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Invasive 3-Dimensional Organotypic Neoplasia from Multiple Normal Human Epithelia

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Abstract

Refined cancer models are required to assess the burgeoning number of potential targets for cancer therapeutics within a rapid and clinically relevant context. Here we utilize tumor-associated genetic pathways to transform primary human epithelial cells from epidermis, oropharynx, esophagus, and cervix into genetically defined tumors within a human 3-dimensional (3-D) tissue environment incorporating cell-populated stroma and intact basement membrane. These engineered organotypic tissues recapitulated natural features of tumor progression, including epithelial invasion through basement membrane, a complex process critically required for biologic malignancy in 90% of human cancers. Invasion was rapid, and potentiated by stromal cells. Oncogenic signals in 3-D tissue, but not 2-D culture, resembled gene expression profiles from spontaneous human cancers. Screening well-characterized signaling pathway inhibitors in 3-D organotypic neoplasia helped distil a clinically faithful cancer gene signature. Multi-tissue 3-D human tissue cancer models may provide an efficient and relevant complement to current approaches to characterize cancer progression.

Keywords

Cancer; Epithelium; Invasion; Gene Expression

High throughput genomics have accelerated identification of genetic alterations in human cancers.[1,2] The numerous mutations in individual tumors and the significant heterogeneity between tumors hinders evaluation of specific genetic changes.[3] This difficulty partially results from technical limitations intrinsic to conventional mouse and cell line-based models commonly utilized for functional analysis of specific genetic alterations. Genetically engineered mice are costly, time-consuming, not amenable to high throughput combinatorial genetic or pharmacologic screens, and can exhibit significant differences from humans with regard to requirements for oncogenic transformation.[4,5] Tumor cell line-based models

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frequently lack key malignancy features and suffer from accumulated mutations that may confound functional studies. The capacity to directly transform primary human cells into genetically defined malignancies provides a platform in which to assess impacts of tumor-associated genes, as well as a benchmark positive control for tumorigenesis assays free of secondary genetic changes. Previous efforts to convert normal primary human cells into neoplasia required different combinations of human and viral oncogenes depending on cell type,[6-10]and a minimal set of oncogenic changes capable of transforming multiple human primary cell types in parallel has not been established.

Here we present a rapid method for malignant conversion of normal primary human epidermal, cervical, esophageal, and oropharyngeal epithelial cells, representing each of the stratified squamous epithelia associated with human cancer. These tissue types were transformed into invasive neoplasias through introduction of a physiologically relevant two genetic element pair consisting of active human Ras and Cdk4 proteins. These elements mediate Ras pathway activation and bypass of Rb-mediated G1 cell cycle restraints, which reflects alterations commonly observed in spontaneous human epithelial cancers. These transformed epithelial cells were used to develop 3-D human tissue models recapitulating key steps in cancer progression, including invasion through intact basement membrane into supporting stroma. As nearly 90% of human malignancies are of epithelial origin[11], understanding elements driving invasion from the in situ environment is critical, as it is only within the supporting stroma that cancer cells gain access to vasculature and lymphatics for systemic tumor spread. Stromal cells and extracellular matrix play important roles in regulating the tumor microenvironment to both promote and antagonize tumor progression.[12-15] We therefore established in vitro organotypic epithelial cancer models comprised of genetically-defined neoplastic epithelium, as well as intact basement membrane, supported by cell-populated living stroma and native extracellular matrix. In this 3–D context, engineered neoplastic epithelial cells progressed from the in situ environment and invaded through basement membrane into stroma within one week. These model tissues enabled rapid assessment of potential pharmacologic inhibitors of invasion and highlighted the Ras effector pathway and gene expression signature associated with neoplastic invasion. Organotypic human neoplasia models may facilitate translating the cancer genome into future strategies for cancer therapy and prevention.

Results

Oncogenic Ras and bypass of Rb, in the form of enforced Cdk4 expression, were effected in primary human epithelial cells from epidermis, cervix, esophagus, and oropharynx via high efficiency retroviral transfer without drug selection[16]. Twenty four hours after transduction, cells were injected into the subcutaneous space of immunodeficient mice. By 30 days, tumors were present at each injection site (Supplementary Table 1). Tumors displayed epithelial neoplasias with squamous morphology, reflecting typical histologic features of spontaneous carcinomas arising at these sites (Supplementary Figure 1). Thus, oncogenic Ras and Rb bypass efficiently induces tumorigenic conversion of primary epithelial cells from multiple human stratified epithelia.

