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Age-Related Dysfunction in Mechanotransduction Impairs Differentiation of Human Mammary Epithelial Progenitors

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SUMMARY

Dysfunctional progenitor and luminal cells with acquired basal cell properties accumulate during human mammary epithelial aging for reasons not understood. Multipotent progenitors from women aged <30 years were exposed to a physiologically relevant range of matrix elastic modulus (stiffness). Increased stiffness causes a differentiation bias towards myoepithelial cells while reducing production of luminal cells and progenitor maintenance. Lineage representation in progenitors from women >55 years is unaffected by physiological stiffness changes. Efficient activation of Hippo pathway transducers YAP and TAZ is required for the modulus-dependent myoepithelial/basal bias in younger progenitors. In older progenitors, YAP and TAZ are activated only when stressed with extraphysiologically stiff matrices, which bias differentiation towards luminal-like phenotypes. In vivo YAP is primarily active in myoepithelia of younger breasts, but localization and activity increases in luminal cells with age. Thus, aging phenotypes of mammary epithelia may arise partly because alterations in Hippo pathway activation impair microenvironment-directed differentiation and lineage specificity.

INTRODUCTION

The aging process is often correlated with changes in stem cell activity with consequences ranging from reduced regenerative capacity to increased cancer incidence. Human hematopoietic stem cells accumulate with age (Kuranda et al., 2011; Pang et al., 2011) and exhibit a differentiation bias toward defective myeloid lineages (Cho et al., 2008), making individuals more prone to autoimmune problems and myeloid leukemias (Henry et al., 2011). In mice, the proportion of mitotic neural stem cells increases with age, whereas numbers of adult-born neurons decrease (Stoll et al., 2011). Human hippocampus shows patterns of age-related changes similar to mice that may underlie age-related cognitive decline (Knoth et al., 2010). Transit amplifying cells, not stem cells, accumulate in epidermis with age and delay wound healing (Charruyer et al., 2009). Mammary epithelium is maintained by a hierarchy of lineage-biased and multipotent progenitor and stem cells (Nguyen et al., 2014; Rios et al., 2014; Villadsen et al., 2007). In human mammary gland, differentiation-defective cKit-expressing multipotent progenitors (MPPs) accumulate with age, and proportions of daughter myoepithelial (MEP) and luminal epithelial (LEP) cells shift with age. We hypothesized that these age-associated changes make aged breast tissue susceptible to malignant progression (Garbe et al., 2012). Accumulation of defective stem or progenitor cells may be a common phenotype among aging tissues, and we hypothesize that aged MPPs accumulate because they do not correctly perceive microenvironmental differentiation cues.

The molecular composition of microenvironments impose specific cell fate decisions in normal and immortal nonmalignant mammary MPP (LaBarge et al., 2009). Cell culture substrata tuned to elastic moduli that mimicked normal breast tissue also biased the differentiation of an immortal nonmalignant MPP cell line into LEP (Lui et al., 2012). Matrix stiffness is mechanistically important in breast cancer progression as well; rigid breast tissue correlates with high breast cancer risk and drives malignant phenotypes in breast cancer cell lines (Yu et al., 2011). The physiological range of elastic modulus in breast likely plays an instructive role in the differentiation of normal mammary epithelial progenitors.

Membrane and cytoskeleton proteins sense mechanical cues and trigger transduction cascades that relay information throughout the cytoskeleton and to the nucleus. Responses can include changes in morphology and gene expression (Vogel and Sheetz, 2006). Sensing matrix elasticity occurs through cell-cell and cell-extracellular matrix (ECM) interactions mediated by adherens, integrins, vinculin, focal adhesion kinase (FAK), and others (Beningo et al., 2001; Bershadsky et al., 2003; Tamada

et al., 2004). The actinomyosin network includes RhoA, which regulates the actin cytoskeleton in the formation of stress fibers (SFs) and focal adhesions (FAs). Activation of ROCK1/ROCK2 causes increased activity of the motor protein myosin II by phosphorylation of the myosin light chain (MLC) and inactivation of the MLC phosphatase (Ishizaki et al., 1997; Kimura et al., 1996). YAP and TAZ are Hippo pathway transcriptional coactivators that are thought to interact with the Rho pathway to transduce mechanical information about the microenvironment to the nucleus (Halder et al., 2012). As stiffness increases, YAP/TAZ relocates from cytoplasm into nucleus, where they generate gene expression patterns that underlie cellular functions like proliferation, migration, epithelial-to-mesenchymal transition, and differentiation (Dupont et al., 2011; Kanai et al., 2000; Zhao et al., 2007).

Differentiation of mesenchymal stem cells down neurogenic, myogenic, or osteogenic pathways was directed by exposure to a wide range of tissue-relevant elastic moduli, from 100 to ~40,000 Pa (Engler et al., 2006). In comparison, mammary MPP differentiation should be responsive to a much narrower range of modulus relevant to normal and malignant breast (100~4,000 Pa; Paszek et al., 2005). The impact of aging on modulus-directed differentiation is unknown. Addressing these issues required a culture-based platform for functional analyses of primary normal human mammary MPP from many individuals. Here, we used such an approach to demonstrate that differentiation patterns of MPP from women aged <30 years cultured on tunable 2D and 3D substrata were exquisitely responsive to a physiologically relevant range of elastic modulus in a YAP/TAZ-dependent manner, whereas MPP from women >55 years were relatively unresponsive to changes in rigidity due to inefficient activation of the Hippo pathway transducers.

