our preclinical experiments implanting differentiated ESCs into the injured lung are also described here. Future efforts will focus on maximizing ESC differentiation efficiency and yield of the target phenotype, as well as characterizing the function of derived cells *in vivo* and *in vitro*.

Keywords: embryonic stem cells; lung epithelium; differentiation; endoderm

Stem cells are defined by two minimal criteria: the capacity for long-term self-renewal without senescence and the ability to differentiate into one or more specialized somatic cell types given the appropriate stimuli. In the postnatal organism, stem cells deposited in numerous niches throughout the body play essential roles in tissue growth, maintenance, and repair. Most organs have been found to contain one or more pools of tissue-committed stem cells, which act to renew local cell populations. For example, epithelial stem cells located within the respiratory niche include the basal cells of the proximal airways (1), a subset of Clara cells in the distal airways (2), bronchioalveolar stem cells located at bronchiolar-alveolar duct junctions (3, 4), and a subset of type II pneumocytes in the alveolar epithelium (5). Furthermore, evidence suggests that bone marrow-derived stem cells can be recruited into many tissues, including the lung, under particular injury conditions, and this may represent an additional, bodywide repair pathway (6–10).

In contrast, embryonic stem cells (ESCs) are an *in vitro* phenomenon, derived by dissection and culture of the inner cell mass of the preimplantation blastocyst (5–8 d postconception for human embryos). During normal embryonic development, the inner cell mass exists only transiently in the undifferentiated, pluripotent state until gastrulation forms the three embryonic

germ layers and initiates somatic differentiation. Thus, the inner cell mass does not display a capacity for long-term self-renewal *in vivo*. *In vitro*, however, specialized culture conditions have been identified that prevent onward development of the inner cell mass such that the cells can be extensively expanded without loss of pluripotency. It is now well established that both murine and human ESC lines can be cultured continuously in the undifferentiated state for many months without the loss of differentiation capability, as demonstrated by both *in vivo* and *in vitro* differentiation assays. ESCs therefore represent a potentially inexhaustible source of any somatic cell type.

HISTORY OF ESCs

The majority of culture techniques used for ESCs are based on those originally developed for embryonal carcinoma (EC) cell lines, the first pluripotent cells to be established in culture. These are derived from the undifferentiated compartment of germ cell tumors and can be expanded continuously in culture as well as induced to differentiate into derivatives of neuroectoderm, endoderm, and mesoderm, similar to ESCs (11). EC cell lines have proven a useful model system to study cellular differentiation in the laboratory; however, they are of a malignant nature and therefore not phenotypically normal. Murine ESCs were first isolated in 1981 and propagated as undifferentiated colonies on a feeder layer of murine embryonic fibroblasts (12, 13). Later, leukemia inhibitory factor (LIF) was identified as a specific differentiation-inhibiting cytokine that could replace the fibroblast feeder layer in the maintenance of pluripotency (14). The establishment of murine ESC lines had an enormous impact on many fields of research. Perhaps the most profound was the ability to manipulate the early mouse embryo and produce genetically modified mice, a technology for which Martin Evans was jointly awarded the 2007 Nobel Prize in Medicine along with Oliver Smithies and Mario Capecchi. Furthermore, murine ESCs continue to provide an important cellular model to dissect the fundamental mechanisms underlying pluripotency and cell fate specification.

The establishment of human ESC lines was finally achieved in 1998 (15) using similar, but not identical, methods to the murine lines. Human ESCs exhibit several differences from murine ESCs in both culture requirements and phenotype, and recent evidence indicates that they represent a subtly different developmental stage (16). Nevertheless, 10 years of research have conclusively demonstrated that they possess the same essential characteristics of indefinite self-renewal and pluripotency. For more details on the derivation and maintenance of ESC lines, see the comprehensive reviews by Andrews (11), Bodnar and colleagues (17), and Ohtsuka and Dalton (18).

