Xenotransplantation is a widely used technique to test the tumorigenic potential of human cells in vivo using immunodeficient mice. Here we describe basic technologies and recent advances in xenotransplantation applied to study squamous cell carcinomas (SCCs) of the skin. SCC cells isolated from tumors can either be cultured to generate a cell line or injected directly into mice. Several immunodeficient mouse models are available for selection based on the experimental design and the type of tumorigenicity assay. Subcutaneous injection is the most widely used technique for xenotransplantation because it involves a simple procedure allowing the use of a large number of cells, although it may not mimic the original tumor environment. SCC cell injections at the epidermal-to-dermal junction or grafting of organotypic cultures containing human stroma have also been used to more closely resemble the tumor environment. Mixing of SCC cells with cancer-associated fibroblasts can allow the study of their interaction and reciprocal influence, which can be followed in real time by intradermal ear injection using conventional fluorescent microscopy. In this article, we will review recent advances in xenotransplantation technologies applied to study behavior of SCC cells and their interaction with the tumor environment in vivo.

### BENEFITS

- Allows measurement of the tumorigenic potential of human skin cancer cells in a complex in vivo environment.
- Allows study of the consequences of specific genetic alterations in vivo.
- Can be used for screening the therapeutic potential of novel chemical compounds on human tissues.
- Interactions between epithelial tumor cells, stroma, and other cell types can be studied in combination.
- Recent techniques allow growth of primary SCC tissue and SCC single-cell suspensions, and/or monitoring growth in real time using in vivo imaging.

### LIMITATIONS

- Only partially recapitulates tumor organization and function, and its interaction with the immune, vasculature, and lymphatic systems.
- As with other in vivo assays, it is subject to high variability, and appropriate statistical analyses must be used to obtain reliable results.
- Tumor development occurs slowly, and may take up to 4 or more than 4 months.
**IMMUNODEFICIENT MOUSE MODELS FOR XENOTRANSPLANTATION**

Immunodeficient mouse strains are used for human cell studies to avoid rejection of the human cells. Here we will focus on immunodeficient mouse models that have been used in dermatological research for studying SCC (for a more comprehensive description of recipient mouse strains, see Russell et al., 2015). Athymic Foxn1nu (nude) mice lack functional T cells, but have an intact humoral adaptive and innate immune system (Price, 2014). In spite of only partial impairment of the immune system, nude mice effectively support tumor growth because of a paradoxical role of both the adaptive and innate immune responses in inducing inflammation, which can be protumorigenic (Patel et al., 2012). In severe combined immune deficiency (SCID) mice, the development of mature T- and B-lymphocytes is abolished, but innate immunity is conserved. Similarly, RAG1/2 null mice lack both functional T and B cells. Crossing SCID mice with nonobese diabetic (NOD) mice confers partially defective innate immunity. To further weaken the innate immune system, immunodeficient mice carrying a targeted mutation in the IL2 receptor common gamma chain gene (IL2rγ) have been crossed with NOD/SCID or NOD/RAG1/2 mice, generating the NOD/SCID/IL2rγ null or NOD/RAG1/2/IL2rγ null mice. Adaptive immunity is completely lacking in NOD/SCID/IL2rγ null and NOD/RAG1/2/IL2rγ null mice, and they are severely deficient in innate immunity, thus being highly receptive to the engraftment of human cells, tissues, and primary tumors.

**INJECTIONS OF HUMAN CELLS IN IMMUNODEFICIENT MICE**

The most widely used tumorigenicity assay involves ectopic injection of neoplastic cells into the subcutaneous space on the back of immunodeficient mice. Depending on the experimental design, tumor cells and relative controls are frequently injected into the two dorsal flanks to compare their tumorigenicity in the same biological environment.

