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EGF-receptor regulates salivary gland branching morphogenesis by supporting proliferation and maturation of epithelial cells and survival of mesenchymal cells

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ABSTRACT

Epidermal growth factor receptor (EGF-R) regulates epithelial morphogenesis during development and is important for the proper branching of the lung, mammary gland, and pancreas. We analyzed the salivary gland phenotype of EGF-R-deficient mice and showed impaired growth, branching, and maturation of the epithelium. Furthermore, treatment of wild-type E13 salivary glands with gefitinib, a small molecular inhibitor of EGF-R, led to apoptosis of the mesenchyme. Interestingly, MMP2 and plasminogen activators were upregulated upon inhibition of EGF-R signaling. To summarize, we show that EGF-R is a physiological regulator of salivary gland development and its main function is to support the proliferation and maturation of the epithelium and the survival of the mesenchyme.

1. Introduction

Epidermal growth factor receptor (EGF-R) is an evolutionary conserved tyrosine kinase receptor known to be involved in epithelial morphogenesis from *Caenorhabditis elegans* to man (reviewed in Citri and Yarden (2006)). The binding of EGF family ligands [amphiregulin (AR), betacellulin (BTC), EGF, epigen (EPG), epiregulin (EPR), heparin-binding EGF (HB-EGF), and transforming growth factor-α (TGF-α)] to EGF-R induces homo- or heterodimerization with the structurally related family of erbB homologs and activates the ERK-1/2-, PI3-kinase, and PLC-gamma pathways (Holbro and Hynes, 2004). Depending on the cell type, this may lead to cell proliferation, migration, and protection from apoptosis. Interestingly, the phenotypes of mice with targeted inactivation of the various EGF ligands are fairly mild, suggesting a great level of redundancy in their functions (Mann et al., 1993; Troyer et al., 2001; Luetteke et al., 1999). Nevertheless, the developmental role of EGF-R is crucial, since mice lacking EGF-R either die before implantation, either embryonically or soon after birth (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). The EGF-R (−/−) mice suffer from various defects, particularly in organs developing through branching morphogenesis (i.e. the lung, pancreas, and mammary gland) (Miettinen et al., 1995, 1997, 2000).

The salivary gland also develops through branching morphogenesis. Morphogenesis of the submandibular salivary gland (SMG) begins at the age of embryonic day 12 in mice, when an epithelial stalk invaginates into the mesenchyme (Borghese, 1950; Jaskoll and Melnick, 1999; Patel et al., 2006). The growing stalk begins to branch on embryonic day 13 (E13) forming three buds. These grow with continuous proliferation throughout the epithelium. Thereafter, branching morphogenesis rapidly proceeds through repeated clefting, budding, and branching. By E15, the epithelium has formed a tree-like structure filling the mesenchyme. In this stage, the first alveolar and acinar structures form in the buds, eventually leading to differentiated glycoprotein-producing and -conducting structures.

Reciprocal signaling between epithelial–mesenchymal components is essential for proper branching morphogenesis (Sakakura et al., 1976; Nogawa and Mizuno, 1981; Nogawa, 1983). Exact molecular mechanisms are unknown, but the role of basement membrane components, including laminins and fibronectin

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(Kadoya and Yamashina, 2005; Larsen et al., 2006) and many signaling pathways, including FGF- and EGF-receptor signaling, have been implicated in this process (Kashimata and Gresik, 1997; Umeda et al., 2001; Hoffman et al., 2002; Steinberg et al., 2005). EGF-R is expressed in the developing salivary gland epithelium (Gresik et al., 1997; Miyazaki et al., 2004) together with most EGF family growth factors. Inhibition of EGF-R signaling in organ cultures impairs branching (Kashimata and Gresik, 1997), but the *in vivo* effects have not been described in detail. Koyama et al. (2003) showed that EGF-R triggers salivary gland branching through ERK-1/2 and PI3K pathways but it is also able to inhibit branching by activation of PKC signaling. EGF-R also regulates the expression of α6-integrin in the fetal mouse salivary glands of mice, suggesting that EGF-R may modulate extracellular matrix (ECM) interactions (Kashimata and Gresik, 1997).

Using EGF-R-deficient mice and gefitinib, a small-molecular EGF-R inhibitor, we studied the role of EGF-R in salivary gland development. In this paper, we show that functional EGF-R is crucial for proper salivary branching morphogenesis. In particular, EGF-R signaling supports the survival of mesenchymal cells, which produce ECM required for branching.