ESCs IN MEDICINE

Effecting tissue repair or replacement using cell or tissue constructs derived from stem cells would revolutionize the field

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of medicine. The ability to produce any cell or tissue type virtually “to order” in the laboratory offers the opportunity to treat an enormous array of end-stage diseases as well as to regenerate aging tissues and heal serious injuries. Currently, stem cell–based therapies in clinical trials are almost exclusively based on autologous adult stem cell populations due to immunocompatibility and the perception of comparative safety. Major hurdles, primarily the potential immunogenicity and risk of tumor formation, stand between the transition of ESC research from bench to bedside. However, the widespread use of adult stem cell populations in clinical medicine is hampered by their low frequency in tissue, insufficient availability of primary tissue, difficulties in isolation, restricted lineage potential, and poor expansion in cell culture. In the future, assuming that the safety issues can be addressed, ESCs may provide an alternative, more practical cell source.

In the shorter term, ESC technology is likely to be exploited for the development of *in vitro* cell and tissue models for drug development, for safety testing, and as models for basic research. In the European Union, this line of research may be particularly timely; the Seventh Amendment to the EU Cosmetic Directive 76/768/EEC will outlaw all animal testing of cosmetic and household products and ingredients as of 2013 despite the lack of validated *in vitro* models to replace traditional *in vivo* toxicology tests.

Respiratory toxicity testing is clearly crucial for products incorporated into sprays because frequent inhalational exposure is probable. Unfortunately, current *in vitro* lung models are very limited in their capacity to model *in vivo* tissue. There is a paucity of healthy human lung tissue available for organotypic cultures or for the isolation of primary cells; furthermore, many differentiated pulmonary cell types are laborious to purify and difficult to maintain *in vitro*. Conversely, immortalized cell lines provide a robust and abundant cell source, but these cannot be assumed to be phenotypically normal and often display a loss of differentiated characteristics. For example, the frequently used alveolar epithelial cell line, A549, cannot form a tight junction-bound epithelial layer (19), which is crucial in assessing permeability effects on the blood–air barrier. However, the immortalized ESC phenotype is inherent only to the undifferentiated state; upon differentiation, somatic cell progeny undergo terminal differentiation and become subject to the Hayflick limit. If methods were established to efficiently derive pulmonary cells and tissue from ESCs, these could provide abundant, fully characterized, consistent, and phenotypically normal *in vitro* models.

DIFFERENTIATION OF ESCs

Initiating differentiation of ESCs is straightforward. Simply eliminate the culture conditions that sustain the undifferenti-

ated state, namely, the feeder layer and LIF/basic fibroblast growth factor (bFGF), and ESCs will spontaneously differentiate to a highly heterogeneous mixture of phenotypes containing derivatives of all three germ layers together with large quantities of extraembryonic endoderm. Three-dimensional culture of differentiating ESC as organoids in suspension, known as embryoid bodies (EBs), is also traditionally used as an instructive cue (Figure 1). This promotes differentiation along embryonic, rather than extraembryonic, lineages by partially mimicking embryonic structure.

To derive significant yields of a target cell type and minimize culture heterogeneity, ESC differentiation must be tightly regulated such that the cells are driven along the appropriate developmental lineage. The remainder of this article discusses the variety of methods that are effective in enhancing the derivation of pulmonary epithelium from murine and human ESCs.

DERIVATION OF LUNG EPITHELIUM FROM ESCs

In the early days of ESC research, it became quickly apparent that it was considerably harder to derive endodermal lineages than those of the mesoderm and ectodermal germ layers. Endoderm is the last of the three germ layers to form during embryonic development *in vivo*; therefore, endodermal specification is a considerably more complex process (20). Consequently, progress in deriving lung epithelium has lagged considerably behind that of lineages such as cardiomyocytes and neurons. Nevertheless, a number of different approaches have been established that markedly up-regulate differentiation, although in many cases the yield of lung epithelium still remains comparatively low.

Small Airway Growth Medium

The first report of the detection of a lung epithelial phenotype, type II pneumocytes, in differentiated murine ESC cultures was made in 2002 (21). The protocol used in this study was based on a simple premise: that a commercial cell culture medium (Small Airway Growth Medium; Cambrex, College Park, MD) optimized for the growth of primary distal lung epithelial cells would contain a cocktail of soluble factors that could also increase the yield of lung epithelial cells from ESCs. This was indeed the case, although ESCs were unresponsive to the serum-free medium until they had achieved a relatively advanced state of differentiation, suggesting that it selected out spontaneously differentiated type II pneumocytes rather than actively drove differentiation. Derived pneumocytes were shown to express surfactant protein C (SPC) mRNA and protein, and contained sparse lamellar body–like vesicles in the cytoplasm, but unfortunately were present in only very low numbers in the final culture. Subsequent attempts to optimize the growth factor composition of Small Airway Growth Medium (SAGM) and increase pneumocyte differentiation yielded only