RESULTS

Aging Alters Modulus-Dependent Differentiation

To test whether microenvironment rigidity directed differentiation in MPP, we enriched receptor tyrosine kinase cKit-expressing (cKit+) human mammary epithelial cells (HMECs) by flow cytometry (fluorescence-activated cell sorting [FACS]) from fourth passage primary prestasis HMEC (strains) derived from five women aged <30 years and five from women >55 years (Figure S1; Table S1). Prestasis HMEC strains are normal and not treated with any immortalizing agents, have finite lifespans, and we previously demonstrated that they retain molecular, biochemical, and functional properties consistent with chronological aging in vivo (Garbe et al., 2012). The cKit+ MPP were cultured for 48 hr on type 1 collagen-coated polyacrylamide (PA) gels. The Young's elastic modulus (E[Pa]scals) of the PA gels was tuned from 200 Pa to 2,350 Pa. The lineage of each daughter cell was confirmed by immunofluorescence (IF) of intermediate filament proteins keratin (K)14 and K19, CD227 (sialomucin-1), and CD10 (Calla; Figures 1A, 1B, 1E, and 1F). Computer image analysis identified the different lineages; LEP are $CD227^+/CD10^-/K14^-/K19^+$, MEP are $CD227^-/CD10^+/K14^+/K19^-$, and $K14^+K19^+$ expression is consistent with MPP states (Villadsen et al., 2007). cKit+ MPP from women <30 years generated proportionately more LEP on soft substrata, but generation of MEP increased with higher E (Figures 1C, 1C', 1G, and S2A). cKit+ MPP from women >55 years did not generate different lineage proportions in response to changes in E (Figures 1D, 1D', 1H, and S2B). Primary first passage cKit+ MPP from three women <30 years, embedded in tunable 3D hydrogels with some type 1 collagen for 7 days (Figure S3A; Ananthanarayanan et al., 2011), gave rise to more LEP at 120 Pa versus more MEP at 3,800 Pa (Figures 1K, 1M, 1M', and S3B). In contrast, cKit+ MPP from three women >55 years did not display modulus-dependent differentiation patterns (Figures 1L, 1N, 1N', and S3C). The proportion of $K14^+/K19^+$ MPP decreased with elastic modulus on 2D PA gels and in 3D gels <30 years HMEC (Figures S4A–S4C), but no change in proportions of MPP was observed in >55 years HMEC (Figures S4B, S4D, and S4F). These results suggested that differentiation was modulus dependent in younger MPP and that this response was lost with age.

To test if changes in lineage proportions were due to lineage-biased proliferation, incorporation of 5-ethynyl-2′-deoxyuridine (EdU) into DNA was measured as a proxy for proliferation. All lineages derived after 48 hr from cKit+ MPP on PA gels exhibited similar proportions of EdU incorporation (EdU^+), irrespective of substrate rigidity or age (Figure 1I). In contrast, EdU incorporation in unsorted HMEC strains, which are primarily composed of more mature LEP and MEP, revealed age- and lineage-specific replicative behaviors. Proportions of EdU^+ MEP from <30 years HMEC significantly increased with greater E whereas EdU^+ LEP trended downward (Figure 1J). Unsorted HMEC from women >55 years exhibited neither lineage- nor modulus-dependent proliferation (Figure 1J), underscoring the lack of mechanoresponse with age. Thus, changes in lineage proportions exhibited by <30 years MPP after 48 hr were likely due to modulus-dependent differentiation, and only after the lineages matured in <30 years HMEC strains, did they show evidence of modulus-dependent proliferation.

Mechanosensing Functions Were Unaltered by Age

To determine whether mechanosensing was age dependent, F-actin SF formation and FA assembly activities were measured in cKit+ MPP from three <30 years and three >55 years strains. Irrespective of age, SF formation increased with greater E (Figure 2A). Homogeneity measurements were used to quantify formation of the F-actin cables (Haralick et al., 1973; Pantic et al., 2012); SF homogeneity was inversely proportional to E in both age groups, which showed similar slopes (Figures 2B and 2C). Progenitors from both age groups were stained for pFAK and vinculin, which colocalize at FA. More FA assemblies were observed with increased E at the interfaces of MPP and gels, and FA formation was not impaired with age (Figures 2D and 2E). FA homogeneity was inversely proportional to E with comparable slopes in both age groups (Figures 2F and 2G). Thus, similar SF and FA phenotypes were observed both in younger and older cKit+ MPP.

Extracellular signal-regulated kinase (ERK) is phosphorylated in response to increased elastic modulus in some adherent cell lines (Provenzano et al., 2009), and because it is a key effector of serum responses, changes in its modulation could cause pleiotropic cellular responses. Both young and old cKit+ MPP exhibited a low level of ERK phosphorylation on 200 Pa PA