K5/K14-positive cells contribute to salivary gland-like breast tumors with myoepithelial differentiation

Werner Boecker^{1,2}, Goeran Stenman³, Thomas Loening⁴, Mattias K Andersson³, Agnes Bankfalvi⁵, Sarah von Holstein^{6,7}, Steffen Heegaard^{6,7}, Alina Lange¹, Tobias Berg¹, Vera Samoilova¹, Katharina Tiemann¹ and Igor Buchwalow¹

¹Institute for Hematopathology, Reference Center for Gynaeco- and Breast-pathology, Hamburg, Germany;

²Gerhard-Domagk-Institute of Pathology, University of Muenster, Münster, Germany; ³Department of Pathology, Sahlgrenska Cancer Center, University of Gothenburg, Gothenburg, Sweden; ⁴Albertinen Pathology, Hamburg, Germany; ⁵Institute for Pathology and Neuropathology, University Hospital Essen, Essen, Germany; ⁶Institute of Neuroscience and Pharmacology, Eye Pathology Institute, University of Copenhagen, København Ø, Denmark and ⁷Department of Ophthalmology, Glostrup Hospital, København Ø, Denmark

Salivary gland-like tumors of the breast show a great variety of architectural patterns and cellular differentiations such as glandular, myoepithelial, squamous, and even mesenchymal phenotypes. However, currently little is known about the evolution and cellular differentiation of these tumors. For that reason, we performed an *in situ* triple immunofluorescence lineage/differentiation tracing (*isTILT*) and qRT-PCR study of basal (K5/K14), glandular (K7/K8/18), and epidermal-specific squamous (K10) keratins, p63, and smooth muscle actin (SMA; myoepithelial marker) with the aim to construct and trace different cell lineages and define their cellular hierarchy in tumors with myoepithelial differentiation. *isTILT* analysis of a series of 28 breast, salivary, and lacrimal gland tumors, including pleomorphic adenomas ($n = 8$), epithelial-myoepithelial tumors ($n = 9$), and adenoid cystic carcinomas ($n = 11$) revealed that all tumor types contained K5/K14-positive progenitor cells in varying frequencies from a few percent up to 15%. These K5/K14-positive tumor cells were found to differentiate to glandular- (K8/18-positive) and myoepithelial-lineage (SMA-positive)-specific cells and were also shown to generate various heterologous cell differentiations such as squamous and mesenchymal progenies. p63 was co-expressed with K5/K14 in basal-like progenitor cells, myoepithelial, and squamous cells but not in glandular cells. Our results show that the corresponding counterpart tumors of breast and salivary/lacrimal glands have identical cellular compositions. Taken together, our *isTILT* and RNA-expression data indicate that look-alike tumors of the breast represent a special subgroup of basal-type tumors with benign or usually low malignant potential.

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A small proportion of benign and malignant tumors of the breast have a morphological appearance analogous to tumors of the salivary, lacrimal, and

sweat glands.^{1,2} Altogether these tumors account for about 1% of all breast cancers.³ In contrast, these tumors are common in the salivary glands, accounting for as much as 60% of the tumors.⁴ These look-alike tumors include pleomorphic adenomas, epithelial-myoepithelial tumors, adenoid cystic carcinomas, mucoepidermoid carcinomas, adeno-squamous carcinomas, and others. They are characterized by a remarkable morphological heterogeneity both between and within individual tumors of both sites. Thus, different architectural patterns and cell populations with different phenotypes can

Correspondence: Dr W Boecker, MD, Institute for Hematopathology, Immunohistopathology, Fangdieckstreet 75a, 22547 Hamburg, Germany.

E-mail: boecker@me.com

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be found including tumor cells showing glandular, myoepithelial, squamous, and mesenchymal phenotypes.^{4–8} However, the evolution of these lesions in both breast and salivary glands in terms of different lineage differentiations and cells of origin are still contradictory and poorly understood. Several authors have suggested that some of these tumors arise from a single cell with the potential for divergent differentiations.^{2,9,10} It has also been proposed that the myoepithelial cell might have a role in this context, at least in some of these lesions.^{2,11}

In order to investigate this further, we have now analyzed a series of salivary gland-like tumors of the breast and their salivary/lacrimal gland counterparts, including pleomorphic adenomas, adenoid cystic carcinomas, and epithelial-myoepithelial tumors, using *in situ* triple immunofluorescence lineage/differentiation tracing (*isTILT*) experiments, which allow direct observation of cells at different stages of differentiation. By tracing the sequential expression of basal (K5 and K14), glandular (K7 and K18, K8/18), and epidermal-specific squamous keratin (K10)^{12–14} in combination with p63^{15,16} and discriminatory myoepithelial markers such as SMA,^{7,17–19} we can infer the developmental capacities of K5/K14-positive tumor cells and construct differentiation lineage trees for these tumor types.