Establishment of invasive 3–D organotypic tissues

Although subcutaneous tumorigenesis is a standard assay for oncogenic transformation, it lacks features of native cancer progression, including the key step of basement membrane invasion. To model this, we regenerated the 4 types of human stratified epithelia on intact basement membrane with cell-populated human stroma in organotypic tissue. The structural tissue framework for the regenerated tissues was an acellular derivative of cutaneous dermis, a stratified epithelial stroma containing extracellular matrix proteins as well as secreted basement membrane elements. Tissue generated from normal cells of each cell type attached to and respected the basement membrane (Fig. 1, 2). Epidermal organotypic tissue displayed normal stratification and differentiation programs (Fig. 1c), including a fully-formed basement membrane with normal ultrastructural elements (Fig. 1d–f). Epithelial tissues engineered with oncogenic Ras and Cdk4, in contrast, displayed disrupted polarity and epithelial cell invasion through basement membrane, hallmarks of early invasive neoplasia (Fig. 2). Neoplastic changes occurred within 6 days, indicating that multiple stratified human epithelia can be rapidly converted to invasive neoplasms in organotypic tissue.

To mimic somatic mutation-induced oncogene activation within normal tissue *in situ*, we also co-expressed a 4-hydroxytamoxifen (4OHT) inducible ER-Ras fusion protein[17] with Cdk4 in epidermal keratinocytes. Normal differentiated multilayer tissue was first established, then Ras subsequently activated with 4OHT. Inducible oncogene activation also led to invasion (Supplementary Fig. 2), similar to that seen with constitutively active Ras. Stromal invasion was associated with focal gaps in the previously continuous basement membrane (Fig. 3). Stromal fibroblasts secrete proteins important for basement membrane structural integrity[18], however, they also secrete tumor-enabling growth factors and chemotactic agents, raising the question of whether they would impair or enable invasiveness. While not required for invasion, stromal dermal fibroblasts enhanced it >400% (Fig. 3e–g). To assess if fibroblasts from other stratified epithelia would have similar effects to those from skin, cervical stromal fibroblasts were tested for their capacity to enhance cervical epithelial invasion. As with the dermal fibroblasts, a similar invasion-potentiating effect was noted (Supplementary Figure 3).

Human organotypic tissue neoplasia models containing primary human cells and intact basement membrane represent an alternative to conventional *in vitro* invasion assays. The latter measures migration through Matrigel™-coated perforated synthetic filters in Boyden chamberbased transwells as an invasion surrogate. Such assays allow some migration by normal cells; in contrast, normal cells in organotypic tissue never invaded through the intact basement membrane. Also consistent with major differences in these approaches, the human stratified epithelial tumor line, A431, highly invasive in Boyden chamber assays[19], was unable to invade through intact basement membrane (Supplementary Fig. 4). Inclusion of an intact basement membrane and stroma within organotypic neoplasia models provides an alternative approach for studying the complex process of epithelial tumor invasion that may complement traditional synthetic filter-based assays.

3–D organotypic human tissue neoplasia may support cancer target screens. A panel of 20 inhibitors (Supplementary Table 2), was tested for capacity to alter organotypic epidermal

neoplasia. Use of many of these research agents *in vivo* is hindered by an inability to deliver them efficiently and by systemic toxicity. In this initial screen, three inhibitors impeded invasive neoplasia. Inhibition of the Ras–Raf MAPK cascade at the level of Mek and Erk with U0126 completely blocked invasion at concentrations that had no apparent adverse effects on normal tissue, while blockade of another Ras effector pathway, PI3K had no detectable effect on invasion (Fig. 4a), suggesting that the Ras–Raf–MAPK cascade is a primary pathway driving this process. Although U0126 treated neoplastic cells did not invade, they did continue to proliferate (Supplementary figure 5). The broad-spectrum protease inhibitor GM6001, and the JNK inhibitor SP600125 also both diminished invasion depth, but did permit some epithelial cells to penetrate the basement membrane into stroma (Supplementary Table 2). Organotypic neoplasia thus offers a means of screening inhibitors of neoplastic invasion within a 3-D human tissue context.

