Evo-Devo of the Mammary Gland

Olav T. Oftedal & Danielle Dhouailly

Journal of Mammary Gland Biology and Neoplasia

ISSN 1083-3021

J Mammary Gland Biol Neoplasia
DOI 10.1007/s10911-013-9290-8

Springer

J Mammary Gland Biol Neoplasia
DOI 10.1007/s10911-013-9290-8

Evo-Devo of the Mammary Gland

Olav T. Oftedal · Danielle Dhouailly

Received: 4 March 2013 / Accepted: 30 April 2013

Abstract We propose a new scenario for mammary evolution based on comparative review of early mammary development among mammals. Mammary development proceeds through homologous phases across taxa, but evolutionary modifications in early development produce different final morphologies. In monotremes, the mammary placode spreads out to form a plate-like mammary bulb from which more than 100 primary sprouts descend into mesenchyme. At their distal ends, secondary sprouts develop, including pilosebaceous anlagen, resulting in a mature structure in which mammary lobules and sebaceous glands empty into the infundibula of hair follicles; these structural triads (mammolobular-pilo-sebaceous units or MPSUs) represent an ancestral condition. In marsupials a flask-like mammary bulb elongates as a sprout, but then hollows out; its secondary sprouts include hair and sebaceous anlagen (MPSUs), but the hairs are shed during nipple formation. In some eutherians (cat, horse, human) MPSUs form at the distal ends of primary sprouts; pilosebaceous components either regress or develop into mature structures. We propose that a preexisting structural triad (the apocrine-pilo-sebaceous unit) was incorporated into the evolving mammary structure, and coupled to additional developmental processes that form the mammary line, placode, bulb and primary sprout. In this scenario only mammary ductal trees and secretory tissue derive from ancestral apocrine-like glands. The mammary gland appears to have coopted signaling pathways and genes for secretory products from even earlier integumentary structures, such as odontode (tooth-like) or odontode-derived structures. We speculate that modifications in signal use (such as PTHrP and BMP4) may contribute to taxonomic differences in MPSU development.

Keywords Evolution · Mammary gland · Lactation · Signaling · Morphogenesis

Abbreviations

APSU	Apo-pilo-sebaceous unit
BMP	Bone morphogenic protein
CRL	Crown-rump length
EDA	Ectodysplasin
FGF	Fibroblast growth factor
Ihh	Indian hedgehog
MB	Mammary bulb
MG	Mammary gland
ML	Mammary line
MP	Mammary placode
MPSU	Mammolobular-pilo-sebaceous unit
mya	Million years ago
PS	Primary sprout
PTHrP	Parathyroid hormone-related protein
SG	Sebaceous gland
Shh	Sonic hedgehog
SS	Secondary sprout

Introduction: Mammary Evolution is an Ancient Story

The origin of the mammary gland (MG) is buried deep in time, as many of its evolutionary novelties—such as caseins and other milk-specific proteins, and the method of sugar synthesis—appear to have originated more than 300 million years ago (mya) in the Carboniferous geological period [1, 2]. This was a time (Fig. 1) when the first fully terrestrial vertebrates, the basal amniotes, were evolving from earlier tetrapods (ancestors of amphibians and other terrestrial forms) from which they

Electronic supplementary material The online version of this article (doi:10.1007/s10911-013-9290-8) contains supplementary material, which is available to authorized users.

O. T. Oftedal (✉)
Smithsonian Environmental Research Center, Edgewater, MD 21037, USA
e-mail: oftedalo@si.edu

D. Dhouailly
FRE 3405 CNRS/UJF/EPHE Université Joseph Fourier Site Santé, La Tronche 38706, France

Published online: 17 May 2013

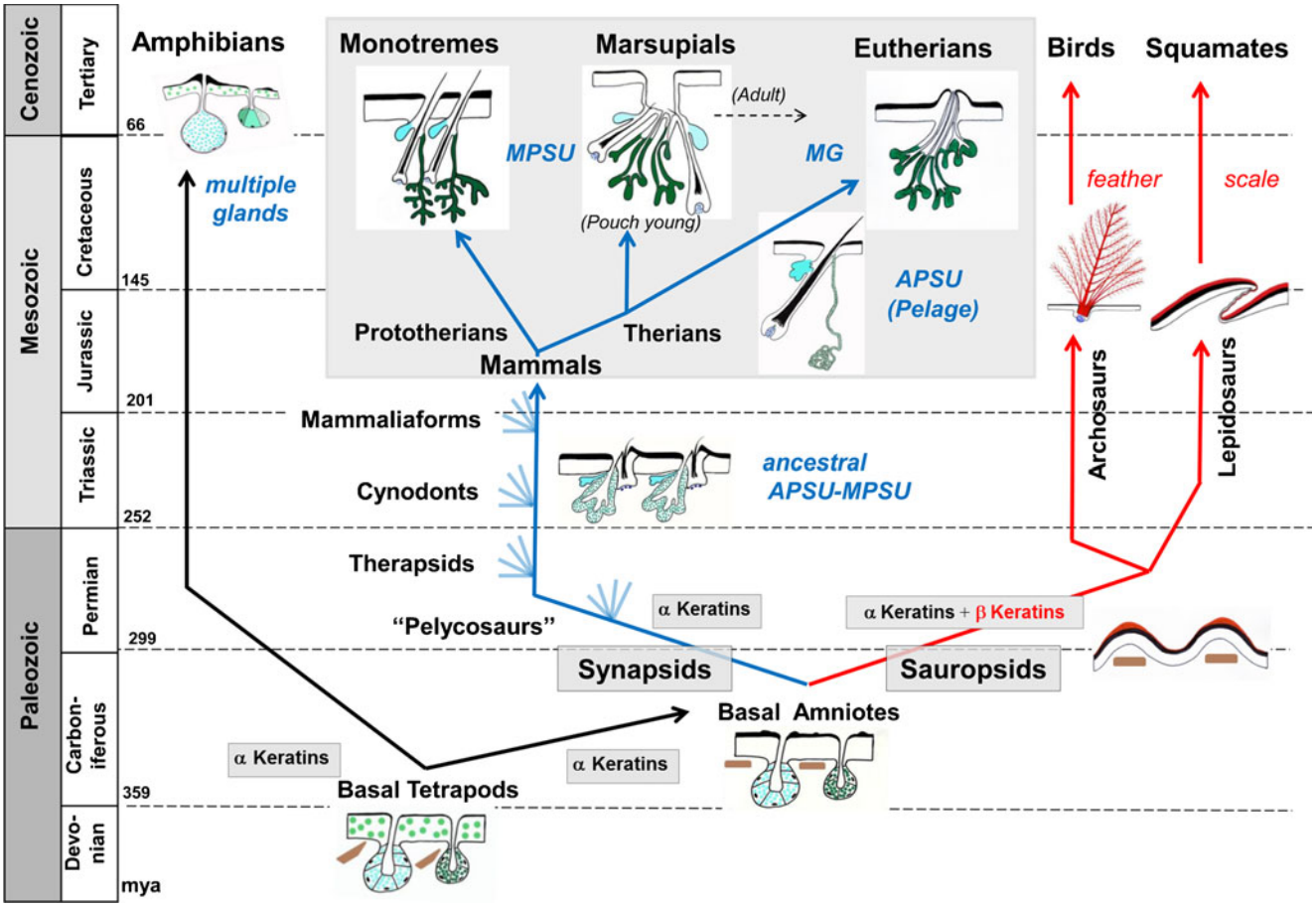

Fig. 1 Evolution of mammary glands (MG) and other integumentary structures. Representative mammary structures are illustrated for monotremes, marsupials (pouch young; transition to adult indicated by *dashed arrow*) and eutherians (see text for species variation). The synapsid lineage (*blue lines of descent*) is characterized by evolution of hair—possibly derived from α-keratin bumps in basal amniotes—and specialized glands in apo-pilo-sebaceous units (APSU) and mammolobular-pilo-sebaceous units (MPSU), but the time, taxon and structure of the ancestral APSU-MPSU are hypothetical. The four sequential synapsid radiations (*blue starbursts*) became increasingly mammal-like in morphology. The sauropsid lineage (*red lines*) is characterized by synthesis of β- keratin in epidermal scales and feathers; only a few complex glands occur (*not shown*). Branch points and line junctions represent estimated times of divergence or of first appearance in the fossil record; only major taxa (but not all radiations nor turtles) are included. Schematics for APSU, scale and feather are placed in the Mesozoic as fossilized impressions have been found. Schematics for basal amniote and basal tetrapod structures are hypothetical. See http://en.wikipedia.org/wiki/Geologic_time_scale for details on geologic periods and [3–5] for details on taxa. mya = millions of years ago. Color key for schematics: **I**. **Solid colors**: *black* = α-keratin; *red* = β- keratin; *brown* = dermal scale (bone), *dark green* = mammary secretory cells, *cyan* = sebaceous gland, *seagreen* (mesh) = apocrine gland, *turquoise* = granular gland, *medium seagreen* (large dot) = single-celled gland. **II**. **Stipling**: *dark blue* = dermal papilla, *dark cyan* = apocrine-mammary transitional cells, *pale turquoise* = mucous gland

inherited a glandular integument—long before the first appearance of mammals (*ca*. 190 mya) or even of dinosaurs (*ca*. 230 mya). Key evolutionary events among Carboniferous tetrapods likely included the evolution of a keratinized integument with ducted multicellular glands (Fig. 1), the ability to lay large eggs on land (rather than small eggs in the water), and parental care of these eggs—traits seen in living amphibians with terrestrial reproduction [6, 7]. In its earliest evolutionary form, the glandular structure ancestral to the MG may have functioned as a source of dilute secretion that helped eggs withstand desiccation associated with incubation on land [8]. This reproductive function of glandular secretion may have evolved among the tetrapods or among the basal amniotes—vertebrates that evolved eggs with an amnion and other specialized extraembryonic membranes that facilitate gas exchange, waste compartmentalization, and water and nutrient uptake by the egg [2, 8]. Living descendants of these basal amniotes (Fig. 1) include mammals, archosaurs (crocodiles and birds), squamates (lizards and snakes), tuataras and turtles (not illustrated).

Two separate lineages of amniotes emerged in the Carboniferous: synapsids and sauropsids (Fig. 1). During the sequential radiations of synapsids ("pelycosaurs", therapsids, cynodonts, mammaliaforms and mammals) glands in the integument became more specialized, leading to MGs [9]. In contrast, among sauropsids ("reptiles" and birds) a novel

integumentary protein (β-keratin) evolved that was used to develop water-resistant epidermal scales and feathers (Fig. 1), and the glandular component of skin became much reduced. Another evolutionary novelty of sauropsids is a calcified eggshell, which greatly reduced egg moisture loss [8]. Note that mammalian features such as hair and lactation evolved in a synapsid lineage with glandular skin and parchment-shelled eggs (subject to moisture loss) [9, 10], not in the "reptilian" lineage (sauropsids) that developed an impervious scaly skin and more-or-less calcified eggshells.