Figure 1. Differentiating embryoid bodies from murine embryonic stem cells (ESCs). Traditional approaches to ESC differentiation involve initially culturing the cells as three-dimensional spheroids (a). To obtain lung epithelial cells, these spheroids are then adhered whole onto gelatin-coated tissue culture plastic and outgrown for at least 10 days (b).

modest results (22). SAGM treatment has since been shown to enhance type II pneumocyte differentiation from human ESCs with similar results (23, 24).

Conditioned Media

To develop a complex differentiation medium that can be applied to ESCs earlier in the differentiation process, our laboratory has investigated the possibility of preconditioning serum-containing media on pneumocyte cell lines. Media conditioned on the A549 human lung adenocarcinoma cell line are particularly effective in driving type II pneumocyte differentiation from both murine and human ESCs (25). Type II pneumocyte-like cells derived by this method have a well-differentiated ultrastructure containing lamellar bodies and apical microvilli, and also express SPC (Figure 2). Preliminary, subjective quantification of SPC-expressing cells suggests that this may represent one of the most efficient methods to derive type II pneumocytes, achieving up to 25% yield with optimal batches of conditioned media.

Coculture

A more complex, but potentially very effective, method to drive differentiation is to recapitulate the embryonic environment by direct coculture of ESCs with developmentally relevant cell types. The profound role that embryonic lung mesenchyme plays in the specification of regional epithelial phenotypes along the pulmonary tree has been long established in developmental biology (26, 27). Correspondingly, coculture of murine EBs with mesenchyme dissected from the distal tips of midgestation murine fetal lungs (Embryonic Days E11.5–E13.5) directed the differentiation of epithelial cells expressing thyroid transcription factor (TTF)-1, a marker of early pulmonary epithelium, and SPC in three-dimensional channels reminiscent of the embryonic lung (24, 28). This was also effective when mesenchyme and EBs were separated by a porous membrane, suggesting that the mechanisms involved paracrine signaling. Although the coculture differentiation method is not amenable to scale up, as it is highly labor intensive, it does provide a useful model system to dissect the developing respiratory epithelial niche. Accordingly, more recent work indicated that the inductive power of embryonic lung mesenchyme is stage specific, being most effective at Embryonic Day E11.5, just before the initiation of distal lung branching morphogenesis (29). Similarly, attempts have been made with human ESCs, but murine lung mesenchyme was largely unable to initiate lung-specific marker expression, suggesting that the pulmonary niche exhibits species-specific differences (30).

Cell Extract-based Reprogramming

Although the majority of approaches to ESC differentiation are based on coaxing cells along a developmental pathway by recapitulation of the appropriate niche, a small number of methods modify cell phenotype more directly and immediately. One of these is a cellular reprogramming method using complex cell extracts derived from the mature target cell type. Originally, this was developed to switch the phenotype of mature cells—for example, by deriving T lymphocytes from the 293T fibroblast cell line (31). However, it also proved a highly effective method to differentiate ESCs into alveolar epithelial cells, obtaining yields of approximately 10% SPC-expressing cells within 7 days (32). Briefly, ESC differentiation was initiated by EB formation, then 10-day-old EBs were dissociated and permeabilized with streptolysin O, before exposing to an extract of murine type II pneumocytes (MLE-12 cell line). SPC-expressing cells appeared between Days 3 and 7 after cellular reprogramming; this observation was specific to the MLE-12 cell extract and

Figure 2. Type II pneumocytes obtained from embryonic stem cells (ESCs) using A549 conditioned medium. Comparatively high proportions of type II pneumocyte-like cells can be obtained by differentiation of murine or human ESCs in media preconditioned on the A549 cell line. Surfactant protein C (SPC) expression is usually detected within 2–3 weeks of continual exposure to conditioned media. (a) Immunofluorescent staining for pro-SPC (green) with DAPI nuclear counterstain (blue) shows a cluster of type II pneumocyte-like cells derived from murine ESCs. (b) Well-formed lamellar bodies observed by transmission electron microscopy on differentiated murine ESC cultures (arrows). (c) Electron micrograph of differentiated human ESCs reveals the presence of apical microvilli, another feature of type II pneumocytes.