Correlation between organotypic tissues and human tumors

To begin to characterize stratified epithelial organotypic neoplasia models, Affymetrix oligonucleotide microarrays were employed to determine gene expression profiles from invasive organotypic epidermis and cervix tissues expressing constitutively active H–Ras and Cdk4 with and without U0126 mediated Mek and Erk blockade. In both epidermis and cervix, U0126 had a dominant effect, reverting the majority of the gene changes introduced by the oncogenic stimulus (Fig. 4b, Supplementary Fig. 6). In organotypic epidermal neoplasia, 483 genes were differentially expressed (Supplementary Table 3), and 74% (356) of these genes were reverted by Mek and Erk blockade with U0126 (Supplementary Table 3). In organotypic cervical neoplasia, 366 genes were differentially expressed (Supplementary Table 4) and 71% (259) of these genes were reverted by U0126. There was a highly significant overlap ($p=1.2 \times 10^{-79}$, n=113) between the differentially expressed genes from the two tissue types, indicating that the tissues respond to oncogenic Ras with both common and tissue-specific transcriptional responses. The reverted genes were enriched in gene ontology (GO) terms associated with cancer, including angiogenesis, metabolism, cell motility, differentiation, wounding, and cell adhesion (Supplementary Table 5). The relative expression of these genes in organotypic epidermal neoplasia highly correlated with a series of spontaneous head and neck squamous cell carcinomas (SCC)[20] (Pearson coefficient 0.41, $p= 4.0 \times 10^{-16}$) (Fig. 4c), indicating that organotypic neoplasia engages gene regulatory changes similar to those seen in naturally occurring cancers.

To quantitatively determine the degree to which organotypic neoplasia reflects transcriptional changes in spontaneous human SCCs, and to compare the correlation with other available SCC models, we first generated 22 separate gene lists consisting of differentially expressed genes in each of 22 previously reported[21] spontaneous SCCs compared to matched adjacent normal tissue. The relative expression of each gene in these 22 gene sets was then examined in multiple SCC models including: A) 3–D organotypic neoplasia B) 2-D culture of the same epithelial cells used to generate organotypic neoplasia C) an in vivo Ras-driven SCC model[22] in which regenerated normal and neoplastic human skin is grafted on immunodeficient mice D) 2–D culture of a series of 10 human SCC cell lines[23] and E) a second independent set of human SCC tumors matched to adjacent normal control tissue[20]. The expression of each gene was examined in each of the models and

compared to its expression level in the corresponding control samples (tissue or primary cell culture) for each model system. Pearson coefficients were calculated between each of the 22 gene sets and each of the individual samples from the five model systems (Fig. 4d). As expected, the highest correlation was between the two sets of spontaneous human clinical specimens (median Pearson coefficient = 0.45, $p = 1.3\times10^{-12}$), indicating that the most faithful replicate of a given spontaneous clinical SCC is another human SCC. The 3–D organotypic model was also highly correlated (Pearson coefficient 0.34, $p = 1.1\times10^{-6}$) to a degree nearly identical to the in vivo grafting model, indicating that the transcriptional response to oncogenic signaling in organotypic neoplasia resembles that occurring within the orthotopic in vivo model, as well as in spontaneous clinical SCC.

In contrast to Ras and Cdk4 co-expression within 3–D organotypic tissue, 2–D culture of the same epithelial cells displayed a Pearson coefficient of 0.0 with natural SCC tumors, indicating profound differences between the 2–D and 3-–D environment. Consistent with this, established SCC cell lines grown in 2–D[23] also displayed a median Pearson coefficient of 0.0, indicating that adaptation to 2–D culture resulted in cell populations that may no longer reflect the gene expression patterns of human SCC tumors in vivo. To verify these results, we utilized the same analytic approach to quantitate the correlation between gene expression in a second set of natural human SCC[20] and the multiple SCC models. These results were nearly identical to those obtained with the first SCC tumor set (Supplementary Fig. 7b). Together, this indicated that genes altered in spontaneous tumors are similarly regulated in the 3–D organotypic neoplasia model but not in 2–D cultured cells. In a faithful model, genes significantly altered in the model system should also be similarly regulated in the spontaneous tumors. This was indeed the case with organotypic neoplasia. The relative expression of genes altered in the models correlated significantly with their expression in both sets of spontaneous tumors (Supplementary Fig. 7c, d); in contrast, the 2–D cultures were not correlated. Thus, 3–D organotypic neoplasia may offer distinct advantages over 2–D models with regard to physiologic relevance, and may represent a useful complement to conventional cancer models.

Discussion

These data provide an approach to produce organotypic human neoplasia models using primary epithelial cells from multiple stratified epithelial tissues. To our knowledge, this is the first generation of genetically defined tumors from primary human esophageal, oropharyngeal, and cervical epithelial cells. Organotypic neoplasia developed within one week, and could be induced by regulated Ras activation *in situ.* Organotypic neoplasia displayed enhanced invasion in the presence of stromal cells, as well as a capacity to serve as a platform for assessing invasion inhibitors. Finally, global gene expression in organotypic neoplasia was significantly correlated to spontaneous human cancer, suggesting that it represents a useful complement to currently available cancer models.