The evolution of complex organ systems involving many evolutionary novelties requires a long period of time for natural selection to operate. The fact that MGs and their secretory products (including milk fat globules, casein micelles, whey proteins and sugars) are structurally similar across all mammals—whether egg-laying monotremes (e.g., platypus), pouched or pouchless marsupials (e.g., opossums and kangaroos), or altricial/precocial eutherians (e.g., mice, humans)—is evidence that MGs were fully developed prior to the emergence of mammals [9]. Comparison of mammary-expressed genes across monotreme, marsupial and eutherian genomes also reveals the shared presence and high degree of conservation of these genes across mammalian groups, indicating a common ancestral MG origin [11]. The time period over which MGs evolved their current mammalian form probably spans 130 million years, from Carboniferous tetrapods/early amniotes (*ca*. 310–330 mya) until the mammaliaforms and earliest mammals (*ca*. 190 mya) (Fig. 1). While many intermediate glandular forms may have arisen in the sequential radiations of synapsids, there is one that is suggested by comparative analysis: an ancestral apocrine-like gland.

Although not identical, MG and apocrine glands in mammalian integument bear many similarities: 1) the secretory portion is bilayered (secretory and myoepithelial cells), 2) they penetrate deep into hypodermis, 3) secretion includes both apocrine and merocrine (exocytosis) pathways, 4) active secretion requires hormonal maturation (puberty), and 5) ontogenetic development entails a transitory or permanent association with hair follicles and sebaceous glands (SG), at least in some mammalian taxa [9]. During integumental development the hair peg in most mammals produces lateral "bulges" that proliferate and differentiate into SG and apocrine glands. The apocrine gland begins to develop at or before the time that basal invagination encloses the dermal papilla, but the onset of SG development varies greatly among species [12]. As the entire complex derives from one epidermal downgrowth it is known as an apo-pilo-sebaceous unit (APSU; Fig. 1) [13]; in this paper we use APSU to refer to both apocrine and ancestral apocrine-like structures. A similar phenomenon occurs in the ventral skin of monotremes, where a downward growing epithelial sprout produces a mammary hair, a SG and a MG lobule; in the lactating female the ducts for both the MG lobule and SG open into the infundibulum of a hair follicle. By analogy, Oftedal [9] termed this a mammo-pilo-sebaceous unit (MPSU; Fig. 1). However, as will become clear in this review, the MPSU is a component of the MG, not the converse, and thus the MPSU is more accurately described as a mammolobular-pilo-sebaceous unit.

Evolution is known to borrow and repurpose existing genetic products, processes and structures rather than creating everything de novo. When discussing MG evolution, one should consider all levels—gene sequences, signaling pathways, morphogenic stages, mature gland structure, secretory pathways, secreted constituents, hormonal controls and reproductive strategies—for evidence of the ancestral traits that have been modified. In this paper we review what is known about morphogenesis of MG (and MPSU) in diverse mammalian taxa, including monotremes, marsupials and eutherians (gray box in Fig. 1). We also consider some of the signaling pathways that determine developmental fate of ectodermal cells in MG in comparison to other ectodermal organs, such as teeth and hair (unfortunately, very little is known about apocrine glands). Our goal is to shed light on which structures, processes and signals have been coopted, modified and repurposed in the evolution of mammary development.

Early Mammary Gland Morphogenesis Proceeds via Phases, but these Differ in Detail among Taxa

One problem in comparing taxa with long separate evolutionary histories (e.g., *ca*. 190 mya since split of monotremes and therians, and *ca*. 160 mya since split of marsupials and eutherians) is that change may be so great that determining what is homologous—that is, derived from a common ancestral structure or process—may be difficult. It is crucial to distinguish the primary phases that MG development undergoes. The phases described herein are not intended to replace the precisely defined stages of MG development in individual species, such as the 8, 10 and 12 defined stages in mouse, human and bovine MG development, respectively [14–16], but rather to allow comparison of homologous phases across diverse taxa.

The phases are described in terms of epithelial (ectodermal) structure, but the underlying mesenchyme (mesoderm) also undergoes structural change and development, and is engaged in active cross-talk with the ectoderm via signaling pathways. In brief, the first phase is formation of an ectodermal field committed to MG identity, the **mammary line** (ML). After epithelial cell migration one or more **mammary placodes** (MP) are formed in the second phase. Then the MP cells reorganize—and in some species proliferate—and undergo some differentiation to form what may be termed a **mammary bulb** (MB), in that, like a garden bulb, it becomes a quiescent structure from which sprouts subsequently emerge; however, in some taxa the MB is not bulb-shaped. The downward

epithelial column(s) that emerge from the MB initiates the fourth phase known as the **primary sprout** (PS). In many taxa, a fifth phase occurs when a set of **secondary sprouts** (SS) emerge from the terminal end of the PS, including anlagen for both MG ductal/secretory tissue and pilosebaceous units (i.e., MPSUs). However, in many eutherians, including the mouse and cattle, this phase is bypassed, and the PS continues to penetrate downward into the developing dermis. The sixth phase entails **sprout canalization**. The seventh phase is **terminal branching** of sprouts that penetrate into a developing fat pad, or other suitable hypodermal structure that can support a developing ductal tree. This rudimentary ductal tree then enters a quiescent period which represents the end of early mammary morphogenesis; the rudimentary tree subsequently undergoes isometric growth to keep pace with body growth, but does not undergo significant further morphogenesis until puberty. We will briefly review the early transitions in monotreme, marsupial and eutherian MG development, and examine the differences indicative of mammary evolution.

Early Mammary Gland Development in Monotremes

The reproductive pattern of extant monotremes—egg formation, egg incubation and extended lactation—is considered the ancestral mammalian condition [1, 9, 17]. Embryonic MG development has been examined in embryos of short-beaked echidnas in late egg incubation and during the first few weeks of pouch-life (Fig. 2) [18]. A 0.4×0.1 mm ectodermal ML is first evident on the lateral surface between the limbs, but transits via medioventral movement until it lies adjacent to the amniotic fold where it assumes the more rounded lenticular shape of a placode (MP). At about the time of hatching (ca. 45 somite stage) the mammary anlage spreads out and the MB assumes the shape of a 0.4×0.3 mm oval mammary plate (Fig. 2), which increases to about 1.7×1.1 mm as crown-rump length (CRL) doubles from 15 to 30 mm. This MB can be recognized by the underlying condensed mesenchyme as well as by the absence of hair placodes, which form in adjacent ventral skin, but not in the MB (Fig. 2), suggesting inhibition of hair follicle formation. Plate growth ceases, as the MB enters a quiescent period. At about 45 mm CRL, the basal epithelial layer of the MB develops crenulation, followed by downward sprouting of solid cellular columns (PS) into the mesenchyme; based on final MG lobule number there appear to be 100–150 PS per MB. Bresslau [18] refers to these as hair buds, because of their superficial resemblance to true pilosebaceous anlagen outside the plate (Fig. 2), but we consider them PS, as they develop SS at their distal termini before 70 mm CRL. The SS include both mammary and pilosebaceous anlagen. Indentations above each PS at 70 mm CRL indicate the beginnings of cornification and by 95 mm CRL both PS and mammary SS (now about 3× the length of PS) are independently canalizing. The oldest (95 mm) pouch young of Bresslau [18] were probably about 20 days post-hatching. Subsequent development of the two types of SS to produce ductal trees/lobules, and mammary hairs and SG, respectively, has not been studied. However, we surmise that each PS generates a complete MPSU (Fig. 1), because during lactation each lobule opens via a galactophore into the infundibulum of an enlarged mammary hair follicle, as does an associated SG. At this time the single MG on each side contains a fan-shaped group of 100–150 (in the echidna) or 100–200 (in the platypus) club-shaped lobules that deliver milk to the skin surface via the large mammary hairs in an abdominal mammary patch; there is no nipple [9, 17]. During mid-lactation, MG lobules are highly branched and densely alveolar [19], although when the young hatch the lobules are still small, minimally branched and tubular in shape such that individual MPSUs bear a superficial resemblance to APSUs in the pelage [9].

In summary, monotreme MG development is characterized by a plate-like mammary bulb that generates 100–200 primary sprouts on each of which an MPSU develops. All three MPSU components are fully developed and functional in the mature mammary patch; there is no nipple.

Early Mammary Gland Development in Marsupials

Marsupials diverged about 160 mya (Fig. 1) and radiated into seven different orders [20]. Marsupials are very altricial at birth [21], especially litter-bearing species of order Dasyuromorphia (e.g., native marsupial cats, *Dasyurus*), whereas singleton-bearing species of Diprotodontia (e.g., kangaroos and wallabies, *Macropus*) are somewhat more developed. Both mammary structures and lactation strategies differ among taxa. For example, in pouch-less species of mouse opossums and short-tailed opossums (*Marmosa* spp., *Monodelphis* spp., Didelphimorphia) the 6–14 altricial young attach to nipples arranged in a circular pattern, including a single or multiple nipples in the middle [22]. These central nipples apparently derive via ventro-medial migration and overlap of parallel lines of mammary primordia [22]. By contrast, kangaroos and wallabies give birth to one young that attaches to a nipple within a pouch, but an older young may still be suckling milk of very different composition from a larger nipple and MG [23, 24].

Tyndale-Biscoe and Renfree [23, p. 344–345] assert that marsupial MG "do not differentiate from a mammary line, as in Eutheria, but each begins as a separate anlage, the number varying from 2 to 25 in concordance with the adult teat number for the species". This is incorrect. In a 17.5 mm intrauterine embryo of an opossum (*Didelphis* sp.), Bresslau [22] observed a ML anterior to the hind limbs and about one third of the way up the lateral body wall. The ML was a ca. 1 mm irregular ridge of ectoderm that was 5–7 cell layers deep

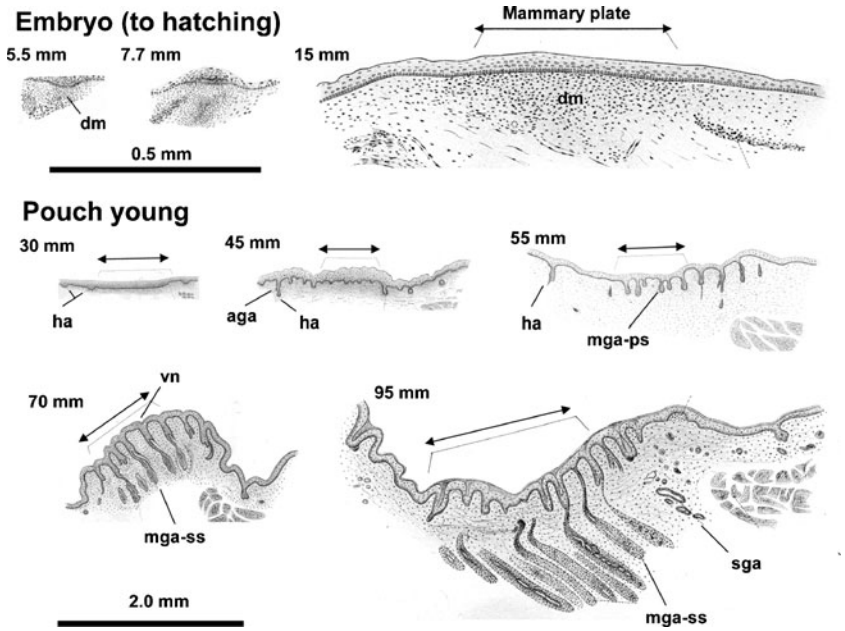

Fig. 2 Early mammary development in a monotreme (echidna). *Upper box* illustrates from left to right: transverse cross sections of a mammary line (5–6 cell layers, stage 42 embryo), placode or somewhat more advanced structure (8–9 cell layers, stage 44–45 embryo) and a plate-like mammary "bulb" in a hatchling (note mesenchymal condensation); all to scale of 0.5 mm bar. *Lower box* illustrates from left to right, top to bottom: expanded plate in 30 mm CRL pouch young (note hair anlage **[ha]** beyond border of plate); crenulation of basal surface in 45 mm young indicating initiation of primary sprouting (note apocrine gland anlage **[aga]** outside of plate); downward projection of primary sprout [PS, **mga-ps**] in 55 mm young (note plate is relatively unchanged in size); formation of secondary sprouts [SS, **mga-ss**] at distal terminus of PS in 70 mm young (note v-notch [**vn**] and narrow presumptive pilosebaceous anlagen at end of PS [not labeled]); canalization of PS and SS in 95 mm young (note sectioned sebaceous gland anlagen [**sga**] outside mammary area); all to scale of 2.0 mm bar. Sections of mammary line and placode are caudal views of right MG (mid-ventrum to right, as illustrated); others are of left MG (mid-ventrum to left). Illustrations of *Tachyglossus aculeatus* adapted from [18]