not observed with a fibroblast cell extract. Putative type II pneumocytes derived by this method were shown to coexpress TTF-1 and SPC, contained abundant lamellar bodies, and over time appeared to transdifferentiate to type I pneumocytes. Together, these data argue against the SPC-expressing ESCs representing merely a transient transfer of phenotypic markers from the cell extract, and instead suggest that the ESCs acquired

a type II pneumocyte phenotype by differentiation. Accordingly, recent data from our laboratory show that reprogramming of human ESCs with MLE-12 extracts is also effective, and species-specific reverse transcriptase-polymerase chain reaction proved that the majority of SPC mRNA in reprogrammed cells was human, not murine (M. Qin, personal communication).

Activin A-mediated Induction of Endodermal Lineages

Approximately 4 to 5 years ago, it became apparent to ESC researchers that the derivation of all endodermal lineages was proving technically difficult, including derivation of pancreatic β -cells and hepatocytes as well as pulmonary epithelium. Therefore, attention turned to developing means by which endodermal differentiation could be specifically enhanced and the other two germ layers suppressed during the very early stages of ESC differentiation, before the emergence of mature somatic lineages. The most effective strategy yet developed is to mimic embryonic signaling events and expose ESCs to saturating concentrations of activin A, a member of the Nodal signaling family, during the first 7 days of differentiation. In 2004, a landmark paper clearly demonstrated that high levels of activin A promoted the sequential emergence of definitive endoderm, followed by the pancreatic β -cell and hepatocyte lineages (33). Accordingly, lung epithelial differentiation was also found to be enhanced by early treatment of ESCs with 100 ng/ml activin A (34). In this study, the use of fetal bovine serum was largely replaced by a synthetic, partially defined additive (KnockOut Serum Replacement; Invitrogen Ltd., Paisley, UK). Interestingly, the absence of serum appeared to cause the premature arrest of pulmonary epithelial differentiation at a primitive fetal-like phenotype.

Air-Liquid Interface

Clearly, the majority of attempts to derive pulmonary epithelium from ESCs have focused on obtaining type II pneumocytes. This has been primarily a pragmatic decision; type II pneumocyte differentiation can be easily tracked using SPC expression because it is a highly specific phenotypic marker. However, the derivation of airway epithelium from murine ESCs has also been achieved (35). In this work, a proportion of ESCs were induced to differentiate into nonsecretory Clara cells simply by culture on a collagen I matrix. When these cells were raised to an air-liquid interface, they then gave rise to a fully differentiated airway epithelium containing Clara cells, ciliated cells, and basal cells arranged in a structure strikingly reminiscent of murine airway epithelium *in vivo*. This demonstrates not only that pulmonary phenotypes other than alveolar epithelium can be obtained from ESCs but also that physical cues can be as effective as chemical stimuli in initiating and directing differentiation.

ESC-DERIVED PULMONARY EPITHELIUM *IN VIVO*

Recently, our laboratory initiated studies to investigate the capacity of ESC derivatives to repair injured lung tissue *in vivo*. The following preliminary data were reported at the American Thoracic Society annual meeting in 2007 (36).

Implantation of Purified SPC-Positive Cells

In our initial implantation experiments, SPC/enhanced green fluorescent protein (eGFP)-positive cells were isolated from mixed populations of differentiated using fluorescence activated cell sorting (FACS) (Figure 3a). ESCs were differentiated according to the activin A protocol described in Reference 34; this method was chosen because it yields a progenitor-like phenotype that we judged to have more potential for *in vivo*