Primary human epithelial cells were converted to neoplastic cells using oncogenic Ras and Cdk4-mediated bypass of Rb cell cycle restraints. This combination is relevant to spontaneous human cancer. Large scale efforts human tumor sequencing efforts, including the Cancer Genome Atlas (TCGA) and the Catalog of Somatic Mutations in Cancer

(COSMIC), identified the Ras and Rb pathways as among the most commonly mutated pathways in human cancers; and *Ras*, among the most frequently mutated oncogenes, is frequently activated in stratified epithelial cancers[24,25]. Cdk4 itself is activated in epithelial cancers, including by gene amplification and via activating point mutations. The use of these genetic elements found in spontaneous human tumors represents an important line of experimentation that is likely to increase the relevance to spontaneous malignancy. This oncogenic gene set proved to be remarkably efficient, rapidly transforming all 4 cell types to invasive-tumor forming populations without the requirement for drug selection, in vitro passage, viral oncoproteins, or clonal selection. This allowed use of primary human cells as the starting substrate for interrogation, as opposed to cell lines adapted for long-term culture. Ras and Cdk4 may thus be useful as a positive control benchmark in organotypic neoplasia studies designed to characterize the function of other genetic changes identified in human tumor samples.

While unable to drive invasive behavior in the absence of epithelial-intrinsic oncogenic drivers, stromal fibroblasts clearly augmented invasion. In this context, they may be playing a role analogous to the enabling supportive function played by non-oncogene addicted (NOA) genes[26] in the tumor cells themselves. Systematically characterizing stromal factors enabling invasion may highlight potential therapeutic targets for a range of epithelial tumors. Directing therapeutics toward the stroma directly, rather than the cancer cells, is particularly attractive, as stromal cells represent a more genetically stable target that is less likely to develop resistance.

We noted significant differences between neoplastic cells' capacity to invade through basement membrane and their ability to migrate through Matrigel. Invasion through intact basement membrane requires that cells move through a dense highly-ordered structure, which may represent a more formidable obstacle than commonly used semisolid gels. In that regard, the invasion assay described here may more accurately recapitulate the process of epithelial tumor cell invasion in vivo than Boyden chamber migration assays. The ultimate merit of one method over another may need to await identification and functional validation of invasion inhibitors and cancer therapeutics that prove to be useful both in pre-clinical models and in clinical use. Matrigel based 3–D culture systems have proven useful however, for distinguishing normal from malignant mammary epithelial cells based on their ability to form organized spheroids and deposit the basement membrane component collagen IV.[27]

Organotypic tissue proved a useful platform for drug assessment, and allowed us to implicate the Ras downstream Raf–Mek–Erk MAPK cascade as required for the invasive phenotype. While the Mek inhibitor U0126 abrogated invasion and reversed the majority of the associated gene changes, the Raf inhibitor did not have similar effects. While the mechanisms underlying this difference are not clear, it most likely reflects functional redundancy at the level of Raf. There are only two isoforms of Mek, which are both inhibited by U0126. Raf however has multiple isoforms including a–Raf, b–Raf and c–Raf. The inhibitor used is targeted to c–Raf, and there may be compensation by other isoforms sufficient to transmit the Ras signal and drive invasion. Other possibilities exist, however, which reflect on the complexity of Raf action, including recent work demonstrating that Raf inhibition can paradoxically stimulate MAPK activity in the context of active Ras[28].

To establish the degree to which the organotypic neoplasia reflects the biology of spontaneous tumors, we developed an analytical method of quantitative transcriptome analysis based on the Pearson correlation between transcriptional responses to oncogenic stimuli in the model tissue and spontaneous human tumors. This approach quantitated the baseline degree of correlation and heterogeneity between individual spontaneous human SCC tumors, and demonstrated a greater relative correlation of the 3–D organotypic and in vivo models to spontaneous cancers compared to 2–D culture. This may be an important point to consider when designing experiments based on cancer cell lines grown *in vitro.*

While it would also be of interest to compare gene expression profiles between organotyic tissues and orthotopic in vivo grafts from tissue types other than epidermis, this is unfortunately currently not possible given the lack of a suitable normal control tissue for the analysis. In the case of epidermis, tissue grafts consisting of normal control keratinocytes are also grafted onto mice and used as a baseline for comparison to the invasive Ras-expressing grafts. In this regard, the epidermis presents a unique opportunity to conduct these types of comparison analyses. There are currently no available methods, to our knowledge, that would allow us to regenerate normal human esophagus, cervix, or oral mucosa in the orthotopic site on a mouse to make an “in vivo model” analogous to that which we have developed for skin. It is because of that technical limitation that we utilized the established standard of subcutaneous injection for these other tissues. While tumors form with all four Ras/Cdk4-expressing cell types, the control primary cells do not form tumors. Therefore, while we could obtain mRNA from the subcutaneous tumors, there was no corresponding *in vivo* tissue to use as an appropriate baseline for comparison for expression profiling studies, and the more in-depth profiling studies have thus focused on skin. Organotypic neoplasia therefore complements both traditional subcutaneous tumor formation assays and transgenic mouse studies by providing a standard experimental platform that is relatively inexpensive, rapid, and based on primary human epithelial cells on intact basement membrane within 3–D tissue.