and narrow (0.1 mm) close to its caudal end, but shallower and broader at its cranial end. In a 20 mm opossum embryo (estimated 18 h pre- birth) each ML had moved ventromedially, purportedly due to the advancing myotome [22], and split into six lens-shaped placodes (plus an extra unpaired MP on the left; opossums normally have 13 MG). ML are also present in intrauterine and newborn *Dasyurus viverrinus* of 6 mm CRL [22]; *Dasyurus* is the only mammal known to be born prior to MP formation, indicative of extreme immaturity. The MG of *Dasyurus* attain a sunken flask or bulb shape (MB) about 14 days postpartum but in other marsupials, such as bandicoots, wombats, squirrel gliders, koalas and rat kangaroos, such MB were already forming, or had formed, by birth. In some taxa caudal mammary anlagen develop prior to cranial anlagen [22]. The MB enter a lengthy quiescent period during which pouch structures—if present—undergo development [22, 25].

Downward projection of a single PS follows (Fig. 3a), although in opossums (*Didelphis*) the downward projection is modest as expansion is mostly lateral. Cornification at the upper surface produces an indentation by the time SS appear at the distal end of the PS (Fig. 3b). Hair anlagen emerge first [22], followed by mammary sprouts and then separate SG anlagen (forming MPSU triads), although figures provided by Bresslau [22] suggest relative timing may vary somewhat. The PS hollows out, forming a "nipple-pocket", while developing hair anlagen penetrate the distal end of this cavity (Fig. 3c), generating hair-filled ostia. The nipple-pocket subsequently everts through its hollowed-out opening (Fig. 3d) so that the inner surface of the nipple-pocket supplies much or most of the outer wall of the nipple [22]. Mammary hairs are usually shed prior to eversion (except in the koala, Fig. 3d), but their ostia (into which galactophores empty) and associated SG remain [22]. The number of MPSU triads per nipple-pocket is species-specific, ranging from 3 to about 27, generating varied numbers of galactophores and mammary ductal trees per nipple (see Supplemental Table 1). In species with very large numbers of MPSUs per nipple, such as kangaroos, some components regress without forming mature structures [26]. In *Didelphis*, although the apparent PS partially hollows out, it does not evert, which Bresslau [25] considered an intermediate evolutionary stage. However, the eversion process occurs in at least nine genera, representing four marsupial orders and including other species of opossums (*Marmosa*) [22]. The mammary SS penetrate into underlying adipose layers and undergo branching (Fig. 3d), producing a rudimentary ductal tree before entering a quiescent period.

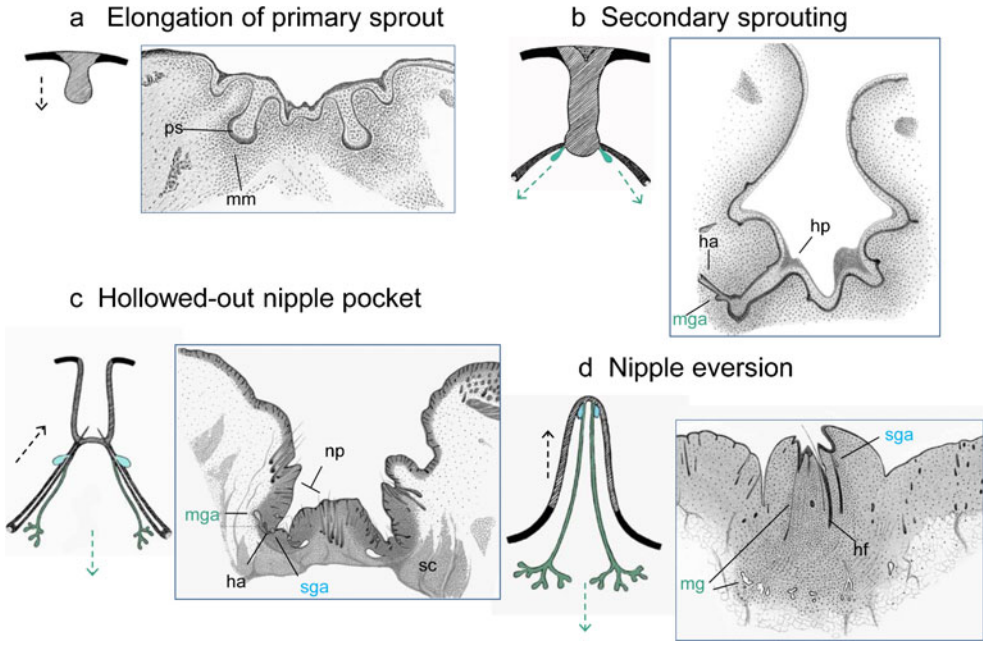

Fig. 3 Early mammary morphogenesis in marsupials. Each figure represents a partial pouch cross-section with either two (**a**–**c**) or one (**d**) developing MG. Schematic to left of each figure illustrates the developmental process: **a** downward growth of primary sprout (**ps**) into mammary mesenchyme (**mm**); **b** cornification of a horny plug (**hp**) in the PS accompanied by secondary sprouting (SS) of hair (**ha**) and mammary gland anlage (**mga** *in green*); **c** hollowing out of nipple-pocket (**np**), growth of mammary hair anlagen and formation of sebaceous gland anlagen (**sga**, *in blue*); and **d** nipple eversion and continued downward MG (**mg**) growth. Main figures, not to same scale, illustrate **a** pouch young of a brush-tailed possum, *Trichosurus* sp. (69 mm CRL), **b** older pouch young of a brush-tailed possum (102 mm), **c** pouch young of a short-nosed bandicoot, *Isoodon obeselus* (130 mm), and **d** pouch young of a koala, *Phascolarctos cinereus* (235 mm). Note, in **c**, that two mga-ha-sga triads (MPSU) have formed in the left nipple pocket while others are forming in a second nipple pocket to the right, and subcutaneous adipose tissue (**sc**) is also developing, and in **d** that sectioned distended mammary ducts are apparent in the dermis while a mammary hair projects from a follicle (**hf**) through the surface of the everted nipple. All illustrations adapted from [22]

In summary, early marsupial MG development is characterized by a single primary sprout per MG, but after MPSUs have appeared the hollowed-out sprout everts to form a nipple. Hair follicles and sebaceous glands mature but the mammary hairs are shed prior to nipple eversion. Each mature MG is constructed from many MPSUs, all draining to one nipple.

Early Mammary Gland Development in Eutherians

Eutherians encompass a wide range of lactation strategies, in part because placental evolution has freed lactation from the constraints of nourishing extremely altricial offspring [2]. Mammary development also varies, producing MG that differ greatly in number, galactophores per nipple, and numbers of discrete mammary trees (Supplemental Table 1).

Mammary development is best known in the mouse (*Mus musculus*) model [14, 27]. In brief, each ML produces 5 MP. These develop into elevated buds that sink into the mesenchyme, some cells of which reorient to condense around the MB. After a brief quiescent period, the bulb elongates as a single down-growth (PS) that penetrates deeply into the differentiating fat pad; SS (as defined above) do not appear. The surface indents as the sprout canalizes by apoptosis, and the sprout branches and rebranches (terminal branching) generating a rudimentary ductal tree within the fat pad. Each MB produces one PS and one ductal tree of about 10–20 branches but no pilosebaceous anlagen are formed.

Mammary development in ruminants, such as cattle (*Bos taurus*), is similar [28–30]. Per histological observation, the ML forms at about 15 mm (5 weeks), two MPs form per ML at 20 mm (5.5 weeks), and sunken MBs surrounded by mammary mesenchyme develop at about 40 mm CRL (7 weeks). After a lengthy quiescent period a single PS appears from each bulb at about 12 weeks, but no SS are formed. Branching occurs at the distal end of the PS at ca. 13 weeks, and at that time, or within about a week, the PS begins to canalize, initiating such specialized structures as the streak canal, teat cistern and gland cistern. About 8–12 large ducts empty into the gland cistern, but from a developmental perspective these are branches of one mammary tree. The teat forms by epithelial proliferation, but differently than nipples in mice [16].

The horse (*Equus caballus*) differs in that MPSUs form during MG development. A ML has been reported (but disputed) at about 2 cm length [31, 32]. One MP—or a somewhat more developed structure—forms per side at 7.9 cm CRL

[31], a MB at 8 cm [32], and two PS per MB at 9.5 cm (Fig. 4). Each PS produces a pair of SS: a descending mammary sprout and a lateral pilosebaceous sprout. The descending SS canalizes and widens as it descends, forming a teat cistern, and via terminal branching and rebranching generates a mammary ductal tree. Concurrently, the pilosebaceous sprout differentiates into a primary and sometimes secondary hair follicles as well as a SG (Fig. 4). The enlarged mammary hair(s) exit the teat adjacent to the galactophore derived from the same initial PS, and the duct of the SG opens into the follicular infundibulum; thus this triad comprises a MPSU, two of which occur in each teat. Each teat thus develops two mammary trees that do not interconnect [28]. The mammary hair(s) and SG persist postnatally [31] and in mature teats [28]. Similarly, in the domestic cat each of five downward-penetrating PS per MB develops pilosebaceous anlagen that are present at birth, and that by 1 week postpartum have developed lobed sebaceous glands on maturing hair follicles, representing MPSU triads, but the pilosebaceous components subsequently regress and are absent by 3 months postpartum, about one month after weaning [35].