The data presented here illustrate the cellular organization of look-alike tumors with myoepithelial differentiation of the breast and salivary/lacrimal glands from a novel point of view. The full extent of K5/K14-positive tumor cells with their complex lineage differentiations can be appreciated and shown to be an important clue for the understanding of the evolution of these tumors. On the basis of our findings, we postulate that K5/K14-positive progenitor cells contribute to the development and differentiation of these lesions and thus confirm their basal type nature.

Materials and methods

Case Selection

Routinely processed formalin-fixed paraffin-embedded tissues from 8 pleomorphic adenomas, 9 epithelial-myoepithelial/myoepithelial tumors, and 11 adenoid cystic carcinomas of the breast and their counterparts of salivary/lacrimal gland origin were retrieved from the consultation files of the Department of Pathology of the Muenster University (WB) and Albertinen Pathology, Hamburg/Salivary Gland Registry, Hamburg (ThL). All cases were reviewed at least by two pathologists independently (WB, ThL) and classified according to previously published criteria described in the WHO Breast Tumor Classification from 2012^{5,7,8} and the WHO Classification of Head and Neck Tumors.^{20–26} Normal human breast tissue obtained from plastic surgery and normal salivary glands were used as controls.

Tissue Probe Processing and Primary Antibodies

Paraffin tissue sections (4 μ m thick) were pretreated and stained as described elsewhere.²⁷ For immunostaining, we used primary antibodies raised against keratin K5 (rabbit monoclonal, MEDAC Diagnostica), keratins K5/6, K14, K7, K8/18, K18 and K10 (mouse monoclonals, DAKO, Sigma and Dianova), SMA (rabbit polyclonal, Abcam), vimentin (mouse monoclonal, Abcam and DAKO), Ki-67 (Rabbit Monoclonal, Thermo Fisher Scientific), and p63 (DAKO). The exclusion of the primary antibody from the immunohistochemical reaction or substitution of primary antibodies with normal IgG (mouse or rabbit) at the same final concentration as that of the primary antibodies resulted in lack of immunostaining.

Bright-field Microscopy

Following the immunoreactions with primary antibodies, the sections were treated for 10 min with methanol containing 0.6% H₂O₂ to quench endogenous peroxidase. For bright-field microscopy, bound primary antibodies were detected with Dako LSAB REAL Detection System (Naphthol phosphate/Fast Red, no. K5005, Dako Corporation, Hamburg, Germany) or with AmpliStain horseradish peroxidase (HRP) conjugate (SDT GmbH, Baesweiler, Germany) according to manufacturers' instructions. The HRP label was visualized using the NovaRed substrate kit (Vector Laboratories, Burlingame, CA, USA). The sections were counterstained with haematoxylin. All steps were preceded by rinsing with PBS (pH 7.4).

isTILT Experiments

Systematic *isTILT*-experiments were performed for basal, glandular (luminal), and squamous keratins as well as for SMA. For these studies, we used secondary antibodies (purchased from Dianova and Molecular Probes) conjugated with Cy3, Alexa Fluor-488, Alexa Fluor-647, or with biotin.^{27,28} For simultaneous visualization of primary antibodies of the same IgG isotype, primary antibodies were non-covalently labeled with a reporter molecule *in vitro* employing monovalent IgG Fc-specific Fab fragments.²⁹ The reporter molecule was either fluorophore Cy3 or biotin. The latter was visualized using fluorophore-labeled streptavidin. Nuclei were counterstained with DAPI (5 μ g/ml PBS, 15 s), and sections were mounted with VectaShield (Vector Laboratories).

Image Acquisition

Immunostained sections were examined using a Zeiss microscope (Axio Imager Z1). Images were captured and processed using an AxioCam digital microscope

camera and the software AxioVision image processing (Carl Zeiss Vision, Germany). The images were imported as JPEG files into PhotoImpact 3.0 (Ulead Systems, Torrance, CA, USA) and submitted with the final revision of the manuscript at 300 DPI.