Methods Summary

Isolation and culture of primary cells

Primary human epidermal and cervical keratinocytes were isolated from fresh surgical specimens. Primary esophageal and oropharyngeal epithelial cells were obtained from commercial sources. Primary human skin and cervix fibroblasts were obtained from fresh surgical specimens.

Retroviral transduction

Retroviruses driving expression of cancer-associated genes were used to transduce early passage primary epithelial cells.

In vivo tumor formation assays

Transduced epithelial cells were injected into the subcutaneous space of immunodeficient mice with 50% Matrigel according to an experimental protocol approval by the Stanford University Administrative Panel on Laboratory Animal Care.

3-dimensional organotypic culture

Devitalized human acellular dermis was used as the supporting framework for 3–D cultures. Stromal fibroblasts were introduced into the stromal side of the tissue. Epithelial cells were seeded onto the BM side, and tissues maintained in dual chamber supports separating the epithelial and stromal compartments.

Microarray studies

RNA was obtained from organotypic tissues was labeled and hybridized to Affymetrix U133A 2.0 arrays. Analysis was performed as noted in the Supplemental Methods. Array data is available through GEO under accession numbers GSE 22573, and GSE 22382.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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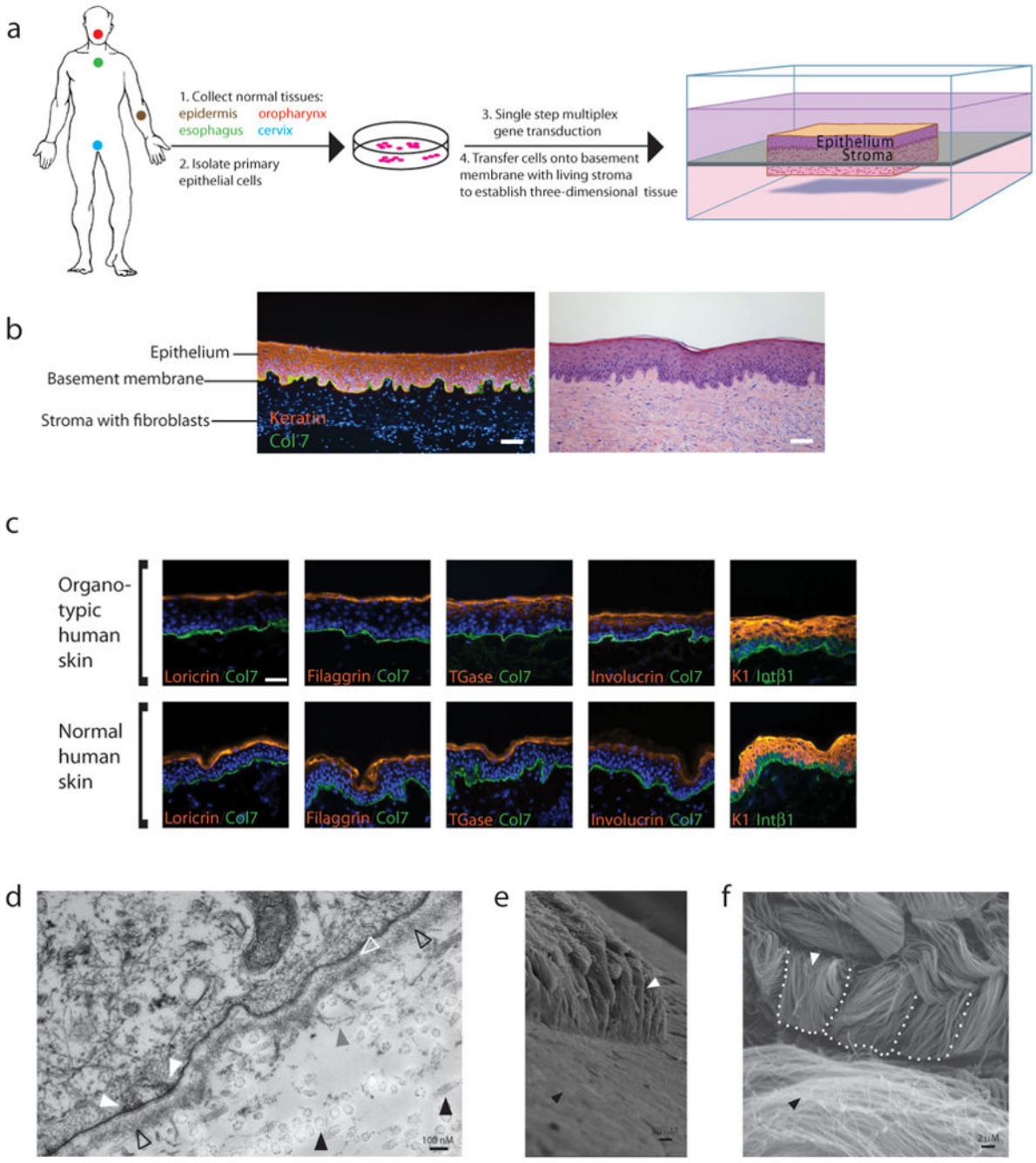