Hair and sebaceous anlagen also arise during development of the human MG [36], although overlooked in recent reviews [37–41]. An elevated ML forms at about the 5th week of gestation (ca. 6–7 mm embryo), each producing one MP at ca. 10–12 mm [15, 36, 38, 42]. The developing structure forms a sunken MB (ca. 4 cm embryo, 7–8th week) which, after a lengthy quiescent period, develops an indented, lobed surface (Fig. 5a; ca. 13–14 cm embryo, 12–13th week). Many (16–25) sprouts appear in the 13th-20th weeks, but usually only 6–15 open as ostia on the nipple surface; many ducts branch just below the ostia in mature breasts [44–47]. This indicates that only a subset of the sprouts are PS; secondarily produced sprouts (SS) emerge from these PS (Fig. 5c, d). The alternative explanation—that ducts anastomose and their surface connections regress—is inconsistent with the lack of anastomosis in MG (as opposed to AG) in other mammals [28], and ignores the appearance of SS; although some anastomoses have been reported in mature breasts, they are rare and may be artifacts [44, 47]. Total apparent sprout number increases with fetal age [36], presumably due to SS. Canalization of sprouts occurs in the 20th–32nd weeks, with some differentiation into simple lobulo-alveolar structures by the end of gestation [15, 38, 40]. Hair and sebaceous anlagen are not seen in the early stages of sprouting [36], but are common in later stages (e.g., 20–40 cm fetuses). In some cases only hair or sebaceous anlagen are observed, but commonly both are seen (Fig. 5b–d). At the peak of their development in about the 8th month, triads of mammary, hair and sebaceous anlagen (MPSUs) are formed (Fig. 5d), with each sebaceous anlage typically an outgrowth from a hair anlage [35, 48]. These hair and sebaceous anlagen remain quiescent in the last month of gestation and first month postpartum [35] but thereafter regress. It is not known if sebaceous anlagen from MPSU

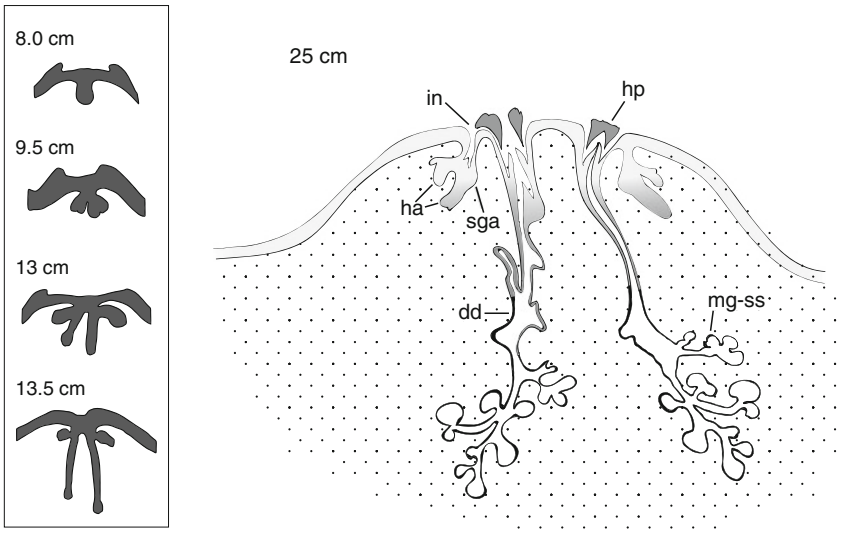

Fig. 4 Dual MPSU in the teat of a fetal horse (*Equus caballus*), including silhouetted early stages. Separate cranial and caudal triads of MG, hair anlagen (**ha**) and sebaceous gland anlagen (**sga**) develop per teat. The main panel illustrates a *ca*. 25 cm CRL fetus in which 1. sebaceous gland anlagen and outbudded secondary hair anlagen have formed on the hair follicle, 2. Shedding of horny plugs (**hp**) has opened multi-layered streak canals adjacent to infundibula (**in**) of hair follicles, and 3. Canalization of distended ducts (**dd**) and ramification of secondary sprouts (**mg-ss**) are forming two independent teat cisterns and mammary trees. Earlier stages, shown top to bottom as silhouettes, illustrate a sunken mammary bulb (8.0 cm CRL), dual primary sprouts (already splitting, 9.5 cm), secondary sprouting of mammary and pilosebaceous sprouts (13 cm), and descent and canalization (*not shown*) of the secondary mammary sprout (13.5 cm). Spatial separation of the mammary trees (*main panel*) occurs due to proliferation of nipple epithelium; all MPSU components are retained in mature mares. Schematics are based on drawings and photographs [31–34]; main panel combines two figures of similar but not identical developmental stage. Artwork prepared by Dr. Regina Eisert

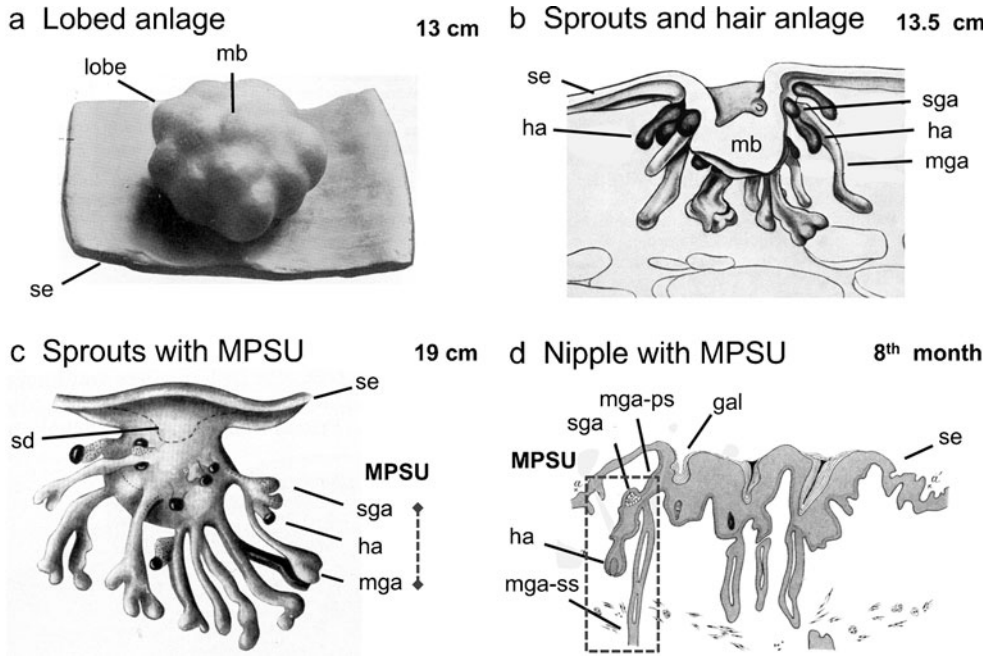

Fig. 5 Development of MPSUs during human mammary development. Fetal length is indicated to upper right of each figure. **a** View from beneath surface epithelium (**se**) of a mammary bulb (**mb**) that is developing lobes and indentations indicative of primary sprout emergence (NB: erroneously labeled as 13 mm by Dabelow [36]); **b** Sectioned bud showing primary/secondary sprouts (**mga**) including hair anlagen (**ha**) and initial sebaceous gland anlagen (**sga**); **c** in older fetus triads of hair, sebaceous and mammary gland anlagen form MPSUs (**sd** = surface depression); **d** nipple cross-section illustrating an MPSU (*dashed box*) with a canalized secondary sprout (**mga-ss**) emerging from a primary mammary sprout (**mga-ps**) connected to a galactophore (**gal**) (fetal CRL not given). Note appearance of differentiated sebaceous cells and dermal papilla on developing hair follicle. Schematics reproduced **a** from [43], **b** and **c** from [36], **d** from [35]; **b** and **c** with copyright permission from Springer Science and Business Media

contribute to the many SG ducts that are observed within nipples and sometimes confused with galactophores [45], or to the compound glands (Montgomery's glands) associated with the areola.

The developmental occurrence of MPSUs in eutherians presumably reflects their shared ancestry with monotremes and marsupials. Hair and sebaceous glands also appear transiently in the nipples of pigs (*Sus scropha*) [28] but whether these derive from MPSUs is unclear. In a squirrel (*Sciurus vulgaris*) ectodermal fragments derived from the ML develop into abdominal vibrissae [22], perhaps derived from ancestral MPSUs. Eutherian species with known MPSU development produce multiple PS per MB but these have shallow dermal penetration. If a correlation exists between number of PS and MPSU development, MPSUs may have a wide eutherian distribution. Multiple PS—as indicated by multiple galactophores per nipple—are characteristic of most eutherian taxa (Supplemental Table 1); only the European mole, some rodents, ruminants, pinnipeds and cetaceans are reported to have a single galactophore per nipple. However, pilosebaceous anlagen do not occur during MG development of two multisprouting species: a bat (*Vespertilio murinus*) and the domestic rabbit (*Oryctolagus cuniculus*) [35].

In summary, eutherian MG development is characterized by one or many primary sprouts per MB; in some species these have shallow penetration but generate secondary sprouts (MPSUs) whereas in other species penetration is deep and no pilosebaceous anlagen are seen.

Comparison of Homologous Phases Reveals Evolutionary Change

A central evolutionary tenet is that change is gradual and cumulative, but when organisms evolve down divergent paths for extended time the cumulative differences may be substantial. Among a monotreme (platypus), marsupial (opossum, *Monodelphis*) and five eutherians the genes expressed in association with mammary development and function are more conserved than other genes [11], suggesting that natural selection has favored MG stability. On the other hand some novel lactation-related genes have been reported in tammar wallabies and other genes have been lost (i.e., are pseudogenes) in eutherians [2, 49]. Developmental patterns support the same conclusion: the phases of MG development are generally similar across taxa, although altered or omitted in some taxa.

The common ancestor of all mammals (Fig. 1) likely underwent the same seven phases of early mammary development as monotremes, marsupials and some eutherians. As all MG originate from MLs which reorganize to form

placodes, this is no doubt the ancestral condition. In some species the initial linear component may be hard to visualize without markers of gene expression [50]. In many species the ML is initially lateral or dorsolateral in location (as in echidna, opossum, rabbit, bat (*Vespertilio*), mouse and human [18, 22, 35, 36, 42, 51]), consistent with the hypothesis that the ML forms at the junction of dorsal and ventral skin, and then shifts medio-ventrally, as documented in diverse species [18, 22, 42, 51]. The purported ventro-lateral ML location in horse embryos [32] has been challenged [31], but a ventral inguinal location of the ML in cattle is generally accepted [28, 30]. It may be that earlier, unidentified anlagen in cattle are initially more lateral in position; additional studies may be needed to resolve this issue.

Evolution has altered the progression from ML to MP(s), both in terms of the final location and number of placodes. MP location determines MG location, which is usually pectoral, abdominal or inguinal, but can be axillary (in manatees) or even dorsolateral (in nutria [52]), reflecting the diverse nursing positions that have evolved among mammals (see [53] for further discussion). The number of placodes formed per ML determines MG number, with the ratio of MG: ML varying from 1:1 (e.g., echidna, koala, marmoset, rhino, Weddell seal and blue whale) to 13:1 (in the short-tailed opossum, *Monodelphis henseli*) [22]) (Supplemental Table 1). One would expect selective pressures to favor a match of supply (MG number) to demand (offspring number). Regressions of median litter size against MG number indicate a slope of about 0.5 young per MG [54, 55], although some species—such as those with communal rearing—deviate from this pattern [56]. In pigs median MG number varies from 10 to 14 according to breed, but is not correlated to litter size at birth [28]. It has been suggested that supernumerary MG—extra, often unpaired MG with varying degrees of glandular development—may reflect an intermediate condition in the loss or gain of normal paired MG, following evolutionary reduction or increase in litter size [57, 58]. Alexander Graham Bell was able to increase functional MG in sheep from 2 to 4 (or more) by selecting for supernumerary MG in breeding experiments, indicating that MG number is highly heritable [59, 60].