Quantitative RT-PCR (qPCR)

Areas of tumor tissue were manually microdissected from tissue sections (WB). Total RNA was extracted from the microdissected tissue using the RNeasy FFPE kit (Qiagen, Germany). mRNA of keratins 5,

14, 7, 8, 18 and 10, and ACTA2 (alpha 2 SMA) was reverse transcribed and amplified using the OneStep RT-PCR kit (Qiagen, Germany). RNA expression was quantified by real time fluorescence detection of amplified cDNA (ABI life technologies, StepOne system) using MGB-probes. Gene expression levels are described as ratios between two absolute measurements (gene of interest/*HPRT* endogenous reference). All samples were run in triplicate for all genes. We compared the differential expression of 6 keratins (K5/K14/K7/K8/K18/K10) and ACTA2 in tumor and normal breast parenchyma or nipple squamous epithelium.

Figure 1 Pleomorphic adenoma of the breast. (a) H/E staining of a typical tumor with chondromyxoid matrix. Normal breast tissue with lobules is shown on the right; (b) *isTILT* for K5/6 (red), K8/18 (green), and SMA (pink) showing the cellular heterogeneity of the tumor; a moderate number of K5/6-expressing cells (red, asterisks) and K8/18-positive cells (green, long arrows) which are typically located in anastomosing cords of more cohesive epithelial cells; Note that there is only a small number of SMA-expressing cells (hollow arrow). (c–e) Small area of (b) with single channels demonstrating glandular and myoepithelial differentiation with typical sequential expression of K5/6 (red) and K8/18 (green) in the glandular lineage and K5/6 (red) and SMA (pink) in the myoepithelial lineage. The expression of only K5/6 characterizes progenitor cells (asterisks). These cells show a sequential expression of K8/18 with occasional intermediary cells expressing both K5/6 and K8/18 (short arrow) and a number of K8/18-expressing differentiated glandular cells (long arrows). The hollow arrow denotes a myoepithelial cell expressing both K5/K14 and SMA, indicating the myoepithelial differentiation lineage. (f–i) Pleomorphic adenoma of the breast with focal squamous cell metaplasia. (f) H/E-stain with an epithelial nest with focal squamous differentiation. (g–i) *isTILT* for K5 (pink), K10 (red), and SMA (green) demonstrating squamous lineage differentiation. Merge (g) and single channels (h) for K5 (pink) and (i) for K10 (red). K5-staining occurs in peripheral progenitor cells. Note the gradual transformation of K5-progenitor cells (asterisks) at the periphery via K5 and K10 co-expression (arrows) to K10-positive squamous cells (hollow arrows).

Figure 1 Continued.

Cell Culture

Tumor cells from a lacrimal gland adenoid cystic carcinoma were maintained in culture as previously described.^{30,31} Briefly, tumor tissue was minced into small pieces and digested in a collagenase solution (1000 U collagenase/ml) (Sigma-Aldrich) for 60 min at room temperature. The tumor cell suspension was washed twice in culture medium and seeded out in 25 cm² flasks. Tumor cells from passage 15 were seeded out on 8-well chambers slides (Nunc) and allowed to attach overnight. On the next day, chambers were rinsed briefly with phosphate-buffered saline (PBS) and cells were fixed in methanol for 5 min at room temperature. Slides were air-dried for 60 min and subsequently subjected to immunostaining.

MYB-NFIB Fusion Gene Screening

Total RNA was isolated from cultured adenoid cystic carcinoma cells (passage 15) as previously described.³¹ DNase treated (DNA-freeTM; Ambion, Austin, TX, USA) total RNA was subsequently converted to cDNA using the SuperScriptTM First-Strand Synthesis System according to the manufacturer's manual (Invitrogen). The MYB-NFIB fusion transcripts were amplified by PCR using previously published primer sets.^{31,32} PCR products were gel-purified and sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Results

Pleomorphic Adenomas

Four pleomorphic adenomas of the breast, one of which was in a papillary lesion, and four salivary pleomorphic adenomas were analyzed. All tumors showed a characteristic histological appearance with anastomosing cords of epithelial cells and abluminal cells, often with a reticular pattern and spread of these cells into the surrounding 'mesenchymal tissue' with mucoid/myxoid and/or chondroid differentiation (Figure 1a and Supplementary Figure 1a).^{9,33–35} In three tumors (two parotid gland and one breast tumor), we found focal squamous cell metaplasia. Conventional immunohistochemical staining revealed a great variability (Table 1) and the dual nature of the tumors with epithelial and myoepithelial differentiation was confirmed.^{36,37} However, in *isTILT*-experiments the most remarkable finding in all cases was small groups of cells with basal keratins K5/K14 as the only expressed keratins (Figures 1b–e and Supplementary Figures 1b–d). These cells were also found to co-express p63. In some tumors, these progenitor cells amounted to 15% of the tumor cell mass. Furthermore, *isTILT* allowed us to follow the divergent differentiation lineages evolving from these K5/K14-positive cells. Thus, Figures 1b–e and Supplementary Figures 1b–d show triple immunostaining with antibodies for K5/6, K8/18 and SMA in pleomorphic adenomas of the breast and