Figure 1. 3-D organotypic human epithelial tissue

(**a**) Experimental approach. (**b**) Normal stratified squamous epithelium and stromal architecture of regenerated human skin. Immunofluorescence image (left) highlighting epidermis (orange=pan-keratin) established on an intact basement membrane (BM) (green=type VII collagen BM marker), with fibroblast-populated dermis below. The corresponding hemotoxylin and eosin stained section is shown (right); scale bar=100μm. (**c**) Normal differentiation and BM protein distribution in organotypic and native human epidermal tissue; scale bar=100μm. (**d**) Transmission electron microscopy (EM) reveals a

continuous intact BM with hallmarks of native tissue. Lamina densa (open black arrow), lamina lucida with anchoring filaments (open white arrow), anchoring fibrils (closed gray arrow), hemidesmosomes (closed white arrows), and dermal type I collagen (closed black arrows). (**e**) Scanning EM of the surface of organotypic human skin tissue at the edge of multilayered epidermal keratinocytes (white arrow) regenerated on intact native BM (black arrow). (**f**) Scanning EM of tissue from (**e)** cut to expose the epidermal-dermal interface with collagen fibers on BM surface (black arrow), and basal keratinocytes (white arrow; note prominent appearance of keratin intermediate filaments). Dots outline individual basal cells sitting on the BM.

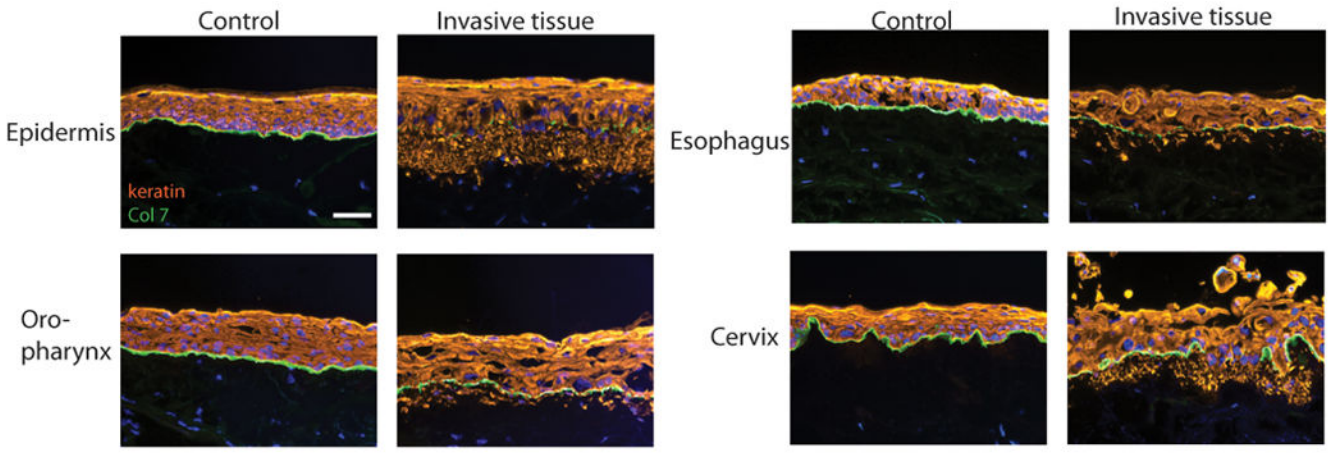

Figure 2. Organotypic neoplasia from multiple stratified epithelia

Ras-driven invasive tissue compared with LacZ-expressing control. Note invasion of epithelial cells derived from four different tissues (orange=keratin) through the BM (green=type VII collagen) into the surrounding stroma. In contrast, control tissues respect the BM; scale bar=100μm.