A plate-like MB was likely the ancestral condition as it would have allowed dispersion of MPSUs across an abdominal mammary patch and thereby promoted both egg care and hatchling nutrition. The evolutionary conversion of the MB from a plate to a condensed epithelial bulb was apparently a therian achievement, predating the eutherian-marsupial divergence (*ca.* 160 mya; Fig. 1). Evolution of a condensed MB and subsequent nipple differentiation may only have been possible when egg retention and live birth eliminated a need for egg tending [8, 9]. Nipple formation is sometimes considered the primary function of the condensed MB [37, 42], but in marsupials it is the emergent sprout (PS) that by eversion produces the nipple. A condensed MB is necessary to consolidate subsequent sprouting (PS and SS) to a localized area, a precondition for nipple formation. In all taxa hair anlagen (incipient APSUs) appear to be absent from the MB vicinity, even in taxa that subsequently develop pilosebaceous components during secondary sprouting.

The PS varies among mammals in size, depth of penetration and structural contribution. The ancestral condition is likely a small PS, as in monotremes; the PS is larger in marsupials, and may be particularly large and deeply penetrating in eutherians without SS formation. The role of the PS in monotremes is not clear, but in marsupials and eutherians the PS may be involved in nipple and cistern formation, respectively.

SS formation is characteristic of monotremes, marsupials and some eutherians; this phase is undoubtedly an ancestral feature of MG development that reflects the evolutionary relationship of APSU and MPSU. The mammary hairs in monotremes may function as wicks that convey moisture to eggs [8] and the adhesiveness of secretions may permit eggs to become "plastered" to hair [19], providing mechanical support during maternal movements [61]; hatchlings also cling to these hairs [17]. In marsupials, developing hair follicles generate the ostia by which galactophores penetrate the nipple, but then the hairs are shed. In eutherians, the function of mammary hair may have been lost (where vestigial and transient) or is unknown (in the horse). The evolutionary loss of SS, coupled with elongation of the primary sprout, has occurred multiple times during eutherian evolution, as the taxa known to lack SS (ruminants, mouse, rabbit and bat) are not closely related. It is not yet clear how this specific type of branching is suppressed, but it may have been a prerequisite before large gland cisterns could evolve in ruminants.

When mammals initiate lactogenesis, they normally have at their disposal a large number of functional mammary trees, in the form of independent mammary lobules (Supplemental Table 1), but these represent outcomes of different developmental sequelae. This number is determined during early mammary morphogenesis by various combinations of the MP: ML ratio, number of PS per MB, and number of mammary SS per PS (in marsupials, where the PS in converted to a nipple).

What Does Signaling Tell Us About the Origin of Mammary Glands?

It is generally recognized that signaling processes are both limited in number and ancient in origin: only seven pathways (Hedgehog, Wnt, transforming growth factor-β, receptor tyrosine kinase, Notch, JAK/STAT and nuclear hormone) are responsible for most of development in both vertebrates and invertebrates [62], including morphogenesis of vertebrate integument [63, 64]. Thus one would predict signaling

pathways employed in mammary development to be inherited from an ancestral tissue, or to have been coopted from other functions, rather than being formed de novo.

Similarity in signal pathways across organ systems reflects the antiquity and conservatism of signaling as much as the evolutionary history of any particular system. Although similarity in signaling and metabolic processes between the MG and the innate immune system may reflect a MG origin from mucous glands, as has been proposed [65, 66], both tissues may simply retain elements of ancient (pre-vertebrate) innate immune and signaling processes [62, 67], independent of their respective origins.

Discrete Domains in the Ectoderm are Necessary for Assignment of Organ Fate

During development, the surface ectoderm has a default competence to make a specific type of integumental appendage, depending on taxonomic class, including feathers in birds and APSUs in mammals (or simply pilosebaceous units in taxa which no longer have apocrine glands in pelage, such as mice (*Mus*) and chinchillas) [10, 68, 69]. Signals that maintain this general competence, such as Noggin and Sonic hedgehog (Shh)—which inhibit bone morphogenetic protein 4 (BMP4) signaling and induce ectodermal cell proliferation, respectively—must be blocked for other skin appendages to develop. Thus formation of scales and cornea in birds, and cornea, teeth and MG in mammals, requires earlier inhibition of feather or APSU potentialities, respectively [10]. In the chick embryo, the plantar field is specified as early as 3.5 days embryonic life (E3.5) via the expression of En-1 [70], long before feathers form at E7 or plantar scales at E11. Likewise, Pax6-expressing cells which segregate in the cephalic ectoderm at 24 h are the precursors for subsequent corneal epithelium (and, after additional induction, lens) [71]. In the mouse embryo the oral ectoderm acquires its competence before E11 when the dental lamina, a horseshoe-shaped epithelial stripe along the mandible and maxilla, becomes established, although the signaling mechanism is not yet known [72]; tooth placodes appear at E12.

In the mouse embryo the ML—the field for MG development—is similarly specified in advance. While mammary and pelage hair placodes form at about E11.5 and E14, respectively, the ML is detectable at E10.75-E11 (Fig. 6). The earliest cue appears to be Gli3-mediated expression of Fibroblast growth factor 10 (FGF10) by the lateral part of the hypaxial dermomyotome of somites from E10.5 onwards [73]. FGF10 triggers, via the receptor FGFR2-IIIb in the ectoderm, the expression of ectodermal *Wnt10b*. Note that this mechanism holds true at least for the region of the mammary line around the prospective middle (third) MP. Molecular mechanisms for the establishment of the axillary and inguinal parts of the ML remain to be determined [73]. Although this mechanism generates a locally specific expression pattern, *Wnt10b* expression and FGF10/FGFR2-IIb signaling occur concomitantly in formation of a variety of integumental appendages. Tbx3 is also expressed very early in mesenchyme underlying the presumptive ML, and via interactions with ventrally-expressed BMP4 may play a role in establishing the dorsal-ventral boundary on which the ML develops [27].

The findings that the initial signal(s) in mammary field (ML) specification is from somite to the ectoderm, and that region-specific signaling is involved in the lateral positioning of the ML, seem inconsistent with the view from classical recombination experiments [76, 77] that uncondensed mesenchyme from the ventrolateral mammary area can, by itself, induce mammary gland development in ectoderm/epidermis from non-mammary forming regions—regions in which ectoderm has not received signals for field specification. The resolution of this seeming conflict may be that the uncondensed mesenchyme had already been "instructed" by ML ectoderm prior to recombination, or that the initial signal, instead of diffusing directly from the hypaxial somite to the ectoderm, is conveyed via migrating somite cells which might subsequently form uncondensed mesenchyme in the mammary area (Fig. 6). It would be instructive to redo these ectoderm-mesoderm experiments using modern methods, molecular tools, and tissues of different embryonic ages.

Comparison of Formation of the MG Primordia to that of Other Appendages

Incipient ectodermal organs such as feathers, avian scales, pilosebaceous units, teeth and MG each develop ectodermal placodes of a distinctive shape and diameter, containing cells of characteristic number and form, but all share great similarity in signaling. This first became apparent from heterospecific tissue recombination studies [77]. For example, chick dermis is able to sustain the formation of hair placodes in mouse epidermis, which in response induce formation of chick dermal papillae, leading to initial differentiation of hair pegs before development is arrested. The take-home message of such experiments [78–80] is that the formation of cutaneous appendages results from an ongoing dialogue between the two skin components, but only the early messages leading to primordia have been tightly conserved in time and space, as Dhouailly pointed out in 1977 [80]. As birds and mammals originate from separate lineages (Fig. 1), the shared sequence of conserved early messages must derive from their common ancestor, a basal amniote, but likely originated much deeper in the evolutionary tree, probably in the earliest vertebrates. By contrast, squamate scale development involves neither a placode nor dermal papilla as these scales form differently from either avian scales or hair; thus when lizard dermis is recombined with chick or mouse epidermis, the epidermis remains flat [79].

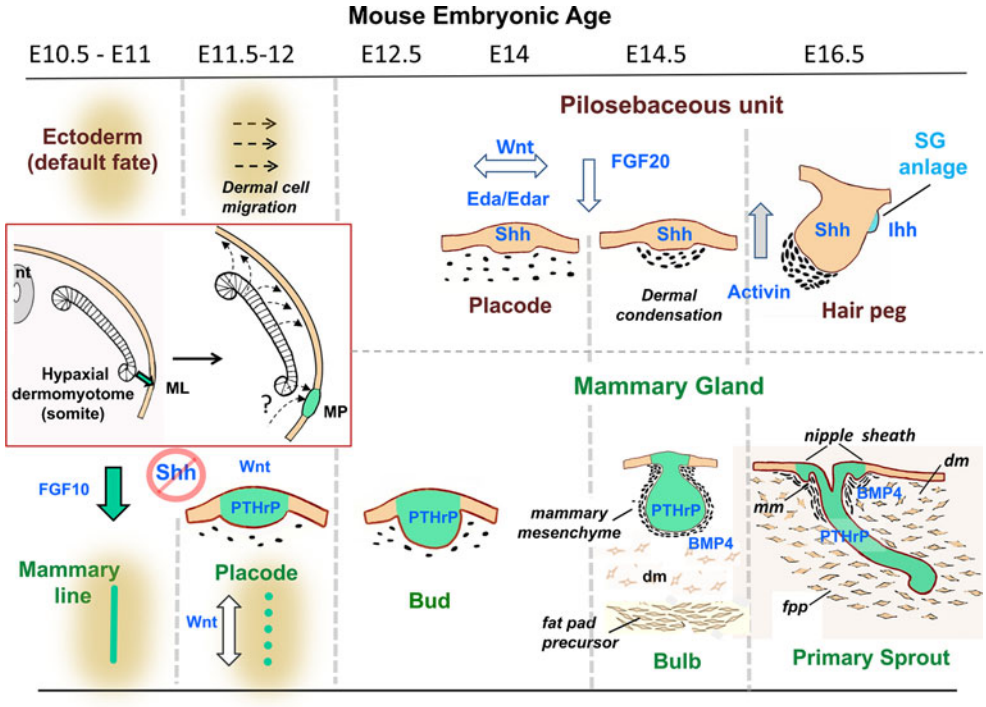

Fig. 6 Timeline comparison of early pilosebaceous and mammary gland development in the mouse. The default fate of ectoderm in the mouse is pilosebaceous unit (PSU) formation (*upper panels*). Early FGF10 expression by the hypaxial dermomyotome (*Inset box*, *left*) triggers a mammary fate (*lower panels*), initially as the mammary line (**ML**; demonstrated for the middle of the ML, see text for details) [73]. This precedes the migration of fibroblasts to constitute a dermis (*Inset box*, *right*), but the origin of mesenchyme associated with mammary ectoderm is uncertain (from the somite or somatopleura). The mammary placodes form before, and subsequently inhibit, hair placode formation, which requires Shh signaling (see text). Fibroblasts condense almost immediately beneath the hair placode in response to FGF20 signaling [74], whereas differentiation and condensation of mammary mesenchyme (**mm**) under the influence of PTHrP signaling is more prolonged. PTHrP and BMP4 signals are essential to primary sprout and nipple sheath formation and to inhibition of pilosebaceous anlagen in the vicinity [75]. The hair peg starts to form a dermal papilla and lateral anlage for a sebaceous gland (**SG**) at about the time that the mammary sprout, after penetrating dermal mesenchyme (**dm**), reaches the fat pad precursor (**fpp**) prior to terminal branching [14]. The timeline (*above*, in days of embryonic life) is approximate as different mammary placodes and areas of integument follow somewhat different chronologies; only a subset of the signaling pathways (*in dark blue*) is illustrated to emphasize differences between PSU and MG formation. See text for signal abbreviations. Illustration of sprouting MG from [14] with copyright permission from Elsevier, Ltd. **nt** = neural tube