Table 1 Immunophenotype of salivary gland-like tumors with myoepithelial differentiation

<i>Pleomorphic adenomas of the breast and the salivary gland</i>									
<i>Case</i>	<i>Organ</i>	<i>Immunophenotype</i>							<i>Remarks</i>
		<i>K5/6</i>	<i>K7</i>	<i>K8/18</i>	<i>K10</i>	<i>P63</i>	<i>SMA</i>	<i>Ki67</i>	
1	Breast	90%	90%	<5%	10%	90%	5%	1%	SCM
2	Breast	90%	NA	20%	Negative	70%	10%	5%	—
3	Breast	K5/6 40% K14 90%	80%	40%	Negative	70%	20%	5%	—
4	Breast	K5/6 60% K14 90%	60%	30%	Negative	60%	20%	15%	—
5	PG	90%	NA	10%	Negative	60%	10%	5%	—
6	PG	90%	80%	80%	5%	70%	10%	5%	SCM
7	PG	80%	60%	20%	Negative	60%	30%	1%	—
8	PG	80%	60%	5%	5%	60%	20%	1%	SCM
<i>Epithelial-myoepithelial tumors of the breast and the salivary gland</i>									
<i>Case</i>	<i>Organ</i>	<i>Immunophenotype</i>							<i>Remarks</i>
		<i>K5/6</i>	<i>K7</i>	<i>K8/18</i>	<i>K10</i>	<i>p63</i>	<i>SMA</i>	<i>Ki67</i>	
9	Breast	K5/6 40% K14 5%	30%	40%	Negative	70% (MEC)	70%	5%	AMT, biphasic SCM
10	Breast	80%	30%	30%	Negative	60%	50%	5%	AMT, biphasic, lobular type
11	Breast	70%	60%	50%	Negative	70%	60%	10%	AMT, biphasic; transition to myoepithelial carcinoma (K5/6 + , SMA + , p63 + , K8/18-)
12	Breast	K5/6 70%	NA	25%	Negative	60%	30%	1%	AMT, biphasic, lobular; associated with sclerosing lesion
13	Breast	90	NA	50	Negative	80%	60%	25%	AMT, spindle type, malignant
14	Breast	80%	20%	50%	Negative	50%	60%	5%	AMT, spinle cell type, malignant
15	PG	50%	40%	30%	Negative	50%	60%	10%	EMC;biphasic
16	PG	20%	50%	60%	Negative	40%	50%	10%	EMC;biphasic
17	Breast	80%	Negative	Negative	Negative	80%	70%	15%	Myoepithelial carcinoma
<i>ACC of the breast, salivary and lacrinmal gland</i>									
<i>Case</i>	<i>Organ</i>	<i>Immunophenotype</i>							<i>Remarks</i>
		<i>K5/6</i>	<i>K7</i>	<i>K8/18</i>	<i>K10</i>	<i>p63</i>	<i>SMA</i>	<i>Ki67</i>	
18	Breast	60%	90%	10%	Negative	15%, ^a	30%	10%	Tubular, cKit 70%
19	Breast	90%	NA	30%	Negative	90%, ^a	40%	<5%	Cribriform
20	Breast	10%	20%	50%	Negative	15%, ^a	20%	10%	Cribriform
21	Breast	30%	25%	40%	Negative	90%	60%	5%	Associated AMT, biphasic, lobular, cKit 50%
22	Breast	90%	20	30%	Negative	70%	10%	10%	cKit 30% tubular
23	Breast	80%	10%	30%	NA	60%	50%	15%	cKit 30%
24	Palate	60%	40%	10%	NA	5%	70%	10%	Cribriform
25	Palate	50%	90%	80%	Negative	80%	80%	15%	cKit 40%
26	LG	40%	80%	40%	Negative	60%	60%	25%	cribriform
27	LG	40%	60%	Negative	Negative	70%	80%	<10%	BM.type
28	LG	20%	10	Negative	Negative	90%	70%	<10%	cribriform

Abbreviations: ACC, adenoid-cystic carcinoma; AMT, adenomyoepithelial tumor; EMC, epithelial-myoepithelial carcinoma; GC, Glandular cells; LG, lacrimal gland; MEC, myoepithelial cells; NA, not analyzed; PG, parotid gland; SCM, squamous cell metaplasia.