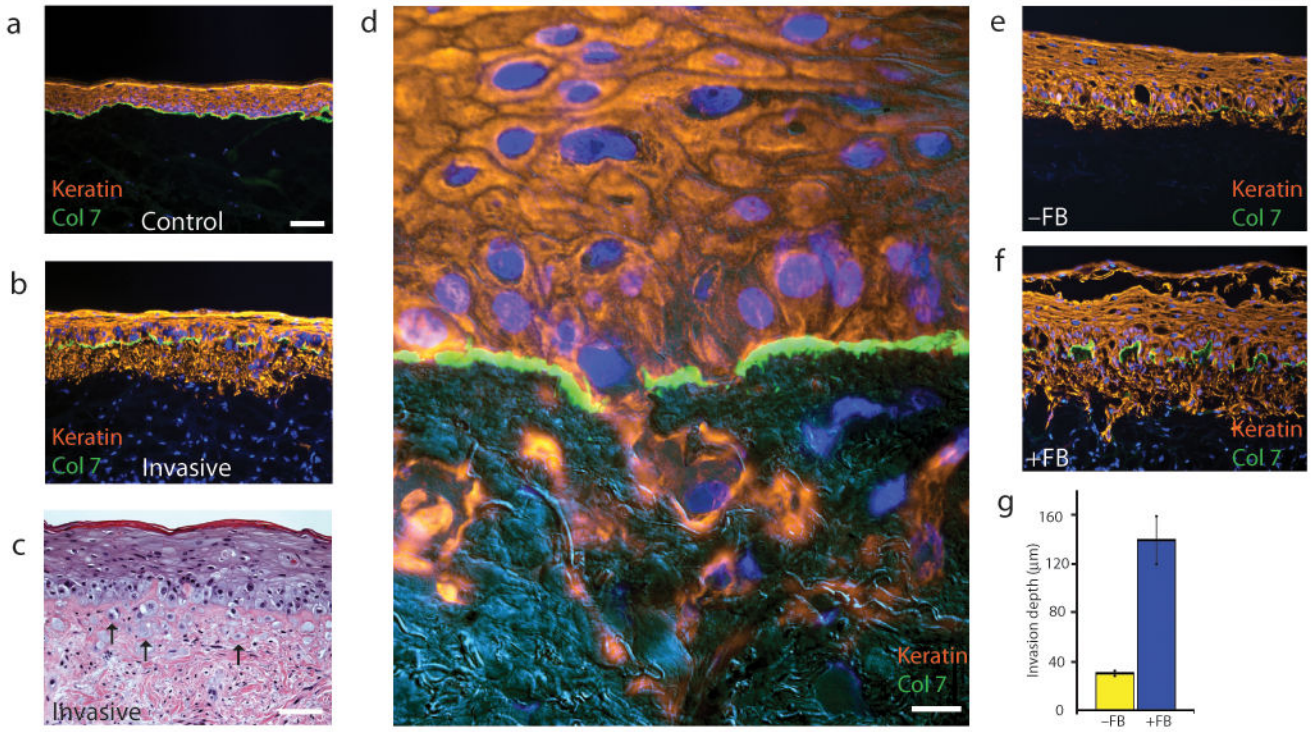

Figure 3. Stromal cells potentiate invasion in organotypic neoplasia

(**a**) Normal control keratinocytes respecting epithelial boundary delimited by the basement membrane. (**b,c)** Invasive Ras-expressing keratinocytes invading into stroma. **(d)** Higher magnification of early epidermal keratinocyte invasion. Note penetration by epithelial cells through disrupted BM; scale bar=10μm. (**e,f**) Invasion is potentiated by the presence of stromal fibroblasts. Compared to control stroma lacking fibroblasts (**e**), Ras-driven organotypic epidermal neoplasia displays significantly deeper invasion in the presence of living fibroblasts (**f**); scale bar=100μm. (**g**) Invasion depth from the overlying basement membrane (collagen VII staining) to the deepest keratin positive cell directly in the stroma was measured. Depth is an average of 6 representative measurements at different points equally spaced across the section.