Wnt ligands expressed in ectoderm initiate Wnt signaling in each placode type. Inhibition of Wnt signaling by overexpression of inhibitors of the Dkk family leads not only to the absence of pilosebaceous placodes [81], but also of mammary placodes [82]. However, default types of appendages, such as hair and feathers, differ from MG in subsequent development. In the formation of hair and feathers, as well as teeth and dermal scales of teleostean fish, interplay between Ectodysplasin (Eda) and its receptor Edar is important to patterning between placodal and interplacodal epithelial cells [83, 84]. The targets of Eda signaling include molecules from other important signaling pathways, such as FGF20 and Shh, making Eda a key initiator for hair formation in the pelage (hair formation in the MPSU has not been studied). FGF20 attracts migration by fibroblasts [85] and Shh allows proliferative downgrowth of the hair bud [86]. Mice with mutated Eda do not form hair or teeth placodes, but do form mammary placodes [87].

At the time of ML and MP formation the ectoderm does not express Shh ligands. The absence of Shh signaling may be a condition that allows a mammary vs. hair placode fate [88], given that MP develop, but hair placodes do not, when Shh signaling is abrogated [89, 90]. This hypothesis has been supported by findings that MP fail to be induced when Gli-activator functions overrule Gli-repressor functions [91] and that incipient pilosebaceous units adopt a mammary-like phenotype in Smo-deleted mouse skin [92]. Whereas Shh plays a crucial role in hair growth [86, 93], another member of the hedgehog family, Indian hedgehog (Ihh) is required in sebaceous gland development [94]. Although both Shh and Ihh are expressed in MB [90], hedgehog signaling is repressed during MB development [91].

Heterogeneity of the epidermis, i.e. the formation of placodes, arises while the dermis is still homogeneous, and prior to the appearance of dermal condensations [80]. In contrast to hair (and feather) primordia in which dermal fibroblasts immediately begin to migrate and condense underneath

the placode, the morphological mesenchymal condensation response to MP appearance takes 1 or 2 days (Fig. 6), although some ectodermal signals and mesenchymal molecular responses probably occur rapidly. This would be worth further study using gene expression and other molecular techniques.

Curiously, the MG may have similarity in signaling with hair vibrissae formation, which likewise precedes pelage hair appearance. In the European red squirrel (*Sciurus vulgaris*) the ML produces both hair vibrissae and MGs [22]. Retinoic acid (RA) treatment is known to transform mouse vibrissae into tubular glands, but this transformation never occurs with pelage hair [95], being strictly dependent on snout epidermis [96]. Given that Raldh2, an enzyme that generates RA, is expressed in the lateral somite, overlapping that of FGF10 [97], it would be worth exploring whether RA plays a role in the specification of MG vs. APSU fates.

Does Signaling Play a Role in the Different Morphologies of the MG?

The developmental process of branching morphogenesis is important in mammals not only in MG but also in a variety of organs of endodermal (salivary gland and lung) and mesodermal (kidney) origin. Regardless of the type of organ, two gene families have been shown to interact in this process in mice: FGFs (mostly FGF10) and Sproutys [98–100]. This interaction was discovered during tracheal branching in *Drosophila* [101], is responsible for feather branching in birds [102], and was presumably coopted by the MG from an earlier structure.

Among the many signal pathways involved in MG development, parathyroid hormone-related protein (PTHrP) is of particular interest. PTHrP is expressed in mammary epithelial cells in MP and MB; it acts on its type I receptor (PTHR1) in immature mesenchyme, inducing differentiation into condensed mesenchyme and augmenting BMP signaling [75]. In the mouse, the pattern of PTHrP expression in embryonic MG has been studied up to about E15-E16 (Fig. 6). The interaction of PTHrP and BMP4 is necessary for PS and nipple formation and to inhibit pilosebaceous development [75, 103]. In PTHrP knockout mice presumptive hair follicles appear to form on the neck of the MB in place of nipple epithelium and/or PS formation [103]. Genetic defects in PTHR1 expression result in failure of PS formation in human fetuses, but APSUs develop normally [104]. Inhibition of BMP4 by Noggin overexpression converts cells from a nipple epithelium fate to a pilosebaceous fate [105], while BMP4 treatment can rescue sprouting of *PTHrP*$^{-/-}$ mammary buds in organ culture [75]. A coupling of PS formation and inhibition of hair follicle formation appears to occur in all taxa at the MB-PS transition, even in those species that subsequently develop pilosebaceous anlagen during secondary sprouting. We speculate that a progressive decline in PTHrP and BMP signaling may a prerequisite for SS, and that species with differing degrees of MPSU formation may vary in the timing, extent of expression and/or sensitivity to these signals, although other signaling pathways are certainly also involved.

PTHrP signaling may have evolutionary significance in that it has major developmental roles in endochondral bone formation, tooth eruption and MG development [75, 106]. PTHrP is highly expressed in enamel epithelium (ameloblasts) and appears to bind PTHR1 in osteoblasts and in dental mesenchyme; failure to express either PTHrP or its receptor results in aberrant bone formation and failure in tooth eruption [104]. All odontodes (oral and pharyngeal teeth, dermal tooth-like structures) are homologous, undergo similar developmental stages and are under the control of the same set of *Dlx* genes [107]. As such structures predate multicellular glands in vertebrate evolution, PTHrP signaling in the MG may have been coopted from odontodes or a tissue descended from them. This parallels the hypothesized odontode source of a calcium-binding phosphoprotein gene (*ODAM*) that was duplicated and modified during the origin and evolution of α_{s-} and β-caseins, the major calcium transport proteins in milk [108]. In Carboniferous tetrapods the integumentary skeleton included osteoderms that became localized in ventral and ventrolateral areas in the form of gastralia [6, 109], but whether these retained an odontode-like signaling pattern—and thus could play a role in origin of the ancestral MG—is unknown.

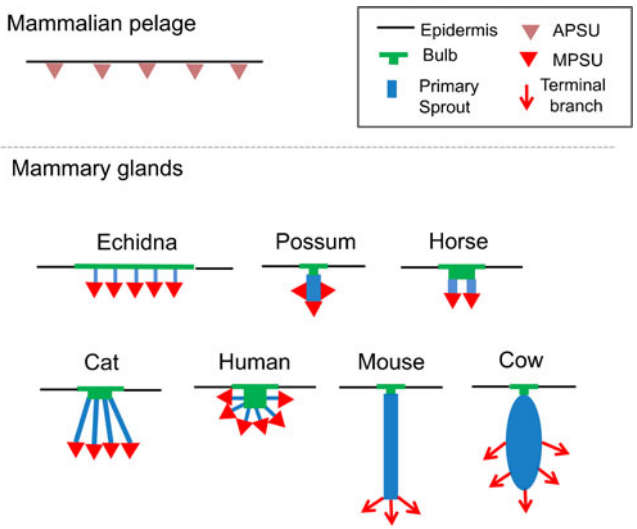

Fig. 7 Comparison of mammary developmental structures at the time of MPSU formation or terminal branching (where no MPSUs form). Simple schematics for each species represent the approximate relative size (not to scale) and number (not necessarily complete) of the mammary bulb (*in green*), primary sprout (*in blue*) and mammolobular-pilo-sebaceous units (MPSU, *red triangles*); where no MPSUs form, terminal branches are illustrated (*red arrows*). See text for more specifics. Although only one marsupial is illustrated (brush-tailed possum), this schematic applies to most marsupials but they differ in the numbers of MPSUs associated with the primary sprout. Note that at this time apo-pilo-sebaceous units (*pink triangles*) are also forming in the integument, although in some species (such as the mouse) the apocrine component has been lost

Conclusion—The Importance of MPSUs in Mammary Gland Evolution

The developmental evolution of the MG has been central to the evolution of synapsids, and their surviving mammalian radiations. There are close evolutionary ties between MG, hair follicles and sebaceous glands. Although several authors have focused separately on the evolution of hair [10, 110, 111] or of MG and lactation [1, 2, 64, 65, 112, 113], hair follicles and glands co-evolved, and should be considered jointly.

We were surprised to discover, in German and French publications from the early 20th century, that pilosebaceous anlagen appear during MG development in eutherians such as the cat, horse and human [31, 35, 48]. Although not recognized at the time, these are components of MPSU triads, and as such directly link eutherian MG development to that of monotremes and marsupials. In particular, eutherian MPSUs provide strong support for two important hypotheses: 1) that the MPSU represents an ancestral developmental triad (derivation of three ectodermal structures from one rudiment) that predates the divergence of monotremes, marsupials and eutherians (Fig. 1), and 2) that the MG evolved via incorporation of an ancestral APSU-MPSU into a more complex structure. We provide a schematic comparison of early MG structures of diverse species in Fig. 7. Note that whereas APSUs develop individually at the skin surface, MPSUs only appear *after bulb/sprout development* at the terminal ends of PS. We believe this represents a critical evolutionary novelty.

How this was accomplished is not known. Are these initial components (bulb and sprouts) in MG development derived from an earlier structure—that somehow managed to capture ancestral APSUs—or did they evolve de novo, using signaling pathways already employed by other structures? Is there evolutionary significance to the fact that the ontogenetic fate of mammary cells is determined so very early via specification of the ML (Fig. 6)? It is almost as if the initial developmental steps have been inserted so early in ontogeny so that MPSUs can still follow the ontogenetic timetable of APSUs. Compare pilosebaceous and MG development of the mouse in Fig. 6; we predict MPSUs would appear at about E16 if not inhibited. Perhaps there are developmental features of the biochemical or signal milieu in an integumental region that favor or require concurrent development of the apo/mammo-pilo-sebaceous triads whether as APSU or MPSU. If so, the relative timing of early development of the MG may reflect the evolutionary history of its MPSU constituents—even in species, such as the mouse, in which MPSU no longer develop.

Finally, we must qualify an earlier hypothesis that the MG evolved from an ancestral apocrine-like gland [9]. While it appears that the mammary ductal tree and secretory tissue (lactocytes and myoepithelial cells) evolved from an ancestral apocrine gland embedded within an APSU, the earlier developmental structures, including the ML, MP, MB and PS—and the nipple and gland cistern derived from these structures—have a separate evolutionary origin.