engraftment than mature, terminally differentiated lung epithelial cells. A total of 10^5 purified cells were injected via the tail vein into wild-type C57BL/6 mice or mice that had received 50 ng to 25 μ g intratracheal LPS 4 to 5 hours before implantation. This model of acute lung injury was chosen under the hypothesis that inflammatory stimuli would promote the recruitment of circulating cells into damaged lung tissue. Mice were killed 1 to 5 days postimplantation and multiple tissues fixed, sectioned, and examined by fluorescence microscopy for the presence of ESCs. Cell tracking was achieved by prelabeling cells with the fluorescent dye carboxylfluorescein diacetate, succinimidyl ester (CFDA-SE). Pulmonary localization of labeled cells was observed at a very low frequency in injured animals (Figure 3b), but healthy lungs did not appear to contain any implanted cells. No labeled cells were found in the liver, brain, spleen, heart, or kidney in either healthy or injured animals. This suggested that pulmonary injury was critical to the recruitment of implanted ESCs; however, even in the presence of injury, the rate of ESC engraftment was so low as to be barely detectable—only 10 to 20 cells per lung. Subsequently, we discovered that at least 50% of the cells activated caspase-3 shortly after FACS sorting (Figure 3c), suggesting the widespread induction of apoptosis. A less traumatic method of purification, such as the antibiotic resistance used in Reference 36, may be required to maintain viability of ESC-derived lung epithelium after isolation.

Implantation of Unpurified Differentiated ESCs

To attempt to improve the frequency of pulmonary engraftment, we then delivered 10^6 cells from mixed differentiated cultures without purification. Twenty-four to 48 hours after implantation, labeled cells were abundant in the distal lung parenchyma and were localized by histologic examination to both the epithelial and mesenchymal compartments (Figure 4). Cells were generally not associated with large blood vessels or airways. After 48 hours, the frequency of labeled cells in lung tissue progressively declined and cells were virtually undetectable in both wild-type and injured animals at 7 days. Cell engraftment was therefore transient regardless of the presence or absence of injury. No extrapulmonary migration of cells has yet been detected in any animal at any time point. Work is ongoing to immunophenotype engrafted cells and to determine whether the transient presence of a mixed population of ESCs ameliorates LPS-mediated lung injury.

ESC *IN VIVO*: A CAUTIONARY NOTE

Although preclinical studies are underway, as alluded to earlier there are several outstanding safety issues that must be overcome before ESCs can be used in humans. First and foremost, undifferentiated ESCs form benign tumors called teratomas *in vivo*; therefore, the accidental implantation of pluripotent cells would be a significant safety hazard. Even if no pluripotent cells were present in implanted populations, stem cells could differentiate aberrantly in host tissues, leading to the presence of inappropriate, potentially harmful, cell types. This risk may be particularly compounded in diseased tissue in which pathologic changes may initiate inappropriate differentiation patterns. For example, a fibrotic environment may promote fibrogenic differentiation of implanted cells, in which case stem cell therapy would risk worsening the disease state. Finally, the current handful of clinical-grade ESC lines represents an allogeneic cell source likely to elicit an immune response. In the future, it may be possible to use autologous ESC-like cells by reprogramming adult somatic cells to produce so-called iPS cells, but at present this requires extensive genetic modification, which would not be acceptable for clinical use (37, 38). Alternatively, multiple ESC

Figure 3. Isolation and *in vivo* implantation of embryonic stem cell (ESC)-derived pneumocytes. Surfactant protein C (SPC)-expressing cells were purified from mixed differentiated cultures by fluorescence activated cell sorting (FACS), using an SPC-enhanced green fluorescent protein (eGFP) reporter murine ESC cell line (a). Tail vein injection of carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE)-labeled SPC⁺ cells into mice with acute LPS lung injury resulted in a very small number of labeled cells (green) embedding in the lung parenchyma (b) (note that orange coloration represents elastin autofluorescence in this red-green overlay). However, on further analysis a high proportion of ESCs were found to contain activated caspase-3 after purification, suggesting that the FACS process induced widespread apoptosis (c). PE-A = red fluorescence; FITC-A = green fluorescence.

lines could be banked and tissue typed similar to existing procedures for organ transplants.