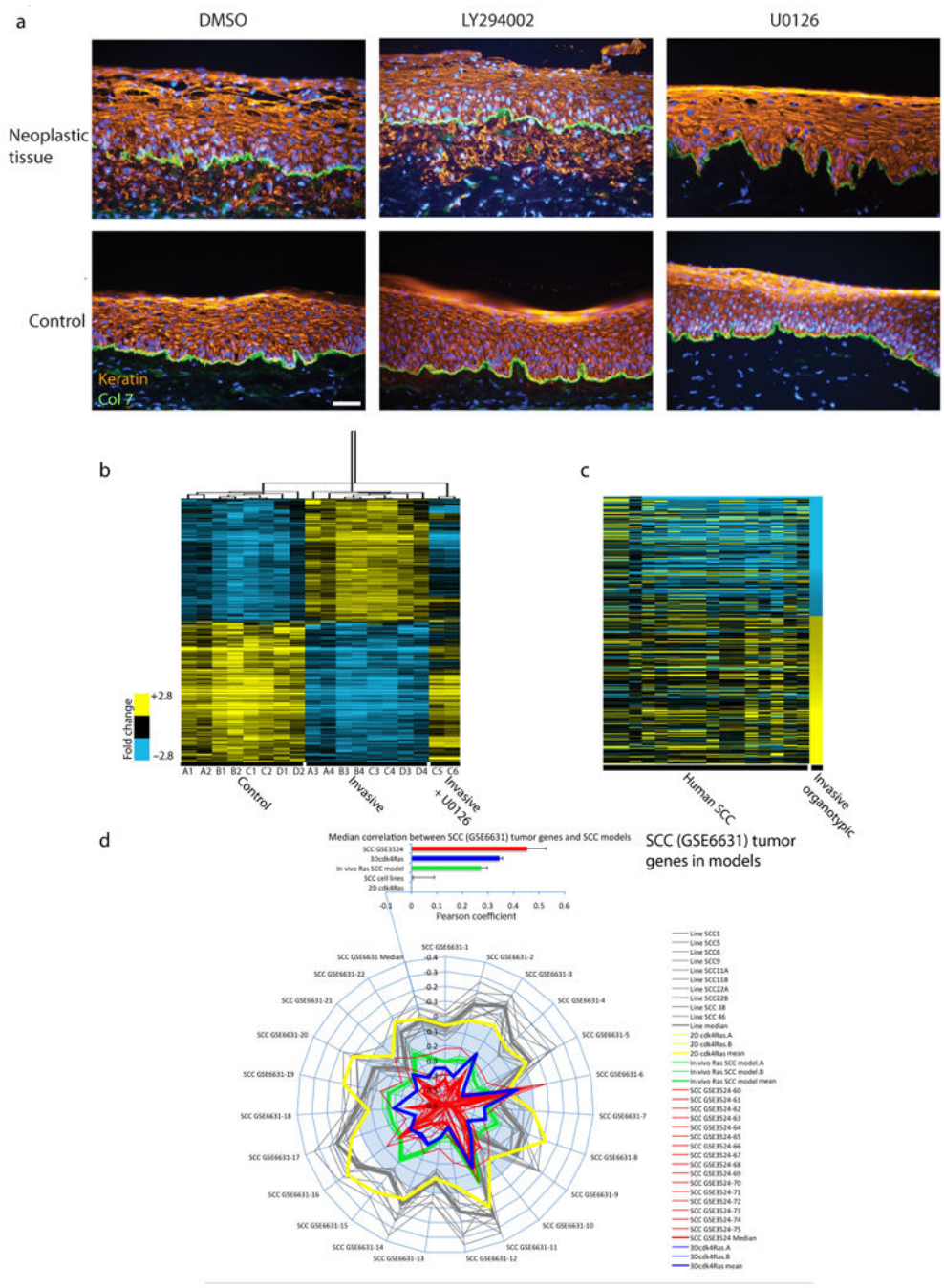

Figure 4. Analysis of organotypic neoplasia

(**a**) Effects of the PI3K inhibitor, LY294002, and the Mek and Erk inhibitor, U0126, on organotypic epidermal neoplasia; scale bar=100μm. (**b**) Expression data from Ras-driven organotypic epidermal neoplasia compared to normal epidermal control tissues (n=8 biologic replicates each) identifies 483 genes differentially expressed (≥2-fold change, FDR<0.03); reversion by U0126 (2 replicates) shown at right. Sample names are listed below the heat map. Cells used for this experiment were derived from 4 different donors (A,B,C,D) and replicate tissues were generated from each cell population. (**c**) Correlation of

relative gene expression in Ras-driven organotypic neoplasia with relative gene expression in spontaneously occurring human head and neck SCC. The heat map displays the expression of the U0126 reverted genes in the tumor specimens relative to matched patient normal. The right column represents the relative expression of the gene set in organotypic neoplasia. (**d**) Pearson correlations between gene expression changes in spontaneous human SCC tumors (radially arrayed around edge of figure) and 5 different model systems (concentric plots within figure). Thicker colored lines represent median (or mean) values for each category.