Acknowledgments We thank Dr. R. Eisert, F. Caraguel and Mrs. B. Peyrusse for assistance in figure preparation, and staff at the Smithsonian Institution Libraries and the History of Medicine Division of the US National Library of Medicine for locating obscure publications. We particularly acknowledge the Biodiversity Heritage Library for online access (www.biodiversitylibrary.org) to scans of old embryological publications. We are grateful to J. Veltmaat and B. Howard for many helpful comments on earlier versions of this paper.

References

1. Oftedal OT. The evolution of milk secretion and its ancient origins. Animal. 2012;6:355–68.
2. Oftedal OT. Origin and evolution of the major constituents of milk. In: McSweeney PLH, Fox PF, editors. Advanced dairy chemistry: volume 1A: proteins. Basic aspects. Boston: Springer US; 2013. p. 1–42.
3. Benton MJ. Vertebrate paleontology. 3rd ed. Malden: Blackwell Publishing; 2005.
4. Kemp TS. The origin and evolution of mammals. New York: Oxford University Press; 2005.
5. Luo Z-X, Yuan CX, Meng Q-J, Ji Q. A Jurassic eutherian mammal and divergence of marsupials and placentals. Nature. 2011;476:442–5.
6. Clack JA. Gaining ground. The origin and evolution of tetrapods. 2nd ed. Bloomington: Indiana University Press; 2012.
7. Wells KD. The ecology and behavior of amphibians. Chicago: University of Chicago Press; 2007.
8. Oftedal OT. The origin of lactation as a water source for parchment-shelled eggs. J Mammary Gland Biol Neoplasia. 2002;7(3):253–66.
9. Oftedal OT. The mammary gland and its origin during synapsid evolution. J Mammary Gland Biol Neoplasia. 2002;7(3):225–52.
10. Dhouailly D. A new scenario for the evolutionary origin of hair, feather, and avian scales. J Anat. 2009;214(4):587–606.
11. Lemay DG, Lynn DJ, Martin WF, Neville MC, Casey TM, Rincon G, et al. The bovine lactation genome: insights into the evolution of mammalian milk. Genome Biol. 2009;10(4):R43. doi:10.1186/gb-2009-10-4-r43.
12. Chapman RE. Hair, wool, quill, nail, claw, hoof, and horn. In: Bereiter-Hahn J, Matolstoy AG, Richards KS, editors. Biology of the integument. 2. Vertebrates. Berlin: Springer Verlag; 1986. p. 293–317.
13. Craigmyle MBL. The apocrine glands and the breast. New York: John Wiley and Sons; 1984.
14. Veltmaat JM, Mailleux AA, Thiery JP, Bellusci S. Mouse embryonic mammogenesis as a model for the molecular regulation of pattern formation. Differentiation. 2003;71:1–17.
15. Vorherr H. The breast. Morphology, physiology and lactation. New York: Academic; 1974.
16. Anderson R. Embryonic and fetal development of the mammary apparatus. In: Larson B, editor. Lactation: a comprehensive treatise. Volume IV. The mammary gland/ human lactation/ milk synthesis. New York: Academic; 1978. p. 3–40.
17. Griffiths M. Biology of the monotremes. New York: Academic; 1978.
18. Bresslau E. Die Entwickelung des Mammarapparates der Monotremen, Marsupialier und einiger Placentalier. Ein Beitrag zur Phylogenie der Saugethiere. I. Entwickelung und Ursprung

des Mammarapparates von Echidna. Denskschr Med-Naturwiss Gesellsch Jena. 1907;7(5):455–518. plates 28–30.
19. Griffiths M, Elliott MA, Leckie RMC, Schoefl GI. Observations of the comparative anatomy and ultrastructure of mammary glands and on the fatty acids of the triglycerides in platypus and echidna milk fats. J Zool. 1973;169:255–79.
20. Nilsson M, Churakov G, Sommer M, Tran N, Zemann A, Brosius J, et al. Tracking marsupial evolution using archaic genomic retroposon insertions. PLoS Biol. 2010;8:e1000436. doi:10.1371/journal.pbio.1000436.
21. Hughes R, Hall L. Structural adaptations of the newborn marsupial. In: Tyndale-Biscoe CH, Janssens P, editors. The developing marsupial. Models for biomedical research. Berlin: Springer Verlag; 1988. p. 8–27.
22. Bresslau E. Die Entwickelung des Mammarapparates der Monotremen, Marsupialier und einiger Placentalier. III. Entwickelung des Mammarapparates der Marsupialier, Insectivoren, Nagathiere, Carnivoren und Wiederkäuer. Denskschr Med-Naturwiss Gesellsch Jena. 1912;7(5):647–874. plates 37–46.
23. Tyndale-Biscoe H, Renfree M. Reproductive physiology of marsupials. Cambridge: Cambridge University Press; 1987.
24. Green B, Merchant J. The composition of marsupial milk. In: Tyndale-Biscoe CH, Janssens PA, editors. The developing marsupial. Models for biomedical research. Berlin: Springer Verlag; 1988. p. 41–54.
25. Bresslau E. Beiträge zur Entwicklungsgeschichte der Mammarorgane bei den Beutelthieren. Z Morphol Anthropol. 1902;4:261–317.
26. Griffiths M, McIntosh D, Leckie RMC. The mammary glands of the red kangaroo with observations on the fatty acid components of the milk triglycerides. J Zool. 1972;166:265–75.
27. Cowin P, Wysolmerski J. Molecular mechanisms guiding embryonic mammary gland development. Cold Spring Harb Perspect Biol. 2011;2:a003251. doi:10.1101/cshperspect.a003251.
28. Turner CW. The mammary gland. I. The anatomy of the udder of cattle and domestic animals. Columbia: Lucas Brothers; 1952.
29. Raynaud A. Morphogenesis of the mammary gland. In: Kon S, Cowie A, editors. Milk: the mammary gland and its secretion. New York: Academic; 1961. p. 3–46.
30. Rowson AR, Daniels KM, Ellis SE, Hovey RC. Growth and development of the mammary glands of livestock: a veritable barnyard of opportunities. Semin Cell Dev Biol. 2012;23:557–66.
31. Uehlinger P. Studien zur Entwicklung der Milchdrüse des Pferdes. 11. Beitrag zum Bau und zur Entwicklung von Hautorganen bei Saugetieren. Inaugural-Dissertation zur Erlangung der Doctor-Würde. Zurich: University of Zurich; 1922.
32. Profé O. Beiträge zur Ontogenie und Phylogenie der Mammarorgane. Anat Hefte. 1899;11:245–86.
33. Rein G. Untersuchungen über die embryonale Enwicklungsgeschichte der Milchdrüse. II. Vergleichend-anatomische Ergebnisse und Schlussresultate. Arch Mikrosk Anat. 1882;21:678–94. plate 30.
34. Hamburger C. Studien zur Entwickelung der Mammarorgane. I. Die Zitze von Pferd und Esel. Anat Anz. 1900;18:16–26.
35. Brouha M. Recherches sur les diverses phases du développement et de l'activité de la mamelle. Arch Biol. 1905;21:459–603. plates 18–20.
36. Dabelow A. Die Milchdrüse. In: von Möllendorff W, Bargmann W, editors. Handbuch der mikroskopischen Anatomie des Menschen. Dritter Band, Haut und Sinnesorgane. Dritter Teil, die Haut die Milchdrüse. Berlin: Springer Verlag; 1957. p. 277–485.
37. Jolicoeur F. Intrauterine breast development and the mammary myoepithelial lineage. J Mammary Gland Biol Neoplasia. 2005;10:199–210.
38. Ba G, Stein T. Human breast development. Semin Cell Dev Biol. 2012;23:567–73.
39. Howard BA, Gusterson BA. Human breast development. J Mammary Gland Biol Neoplasia. 2000;5:119–37.
40. Russo J, Russo IH. Development of the human mammary gland. In: Neville MC, Daniel CW, editors. The mammary gland. Development, regulation and function. New York: Plenum Press; 1987. p. 67–93.
41. Hassiotou F, Geddes D. Anatomy of the human mammary gland: current status of knowledge. Clin Anat. 2013;26:29–48.
42. Hughes ESR. Development of the mammary gland. Ann R Coll Surg Engl. 1950;6:99–119.
43. Broman I. Normale und abnorme Entwicklung des Menschen. Wiesbaden: Verlag von JF Bergmann; 1911.
44. Cooper AP. On the anatomy of the breast. London: Longman, Orme, Green, Brown and Longmans; 1840.
45. Love SM, Barsky SH. Anatomy of the nipple and breast ducts revisited. Cancer. 2004;101:1947–57.
46. Rusby J, Brachtel E, Michaelson J, Koerner F, Smith B. Breast duct anatomy in the human nipple: three-dimensional patterns and clinical implications. Breast Cancer Res Treat. 2007;106:171–9.
47. Going JJ. Lobar anatomy of human breast and its importance for breast cancer. In: Tot T, editor. Breast cancer. London: Springer London; 2011. p. 19–38.
48. Eggeling H. Über ein wichtiges Stadium in der Entwicklung der menschlichen Milchdrüse. Anat Anz. 1904;24:595–605.
49. Renfree MB, Papenfuss AT, Deakin JE, Lindsay J, Heider T, Belov K, et al. Genome sequence of an Australian kangaroo, *Macropus eugenii*, provides insight into the evolution of mammalian reproduction and development. Genome Biol. 2011;12:R81. doi:10.1186/gb-2011-12-8-r81.
50. Veltmaat JM, Van Veelen W, Thiery JP, Bellusci S. Identification of the mammary line in mouse by *Wnt10b* expression. Dev Dyn. 2004;229:349–56.
51. Rein G. Untersuchungen über die embryonale Entwicklungsgeschichte der Milchdrüse I. Arch Mikrosk Anat. 1882;20:431–501. plates 28–29.
52. Gosling L. The duration of lactation in feral coypus (*Myocastor coypus*). J Zool. 1980;191:461–74.
53. Koyama S, Wu H-J, Easwaran T, Thopady S, Foley F. The nipple: a simple intersection of mammary gland and integument, but focal point of organ function. J Mammary Gland Biol Neoplasia. 2013;18. doi:10.1007/s10911-013-9289-1.
54. Pearl R. On the correlation between the number of mammá of the dam and size of litter in mammals. I. Interracial correlation. Exp Biol Med. 1913;11:27–30.
55. Gilbert AN. Mammary number and litter size in Rodentia: the "one-half rule". Proc Natl Acad Sci U S A. 1986;83:4828–30.
56. Sherman PW, Braude S, Jarvis JUM. Littter sizes and mammary numbers of naked mole-rats: breaking the one-half rule. J Mammal. 1999;80:720–33.
57. Derocher AW. Supernumerary mammae and nipples in the polar bear (*Ursus maritimus*). J Mammal. 1990;71:236–7.
58. Hsu MJ, Moore J, Lin JF, Agoramoorthy G. High incidence of supernumerary nipples and twins in Formosan macaques (*Macaca cyclopis*) at Mt. Longevity, Taiwan. Am J Primatol. 2000;52:199–205.
59. Bell AG. Saving the six-nippled breed. Dr Bell's last contribution to science. J Hered. 1923;14:99–111.
60. Castle WE. The genetics of multi-nippled sheep. An analysis of the sheep breeding experiments of Dr. and Mrs. Alexander Graham Bell at Beinn Bhreagh, N.S. J Hered. 1924;15:75–85.
61. Morrow GE, Nicol SC. Maternal care in the Tasmanian echidna (*Tachyglossus aculeatus setosus*). Aust J Zool. 2013. doi:10.1071/ZO12066.
62. Pires-daSilva A, Sommer RJ. The evolution of signalling pathways in animal development. Nat Rev Genet. 2003;4:39–49.
63. Widelitz R, Chuong C-M. Early events in skin appendage formation: induction of epithelial placodes and condensation of dermal mesenchyme. J Invest Dermatol Symp Proc. 1999;4:302–6.