FUTURE DIRECTIONS OF ESC RESEARCH

Several diverse methods have now been established for the derivation of pulmonary epithelium and the next challenge will be the combination of individual approaches to provide ESCs

Figure 4. *In vivo* implantation of unpurified differentiated embryonic stem cells (ESCs). When mixed populations of differentiated ESCs were injected into mice, labeled cells (bright green) were detected frequently in the distal lung of both healthy and injured animals. This image shows carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE)-labeled cells scattered throughout the lung of an uninjured mouse, 24 hours after injection with 10⁶ differentiated ESCs.

with a more complex and tightly regulated microenvironment. For example, any of the methods described here could easily be applied to activin A-induced rather than raw, undifferentiated ESCs. Thought should be given to enriching the physical culture environment, which could ultimately incorporate air-liquid interfaces, together with a three-dimensional scaffold and cyclic stretch, to provide a more lunglike configuration. Furthermore, it is likely that the ESC differentiation methods described here also yield pulmonary phenotypes other than epithelium—for example, endothelium and mesenchyme—but this has yet to be investigated.

Efforts will continue to maximize differentiation efficiency from ESCs, but it seems inevitable that strategies to purify the target cell type from a heterogeneous population will always be necessary. Unidirectional differentiation has not yet been achieved for any target cell type, even those that spontaneously generate high yields, such as neural phenotypes. Current approaches to purification require the generation of genetically modified lines expressing cell-specific reporters or antibiotic resistance (34, 36), but characterization of ESC-derived cells may identify suitable surface markers that can be targeted by FACS or magnetic cell sorting (MACS).

Finally, further *in vivo* and *in vitro* studies are required on purified ESC-derived lung epithelium to conclusively demonstrate functionality. For the purposes of *in vitro* testing, responses to established pulmonary toxins and drugs should be quantified and compared with those of primary tissue. For future clinical use, ESC-derived cells must undergo rigorous pre-clinical testing to establish a safety and efficacy profile for a variety of pulmonary injuries and diseases.