A range of 0.5 × 10⁵ to 5 × 10⁶ tumor cells are injected, depending on their tumorigenic potential and the experimental plan. A smaller number of cells can be utilized when testing the tumor-initiating capability of selected cell populations. In this case, it is crucial to use NOD/SCID/IL2rγ null mice where adaptive immunity is completely lacking, because a small number of cells are more prone to be destroyed by the immune system. In addition, tumorigenic cells are mixed with Matrigel, a complex mixture of extracellular matrix proteins that enhances the engraftment of primary epithelial cancer cells by promoting perfusion of nutrients and holding cells in place in the subcutaneous tissue (Quintana et al., 2008). Matrigel is used at a high concentration (20 mg/ml) with the cells mixed in a 1:1 ratio. After injection, tumor size is monitored once a week, and two diameters of the tumors are measured to estimate changes in tumor volume over time. Immunohistological and immunohistochemical analysis is required to determine the tumor grade by examining tissue morphology, degree of cell proliferation, differentiation, and number of cells undergoing senescence or apoptosis.

**ADVANCES IN SCC XENOTRANSPLANTATION**

Established SCC cell lines are mostly tumorigenic and retain features of primary cutaneous SCC with variable degree of differentiation, ranging from well-differentiated cysts to more aggressive tumors, whereas grafting of freshly isolated SCC cells is more challenging. Recently, Patel et al. (2012) established a method to obtain reproducible and robust growth of xenografted primary SCC tissue and SCC single-cell suspensions in athymic nude mice by preimplantation of a humanized stromal bed. In this assay, a glass disk or Gelfoam dressing is implanted into the dorsal subcutaneous space, together with 10⁶ primary human dermal fibroblasts (HDFs) suspended in Matrigel. After 2 weeks, the glass disk is removed and intact tumor tissue or primary human SCC cells suspended in Matrigel are injected into the subcutaneous space or into the in situ Gelfoam dressing (Patel et al., 2012). The glass disk promotes a stromal reaction and vascularization sufficient to induce reproducible tumor growth from SCC cell lines. However, preimplantation of HDFs is essential to achieve robust growth of primary SCC cells. With this method, xenografts from all tumor grades maintain the histological and growth characteristics of the original tumors even through serial transplantation (Figure 1). Interestingly, xenografts of freshly isolated SCC cells are consistently larger in athymic nude mice than in more immunocompromised mice (SCID), possibly due to a higher inflammatory response elicited in athymic nude mice, in which only T cells are compromised, but the humoral and innate responses are still active.

**Subepidermal injections**

Although subcutaneous injections are widely used, the subcutaneous microenvironment is different from the one in which skin tumors originally develop. To promote the interaction of cancer cells with a more physiological environment, SCC cells can be mixed with Matrigel and injected at the dermal-epidermal junction. An advantage of this technique is that SCC cells diffuse less, and proliferative centers originating from single cells can be counted (Wu et al., 2010). Although injecting in the subepidermal environment is insufficient to enhance SCC tumorigenic potential, it can be useful to test cancer-inducing factors. Indeed, using this assay, Wu and co-workers demonstrated that inhibition of the calcineurin/nuclear factor of activated T-cell pathway induced a more aggressive, moderately infiltrating tumor phenotype with high cellularity, thus suggesting a reason for why treatment with calcineurin inhibitors, used as immunosuppressive treatment for organ transplantation recipients, leads to an increased risk of SCC formation.

**Ear injections**

More recently, Procopio et al. (2015) described a novel assay for studying SCC and stromal cell expansion by intradermal ear injection in NOD/SCID/IL2rγ null mice. The ear thickness enables tumor formation to follow in real time and quantification of its growth rate by in vivo imaging using conventional fluorescence stereomicroscopy (Figure 2). A very low volume can be injected, allowing a low number of cells (10⁵) to be used. This novel technique has proven useful to test the function of the Notch effector.
The expansion of SCC13 cells admixed with HDFs depleted for CSL, p53, or both was monitored in time. Cancer cell expansion was significantly enhanced in the presence of HDFs with concomitant silencing of CSL and p53, as compared with CSL or p53 alone. Interestingly, stromal cells themselves missing both CSL and p53 expanded to a much greater extent than those with silencing of CSL alone. Thus, loss or reduction of CSL in HDFs coupled with p53 inactivation induces SCC expansion, demonstrating a crucial contribution of stromal CSL in tumor formation. Similar to the observations obtained by Patel et al. (2012), these findings demonstrate a fundamental role of the stroma in SCC expansion.