^aNotes: periphery.

salivary gland. Clusters of K5/6-positive cells were identified. In addition, there were also a number of cells with coexpression of K5/6 and K8/18 (intermediary glandular cells) as well as differentiated cells that only expressed the glandular-specific keratins 8/18 (Figures 1c and d). A similar sequential expression of K5 or K14 and other lineage markers could also be observed in the myoepithelial (Figures 1a and d and Supplementary Figures 1c and d), squamous (Figures 1f–i), and mesenchymal lineages (Supplementary Figure 2). p63-expression was seen only in the myoepithelial and squamous lineages and not in the glandular lineage.

Epithelial-myoepithelial Tumors

We analyzed six epithelial-myoepithelial tumors of the breast and two of the salivary glands. Four breast tumors and two salivary gland tumors showed the typical biphasic pattern with prominent myoepithelial proliferations intermingled with glandular

structures (Figure 2a and Supplementary Figure 3a). Myoepithelial cells with spindled appearance and clear or eosinophilic cytoplasm were evenly distributed in the background. Immunohistochemically, a multilayering of myoepithelial cells was usually observed outside of the glandular cells (Supplementary Figures 3b–d), which were partly surrounded by a deposition of collagen IV- and laminin-positive basement membrane (data not shown). In other cases, we observed a solid proliferation of myoepithelial cells between glandular spaces with irregular formation of basement membrane-like material between the tumor cells (Supplementary Figure 3e). In *isTILT* experiments, we observed in all tumors K5/K14-positive progenitor cells amounting to about 1% of the tumor cell population (Figures 2c and d and Supplementary Figure 4). Furthermore, a sequential expression of K5/K14 and glandular lineage markers K8/18 in glandular cells and K5/K14 and the myoepithelial marker SMA in spindle cells were observed in all

Figure 2 Adenomyoepithelial tumor of the breast. (a) H/E stain of a biphasic tumor with some tubular structures in the lower part of the figure and a predominant proliferation of polygonal myoepithelial cells with clear cytoplasm. Note the reticular basement membrane-like material between the tumor sheets. (b–e) *isTILT* for basal keratin K14 (red), glandular keratins K8/18 (green), and SMA (pink) demonstrating glandular and myoepithelial differentiation. (b) Merge image. (c–e) Single channels at higher magnification showing glandular structures with expression of glandular keratins 8/18 (green), surrounding spindle cells expressing K14 (red), and SMA (pink). A few cells express only K14 indicating that they are progenitor cells (asterisk). The glandular cells co-express K14 and glandular keratins 8/18 (arrows). There are also cells co-expressing K14 and SMA (hollow arrow) but many myoepithelial cells only express SMA. The sequential expression of basal- and lineage-specific markers indicates the lineage differentiation from K14-positive progenitor cells. (f–i) Adenomyoepithelial tumor of the breast with focal squamous metaplasia. *isTILT* for K14 (f, green), K10 (g, Cy3), and K5 (h, pink). (i) Merge image. Nuclei are counterstained with DAPI. Note the transition of K5- and/or K14-positive cells to K10-positive squamous cells. Inset in (g): H/E-staining of squamous cell metaplasia in an otherwise typical biphasic AME.

Figure 2 Continued.

tumors investigated (Figures 2c–e and Supplementary Figures 4c–e), indicating the evolution of these two cell lineages from K5/K14 progenitor cells. The same staining pattern could be observed in two cases of malignant adenomyoepithelioma of the breast of the spindle cell type (Supplementary Figure 5). Interestingly, the two adenomyoepitheliomas with spindle cell pattern contained a number of hybrid-cells co-expressing keratins K5/K14, K8/18, and the myoepithelial marker SMA as well as p63. One case of adenomyoepithelioma displayed squamous differentiation evolving from K5 and/or K14-positive progenitor cells (Figures 2f–i).