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References

- Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *Am J Pathol* 2004;164:577–588.
- Reynolds SD, Giangreco A, Hong KU, McGrath KE, Ortiz LA, Stripp BR. Airway injury in the pathophysiology of lung disease: selective depletion of airway stem and progenitor cell pools potentiates lung inflammation and alveolar dysfunction. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L1256–L1265.
- Giangreco A, Reynolds SD, Stripp BR. Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. *Am J Pathol* 2002;161:173–182.
- Bender Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 2005;121:823–835.
- Reddy R, Buckley S, Doerken M, Barsky L, Weinberg K, Anderson KD, Warburton D, Driscoll B. Isolation of a putative progenitor subpopulation of alveolar epithelial type 2 cells. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L658–L667.
- Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* 2005;105:369–377.
- Herzog EL, Van Arnem JS, Hu B, Krause DS. Threshold of lung injury required for the appearance of marrow-derived lung epithelia. *Stem Cells* 2006;24:1986–1992.
- Francois S, Bensidhoum M, Mouisseddine M, Mazurier C, Allenet B, Semont A, Frick J, Sache A, Bouchet S, Thierry D, et al. Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promote their widespread engraftment to multiple organs: a study of their quantitative distribution after irradiation damage. *Stem Cells* 2006;24:1020–1029.
- Rojas M, Xu J, Woods CR, Mora AL, Spears W, Roman J, Brigham KL. Bone marrow-derived mesenchymal stem cells in repair of the injured lung. *Am J Respir Cell Mol Biol* 2005;33:152.
- MacPherson H, Keir P, Webb S, Samuel K, Boyle S, Bickmore W, Forrester L, Dorin J. Bone marrow-derived sp cells can contribute to the respiratory tract of mice in vivo. *J Cell Sci* 2005;118:2441–2450.
- Andrews PW. From teratocarcinomas to embryonic stem cells. *Philos Trans R Soc Lond B Biol Sci* 2002;357:405–417.
- Evans MJ, Kaufman M. Establishment in culture of pluripotent cells from mouse embryos. *Nature* 1981;292:154–156.
- Martin G. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma cells. *Proc Natl Acad Sci USA* 1981;78:7634–7638.
- Smith A, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 1988;336:688–690.
- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cells derived from human blastocysts. *Science* 1998;282:1145–1147.
- Brons IG, Smithers LE, Trotter MW, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM, Howlett SK, Clarkson A, Ahrlund-Richter L, Pedersen RA, et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* 2007;7150:191–195.
- Bodnar MS, Meneses JJ, Rodriguez RT, Firpo MT. Propagation and maintenance of undifferentiated human embryonic stem cells. *Stem Cells Dev* 2004;13:243–253.
- Ohtsuka S, Dalton S. Molecular and biological properties of pluripotent embryonic stem cells. *Gene Ther* 2008;15:74–81.
- Elbert KJ, Schafer UF, Schafers HJ, Kim KJ, Lee VH, Lehr CM. Monolayers of human alveolar epithelial cells in primary culture for pulmonary absorption and transport studies. *Pharm Res* 1999;16:601–608.
- Gadue P, Huber TL, Nostro MC, Kattman S, Keller GM. Germ layer induction from embryonic stem cells. *Exp Hematol* 2005;33:955–964.
- Ali NN, Edgar AJ, Samadikuchaksaraei A, Timson CM, Romanska HM, Polak JM, Bishop AE. Derivation of type II alveolar epithelial cells from murine embryonic stem cells. *Tissue Eng* 2002;8:541–550.
- Rippon HJ, Ali NN, Polak JM, Bishop AE. Initial observations on the effect of medium composition on the differentiation of murine embryonic stem cells to alveolar type II cells. *Cloning Stem Cells* 2004;6:49–56.
- Samadikuchaksaraei A, Cohen S, Isaac K, Rippon HJ, Polak JM, Bielby RC, Bishop AE. Derivation of distal airway epithelium from human embryonic stem cells. *Tissue Eng* 2006;12:867–875.
- Van Vranken BE, Rippon HJ, Samadikuchaksaraei A, Trounson A, Bishop AE. The differentiation of distal lung epithelium from embryonic stem cells. In: Bhatia M, Elefanti A, Fisher S, Patient R, Schlaeger T, Snyder E, editors. Current protocols in stem cell biology. Hoboken, NJ: John Wiley & Sons; 2007. pp. 1G.1.1–1G.1.22.
- Ismail NS, Bishop AE, Mantalaris A. Engineering alveolar epithelial cells from murine embryonic stem cells (MESC) in static and rotating bioreactor systems [abstract]. Presented at the International Society of Stem Cell Research Annual Conference, Cairns, Australia; 2007.
- Shannon JM. Induction of alveolar type II cell differentiation in fetal tracheal epithelium by grafted distal lung mesenchyme. *Dev Biol* 1994;166:600–614.
- Shannon JM, Nielsen LD, Gebb SA, Randell SH. Mesenchyme specifies epithelial differentiation in reciprocal recombinants of embryonic lung and trachea. *Dev Dyn* 1998;212:482–494.
- Van Vranken BE, Romanska HM, Polak JM, Rippon HJ, Shannon JM, Bishop AE. Co-culture of embryonic stem cells with pulmonary mesenchyme: a microenvironment that promotes differentiation of pulmonary epithelium. *Tissue Eng* 2005;11:1177–1187.
- Denham M, Cole TJ, Mollard R. Embryonic stem cells form glandular structures and express surfactant protein C following culture with dissociated fetal respiratory tissue. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L1210–L1215.
- Denham M, Conley BJ, Olsson F, Gulluyan L, Cole TJ, Mollard R. A murine respiratory-inducing niche displays variable efficiency across human and mouse embryonic stem cell species. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1241–L1247.
- Hakelien AM, Landsverk HB, Robl JM, Skälhegg BS, Collas P. Reprogramming fibroblasts to express T-cell functions using cell extracts. *Nat Biotechnol* 2002;20:460–466.
- Qin M, Tai G, Collas P, Polak JM, Bishop AE. Cell extract-derived differentiation of embryonic stem cells. *Stem Cells* 2005;23:712–718.
- Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G. Development of definitive endoderm from embryonic stem cells in culture. *Development* 2004;131:1651–1662.
- Rippon HJ, Polak JM, Qin M, Bishop AE. Derivation of distal lung epithelial progenitors from murine embryonic stem cells using a novel three-step differentiation protocol. *Stem Cells* 2006;24:1389–1398.
- Coraux C, Nawrocki-Raby B, Hinnrasky J, Kileztky C, Gaillard D, Dani C, Puchelle E. Embryonic stem cells generate airway epithelial tissue. *Am J Respir Cell Mol Biol* 2005;32:87–92.
- Wang D, Haviland DL, Burns AR, Zsigmond E, Wetsel RA. A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 2007;104:4449–4454.
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;131:861–872.
- Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007;318:1917–1920.