Grafting of human engineered skin
To study specific genetic contributors to SCC, genetic manipulation of primary human keratinocytes using high-efficiency gene transfer can be achieved using retroviral infections repeated at 8- to 12-hour intervals in rapidly dividing cells (Lazarov et al., 2002), which can then be seeded onto devitalized human dermis containing extracellular matrix and stromal proteins (Khavari, 2006). In contrast to subcutaneous or subepidermal injections where an underlying extracellular matrix and intact epithelial basement membrane is lacking, this method allows reconstitution of a human skin-like environment, although it does not fully recapitulate the stochastic nature of mutations that occur in spontaneous cancers and the genomic instability typical of most human cancers.

Recently, Monteleon et al. (2015) used this model to study the function of the protein domains of the IQ motif-containing GTPase-activating protein (IQGAP1), a modulator of mitogen-activated protein kinase signaling, using exogenous expression of IQGAP1 decoy peptides. Ras/CDK4 transformed keratinocytes were infected with lentiviruses designed to drive expression of a single IQGAP structural domain to interfere with specific IQGAP functions. Using this model, a single decoy peptide designed to interfere with binding to the Ras effector Raf (IQGAP-IQM) was found to significantly suppress tumor formation and mitogen-activated protein kinase phosphorylation (Figure 3), whereas the other peptides were ineffective.

CONCLUDING REMARKS
In conclusion, xenotransplantation represents a unique tool to study human SCC progression in an in vivo environment. Recent advances in this technique focus on reproducing the
tumor microenvironment and following tumor growth in real time. In the future, engineered human skin and subepidermal and ear injections may become increasingly useful for preclinical investigations of novel therapies, because these models are amenable to topical treatments.

CONFLICT OF INTEREST
The authors state no conflict of interest.

CME ACCREDITATION
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SUPPLEMENTARY MATERIAL
Supplementary material is linked to this paper.

Figure 3. Expression of the IQGAP1-IQM decoy peptide inhibits invasive squamous cell carcinoma (SCC). (a) IQGAP1-IQM expression inhibits keratinocyte proliferation and invasion in an SCC skin xenograft, bar = 250 μm. (b) Relative tumor cross-sectional area of xenograft control versus IQGAP1-IQM SCC tumors. (c) MAPK signaling as indicated by phospho-ERK expression is markedly diminished in the IQM-expressing tissue, bar = 100 μm. ERK, extracellular signal-regulated kinase; IQGAP, IQ motif-containing GTPase-activating protein; MAPK, mitogen-activated protein kinase. Reprinted with permission from Monteleon et al. (2015).

MULTIPLE CHOICE QUESTIONS

1. Which of the following murine models is more receptive for the engraftment of human tumor cells?
   A. NOD/SCID
   B. SCID
   C. NOD/SCID/IL2γ null (NSG)
   D. Athymic Foxn1nu (nude)

2. Are SCC cells highly tumorigenic when used in xenotransplantation assays?
   A. SCC cells are less tumorigenic when injected with human dermal fibroblasts
   B. SCC cells form aggressive tumors when injected at high concentration
   C. SCC cells form aggressive tumors when injected in the ear
   D. Freshly isolated SCC cells are not highly tumorigenic when injected subcutaneously in nude mice without a stromal bed

3. Which of the following sentences is false?
   A. Subepidermal injections enhance SCC tumorigenic potential and can be used to test cancer inhibiting small compounds
   B. Ear injections can be used to monitor expansion of tumorigenic cells in real time using conventional fluorescence stereomicroscopy
   C. In subcutaneous injections, the presence of human dermal fibroblasts enhances tumor growth of human SCC primary cells
   D. Grafting of human engineered skin onto mice reconstitutes a human skin-like environment

4. What is the advantage of injecting SCC cells in the subepidermal compartment?
   A. Allows injection of a low number of cells
   B. Allows interaction of cancer cells with a more physiological environment
   C. Induces a higher inflammatory response
   D. Promotes rapid tumor growth

5. Why does preimplantation of a glass disk or Gelfoam improve tumorigenicity of SCC cells in subcutaneous injections in nude mice?
   A. It generates a wound-like environment, allowing easier access for implantation of SCC cells
   B. It induces secretion of T-lymphocyte–secreted chemokines, thus favoring tumor growth
   C. It allows the SCC cells to grow more efficiently in clusters
   D. It creates a favorable environment by generating a stromal reaction

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REFERENCES