Myoepithelial Carcinoma (Malignant Myoepithelioma)

We have found only one convincing case classified as a pure myoepithelial carcinoma, which was included in this series. Monophasic tumors with additional glandular differentiation, as identified immunohistochemically by K8/18 expression, were classified as monophasic adenomyoepitheliomas (see above). The case with pure myoepithelial differentiation contained K5/14-positive cells, which coexpressed the myoepithelial marker SMA

(Supplementary Figure 6). p63 was also highly expressed in these lesions. No other lineage differentiation was observed.

Adenoid Cystic Carcinoma

Six primary adenoid cystic carcinomas of the breast-, and two salivary and three lacrimal adenoid cystic carcinomas were analyzed. All cases displayed a typical cribriform pattern dominated by basal-like cells. Many pseudoglandular lumina with eosinophilic alcian blue-positive globules and true glandular lumina containing H/E basophilic and diastase-PAS-positive secretions were found. The immunohistochemical profile observed in all cases included positivity for K5 and K14, SMA, K7/K8/18, and p63 (Table 1). The glandular keratins K7/K8/18 were focally found in glandular structures. The glands were nearly always p63-negative, but a number of glandular cells expressed K5/K14. All tumors displayed myoepithelial differentiation, as indicated by coexpression of K5/K14, p63 and SMA. Using *isTILT*, the cellular hallmark of these tumors was a mixture of few K5/K14-positive progenitor cells (about 1% of the tumor cells) in a bulk of cells

differentiating into myoepithelial cells (positive for K5/K14/SMA/p63) and focally into glandular cells (sequential expression of K5/K14 and K7/K8/18) (Figures 3a–d). The myoepithelial differentiation was observed in basal-like cells and in cells in the vicinity of pseudoglandular spaces that were filled with collagen IV- and laminin-positive hyaline basement membrane-like globules or membranes.

We also studied the cellular composition and differentiation of cultured tumor cells from a lacrimal gland adenoid cystic carcinoma as well as from the primary tumor from which the cells were derived. The cultured cells had a typical epithelial morphology with small to medium-sized, rounded or cuboidal cells. Both the primary tumor and the cultured cells expressed the adenoid cystic carcinoma-specific *MYB-NFIB* gene fusion (data not shown). *isTILT*-analysis revealed that the cultured adenoid cystic carcinoma cells showed the same cellular differentiation pattern as the primary tumor (Figures 3e–h and Supplementary Figure 7).

Normal Breast and Salivary Gland Epithelium

Based on the assumption that the cellular features of these tumors reflect (mimic) the differentiation potential of their cells of origin, we analyzed the

epithelium in both organs using the *isTILT* experiments described above.

The ducts of both glands contained K5/14-positive progenitor cells, which differentiated to glandular cells (Supplementary Figure 7). However, the interlobular ducts of the breast contained a luminal layer showing a mixture of K5 and/or K14-positive progenitor cells, intermediary glandular cells (K5/K14 and K7/K8/18) and glandular cells (only K7/8/18). In contrast, in striated ducts of the salivary gland, K5/K14-positive progenitor cells could be found only in a basal position which co-expressed p63. The luminal layer contained glandular cells expressing K7 and K8/18. Occasionally, a sequential expression of glandular keratins K7/K8/18 and basal keratins 5/14 could be observed in striated ducts with formation of intermediary (K5/K14 and K7/K8/18) and glandular cells (K7/8/18 only). These data clearly speak for a differentiation pathway of glandular duct cells from K5/K14-positive progenitor cells.

qPCR Analyses

The expressions of basal (K5/K14) and glandular (K7/K8/K18) keratins, epidermal specific squamous keratin (K10), and ACTA2 were studied by qPCR in seven tumors with myoepithelial differentiation. As

Figure 3 Adenoid cystic carcinoma of the lacrimal gland. *isTILT* for keratins K5 (green), K7 (red), and SMA (green). (a) Merge image. (b–d) Single channels showing a smaller number of tumor cells co-expressing K5 (green) and K7 (red). The bulk or tumor cells co-express K5 (green) and SMA (pink). Occasionally, cells are observed that only express K5 (arrows). The sequential expression of K5 and K7 on one side and K5 and SMA on the other side indicates the evolution of glandular and myoepithelial cells from K5-positive progenitor cells. (e–h) Cultured tumor cells derived from the adenoid cystic carcinoma shown in (a) demonstrating one K5-positive cell (arrow), and cells co-expressing K5 and K7 (tailed arrows). One cell shows K5 and a weak co-expression of both K7 and SMA (hollow arrow).