2. Results

2.1. Impaired branching and delayed differentiation after EGF-R inactivation

Macroscopically, the newborn EGF-R (−/−) salivary glands (SMG) were slightly, but not significantly smaller than the wild-type salivary glands (SMG). To analyze the effect of EGF-R inactivation on salivary gland branching, we analyzed the SMGs using routine histological and epithelium-specific cytokeratin K8 (CK8) staining. On embryonic day 15 (E15), the wild-type salivary glands expressed CK8 only in the linings of the ducts and developing alveoli while most of the developing epithelium failed to express it (Fig. 1A–B). However, the epithelial buds could still be identified morphologically from the mesenchyme (i.e. they were closely packed and formed well-defined branches). From E15 to the newborn stage (postnatal day 0), progression from the pseudocanalicular stage to the terminal bud stage (Jaskoll and Melnick, 1999), CK8 expression spread from the proximal parts toward the distal buds (Fig. 1C–F), and could be detected at P0 in nearly every epithelial cell, even in the most distal parts. In the EGF-R (−/−) SMG, however, general development was slower than the wild-type SMG and on E15, only a few buds expressed CK8. The EGF-R (−/−) SMG contained significantly fewer CK8-positive end buds per all buds compared to the wild-type (46% vs. 66%, $p<0.001$; Fig. 1G). Even at P0, several EGF-R (−/−) buds were CK8-negative. Furthermore, tracing the epithelium from the total gland area clearly showed that the ratio of epithelium to mesenchyme was reduced by 20–30% in the EGF-R (−/−) salivary glands at all of the time points studied (Table 1 and Fig. 1). To conclude, the data suggest delayed and reduced branching morphogenesis after EGF-R inactivation.

To better characterize the EGF-R (−/−) salivary phenotype, we performed organ culture experiments. SMG development is well characterized in *in vitro* settings (Borghese, 1950; Kashimata and Gresik, 1997; Larsen et al., 2006). For this purpose, E13 salivary glands (early canalicular stage) from EGF-R (−/−) embryos and their wild-type littermates were dissected and cultured for 48 h. After the culture period, the glands were stained with anti-CK8, and the number of end buds per gland calculated. Again, we observed a significant 26% reduction in the number of the EGF-R (−/−) end buds compared to the wild-type salivary glands (21 ± 1.4 vs. 28 ± 1.7; $p<0.005$; Fig. 2). Thus, due to the loss of both the number of branches and the size of the epithelium, the secretory capacity of the developing gland will be reduced even more.

To further elucidate the mechanisms of EGF-R-regulated branching morphogenesis, we performed organ culture experiments in which EGF-R was inhibited with gefitinib, a specific EGF-R inhibitor that is also used in clinical oncology (Denny, 2001). With this approach, the inactivation of EGF-R can be timed to the early stages of branching morphogenesis, and its systemic effects can be avoided. E13 SMGs were cultured in serum-free conditions with 10 or 20 μM gefitinib added to the medium, and we again counted the number of end buds after 2 days, and then processed the glands for histological analysis. Gefitinib impaired branching both dose and stage dependently (Fig. 3). Compared to control cultures with no inhibitor, the lower concentration of gefitinib (10 μM) reduced the number of end-buds at E13+2 SMGs to 42% (116 ± 27 vs. 277 ± 43, $p<0.01$). A 20 μM concentration of gefitinib further reduced the number of end buds to 26% (73 ± 17 vs. 277 ± 43, $p<0.001$). On E12.5, however, this higher concentration either completely stopped branching morphogenesis or destroyed the epithelium. Compared to controls, the lower concentration (10 μM) of gefitinib reduced the number of end buds to 24% (16 ± 4 vs. 66 ± 19, $p<0.05$). At E15-E16, when the salivary gland is in the late canalicular stage, EGF-R inhibition did not affect branching (data not shown).

Inhibition of EGF-R signaling has been shown to delay epithelial maturation (Miettinen et al., 1995, 2000). As cytokeratin expression pattern has been associated with epithelial maturity (Smith et al., 1990), we next analyzed the effect of gefitinib on the developmental stage of the E13+2 cultured salivary epithelium using a PAN-cytokeratin antibody, which in addition to CK8, also recognizes other cytokeratins (1, 4, 5, 6, 10, 13, 18, and 19). We noticed that in the untreated SMGs, as well as in E15 wild-types, PAN-cytokeratin immunoreactivity was expressed in the stalk and in the linings of developing ductal and alveolar structures, but not in the distal part of the end bud (Fig. 4A, C). In the gefitinib-treated salivary glands, however, cytokeratins were localized only in the most proximal parts (Fig. 4B), and appeared unevenly distributed and disorganized (Fig. 4B, D) without a morphologically recognizable distal margin suggesting that the epithelium also in this *in vitro* model is more immature. This result is parallel to the *in vivo* result with the CK8 antibody (Fig. 1).

2.2. Reduced epithelial proliferation in EGF-R (−/−) SMGs

Since the ratio of the epithelium to mesenchyme is reduced in EGF-R (−/−) mice, we analyzed the rate of cell proliferation in branching SMG. Cell proliferation was analyzed from E15, E17, and P0 EGF-R (−/−), and wild-type SMGs using Ki-67 immunohistochemistry and morphometric quantitation. On E15, proliferating cells were present throughout the epithelial buds and ducts, but only scarcely in the mesenchyme. In EGF-R (−/−) mice, the ratio of proliferative cells to all cells was reduced by 27% compared to wild-types (0.49 ± 0.016 vs. 0.67 ± 0.012, $p<0.05$). During later developmental stages, on E17 and P0, cell proliferation appeared more distally and reduced in quantity both in wild-type and EGF-R (−/−) SMGs (data not shown). Cells were still proliferating in the end buds at birth, implicating organ growth even though the most intense phase of branching had finished. It is logical to expect that the epithelium at the tips of the branches would proliferate and that the cells at the cleft would not, thus separating those forming branches. However, we found that cell proliferation in the area of branch formation, including the cleft, was distributed equally throughout the outer layer of the epithelium. Thus, cleft formation did not proceed through differential cell proliferation in the epithelial buds at any stage studied. Our finding is in line with