64. Olivera-Martinez I, Viallet J, Michon F, Pearton DJ, Dhouailly D. The different steps of skin formation in vertebrates. Int J Dev Biol. 2004;48:107–15.
65. McClellan HL, Miller SJ, Hartmann PE. Evolution of lactation: nutrition v. protection with special reference to five mammalian species. Nutr Res Rev. 2008;21:97–116.
66. Vorbach C, Capecchi MR, Penninger JM. Evolution of the mammary gland from the innate immune system? Bioessays. 2006;28:606–16.
67. Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. Phylogenetic perspectives in innate immunity. Science. 1999;284:1313–8.
68. Fliniaux I, Viallet J, Dhouailly D. Signaling dynamics of feather tract formation from the chick somatopleure. Development. 2004;131:3955–66.
69. Fliniaux I, Viallet J, Dhouailly D, Jahoda C. Transformation of amnion epithelium into skin and hair follicles. Int Soc Diff. 2004;72:558–65.
70. Prin F, Dhouailly D. How and when the regional competence of chick epidermis is established: feathers vs. scutate and reticulate scales, a problem en route to a solution. Int J Dev Biol. 2004;48:137–48.
71. Collomb E, Yang Y, Foriel S, Cadau S, Pearton DJ, Dhouailly D. The corneal epithelium and lens develop independently from a common pool of precursors. Dev Dyn. 2013. doi:10.1002/dvdy.23925.
72. Jussila M, Thesleff I. Signaling networks regulating tooth organogenesis and regeneration, and the specification of dental mesenchymal and epithelial cell lineages. Cold Spring Harb Perspect Biol. 2012; 4(4). doi:10.1101/cshperspect.a008425.
73. Veltmaat JM, Relaix F, Le LT, Kratochwil K, Sala FG, van Veelen W, et al. Gli3-mediated somitic FGF10 expression gradients are required for the induction and patterning of mammary epithelium along the embryonic axes. Development. 2006;133:2325–35.
74. Huh SH, Närhi K, Lindfors PH, Häärä O, Yang L, Ornitz DM, et al. Fgf20 governs formation of primary and secondary dermal condensations in developing hair follicles. Genes Dev. 2013;27(4):450–8.
75. Hens JR, Dann P, Zhang J, Harris S, Robinson G, Wysolmerski J. BMP4 and PTHrP interact to stimulate ductal outgrowth during embryonic mammary development and to inhibit hair follicle induction. Development. 2007;134:1221–30.
76. Propper AY. Relations epidermo-mesodermiques dans la differenciation de l'ebauche mammaire d'embryon de lapin. Ann Embryol Morphogen. 1968;2:151–60.
77. Kratochwil K. Organ specificity in mesenchymal induction demonstrated in the embryonic development of the mammary gland of the mouse. Dev Biol. 1969;20:46–71.
78. Dhouailly D. [Dermo-epidermal interactions between birds and mammals: differentiation of cutaneous appendages]. J Embryol Exp Morphol. 1973;30:587–603.
79. Dhouailly D. Formation of cutaneous appendages in dermo-epidermal recombinations between reptiles, birds and mammals. Wilhelm Roux's Arch Dev Biol. 1975;177:323–40.
80. Dhouailly D. Dermo-epidermal interactions during morphogenesis of cutaneous appendages in amniotes. Front Matrix Biol. 1977;4:86–121.
81. Sick S, Reinker S, Timmer J, Schlake T. WNT and DKK determine hair follicle spacing through a reaction-diffusion mechanism. Science. 2006;314(5804):1447–50.
82. Chu EY, Hens J, Andl T, Kairo A, Yamaguchi TP, Brisken C, et al. Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. Development. 2004;131:4819–29.
83. Mikkola ML, Thesleff I. Ectodysplasin signaling in development. Cytokine Growth Factor Rev. 2003;14:211–24.
84. Harris M, Rohner N, Schwartz H, Perathoner H, Konstantinidis P, Nusslein-Volhard C. Zebrafish *eda* and *edar* mutants reveal conserved and ancestral roles of Ectodysplasin signaling in vertebrates. PLoS Genet. 2008;4. doi:10.1371/journal.pgen.1000206.
85. Huh S, Närhi K, Lindfors P, Häärä O, Yang L, Ornitz DM, et al. Fgf20 governs formation of primary and secondary dermal condensations in developing hair follicles. Gene Dev. 2013;27:450–8.
86. St-Jacques B, Dassule HR, Karavanova I, Botchkarev VA, Li J, Danielian PS, et al. Sonic hedgehog signaling is essential for hair development. Curr Biol. 1998;8:1058–68.
87. Pispa J, Pummila M, Barker PA, Thesleff I, Mikkola ML. Edar and Troy signalling pathways act redundantly to regulate initiation of hair follicle development. Hum Mol Genet. 2008;17:3380–91.
88. Lewis MT, Veltmaat JM. Next stop, the twilight zone: hedgehog network regulation of mammary gland development. J Mammary Gland Biol Neoplasia. 2004;9:165–81.
89. Gallego M, Beachy P, Hennighausen L, Robinson G. Differential requirements for Shh in mammary tissue and hair follicle morphogenesis. Dev Biol. 2002;249:131–9.
90. Michno K, Boras-Granic K, Mill P, Hui CC, Hamel PA. Shh expression is required for embryonic hair follicle but not mammary gland development. Dev Biol. 2003;264:153–65.
91. Hatsell SJ, Cowin P. Gli3-mediated repression of Hedgehog targets is required for normal mammary development. Development. 2006;133:3661–70.
92. Gritli-Linde A, Hallberg K, Harfe BD, Reyahi A, Kannius-Janson M, Nilsson J, et al. Abnormal hair development and apparent follicular transformation to mammary gland in the absence of hedgehog signaling. Dev Cell. 2007;12(1):99–112.
93. Chiang C, Swan R, Grachtchouk M, Bolinger M, Litingtung Y, Robertson E, et al. Essential role for Sonic hedgehog during hair follicle morphogenesis. Dev Biol. 1999;205:1–9.
94. Niemann C, Unden A, Lyle S, Zaouboulis C, Toftgård R, Watt F. Indian hedgehog and β-catenin signaling: role in the sebaceous lineage of normal and neoplastic mammalian epidermis. Proc Natl Acad Sci U S A. 2003;30(Supplement 1):11873–80.
95. Hardy M. Glandular metaplasia of hair follicles and other responses to vitamin A excess in cultures of rodent skin. J Embryol Exp Morphol. 1968;19:157–80.
96. Viallet J, Dhouailly D. Retinoic acid and mouse skin morphogenesis. II. Role of epidermal competence in hair glandular metaplasia. Dev Biol. 1994;166:277–88.
97. Cho K, Kwon H, Shin J, Lee J, Cho S. Retinoic acid signaling and the initiation of mammary gland development. Dev Biol. 2012;365:259–66.
98. Parsa S, Ramasamy SK, De Langhe S, Gupte VV, Haigh JJ, Medina D, et al. Terminal end bud maintenance in mammary gland is dependent upon FGFR2b signaling. Dev Biol. 2008;317:121–31.
99. Unbekandt M, del Moral P, Sala FG, Bellusci S, Warburton D, Fleury V. Tracheal occlusion increases the rate of epithelial branching of embryonic mouse lung via the FGF10-FGFR2b-Sprouty2 pathway. Mech Dev. 2008;125:314–24.
100. Lo T, Yusoff P, Fong C, Guo K, McCaw B, Phillips W, et al. The ras/mitogen-activated protein kinase pathway inhibitor and likely tumor suppressor proteins, Sprouty 1 and Sprouty 2 are deregulated in breast cancer. Cancer Res. 2004;64:6127–36.
101. Kramer S, Okabe M, Hacohen N, Krasnow M, Hiromi Y. Sprouty: a common antagonist of FGF and EGF signaling pathways in *Drosophila*. Development. 1999;126:2515–25.
102. Yue Z, Jiang T, Wu P, Widelitz R, Chuong C-M. Sprouty/FGF signaling regulates the proximal-distal feather morphology and the size of dermal papillae. Dev Biol. 2012;372:45–54.
103. Foley J, Dann P, Hong J, Cosgrove J, Dreyer B, Rimm D, et al. Parathyroid hormone-related protein maintains mammary

epithelial fate and triggers nipple skin differentiation during embryonic breast development. Development. 2001;128:513–25.
104. Wysolmerski JJ, Cormier S, Philbrick WM, Dann P, Zhang JP, Roume J, et al. Absence of functional type 1 parathyroid hormone (PTH)/PTH-related protein receptors in humans is associated with abnormal breast development and tooth impaction. J Clin Endocrinol Metab. 2001;86(4):1788–94.
105. Mayer JA, Foley J, De La Cruz D, Chuong C-M, Widelitz R. Conversion of the nipple to hair-bearing epithelia by lowering bone morphogenetic protein pathway activity at the dermal-epidermal interface. Am J Pathol. 2008;173:1339–48.
106. Wysolmerski JJ. Parathyroid hormone-related protein: an update. J Clin Endocrinol Metab. 2012;97:2947–56.
107. Debiais-Thibaud M, Oulion S, Bourrat F, Laurenti P, Casane D, Borday-Birraux V. The homology of odontodes in gnathostomes: insights from *Dlx* gene expression in the dogfish, *Scyliorhinus canicula*. BMC Evol Biol. 2011;11:307. doi:10.1186/1471-2148-11-307.
108. Kawasaki K, Lafont A, Sire J. The evolution of milk casein genes from tooth genes before the origin of mammals. Mol Biol Evol. 2011;28:2053–61.
109. Witzmann F, Scholz H, Mueller J, Kardjilov N. Sculpture and vascularization of dermal bones and implications for the physiology of basal tetrapods. Zool J Linnean Soc. 2010;160:302–40.
110. Wu P, Hou L, Plikus M, Hughes M, Scehnet J, Suksaweang S, et al. Evo-Devo of amniote integuments and appendages. Int J Dev Biol. 2004;48:249–70.
111. Alibardi L. Perspectives on hair evolution based on some comparative studies on vertebrate cornification. J Exp Zool Part B. 2012;318:325–43.
112. Lefèvre C, Sharp J, Nicholas K. Evolution of lactation: ancient origin and extreme adaptations of the lactation system. Annu Rev Genomics Hum Genet. 2010;11:219–38.
113. Widelitz RB, Veltmaat JM, Mayer JA, Foley J, Chuong CM. Mammary glands and feathers: comparing two skin appendages which help define novel classes during vertebrate evolution. Semin Cell Dev Biol. 2007;18(2):255–66.