Figure 3 Continued.

shown in Figure 4, we found upregulation of mRNA of both basal and glandular keratins in all tumors compared with normal breast parenchyma. Similarly, the expression of ACTA2 was lower in the tumors compared with normal breast tissue with the exception of one adenomyoepithelioma (lobulated type) and two adenoid cystic carcinomas that showed the highest levels of ACTA2 expression (cases 9, 19 and 23 in Table 1). The expression of the epidermal-specific squamous keratin 10 was, compared with nipple epidermis, very low with a moderate level only in one pleomorphic adenoma of the parotid gland (case 6 in Table 1), which displayed squamous differentiation in H/E and K10 expression in immunostaining. When comparing the RNA and protein expression levels, we found that the RNA-levels of basal and glandular keratins were positively correlated with the immunohistochemical findings. Thus, the RNA data corroborated the immunohistochemical findings implying that these tumors represent basal type tumors with glandular and myoepithelial differentiation.

Discussion

Here, we present a systematic *isTILT* and qPCR study of a series of look-alike tumors of the breast and salivary/lacrimal glands with myoepithelial differentiation. In agreement with previous observations, our findings revealed the dual epithelial myoepithelial nature of these lesions,^{3,38} including expression of glandular (luminal) keratins K7/K8/18, and myoid markers such as SMA, maspin, CD10, calponin, basal keratins K5/K14, and p63.^{16,38–44} Using *isTILT*, we could demonstrate the cellular organization of these tumors from a novel point of view. Thus, we identified neoplastic cells in all lesions that only expressed the basal keratins K5 and K14. These cells varied in frequency from only a few percent in adenoid cystic carcinomas and up to 15% in pleomorphic adenomas and in epithelial-myoeplithelial tumors. The K5/K14-positive cells co-expressed p63, and, when they lacked specific lineage markers, they were regarded as neoplastic progenitor cells. Moreover, we provide evidence that

Figure 4 mRNA expression of basal keratins K5 and K14, luminal keratins K7, K8, and K18, squamous keratin K10, and ACTA2 in salivary gland-like tumors with myoepithelial differentiation. The expression of basal and luminal keratins are higher in all tumors tested compared with normal breast parenchyma (zero line). In contrast, the expression of ACTA2 is, with the exception of one adenomyoepithelioma of the breast, lower in the tumors compared with normal breast tissue (zero line).

in the glandular and myoepithelial lineages the basal keratins K5/K14 and the corresponding lineage marker (K8/18 or SMA) are sequentially expressed during differentiation. Eventually, the expression of K5/K14 is downregulated in these cells and only the

lineage specific protein is expressed. Thus, our data strongly indicate that the glandular and myoepithelial cell lineages in these tumors are derived from K5/K14/p63-positive neoplastic progenitor cells. These data raise doubts about the

Figure 5 Lineage differentiation model of look-alike tumors with myoepithelial differentiation showing that K5/K14-positive tumor cells are key players in these tumors. K5/K14-positive tumor cells differentiate to glandular and myoepithelial cells. Notably, these cells also give rise to squamous and mesenchymal cells in salivary gland-like breast tumors.

current concept that in these lesions the luminal cell types arise from differentiation of myoepithelial cells.^{45,46} In one case of lacrimal gland adenoid cystic carcinoma, we had access to both cultured MYB–NFIB fusion-positive adenoid cystic carcinoma-cells and the corresponding primary tumor. The immunofluorescence stainings of the cultured cells fully corroborated the results of the primary tumor. They also confirmed the results of our *isTILT*-experiments, which revealed glandular-myoeplithelial hybrid cells with co-expression of both glandular keratins K7/K8/18 and myoid markers such as SMA. Interestingly, the myoepithelial differentiation was usually associated with the formation of collagen IV- and laminin-positive basement membrane material, which has been shown to be a diagnostically important feature in adenoid cystic carcinoma.⁴²

It is well known that look-alike tumors with myoepithelial differentiation of both the breast and salivary glands may show areas of mesenchymal, squamous, and/or sebaceous differentiation.^{41,47–50} It has been suggested that squamous and mesenchymal elements are derived from myoepithelial cells.⁵¹ Using *isTILT*, we found evidence for the origin of the K10-positive squamous lineage from K5/K14-positive progenitor cells, indicating striking similarities to normal squamous differentiation. Finally, sequential expression of K5/K14 and

vimentin as mesenchymal marker in tumor cells of pleomorphic adenomas (in the absence of SMA or other myoepithelial markers) with chondroid changes, support the view that K5/K14-positive cells gradually differentiate to mesenchymal tissues in these tumors. These observations are in line with the view that pleomorphic adenomas arise from a single-cell type capable of divergent differentiation.⁹ We believe that our findings might also explain how at least a part of metaplastic carcinomas with extracellular matrix of the breast may develop.

Based on the data discussed above, we propose a lineage differentiation model of look-alike tumors of the breast and salivary glands with myoepithelial differentiation (Figure 5) using the epithelial cell models of human breast epithelium,^{52–55} eccrine gland ducts,⁵⁶ and squamous epithelium,⁵⁷ as a reference. All our tumors contained K5/K14-positive tumor cells that seem to constitute the progeny cells of the different cell lineages observed in these tumors. Interestingly, K5/K14-positive tumor cells corresponded to K5/K14-positive normal cells of large/nipple and striated/interlobular ducts of breast/salivary gland as well as to the basal layer of squamous epithelium (Boecker *et al*, manuscript in preparation).

However, despite the similarities between these look-alike tumors of the breast and salivary gland, many questions remain unresolved. Thus, most epithelial–myoepithelial tumors of the breast⁵⁸ are thought to possess only a borderline malignant potential,^{43,59–62} whereas the same tumor in salivary glands, known as epithelial myoepithelial carcinoma, are clearly malignant.^{45,63–66} Pure myoepithelial tumors have only rarely been reported in the breast^{3,67–71} and account for about 3% of all tumors of salivary glands.^{72–76} The differential diagnosis include spindle cell tumors with glandular^{72,77–79} and/or squamous differentiation,⁶⁷ spindle cell adenocarcinomas,^{80–84} and mesenchymal tumors.³⁷ Here, we have only studied one malignant myoepithelioma of the breast, which expressed K5/K14 and the myoid marker SMA, but not, or only focally and rather feebly, glandular keratins K7/K8/K18 and/or keratin K10.

Adenoid cystic carcinomas of both the breast and salivary glands are morphologically, immunophenotypically (see above), and genetically very similar. Thus, they are both characterized by the same recurrent t(6;9) translocation resulting in the MYB–NFIB gene fusion.^{31,85} Overexpression of the MYB oncogene is a hallmark of adenoid cystic carcinoma irrespective of anatomical location.^{32,85,86} Despite these similarities, there are important differences between these tumors. Thus, adenoid cystic carcinomas of the breast are very rare (0.1–1% of all breast cancers³⁸) and have an excellent prognosis whereas adenoid cystic carcinomas of the salivary glands account for about 10% of epithelial tumors of the salivary glands and are a highly aggressive disease with a high recurrence rate and long-term

poor survival.^{87,88} The differences in clinical behavior are only partly known. Recent studies using high-resolution array-CGH have shown that adenoid cystic carcinomas of the breast have very few or no recurrent copy number alterations in line with their non-aggressive clinical appearance.⁸⁹ In contrast, salivary gland adenoid cystic carcinomas have multiple recurrent copy number alterations some of which are associated with a poor prognosis.^{90,91}

Dedifferentiation and progression to high-grade lesions (no specific type grade 3, malignant myoepithelial, or of mixed epithelial-myoepithelial type) have been described both in salivary gland and breast tumors.^{59,71,92–97} It has been suggested that new genetic alterations gradually accumulate in subclones of these tumors leading to clonal evolution and disease progression.^{31,90–92} Based on our proposed lineage differentiation model (Figure 5), it seems likely that in addition to specific genetic alterations, the cell lineage and the stage of differentiation of the cells undergoing ‘dedifferentiation’ are important factors that might influence this process.

A key to the understanding of proliferative diseases is the knowledge about the corresponding normal tissues. Our *isTILT* studies revealed that normal breast and salivary glands show striking similarities in their cellular composition. Both glands contain K5/K14/p63-positive progenitor cells, which give rise to glandular cells with subsequent downregulation of basal keratins/p63 and upregulation of glandular keratins K7/K8/18. Furthermore, it has been demonstrated for breast tissue that the myoepithelial cells originate (embryologically, in transplantation studies, and during physiological regeneration) from K5 and/or K14-positive progenitors^{52,53,55} that co-express p63.¹⁶ Our *isTILT* data provide support for similar cellular differentiation processes in the salivary gland (Boecker *et al*, manuscript in preparation). Based on these observations, we suggest that this unique population of K5/K14- and p63-positive progenitor cells of breast, salivary, and lacrimal glands form a core cell population that can spawn these different tumor phenotypes.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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